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An International Journal

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ANALYTICAL BIOCHEMISTRY 2, 1–14 (1961)

A Simple Method for the Determination of Urinary Adrenocortical Steroids (α-Ketols)

GEORGE R. KINGSLEY AND GLORIA GETCHELL

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Received August 5, 1960

INTRODUCTION

The method of Norymberski (1) and its many modifications (2–4) is now generally considered superior to other methods for the determination of corticosteroids in urine. This method employs potassium borohydride to reduce the preformed 17-ketosteroids to alcohols and the 17–20-ketols to glycols. Sodium bismuthate is then used to oxidize the 17-hydroxy steroids (C-21 corticosteroids) to 17-ketosteroids. The ketosteroids are separated by different techniques, such as use of various organic solvents or by chromatography for identification by the Zimmerman reaction or infrared spectographic measurement.

Norymberski *et al.* (5) have stated: "Treatment of the urine with sodium bismuthate in the presence of acetic acid converts 17-ketogenic steroids (types I, II, and IV) into 17-ketosteroids. When this operation is preceded by treatment of the urine with sodium or potassium borohydride all four subgroups of 17-hydroxycorticosteroids (I, II, III, and IV) are converted into 17-ketosteroids while the native 17-ketosteroids of the urine are reduced to the nonchromogenic 17-hydroxy compounds. It follows that the increase of 17-ketosteroids on oxidation with sodium bismuthate gives a measure of 17-ketogenic steroids and that the total amount of 17-ketosteroids found after the reduction–oxidation sequences gives a measure of 17-hydroxy corticosteroids."

The Norymberski method is obviously not a method well suited for rapid routine multiple determinations. Some modifications (2) have been made for this purpose but they are still not rapid or simple to perform. The simplified Reddy method (6) which uses the Porter-Silber reaction determines only steroids with 17,21-diol-20-one side chain and has been shown (7, 8) not to be specific when employed with crude extracts. Recoveries were also poor (7), and considerable difficulty in the purification of *n*-butanol suitable for use in this method has been reported.

Because of the technical difficulties and lack of simplicity of the Norymberski method and its modifications, and the inaccuracies of the simpler Reddy method, the blue tetrazolium methods for the determination of corticosteroids were investigated to find a relatively simple method with sufficient specificity for clinical purposes. Mader and Buck (9) found that blue tetrazolium (3,3′-dianisolebis-4,4′-3,4-diphenyltetrazolium chloride) of the tetrazolium salts was the most sensitive for the detection of corticosterone, 11-deoxycorticosterone, 11-dehydroxycorticosterone, cortisone acetate, and 17-hydroxy-11-deoxycorticosterone. All of these compounds contain a primary α-ketol group which is found in all biologically active corticosteroids. Blue tetrazolium (BT) can also be used to determine both those steroids that react with the Porter-Silber (10) reagent and those that contain only an α-ketol grouping at the C-17 position since its reaction does not depend upon the presence of a 17-hydroxyl group. Chen *et al.* (11) recommended the use of choline to increase the color intensity of the BT corticosteroid reaction; however, this reagent required 2 hr for color development. Wheeler *et al.* (12) compared Chen's BT method with phosphomolybdate reduction, copper reduction, and formaldehyde formation for estimation of sterols and found that it gave similar results on normal and pathological urines. Marks (13) employing Chen's method found: "The reducing power of different adrenocortical hormones with a ketol side chain is mole for mole essentially the same." However, he concluded that the urinary Porter-Silber chromogen procedure is more specific for quantitative measurement of adrenocortical function in man than urinary BT chromogen. Nowaczynski *et al.* (14) used tetramethyl ammonium hydroxide solution to adjust the pH of BT reagent in ethanol to 12.5. This buffered BT reagent was heated until colorless and added while warm to dry corticosteroid residue, then heated in a water bath at 90°C for 2 min in the dark. Acetone was added to dissolve the formazan produced and then read at 600 mμ. Cuvettes were kept covered to prevent precipitation of formazan. Recknagel and Litteria (15), using 0.03 *N* NaOH in ethanol as a solvent buffer for BT, found that room temperature was satisfactory for its reaction with the steroid and light sensitivity was not encountered. These authors (15) abandoned the use of tetramethyl ammonium hydroxide because of the variability of results obtained with its use. Izzo *et al.* (16) using pure steroids and BT in tetramethyl ammonium hydroxide–ethanol determined its reaction rate with over 40 steroids (C-21 and C-19) at 25°C for 2 hr and found that the ketols reacted maximally at 35 min and under correct adjustment of reagents in 15 min, whereas Δ^4-3-ketosteroids produced relatively small amounts of color. This investigator found that the variation of color produced among all the ketols was minimal and probably was not significant.

The blue tetrazolium ketol method in this paper is a modification of the methods of Chen *et al.* (11), Recknagel and Litteria (15), and Izzo *et al.* (16) and was applied to urines after acid and glucuronidase hydrolysis.

METHOD

Reagents

Alcoholic tetrazolium blue[1] [DAJAC, Blue Tetrazolium (BT)]. Dissolve 100 mg tetrazolium blue in absolute ethyl alcohol and dilute to 400 ml with this solvent. Store in refrigerator. (Stable.)

3.6% Alcoholic HCl. Dilute 10 ml of conc. HCl to 100 ml with absolute ethyl alcohol. *Store in refrigerator.* (Stable.)

0.03 N Alcoholic NaOH. Prepare fresh just before use by dilution of 6 *N* NaOH with absolute ethyl alcohol. (Not Stable.)

Stock Cortisone Standard. Dissolve and dilute 100 mg cortisone acetate to 100 ml with absolute ethyl alcohol. *Store at 4°C in glass-stoppered brown bottle.*

Dilute Cortisone Acetate Standard. Dilute 20 ml of stock standard to 100 ml with absolute ethyl alcohol and store in same manner as stock standard. 1 ml = 200 μg or 0.2 mg cortisone acetate.[2]

Procedure

Extraction. Add 1 ml of conc. HCl to 7 ml urine in a glass-stoppered cuvette and heat in a boiling water bath for 3–4 min. Prepare reagent blank by substituting 7 ml saline for 7 ml urine. Cool, add 7 ml chloroform, and shake vigorously for 1 min. Centrifuge if necessary to separate and clarify chloroform extract. Siphon off extracted urine and discard. Wash chloroform with 1 ml of 10% NaOH and then wash free of NaOH with three washes of 5 ml of distilled water. Transfer duplicate 1-ml portions of the urine chloroform extract to standard colorimeter cuvettes and evaporate to dryness on a water bath at 80°C. Remainder of chloroform extract may be used for 17-ketosteroid determination (17) as was done in the analysis of the specimens presented in Table 5.

Color Development. Add 2 ml of absolute ethyl alcohol to the dried residues and dissolve. Add 2 ml of BT reagent and 1 ml of 0.03 *N* alcoholic NaOH. Mix and let stand 15 min. After exactly 15 min add 0.2 ml of 3.6% alcoholic HCl, mix thoroughly, and read at 525 mμ against blank

[1] Borden Chemical Co., Philadelphia, Pa.

[2] The above cortisone reagent solutions may be obtained from Clinton Laboratories, 6010 Wilshire Blvd., Los Angeles 36, Calif.

set at 100% *T*. Prepare the urine blank as directed above except for the substitution of 2 ml of absolute alcohol for 2 ml of the BT reagent.

Standardization

Add 0.0 (blank), 0.2, 0.4, 0.6, 0.8, and 1 ml of dilute cortisone acetate standard to 7 ml saline in glass-stoppered cuvettes (1 ml = 0.2 mg cortisone acetate). Add 1 ml of conc. HCl and continue as described in *Procedure*. Read against blank and determine K from formula.

$$\text{mg/liter cortical steroid} = K^* \times \text{O.D.}$$

where $K^* = 61$ for Junior Coleman No. 6; B cuvettes.

EXPERIMENTAL

Optimum Conditions for Urinary Hydrolysis

Enzymic hydrolysis is generally used to release steroids from their conjugates as this procedure eliminates the possibility of destruction of the free steroid by excessive hydrolysis. However enzymic hydrolysis requires an expensive, unstable reagent (β-glucuronidase) and is time consuming (24–72 hr hydrolysis). There is considerable disagreement as to the optimum time for complete enzymic hydrolysis, and the number of units of β-glucuronidase to use for maximum yield of free or unconjugated sterols (18, 19). Enzymic hydrolysis of inorganic conjugates of β-steroids, such as sulfates, is not satisfactory, and prolonged mild acid hydrolysis (19, 20) with sulfuric acid and ether extraction of several days is also too time consuming to be of practical clinical use. Since it has been generally accepted that preparatory acid hydrolysis of urine for cortical steroid determination is unsatisfactory, the optimum conditions for acid hydrolysis to obtain maximum yield of naturally occurring urinary ketols from their conjugates in urine without loss of active material was carefully studied to obtain, if possible, a practical procedure which would be satisfactory for clinical purposes.

Several urines were subjected to hydrolysis with different concentrations of hydrochloric acid using all other reagents and conditions as described in the above procedure. The same type of hydrolysis curve was obtained with all urines. A composite of these curves is shown in Fig. 1, which indicates that 4.7% HCl produced maximum hydrolysis of the ketols in urine since maximum absorption was obtained with this concentration of acid. Optimum time for this hydrolysis was established from a study of several urines, examples of which are shown in Fig. 2, which indicates that optimum time for hydrolysis of urine occurs in 3–4 min. The cortisone or cortisone acetate standard should not be hydrolyzed over 4 min.

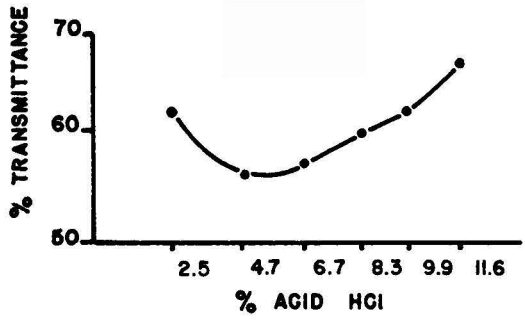

FIG. 1. Determination of optimum concentration of HCl acid for cortical steroid hydrolysis at 100° C.

Since the 17-ketosteroid method of Kingsley and Getchell (17) employs a 10-min HCl acid hydrolysis at 80°C, these conditions were investigated for their adequacy for the hydrolysis of cortical steroid conjugates in order that both α-ketols and 17-ketosteroids be measured on the same sample of urine. Approximately the same results were obtained for both α-ketols and 17-ketosteroids whether the urines were hydrolzyed at 80°C

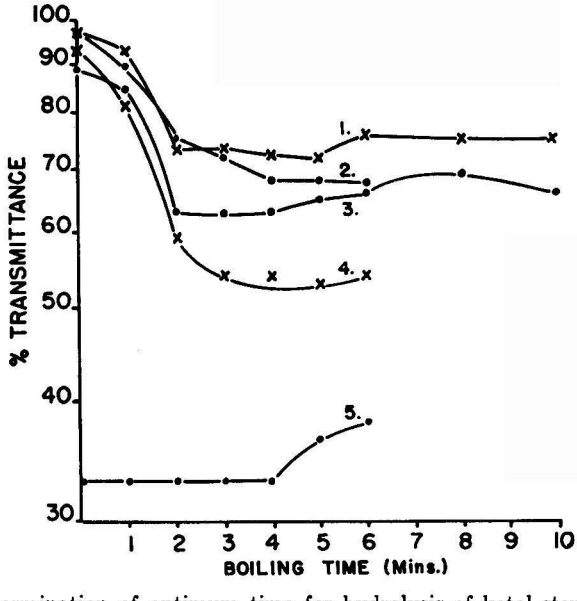

FIG. 2. Determination of optimum time for hydrolysis of ketol steroids with HCl. 1 = urine A, 2 = urine B, 3 = urine C, 4 = urine D, 5 = cortisone and cortisone acetate standards.

for 10 min or at 100°C for 4 min as indicated by the hydrolysis of nine urines which showed standard deviations of from 0.5 to 2.0 with an average deviation of 1.28 for urines ranging from 11.2 to 34 mg/l.

Comparison of HCl and β-glucuronidase hydrolysis of urines indicated the incompleteness of hydrolysis of cortical conjugates with β-glucuronidase in all urines studied (Table 1).

TABLE 1

COMPARISON OF HYDROCHLORIC ACID AND β-GLUCURONIDASE HYDROLYSIS OF URINES IN PREPARATION FOR 17-HYDROXY AND CORTICAL STEROIDS AND 17-KETOSTEROID DETERMINATION (MG/L)

Urine No.	Cortical steroids			17-Hydroxy	17-Ketosteroids (17)		
	HCl	Ketodase[a]		Reddy (6)	HCl	Ketodase[a]	
		36 hr	72 hr			36 hr	72 hr
1	15.1	11.0	11.5	2.4	11.4	12.7	15.1
2	19.2	15.0	16.0	4.5	15.2	13.8	16.3
3	53.0	10.0	12.5	10.5	35.0	10.2	13.5
4	28.0	15.4	18.9	14.1	25.0	11.1	18.0
5	22.0	—	6.3	5.2	15.0	—	11.5
6	19.0	—	8.4	1.2	16.0	—	7.9
7	11.0	—	8.8	2.4	10.0	—	8.8
8	22.3	—	19.6	11.9	14.0	—	9.5
9	10.6	—	5.4	1.4	8.0	—	3.2
		48 hr				48 hr	
10	17.6	4.8	—	0.9	12.5	3.1	—
11	16.5	5.5	—	1.0	10.1	4.3	—
12	40.0	10.0	—	7.5	26.0	10.0	—
13	10.8	5.0	—	3.2	7.5	4.0	—

[a] Two thousand units of Ketodase and 4300 units of penicillin used per milliliter of urine.

The possibility of loss of cortical steroids upon evaporation of the chloroform extract at 80°C was investigated by comparing this temperature of evaporation with 40°C evaporation using the chloroform extracts of eight urines and three cortisone acetate standards. The average values for both urines and standards were the same at 80°C and at 40°C with standard deviation ranging from 0 to 0.5 with average deviation of 0.28, for urines ranging from 4.3 to 31.2 mg/l, indicating that no greater loss of cortical steroid occurred at 80°C than at 40°C.

Optimum Conditions for Maximum Color Development, and Measurement of α-Ketols with BT

Our study (Fig. 3) indicated that 1.25 ml of 0.03 *N* NaOH gave optimum color development (higher concentrations produce turbidity) which

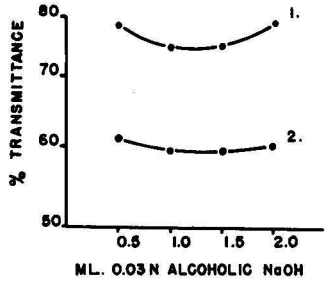

FIG. 3. Effect of variation of concentration of 0.03 N alcoholic NaOH on color development. 1. urine; 2. cortisone acetate standard.

is similar to the 1 ml of 0.03 N NaOH used by Recknagel *et al.* (15). Linear standardization curves with cortisone acetate were also given by 0.03–0.045 N NaOH (Fig. 4). Our comparison of the effect of sodium

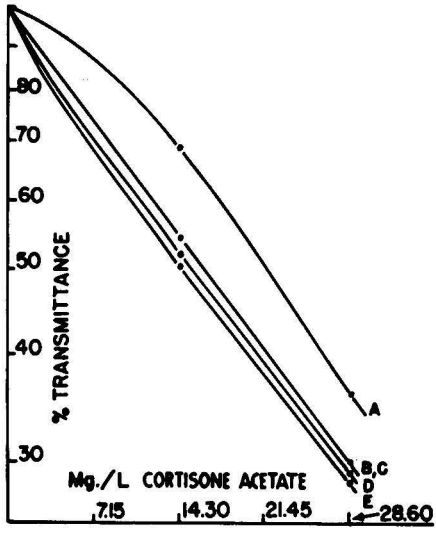

FIG. 4. Effect of different concentrations of alcoholic NaOH on linearity of standardization curves using cortisone acetate. A = .015 N NaOH; B = 0.03 N; C = 0.045 N; D = 0.06 N; and E = 0.09 N. Turbidity developed on standing for curves D and E.

hydroxide and tetramethyl ammonium hydroxide (TMAH) in the color development of reduced BT showed a negligible increase in sensitivity with TMAH (0.166%) as recommended by Izzo *et al.* (16). However, higher concentrations gave nonlinear standardization curves. We have

obtained only slight color enhancement with the use of base choline over that obtained with NaOH.

Some investigators have shown considerable differences in the amount of BT required ranging from 0.5 to 2.5 mg in 1.5–3.0 ml of alcoholic extract of sterol (Table 2). We are in agreement with Chen *et al.* (11)

TABLE 2

REAGENTS AND CONDITIONS OF PUBLISHED BLUE TETRAZOLIUM KETOL METHODS

Reference	Extract (ml)	Base (mg)	BT[a] (mg)	Acid used (ml)	Color dev. time (min)	Maximum absorption spect. (mμ)
(11)	1.8	1 Choline	0.50	none	120	515
(14)	1.5	25 TMAH[b]	1.25	1, G.A.[c]	25	510
(16)	3.0	10 TMAH[b]	2.50	1, 3.6% HCl	15	510
(15)	2.0	1.2 NaOH	0.50	none	30	525
(17)	2.0	1.2 NaOH	0.50	0.2, 3.6% HCl	15	525

[a] BT = blue tetrazolium.
[b] TMAH = tetramethyl ammonium hydroxide.
[c] G.A. = glacial acetic acid.

and Recknagel *et al.* (15) that approximately 2 mg BT is optimal (Fig. 5). We have observed that when larger amounts of BT are used, higher

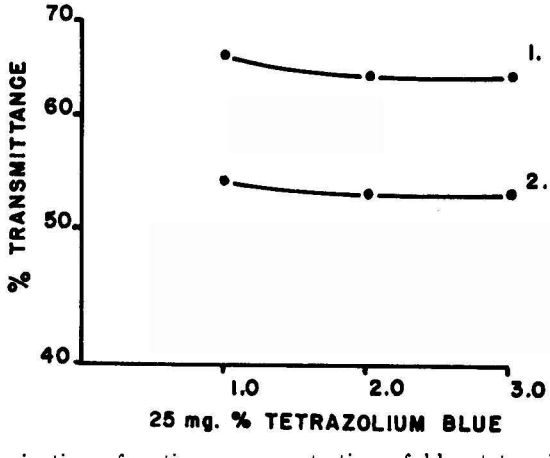

FIG. 5. Determination of optimum concentration of blue tetrazolium. 1 = 11.7 mg/l urinary cortical steroids. 2 = 17.2 mg/l cortisone standard.

blanks are produced. Two-tenths milliliter of alcoholic 3.6% HCl was found to give maximum color stability, and 0.6 ml or more produced less color.

We have found under the conditions of the method outlined that maximum color development occurred at the completion of 15 min (Fig. 6) reaction time. It is important that time for color development not be

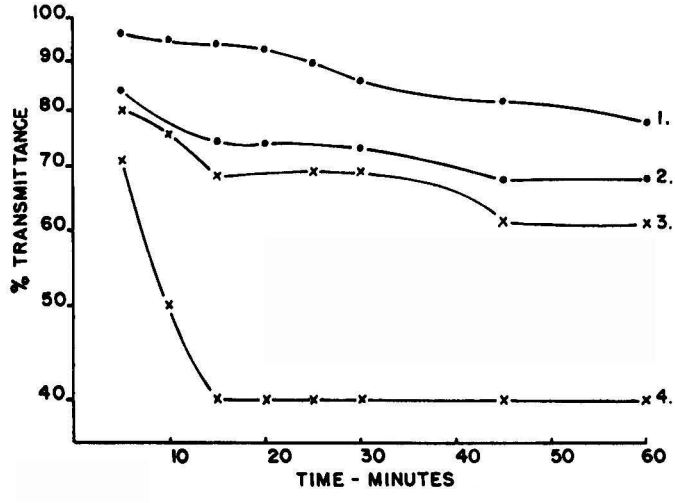

FIG. 6. Effect of time on color development. 1 = Reagent blank read against distilled water. 2 = Urine A. 3 = Urine B. 4 = Cortisone and cortisone acetate standard.

extended since Izzo *et al.* (16) have shown that non-ketolic compounds possessing Δ^4-3-keto groups react slowly but not appreciably during the first 15 min but at a considerably greater rate after this time. We have found that the addition of 0.2 ml of 3.6% alcoholic HCl to the reduced BT is very effective in stabilizing and enhancing the color without increasing the blanks. Nowaczynski *et al.* (14) used 1 ml of glacial acetic acid and 25 min color development time, and Izzo *et al.* (16) employing pure steroids used 1 ml of 3.6% HCl and 15 min color development time (Table 2). Maximum absorption of BT was found at 525 mμ light transmittance as was also observed by Recknagel *et al.* (15). Nitroblue tetrazolium was found not to be as satisfactory as BT for the measurement of cortical sterols due to high blanks.

The effect of time of color development on the linearity of standardization curves prepared from mixtures of equal amounts of 17-OH corticosterone, corticosterone, cortisone, and dehydrocorticosterone showed that 10-, 15-, and 20-min standardization curves were linear and 5, 25, 45, and 60 min nonlinear.

Standardization curves for several α-ketol compounds shown in Fig. 7

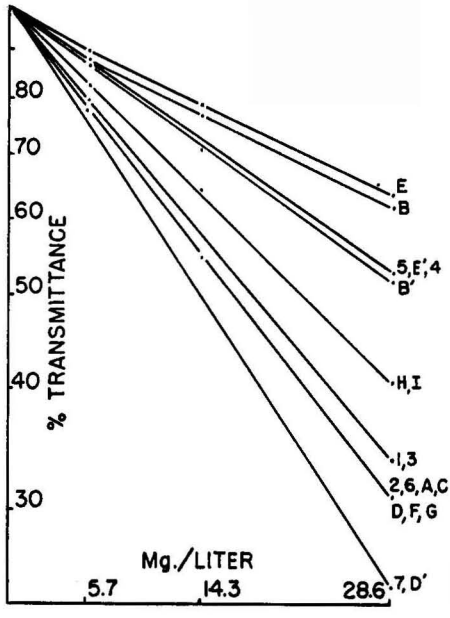

FIG. 7. Comparison of standardization curves obtained with different cortisone compounds. 15-min color development. 1. Merck, 17-OH deoxycorticosterone. 2. Merck, corticosterone. 3. Merck, cortisone. 4. Merck, 17-OH corticosterone (hydrocortisone). 5. Merck, tetrahydrocortisone. 6. Merck, dehydrocorticosterone. 7. Merck, desoxycorticosterone. *A*. K & K Labs. cortisone acetate, 15 and 30 min color development. *B*. K & K Labs. hydrocortisone acetate, 15 min color development. *B'*. K & K Labs. hydrocortisone acetate, 30 min color development. *C*. K & K Labs. corticosterone, 15 and 30 min color development. *D*. K & K Labs. 17, OH corticosterone, 15 and 30 min color development. *D'*. K & K Labs. 17, OH corticosterone, 30 min color development. *E*. K & K desoxycorticosterone acetate, 15 min color development. *F*. Calif. Found. for Biochem. Res., cortisone, 15 min color development. *G*. Calif. Found. for Biochem. Res., cortisone acetate, 15 min color development. *H*. Schering, tetrahydrocortisone, 15 min color development. *I*. Schering, hydrocortisone, 15 min color development.

indicate that all of the biologically active cortical steroids exhibit minimal variation in their reduction of BT and that this colorimetric method is satisfactory clinically for the measurement of the different biologically active cortisones. Cortisone and cortisone acetate obtained from the California Foundation for Biochemical Research gave identical standardization curves (curves *F* and *G*, Fig. 7).

Conditions Which May Interfere with the BT Ketol Method

Glucose was added to a series of three urines to give 2.5 and 5.0% concentration prior to analysis for ketols. The addition of glucose up to

5% was found to have no effect as apparently the short acid hydrolysis is adequate to destroy the glucose (Table 3). Norymberski *et al.* (1)

TABLE 3
THE EFFECT OF THE ADDITION OF GLUCOSE ON THE BLUE TETRAZOLIUM KETOL METHOD

Urine No.	Glucose (%)	17-Ketogenic steroids (mg/l)
1	0	13.4
1	2.5	13.4
1	5.0	13.5
2	0	10.5
2	2.5	10.5
2	5.0	10.5
3	0	7.4
3	2.5	7.4
3	5.0	7.4

observed that the presence of reducing sugars in urine interfered seriously with the bismuthate oxidation method.

Dehydroisoandrosterone, androsterone, estrone, progesterone, pregnandiol, and pregnantriol added to urines or as standards showed no reduction or produced no color development with BT.

Recovery of Cortisone Acetate Added to Urine by the BT Cortical Steroid Method

Good recoveries of cortisone acetate and cortisone added to urines were obtained (Table 4) with the BT method.

TABLE 4
RECOVERY OF CORTISONE AND CORTISONE ACETATE ADDED TO URINE WITH THE CORTICAL STEROID METHOD AS DESCRIBED IN PROCEDURE

Added (mg/l)	Recovered (mg/l)	Recovered (%)
Cortisone acetate		
5.7	5.9	104
11.4	11.3	99
17.2	16.2	94.2
22.9	21.9	96.1
28.2	27.8	97.2
Cortisone		
5.7	5.0	87.7
11.4	11.2	98.2
17.1	17.1	100.0
22.9	23.0	100.4
28.6	28.6	100.0

Normal Range of Ketols (Cortical Steroids) Found by the BT Method

Normal 24-hr urines on thirteen 25–60-year-old males were found to range between 8 and 25 mg ketols/24 hr and averaged 19.9 mg with standard deviation of 3.7; and eight females, 25–40 years old, a range of 7–17 mg and averaged 12.2 mg/24 hr with standard deviation of 4.0.

The new BT method was compared to the ketogenic method of Sobel *et al.* (2) (Table 5) using 24-hr urine of patients. If it is assumed that

TABLE 5

COMPARISON OF THE BLUE TETRAZOLIUM AND THE NORYMBERSKI METHODS FOR URINARY CORTICAL STEROID DETERMINATION AND THE KINGSLEY-GETCHELL AND WARE *et al.* KETOSTEROID METHODS

No.	Sex	Age	Urine volume (ml)	Steroids (mg/24 hr)			
				Cortical		17 Keto	
				Blue tetrazolium	Norymberski (2)[a]	Kingsley-Getchell (17)	Ware *et al.* (21)
1	F	52	2940	27.0	24.1	32.0	
2	F	33	735	7.9	7.7	7.9	
3	F	40	285	3.8	7.1	1.2	1.2
4	F	40	2026	17.3	9.6	9.6	5.3
5	F		1850	17.0	22.2	12.4	
6	F	45	810	16.4	18.8	10.9	
7	F		1580	15.5	13.6	12.0	
8	F	12	850	16.1	8.3	8.4	6.8
9	F	40	1885	31.0	34.0	19.0	16.5
10	F		1060	31.0	34.0	18.0	29.0
11	F		590	11.2	9.0	7.5	
12	F		1200	16.8	15.0	6.5	4.1
13	F		1560	22.8	21.2	21.0	21.7
14	F		1420	13.1	10.7	7.7	4.1
15	F		775	36.4	48.0	17.7	
16	F		1480	9.9	8.4	9.0	9.1
17	F		650	14.7	23.2	12.0	
18	F		1000	22.0	22.6	16.7	28.0
19	F	17	350	7.5	7.3	7.5	7.6
20	F		2490	21.2	16.1	13.4	9.9
21	F		1145	7.6	12.6	3.9	
22	F		2350	7.6	10.4	5.4	4.9
23	F		1230	2.3	4.6	1.2	
24	F		640	8.8	2.2	10.7	0.8
25	F		3110	34.0	31.0	18.0	29.0
26	M		1040	16.2	14.9	8.8	
27	M		1010	14.8	10.8	7.5	2.5

[a] Analyses by the modified Norymberski method (2) were performed by an independent laboratory.

TABLE 5 (*Continued*)

No.	Sex	Age	Urine volume (ml.)	Steroids (mg/24 hr)			
				Cortical		17 Keto	
				Blue tetrazolium	Norymberski (2)[a]	Kingsley-Getchell (17)	Ware *et al.* (21)
28	M		3720	18.3	18.9	18.3	9.5
29	M		1620	39.6	52.0	48.5	84.0
30	M	69	1130	17.7	24.1	11.3	13.3
31	M		1340	33.6	34.0	22.5	18.6
32	M		820	9.8	9.7	7.0	5.2
33	M	7	580	4.2	4.8	2.0	1.0
34	M		1500	16.5	16.6	16.8	
35	M		2300	19.0	30.0	13.2	
36	[b]	2	160	4.0	1.2	3.0	1.0
37	[b]	7	235	3.1	3.0	1.4	0.5
38	[b]	Child	340	3.2	2.9	2.8	1.4
39	[b]		930	9.6	4.8	4.4	3.3
40	[b]		1295	19.2	21.5	15.6	10.9
41	[b]		280	1.5	1.8	1.8	1.3
42	[b]		510	10.4	10.3	7.7	6.1
43	[b]		2000	56.0	66.0	20.2	25.0
44	[b]		3500	28.0	35.0	9.6	12.4
			Avg. =	17.0	17.8		

[b] Sex not known.

the normal range for adult females is 5–18 mg and adult males 8–25 mg of cortical steroids for 24-hr urines, only six out of 44 cases are not in agreement: cases 3, 5, 17, 20, 24, and 35. Four of these cases agree within 5 mg. These data indicate good agreement between the modified BT and the Norymberski ketogenic steroid methods in spite of the fact that the two methods were carried out by two different laboratories several weeks apart on the same specimen. The 17-ketosteroids as determined by the methods of Sobel *et al.* (2) and Kingsley and Getchell (17) (Table 5) are in fair agreement.

SUMMARY

All conditions and concentrations of reagents were carefully assayed and checked to obtain a simple method for practical clinical application for the determination of α-ketols (cortical steroids) in urine by application of acid hydrolysis and reduction of blue tetrazolium by the released ketols.

ACKNOWLEDGMENT

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Simplified Preparation and Properties of Undegraded Rat Liver Transfer Ribonucleic Acid[1]

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INTRODUCTION

It has been established that one of the steps in the pathway of protein biosynthesis involves the activation of amino acids by enzymes of the "pH 5" fraction (1) and their attachment to small molecules of ribonucleic acid (RNA) (2) which have been generally designated transfer RNA. This RNA is part of the "pH 5" fraction and usually is purified by direct extraction [e.g. (3–5)] exposing it to possible enzymic and physical degradation.

In the present paper we propose an alternative and simplified procedure for preparing undegraded transfer RNA, beginning with the direct extraction of whole cells by aqueous phenol (6). The resulting solution, containing most of the cellular RNA, is then fractionated as follows: (*a*) a fraction insoluble in 1 *M* NaCl which contains the high molecular weight RNA (7); (*b*) a fraction insoluble in 70% ethanol, containing the transfer RNA; and (*c*) the remaining material. Some of the properties of these fractions will be described.

METHODS

Extraction of RNA

Livers were removed from anesthetized Wistar rats and immediately frozen at —65°C. The frozen livers were ground to a powder in a cold mortar and then treated with the phenol–buffer–ethylenediamine tetraacetate (EDTA) mixture. For each 10 gm liver, 10 ml of redistilled water-saturated phenol, 10 ml of 0.14 *M* NaCl–0.01 *M* phosphate buffer at pH 7, and 0.2 ml of 1% EDTA (8) solution were used. All operations

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were carried out at 0–5°C. The phenol extraction was done in the 50-ml cup of a Servall Omni-mixer at 8000 rpm for 6–8 min. The phases were separated by centrifugation at 1000 × *g*, and the aqueous phase was extracted three more times by hand shaking for 1–2 min with equal volumes of phenol. The phenol was removed by five extractions with peroxide-free ether, which in turn was removed by bubbling nitrogen through the solution. The optical density of this "original extract" at 260 mμ was measured and converted to RNA concentration by the factor of 50 μg/ml per unit optical density. The RNA yield varied from 5 to 10 mg/gm liver.

Dry NaCl was then added to the "original extract" to a final concentration of 1 *M*, and the solution was kept at 0°C overnight. The white, flocculent precipitate (fraction A) was spun down at 6000 × *g* for 15 min, forming a gelatinous pellet which dissolved very slowly in water or buffer. To the remaining solution was added 2 vol ethanol. Flocculation was complete within several hours at 0°C when centrifugation at 8000 × *g* for 20 min yielded a very soluble, white pellet (fraction B). The ultraviolet absorption spectra of the original extract and the fractions thereof were almost indistinguishable, although the 280/260 mμ optical density ratios showed some differences (Table 1). Also shown in Table 1

TABLE 1
FRACTIONATION OF RAT LIVER RNA

	Relative yield (%)	Ratio of optical densities at 280/260 (mμ)
Original extract	100	0.43
Fraction A (1 *M* NaCl ppt.)	40	0.47
Fraction B (ethanol ppt.)	10	0.47
Fraction C (supernatant fluid)	50	0.38

are the relative yields of the various fractions.

Ultracentrifugal Analysis

The various RNA fractions were studied in a Spinco model E analytical ultracentrifuge, using ultraviolet optics and a 2° sector cell with a Kel-F centerpiece. Solutions were prepared at 20–30 μg/ml in 0.14 *M* NaCl–0.01 *M* phosphate buffer at pH 7. Density tracings of the ultraviolet photographs were obtained on the Spinco Analytrol with the microanalyzer attachment. The tracings were differentiated graphically in order to determine the degree of homogeneity of any sedimenting components and to calculate their sedimentation coefficients. The coefficients

obtained were corrected to the viscosity of water at 20°C. Fraction A was analyzed at 50,740 rpm, taking pictures at 4-min intervals; fractions B and C at 59,780 rpm, taking pictures at 16-min intervals.

The preparation of "pH 5" fraction and of "pH 5" RNA and the determination of amino acid uptake into RNA were adapted, with minor variations, from Hecht *et al.* (9) and Hoagland *et al.* (3). All procedures were carried out at 0–5°C.

Preparation of "pH 5" Fraction

Rat livers were excised, minced, and placed in 10 ml of 0.05 *M* KCl for each 10 gm liver. These were homogenized for 1–2 min at 5000 rpm in the 50-ml cup of a Servall Omni-mixer. The homogenate was diluted with an equal volume of 0.05 *M* KCl and centrifuged at 5000 × *g* for 15 min to remove the coarser particles. The supernatant fluid was then centrifuged at 105,000 × *g* for 90 min to remove the cytoplasmic particles. The resulting cell sap was adjusted to pH 5.2 with 1 *N* acetic acid, and the precipitated enzymes were collected by centrifugation at 10,000 × *g* for 10 min. The pellet was dissolved in medium A (9) (0.25 *M* sucrose, 0.005 *M* $MgCl_2$, 0.025 *M* KCl, and 0.05 *M* Tris-HCl buffer at pH 7.6) and centrifuged again to remove any insoluble material. The enzyme solution was dispensed into glass vials and stored at —80°C, preserving its activity for many weeks.

Preparation of "pH 5" RNA

Freshly prepared "pH 5" fraction was extracted twice with equal volumes of phenol plus 0.1 ml of 1% EDTA, then treated with ether and nitrogen as before, all in the cold. To the resulting RNA solution was added 2 vol ethanol, at 0°C, further to purify and concentrate the soluble RNA. At least 95% of the RNA in the enzyme solution is obtained via the phenol extraction, but only 60–65% is precipitated by the ethanol. The yield of "pH 5" RNA per unit of liver was 50% of that for fraction B. All preparations of transfer RNA were stable for months at 5°C.

Uptake of Amino Acid into RNA

The reaction mixtures generally contained 1–3 mg of enzyme protein [determined by the method of Lowry *et al.* (10)], 0.1–0.2 mg RNA contributed by the enzyme solution itself, 0.2–0.4 mg of added RNA, 10 μmoles adenosine triphosphate (ATP), 0.1 μmole cytidine triphosphate (CTP) (when used), 0.5–2.0 μg of C^{14}-labeled amino acid; all dissolved in medium A to a final volume of 1 ml. The pH of the mixture was about 7.4 and remained unchanged during the reaction.

After incubation for 10–15 min at 37°C, the reaction was stopped by

chilling and adding 1 ml of cold 1 *N* perchloric acid. The precipitated protein–RNA mixture was collected by centrifugation, washed once with cold 0.5 *N* perchloric acid, then twice with cold ethanol and drained well. The RNA was solubilized by treating the residue with 2 ml of 2 *M* NaCl at 100°C for 30 min; insoluble material was removed by centrifugation, and the RNA was then precipitated by adding 4 ml ethanol. After standing overnight at 0°C, the mixture was centrifuged at 12,000 × *g* for 30 min. The pellet was dissolved in 0.2–0.4 ml water, and samples were taken to determine the RNA content [by the orcinol method (11)] and radioactivity. The data thus obtained were used to calculate specific uptake in terms of counts/min/mg RNA.

The effectiveness of the separation by hot NaCl solution was checked by ultraviolet absorption, and the typical spectra of nucleic acid and protein were obtained indicating minimal cross-contamination. At least 60–80% of the RNA in the original reaction mixture could be recovered.

Samples for radioactivity measurement were prepared on metal planchets under conditions of infinite thinness and counted using a thin-window gas-flow detector. There was never any carryover of extraneous radioactivity, since controls were included in which the ATP was omitted, and the RNA isolated from these samples gave no detectable counts above background. In addition, each experiment included a control which indicated uptake of amino acid into the RNA of the enzyme solution alone. This made it possible to calculate the extent of uptake into the added RNA.

RESULTS

The method of fractionating cellular RNA outlined in this paper divided the material into three fractions on the basis of solubility. The physical properties of each fraction were studied in the analytical ultracentrifuge to determine their relative molecular sizes and purity of preparation. Then the amino acid acceptor activity of fraction B was tested in order to measure its transfer RNA content.

Ultracentrifuge Characterization

The sedimentation behavior of each fraction was quite distinct. Fraction A was found to contain two high molecular weight components at 22 and 34 *S*, each a relatively homogeneous molecular species. The major component of fraction B was heterogeneous with an average value of 4.0–4.4 *S*. This latter estimate is in agreement with the value of 4.2 *S* obtained for transfer RNA by Klee and Cantoni (12), who also commented on the heterogeneity of their material. The homogeneity of fraction C could not be ascertained since the bulk of the material did

not sediment under the conditions of these experiments. Table 2 shows the relative proportions of each sedimenting component in the various fractions, and it can be seen that there was little contamination in any fraction by the components of another.

TABLE 2
ULTRACENTRIFUGE CHARACTERIZATION OF RAT LIVER RNA FRACTIONS

	Relative proportions of sedimenting components			
S_{20} =	34	22	3–5	<3
Fraction A (%)	40–50	40–50	5–15	0–5
Fraction B (%)	0–5	0–5	70–90	10–30
Fraction C (%)	0	0	5–10	90–95

The ultracentrifugal behavior of RNA isolated from the "pH 5" fraction was also studied and found to be indistinguishable from that of fraction B with regard to both sedimentation coefficient and heterogeneity. The ultraviolet absorption spectrum and 280/260 mμ ratio of "pH 5" RNA were also found to be identical to fraction B.

Amino Acid Acceptor Activity of Fraction B

It was first determined, using valine and lysine, that the RNA in fraction B would accept amino acids activated by the "pH 5" fraction enzymes. As seen in Table 3, additions of increasing amounts of fraction

TABLE 3
UPTAKE OF VALINE-C^{14a} INTO RNA OF FRACTION B

	Total uptake (counts/min)	Specific uptake (counts/min/mg)
Enzyme	0	0 (enzyme RNA)
Enzyme + ATP	11	90 (enzyme RNA)
Enzyme + ATP + 1 unit fract. B	63	380 (fract. B RNA)
Enzyme + ATP + 2 units fract. B	111	390 (fract. B RNA)
Enzyme + ATP + 3 units fract. B	159	370 (fract. B RNA)

[a] Amino acid concentration 1 μg/ml.

B resulted in proportionately higher uptake of C^{14}-labeled valine (11,800 counts/min/μg). The inferior uptake of the amino acid into the RNA of the enzyme solution was found to be due to an increasing requirement for CTP as the enzyme is stored for longer periods. Presumably the end groups are slowly lost from the RNA, and relatively high concentrations of CTP must be added to reverse the reaction.

A comparison was made between the amino acid acceptor activity of fraction B and "pH 5" RNA (Table 4) in the presence and absence of

TABLE 4
EFFECT OF CTP ON UPTAKE OF C^{14}-LABELED VALINE[a] INTO RNA

	Specific uptake (counts/min/mg)
Enzyme + ATP	160 (enzyme RNA)
Enzyme + ATP + CTP	220 (enzyme RNA)
Enzyme + ATP + pH 5 RNA	80 (added RNA)
Enzyme + ATP + pH 5 RNA + CTP	380 (added RNA)
Enzyme + ATP + fract. B	390 (added RNA)
Enzyme + ATP + fract. B + CTP	360 (added RNA)

[a] Amino acid concentration 1 μg/ml.

CTP. It was noted that unless "pH 5" RNA was prepared immediately from fresh enzyme solution, it too had an increased requirement for CTP. The "pH 5" RNA used in this experiment was prepared from enzyme solution which had been kept for a number of hours at 0°C. The addition of CTP had no significant effect on specific uptake into fraction B, indicating no degradation, i.e., no loss of end groups, during the preparation of this material. Also, since specific uptake by "pH 5" RNA in the presence of CTP was essentially the same as that by fraction B, it was concluded that fraction B represents undegraded transfer RNA at least as pure as that found in the "pH 5" fraction.

DISCUSSION

The fractionation scheme presented in this paper, as applied to the RNA obtained via phenol extraction of rat liver, results in three distinct fractions characterized by solubility and by sedimentation in the ultracentrifuge. Fraction A contains the high molecular weight components insoluble in cold 1 *M* NaCl which are presumably derived from the intracellular particles. Fraction B contains the low molecular weight RNA which is soluble in 1 *M* NaCl but insoluble in ethanol. This apparently represents the RNA of the cell sap, since its behavior was identical to RNA isolated directly from the "pH 5" fraction of the soluble cell material. Fraction C contains lower molecular weight material such as nucleotide pool and coenzymes.

Even though this procedure is based on purely physical properties of RNA, it is biologically valid since it results in metabolically distinct fractions, a goal most desirable in any study of cellular metabolism. An attempt to demonstrate uptake of amino acid into fraction A gave nega-

tive results; fraction C was too dilute to be tested effectively. Thus fraction B may contain all of the transfer RNA of the cell. In addition to its general simplicity, this fractionation method has the advantage of producing undegraded transfer RNA, as evidenced by the lack of CTP requirement showing that the functional end groups are still intact. The yield of transfer RNA is twice as great as that obtainable from the "pH 5" fraction. One also has available for study, in undegraded form, the high molecular weight RNA from the same cells. This is known from the fact that phenol is used to prepare infectious RNA from virus-infected cells.

One question as yet unanswered is whether fraction B contains any RNA from the nucleus. Traces of deoxyribonucleic acid [identified by the diphenylamine reaction (13)] do show up in this fraction, although they amount to less than 1% of the total, indicating very minimal breakage of the nuclear structures. Work is in progress to seek an answer to this question.

The physical and biological properties of transfer RNA obtained here are in excellent agreement with those of Klee and Cantoni (12) using rabbit liver. It is also interesting to note the similarity of mammalian transfer RNA to the soluble RNA obtained from bacteria by Tissières (14) and from yeast by Otaka and Osawa (15). Transfer RNA may well be a common denominator among all cells capable of independent existence. A lack of specificity was observed by Hecht *et al.* (9) who showed that yeast RNA would accept amino acid from Ehrlich ascites cell-activating enzyme. Since all cells use the same amino acids, it would not be surprising to find common functional groups in the transfer RNA from different organisms.

In general agreement with our results, Smith (16) recently described a partial purification of transfer RNA by removing the high molecular weight fraction, with 1 *M* NaCl, from a phenol extract of whole cells.[3]

SUMMARY

A procedure is outlined for separating ribonucleic acid (RNA), as obtained via phenol extraction of whole cells, into three distinct fractions by successive precipitations with 1 *M* NaCl and ethanol. These can be characterized in the ultracentrifuge as A, high molecular weight; B, low molecular weight; and C, nonsedimentable RNA. When tested with amino acid activating enzyme ("pH 5" fraction), fraction B accepts amino acid, establishing its identity as transfer RNA.

[3] Fractionation of RNA by 1 *M* NaCl was also used by Davis and Allen (17) in their isolation from yeast of a fifth nucleotide which was localized in the RNA soluble in 1 *M* NaCl.

ACKNOWLEDGMENT

We wish to express our sincere appreciation to Dr. Angus F. Graham for his excellent and stimulating advice.

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ANALYTICAL BIOCHEMISTRY 2, 23–29 (1961)

Isolation of Radiocarbon-Labeled Glycine as Glycine Copper Picrate from Collagen[1]

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In order to conduct investigations of glycine turnover in connective tissues using glycine-C^{14}, a procedure for isolation and determination of the specific activity of glycine applicable to numerous samples is required. The widely used procedure for separation of glycine from protein hydrolyzates as the dinitrophenyl derivative by column chromatography is time consuming and yields only small amounts of material (1). A macro procedure for the direct isolation of glycine from neutralized gelatin hydrolyzates as glycine copper picrate [$(C_2H_5O_2N)_2 \cdot Cu \cdot (C_6H_2O_7N_3)_2 \cdot 5H_2O$] was described by Selim, El-Wahab, and El-Sadr (2). Procedures have been developed for isolation of labeled glycine from the collagen of connective tissues based upon the above reaction and for determination of the radiocarbon content of the glycine of the isolated complex. The procedure for the specific isolation of glycine from collagen also permits the simultaneous use of glycine-C^{14} and sulfur-35 in investigations of collagen and of sulfated mucopolysaccharides of connective tissue. The methods are readily applied to groups of 12 or more samples of bone, tendon, or skin of 200–250, 50–60, or 50–100 mg dry weight, respectively.

REAGENTS AND APPARATUS

Ion-Exchange Resin

Dowex 2, X-10 anion-exchange resin (200–400 mesh) is processed with repeated alternative treatment with 2 *N* sodium hydroxide and 2 *N* hydrochloric acid (200 gm resin/l solution) and stored in the chloride form. Prior to use for neutralization of acid in sample hydrolyzates, the resin is converted to the hydroxyl form by treating 50 gm of dry resin with 200 ml of 2 *N* sodium hydroxide for 30 min with occasional stirring followed by copious washing with distilled water in a suction funnel. This

[1] This work was supported by a grant from the U. S. Public Health Service.

procedure is repeated two times. After the third sodium hydroxide treatment, the resin is washed with distilled water until the filtrate is neutral. Eight grams of resin is prepared for each milliliter of 8 *N* sulfuric acid used for hydrolysis.

Copper Picrate (2)

To a suspension of 100 gm of commercial picric acid in 1 l of hot water, an excess of copper carbonate (70–80 gm) is added in small portions with stirring. The mixture is boiled several minutes and filtered while hot. The filtrate is placed in a refrigerator overnight, and the resulting crystalline precipitate is separated, washed with aproximately 100 ml of ice water, and air-dried.

Reflux Apparatus

Fifteen-milliliter thin-walled Pyrex centrifuge tubes stoppered with corks fitted with 4-ml pipets as air condensors serve as reflux vessels. The centrifuge tubes are inserted in a sand bath to a distance such that no detectable boiling is observed but such that considerable steam condensation in the pipets is noted (temperature of sand bath, 125°C, 1 in. below surface).

Evaporation Apparatus

A metal manifold designed for use with compressed air is utilized for volume reductions. The air, heated to approximately room temperature, is released through tubes 2 in. long with an internal diameter of 2 mm. A plate containing 30 holes to receive 15-ml centrifuge tubes is raised toward the air outlets as evaporation proceeds.

Counting Planchets

Three centimeter diameter, 2-mm deep, aluminum counting dishes are turned upside down and covered with pressure-sensitive tape Scotch brand No. 666. A single layer of Kleenex brand tissue is applied, and the excess tape and paper are removed.

Counting Equipment

Research Associates windowless Q-Gas flow counter.

PROCEDURE

Separation of Collagen

The soft tissues are removed from bones by dissection. Non-collagenous soluble proteins are removed from fresh tendons and chopped skin by

shaking for 24 hr with 10% sodium chloride (25 ml/200 mg wet weight) and 20% urea (10 ml/gm wet weight), respectively. Tendon and skin samples are washed repeatedly with distilled water and dehydrated with acetone. All samples are extracted with ether–alcohol (1:1 v/v) for 48 hr (Soxhlet extractor).

The bones are cracked and, following removal of the dried marrow, demineralized with 0.33 *N* hydrochloric acid (5 ml/0.2–0.4 gm of dry fat-free bone, room temperature, 24 hr) and washed free of acid with distilled water. The bone matrix residues from above, 50–60 mg of dry fat-free tendon, or 100–200 mg of dry fat-free skin are transferred to 15-ml thin-walled centrifuge tubes containing 3 ml water. The contents are refluxed 16–24 hr and centrifuged. The supernatant liquids are transferred to 15-ml thin-walled centrifuge tubes. The water extractions by reflux are repeated with the bone and skin samples, and the supernatant liquids are combined with the first supernatant liquids. After the last water extraction, all sample residues are heated twice for 5 min in a boiling water bath with 3-ml volumes of water. The washings are combined with the supernatant liquids.

The volume of the combined supernatant liquids and washings is reduced to less than 1 ml, and 14 ml acetone is added to each sample. Following overnight refrigeration, the samples are centrifuged and the acetone decanted. Residual acetone is removed by a cold air stream, and 0.8 ml of 8 *N* sulfuric acid is added to each sample. The mixtures are refluxed 16–24 hr and diluted to 10 ml.

Precipitation of Glycine Copper Picrate and Plating of Samples

The hydrolyzates are adjusted to pH 6–7 (Hydrion test paper) by the addition of hydroxyl-charged Dowex 2, X-10 resin, filtered, and reduced in volume to 0.5–0.6 ml. Care is taken that the final pH is not in excess of 7 in order to prevent adsorption of amino acids by the resin. The samples are placed in a boiling water bath, and approximately 100 mg copper picrate is added to each sample. Heating is continued until a complete solution results. The samples are refrigerated at 0–4°C for approximately 2 hr. The supernatant liquids are separated from the small amount of oily material which collects at the bottom of the tubes, collected in small test tubes, and refrigerated at 0–4°C for at least 24 hr. The resulting glycine copper picrate precipitates are collected by filtration, washed with 0.5 ml of 5% copper picrate, and transferred to small test tubes. The precipitates are recrystallized from a minimum volume of hot water (about 0.2 ml). The tubes containing samples are heated in a boiling water bath until complete solution results and refrigerated at 0–4°C for 25 hr. The recrystallized precipitates are collected by filtration,

washed with a small amount of ice water, and dried 3–4 hr over phosphorus pentoxide. Longer drying periods result in removal of water from the complex which is denoted by deep-green color. Samples of the size indicated usually allow the isolation of greater than 15 mg of recrystallized glycine copper picrate.

From 5- to 20-mg samples of the glycine copper picrate are dissolved in 1 ml of distilled water and a 200-μg aliquot of each solution is spread evenly on a counting planchet. The plates are dried under an infrared lamp. The radioactivities are determined and expressed as counts/min/mg glycine at infinite thinness. The glycine copper picrate complex contains 19.7% glycine.

EXPERIMENTAL AND DISCUSSION

A mixture of amino acids in the proportion reported for gelatin was prepared. Glycine-2-C^{14} in tracer amounts was added, and the glycine was subsequently isolated from the amino acid mixture as glycine copper picrate. The specific activity of the glycine copper picrate, recrystallized 1, 2, and 3 times, was 98, 99, and 98% of the theoretical value, respectively. A similar experiment utilizing pure glycine and the same glycine-2-C^{14} source gave a product with a specific activity 100% of the theoretical value.

Following administration of radiocarbon glycine to experimental animals, the uncombined glycine present in blood and other body fluids frequently has a specific activity different from the glycine of collagen. Consequently if body-fluid-free glycine should form a part of the final precipitate, the observed value of the specific activity of the collagen glycine would be distorted. To test this point, labeled glycine was added to unlabeled samples of cow femur shaft during demineralization. Less than 1% of the added radiocarbon was found in the isolated glycine. Since the amount of uncombined glycine in connective tissues is small compared to that of the collagen glycine, distortion of collagen glycine specific activities by uncombined glycine is not significant.

Possible interference from inorganic anions such as sulfate is eliminated through use of an anion-exchange resin in the neutralization of the acid hydrolyzates. The quantity of ammonium ion resulting from acid hydrolysis of gelatin does not interfere with the precipitation of glycine copper picrate. It is possible that higher ammonium-ion concentrations, relative to glycine content, would occur from acid hydrolysis of some other proteins and result in the formation of ammonium picrate (2).

A sample of labeled glycine copper picrate was prepared. Twenty-two samples ranging in weight from 2.0 to 20.4 mg were dissolved in water, and aliquots were plated for counting as previously described. Each

sample was counted one time, and the results are given in Fig. 1. A group of seven aliquots from the same labeled glycine copper picrate sample utilized for the construction of Fig. 1 were weighed, and their radioactivities were predicted from Fig. 1. The weights in milligrams of the aliquots and the per cent errors from the predicted radioactivities were as follows (statistical error of counting, 1%): 0.96, 2.3; 1.18, 2.8; 2.00, 0.1; 2.44, 0.0; 2.84, 0.1; 3.26, 1.0; 3.64, 1.8.

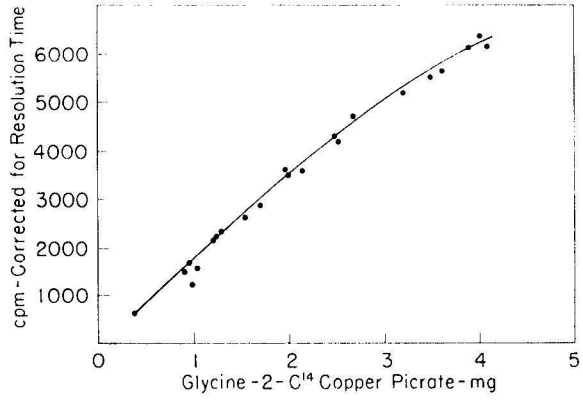

FIG. 1. Specific activity of various weights of a single glycine copper picrate sample.

The symmetry of the plated samples was tested by random selection of several planchets and by repeated determination of the radioactivity of each planchet following stepwise rotation of the planchet by successive 60° displacements. The results of two typical samples are as follows: (counts/min; statistical error of counting, 1%), A, 4829, 4849, 4850, 4799, 4808, 4813; B, 3440, 3487, 3496, 3498, 3470, 3491.

Ten microcuries of glycine-2-C^{14} were injected intraperitoneally into six young adult male rates (average weight, 216 gm). The animals were sacrificed 24 hr later, and both femurs were removed and the collagen glycine was isolated from each femur. The results are recorded in Table 1. The specific activity of the glycine copper picrate resulting from isolation of glycine from femur collagen hydrolyzates, such as those presented in Table 1, did not change significantly upon additional recrystallizations. A sample of 250 mg of dry fat-free rat femur (15% collagen) yields approximately 30 mg glycine copper picrate (80% yield).

On the basis of the above experiments, it is concluded that the procedure described in this paper results in the specific precipitation of glycine from gelatin hydrolyzates of connective tissue specimens as glycine copper picrate in quantities which can rapidly and accurately be handled.

TABLE 1
SPECIFIC ACTIVITIES OF FEMUR COLLAGEN GLYCINE

Animal No.	Specific activity[a]		
	Right femur	Left femur	Average
1	406	449	428
2	353	337	345
3	387	401	394
4	385	400	393
5	356	367	362
6	333	343	338

[a] Counts/min/mg glycine.

No difficulties from possible interfering substances were encountered. The plating and counting technique give reproducible and predictable results. Significant errors occur only when quantities of glycine copper picrate considerably below that normally employed are utilized. Results obtained from analysis of right and left femurs of animals which received glycine-2-C^{14} indicate that the total procedure gives consistent results. No attempt has been made to apply the procedure to tissues with low collagen content. However, the method should be readily applicable to such tissues since collagen may be isolated from tissues such as skeletal muscle in pure form (5).

Residues from the extraction of bone, tail tendon, and skin have been hydrolyzed by acid and analyzed for hydroxyproline by the Neuman-Logan procedure (3, 4). Only traces of hydroxyproline (less than 0.5% of the amount extracted) were found in the residues, and this is probably associated with elastin (6). No loss of hydroxyproline-containing materials could be detected in the processes leading to the hydrolysis of the extracted collagen. Therefore the procedure as outlined results in quantitative extraction and recovery of collagen in the form of gelatin, and the amount of collagen in the tissues investigated may be determined by analysis of the hydroxyproline in the acid hydrolyzates by the Neuman-Logan procedure (3, 4).

SUMMARY

Methodology has been developed which permits the concurrent isolation and determination of the specific activity of radiocarbon-labeled glycine from a large number of connective tissue samples. Collagen is separated from other constituents of the tissues by conversion to gelatin, and the gelatin is then hydrolyzed with sulfuric acid. The acid in the hydrolyzate is neutralized with anion-exchange resin, and the glycine is then precipitated as glycine copper picrate complex. The glycine copper

picrate complex is weighed and dissolved in distilled water. An aliquot of the solution is transferred to a special planchet and, following drying, its radioactivity is determined. Experimental results indicating validity of the procedure are described.

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ANALYTICAL BIOCHEMISTRY 2, 30–38 (1961)

A Fluorometric Method for the Determination of 2-Deoxy-D-glucose[1]

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The colorimetric method of Cramer and Neville (1) for estimating 2-deoxy-D-glucose, based upon a quinaldine reaction for α-methylene aldehydes (2), has achieved widespread use. It has been reported (1) that in the sample range of 10–203 mμmoles/ml DoG[3] the precision of the colorimetric method is only $\pm 10\%$. Although, as will be shown in this paper, the colorimetric procedure can be applied to a much greater range of concentrations of DoG and the precision of the method can be made greater than previously reported (1), the colorimetric method has three drawbacks: (*a*) It is insensitive to the low concentrations of DoG frequently encountered in *in vitro* metabolic studies (1); (*b*) tissue blanks are both high and variable, at least in diaphragm muscle (3); and (*c*) glucose, a metabolite frequently employed in conjunction with DoG in biochemical studies, seriously interferes with the assay (cf. *Results* below).

The quinaldine reaction for acetaldehyde, leading to the formation of a fluorescent product (4), has been adapted into a simple fluorometric method based upon the green fluorescence in ultraviolet light of 2-(1-glycerol)-5-carboxy-7-aminoquinoline, the probable reaction product of the acid-catalyzed condensation of 3,5-diaminobenzoic acid (DABA) with DoG. The fluorometric method is useful for concentrations of DoG ranging from about 2.8 to 125 mμmoles/ml, blanks are zero with tissues that contain high concentrations of deoxyribose, and high concentrations

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[3] The abbreviations used are: DoG, 2-deoxy-D-glucose; DABA, 3,5-diaminobenzoic acid; DABA.2HCl, the dihydrochloride of 3,5-diaminobenzoic acid.

of glucose do not interfere. These factors endow the fluorometric assay for DoG with particular usefulness in studies involving the uptake of DoG by suspensions of cells, e.g., lymphocytes, which contain high concentrations of deoxyribose (as deoxyribonucleic acid) and which are metabolizing simultaneously glycolytic intermediates.

EXPERIMENTAL

Materials and Apparatus

DABA.HCl. DABA (K and K Laboratories, Jamaica, N. Y.) was recrystallized from hot water following decolorization with Norit A. DABA.2HCl was precipitated from concentrated aqueous solutions of DABA by treatment with concentrated HCl. Following isolation by vacuum filtration and washing with cold 10 *N* HCl, white, crystalline DABA.2HCl was dried *in vacuo* at 100° over P_2O_5; decomposition point (uncorr.), 235–240°.

DABA Reagent. A 0.01 *M* solution of DABA.2HCl in 5.0 *M* H_3PO_4.

DoG. A product of the Sigma Chemical Co., used without further treatment; 0.15–15.0 m*M* solutions of DoG in distilled water were prepared fresh before use.

Quinine Reference Standard. A solution containing 1.056 μg quinine sulfate (U.S.P., Merck and Co.) per milliliter 0.10 *N* H_2SO_4.

Coleman Jr. Spectrophotometer. Model 6A, using 12 $\times$ 75 mm matched cuvettes.

Coleman Electronic Photofluorometer. Model 12C, using 19 $\times$ 105 mm matched cuvettes. Primary filter: Colemans #14-216 (Corning No. 5970); this filter has a 35% transmittance at 400 mμ (the approximate excitation maximum) and less than 0.2% transmittance above 430 mμ, and thus eliminates interference with the emitted light (cf. fluorescence spectra below). Secondary filter: although Coleman's #14-218 (Corning No. 3060) can be used, a sharp-cut filter which does not transmit below 430 mμ (cf. fluorescence spectra below), and thus eliminates interference due to scatter, was used; this filter (C.S. 3-72, No. 3387, 3 mm thick) was obtained from the Corning Glass Works, Corning, N. Y.

Procedure

The following procedure represents optimal conditions for the assay of DoG. Allowable variations in the technique, potential causes for interference, and sensitivity and precision of the method will be discussed in the *Results* section.

Solutions containing from 22 to 1000 mμmoles DoG in volumes up to 3.0 ml are made to 3.0 ml with water. Following addition of 3.0 ml of

DABA reagent, mixtures are heated in a boiling water bath for 15 min. Following a 20-min period at 15–17° (preferably in the dark in order to minimize destruction of fluorescence resulting from ultraviolet light), either 1.0 or 2.0 ml (depending on the minimal volume required by the cuvettes for maximal sensitivity) of 2.5 *M* H_3PO_4 is added and the solutions are transferred to cuvettes. Fluorescence is determined in the photofluorometer relative to reaction mixtures obtained with standard reference solutions of DoG; the instrument is set at zero fluorescence with a reagent blank. As a check on reagents and linearity of the method, a series of standard solutions of DoG in the appropriate solvent should be run simultaneously with the unknowns. These standard reaction mixtures are checked against the standard quinine reference solution.

Assay conditions can be subjected to some variation. Reaction mixtures can be heated at 100° for from 10 to 20 min; heating for as long as 30 min resulted in a loss of fluorescence of about 10%. Reaction mixtures can be cooled at 15–17° for from 15 to 30 min; extending this treatment to 60 or 120 min intensified fluorescence to the extent that departure from linearity resulted.

The identical procedure is employed for the colorimetric determination of DoG, with the exception that a higher range of concentrations of DoG must be used (cf. Fig. 2). Optical densities are determined in the spectrophotometer at 420 mμ against a reagent blank. A standard curve should be obtained simultaneously.

Fluorescence Spectrum of 2-(1-Glycerol)-5-carboxy-7-aminoquinoline

The excitation and emission spectra of a reaction mixture, obtained with 64.3 mμmoles/ml DoG by the above procedure, were determined with a Farrand recording spectrofluorometer; tracings of the two uncorrected spectra are reproduced in Fig. 1. When excitation was at 399 mμ, the emission maximum was at 493 mμ. When emission was measured at 496 mμ, maximum excitation was at 410 mμ. Not shown in the figure are small scatter peaks observed at 496 mμ (excitation spectrum) and 399 mμ (emission spectrum). Only negligible fluorescence was observed in reagent blanks.

RESULTS AND DISCUSSION

Proportionality and Precision of the Fluorometric Method

Fluorescence was proportional to concentrations of DoG between 2.8 and 125 mμmoles/ml of final solution (Table 1). Fluorescence obtained with lower concentrations was erratic and could not be measured with accuracy with the Coleman fluorometer. It is likely that the range could

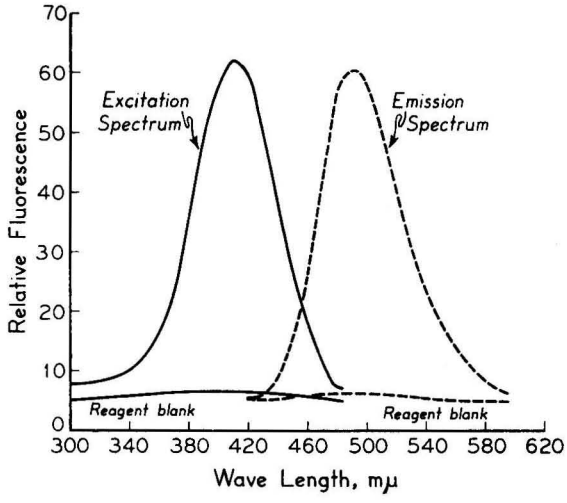

FIG. 1. Uncorrected excitation and emission spectra of 2-(1-glycerol)5-carboxy-7-aminoquinoline. Conditions: 4.0 ml. of reaction mixture in a 10 × 10 × 40 mm cuvette; slit width, 5 mm; meter range, 0.1; voltage divider, 100; temperature, 25°.

TABLE 1

PROPORTIONALITY BETWEEN FLUORESCENCE AND CONCENTRATION OF 2-DEOXY-D-GLUCOSE[a]

Concentrations of DoG (mμmoles/ml)			
Added	Calculated from fluorescence	Added	Calculated from fluorescence
206	190	56.3	55.7
169	157	37.5	38.3
141	137	18.7	18.5
125	126	11.3	11.3
113	112	5.7	5.5
97.5	97.8	2.8	2.8
90.0	89.4	2.25	2.16
75.0	74.4	1.69	2.00

[a] Fluorescence was developed as described in *Procedure*. Measurements were made with 7 ml in 19 × 105 mm cuvettes. The 90 and 113 mμmole/ml samples were used as reference standards for DoG concentrations between 18.7 and 206 mμmoles/ml, and the 56.3 and 75.0 mμmole/ml samples as reference standards for the lower concentrations of DoG; this procedure permitted the use of a fixed slit width on the fluorometer. Each value in the table is the average of from four to ten replicate experiments.

be extended with a more sensitive instrument, e.g., the Farrand fluorometer, model A [cf. Ref. (5)]. At higher concentrations, readings were no longer proportional because of absorption by the sample of a significant fraction of the exciting light.

Five replicate determinations of each of four concentrations of DoG were performed. Over the above range of concentration of DoG, the standard error of the mean relative fluorescence ranged between ±0.2 and ±0.6; the precision (calculated from the mean deviation in relative fluorescence from the sample mean) ranged between ±0.4 and ±2.4%. It is apparent that this method embodies a high degree of precision.

Range and Precision of the Colorimetric Method

Proportionality of color with concentrations of DoG ranging between 10 and 100 mμmoles/ml of colored has been reported (1). In our hands, however, replicate applications of the colorimetric procedure (Fig. 2)

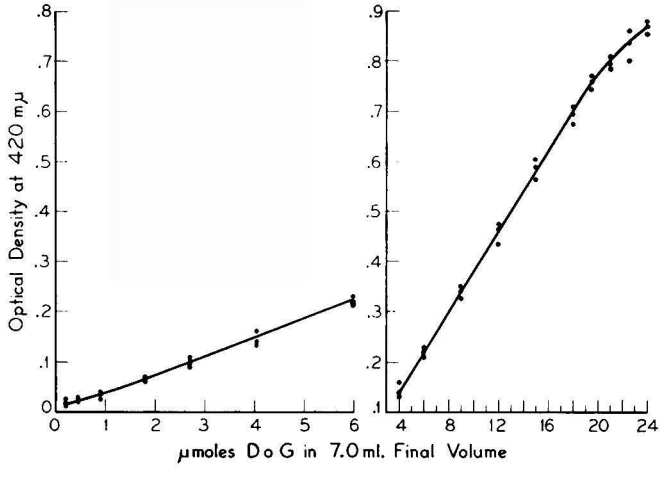

FIG. 2. Standard curves for the colorimetric determination of DoG. Results obtained from three separate experiments are plotted.

indicated the range of proportionality to extend from 300 to 2600 mμmoles DoG/ml. Nevertheless, it is apparent that, although the range of DoG concentrations useful in the colorimetric method may be greater than that for the fluorescence procedure, the lower limit of DoG concentrations for the latter method is only about 1/100 of that necessary for colorimetry. In addition, it should be noted that, in experiments similar to those described above, the precision of the colorimetric procedure over the range of concentrations described in Fig. 2 ranged from ±3 to ±7%.

Effect of Glucose on Fluorometric and Colorimetric Assays for DoG

Fluorometric and colorimetric methods were applied simultaneously to a series of glucose solutions in both the absence and presence of DoG. As indicated in Table 2, small amounts of fluorescence were developed

TABLE 2

EFFECT OF GLUCOSE ON THE DEVELOPMENT OF FLUORESCENCE AND COLOR WITH DoG[a]

Glucose (μmoles/ml)	Relative fluorescence		Optical density at 420 mμ	
	−DoG	+DoG	−DoG	+DoG
0.00	0	74.0	.000	.012
0.71	1.5	75.5	.003	.015
1.43	2.0	74.5	.008	.018
2.86	3.5	74.0	.012	.019
4.28	6.0	74.5	.015	.025
5.72	7.0	73.0	.018	.035
7.14	8.0	74.5	.022	.038
8.57	8.5	75.5	.028	.043
10.00	11.0	73.5	.035	.049
11.42	13.5	73.0	.044	.059
12.86	16.0	74.0	.052	.062
14.28	17.5	72.5	.055	.070

[a] Fluorescence and color were developed as described in *Experimental*. A 57.9 mμmole/ml solution of DoG, carried through the entire procedure, served as reference standard for fluorescence measurements. DoG, where present, 42.8 mμmoles/ml.

with high concentrations of glucose alone, but, in the presence of a low concentration of DoG, fluorescence due to glucose was completely quenched. In contrast, *color* obtained with DoG in the presence of glucose was the summation of that produced separately by each hexose. It is apparent, therefore, that with the fluorometric method, but not with the colorimetric procedure, small amounts of DoG can be measured accurately without interference by glucose.

Effect of Deoxyribose on the Fluorometric Assay for DoG

It was observed that 2-deoxy-D-ribose alone develops a fluorescence which, at its maximum, is about 10% of that produced by the same amounts of DoG (Table 3). Fluorescence produced when both deoxy sugars were present together was the approximate sum of that produced by each separately. This fluorescence due to deoxyribose has been made the basis of a fluorometric method for the determination of deoxyribonucleic acid in animal tissues (5). The fluorescent product of DABA and deoxyribose is maximally excited by light at 420 mμ. Since the trans-

TABLE 3
EFFECT OF 2-DEOXY-D-RIBOSE ON THE FLUOROMETRIC ASSAY FOR DoG[a]

Deoxyribose (mμmoles/ml)	Relative fluorescence	
	−DoG	+DoG
0.0	0	54.5
3.2	0	54.5
4.3	0.5	54.0
7.7	1.0	57.0
12.8	2.0	56.0
19.3	3.5	53.5
25.7	5.5	54.0
32.1	5.5	57.0
38.5	7.0	58.5
45.0	8.0	63.5
51.4	9.5	67.0
57.8	10.5	67.0
64.3	11.0	67.0

[a] Fluorescence, developed as in *Experimental*, was measured relative to a reference standard containing 64.4 mμmoles/ml DoG. Concentrations of deoxyribose (Schwarz Laboratories, lot DRI-5701, crystalline) indicated in table. DoG, where present, 34.3 mμmoles/ml.

mittance of the primary filter used in the present studies is less than 2% at 420 mμ, this may explain the low fluorescence values observed with deoxyribose (Table 3).

Effect of the Solvent for DoG

Data presented thus far have been for solutions of DoG in water. Replacement of water by Krebs-Ringer phosphate buffer, pH 7.4, or by 0.154 *M* KCl, was without effect upon the development and amount of fluorescence. The presence of 1 ml of *freshly prepared* 5% trichloroacetic acid increased the relative fluorescence readings obtained with DoG by from 3 to 10 units. However, volumes of fresh 5% trichloroacetic acid up to 0.5 ml resulted in no detectable effect on the amount of fluorescence. Comparable quantities of *old* solutions of trichloroacetic acid resulted in marked decreases in fluorescence.

Tissue Blanks and Recovery of Added DoG from Tissue Homogenates

Mixtures of DoG and homogenates of a number of tissues were analyzed for DoG by the fluorometric method following deproteinization of the mixtures with trichloroacetic acid. Deviations from theoretical recovery of DoG averaged about 2.6% over a wide range of concentrations (Table

4). The data also indicate that 30 mg (wet weight) of each tissue contained no detectable material reacting in the fluorometric method as did DoG.

TABLE 4
RECOVERY OF DoG FROM TISSUE HOMOGENATES[a]

DoG added (mμmoles/ml)	DoG recovered (mμmoles/ml)			
	Lymphosarcoma	Thymus	Liver	Diaphragm
25	25	25	25	25
50	50	53	50	53
100	110	100	110	103
500	497	517	530	500
750	753	750	753	710
900	900	917	913	913

[a] DoG, in the concentrations listed above, was added to 0.3-ml aliquots of 10% (w/v) tissue homogenates in 0.154 *M* KCl; final volume of mixture, 3.0 ml. Following deproteinization with trichloroacetic acid (final concentration, 5%), 0.5-ml aliquots of the acid extracts were assayed by the fluorometric method. Mixtures containing 1000 mμmoles DoG/ml, carried through the entire procedure, served as reference standards.

SUMMARY

1. A fluorometric assay for 2-deoxy-D-glucose (DoG) is described which is based upon the fluorescence of 2-(1-glycerol)-5-carboxy-7-aminoquinoline, the reaction product of the condensation of deoxyglucose with 3,5-diaminobenzoic acid. The quinoline derivative is maximally excited at about 405 mμ and has an emission maximum at about 495 mμ.

2. Fluorescence is proportional to concentration of DoG between 2.8 and 125 mμmoles/ml solution. The method is highly reproducible and precise.

3. With the fluorometric method, small amounts of DoG can be determined accurately in the presence of very high concentrations of glucose. Interference due to high concentrations of deoxyribose is small, and that due to tissue components is non-existent. DoG can be quantitatively recovered from tissue homogenates by extraction with trichloroacetic acid.

4. The same reaction, upon which is based the fluorometric assay, can also be used for the colorimetric estimation of DoG. However, the colorimetric method is insensitive to low concentrations of DoG, has a low degree of reproducibility and precision, and is subject to serious interference by glucose and deoxyribose.

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ANALYTICAL BIOCHEMISTRY 2, 39–44 (1961)

A Rapid Method for the Quantitative Assay of Proteolytic Enzymes[1]

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Received October 25, 1960

Insoluble dye-protein complexes offer many advantages when used as substrates for proteolytic enzymes. The proteolytic split products can be separated from the starting substrate conveniently by filtration, and the concentration of the soluble colored products of hydrolysis can easily be measured at various time intervals. Dye–protein complexes have been used since 1874 for the qualitative or semiquantitative determination of proteolytic enzymes (2–5). This paper describes a quantitative assay for proteolytic enzymes using insoluble dye–protein complexes and a convenient method for the preparation of stable substrates.

EXPERIMENTAL MATERIALS

Enzymes

Pepsin and trypsin were three-times recrystallized salt-free enzyme preparations obtained from Pentex, Inc.[2] Pepsin, N.F. granular, was also used in some experiments.

Preparation of Blood Fibrin

Difco desiccated blood fibrin[3] has been found suitable for use. The following modification of the method of Cullen and Van Slyke (7) was used in making laboratory preparations: *Fresh,* oxalted (0.5%), bovine plasma is diluted 30 times with 0.8% sodium chloride solution and then is recalcified by the addition of 7.3 ml of 1 *M* calcium chloride/l solution. The gel is allowed to form without stirring and is removed either by pressing into a more solid form or by stirring with a glass rod. The fibrin

[1] Supported in part by grants No. A-1316 and RG-4884 from the National Institutes of Health, Public Health Service. A preliminary report of this investigation has been published (1).

[2] Pentex Incorporated, Kankakee, Illinois.

[3] Difco Laboratories Inc., Detroit, Michigan.

is washed with stirring, first with 0.8% sodium chloride (three times) and lastly with distilled water (three times) for periods of 1 hr each. The washed fibrin is air-dried at room temperature and then ground in a Micro Wiley mill using a 20-mesh screen. The product is white, opaque, and apparently homogeneous.

Preparation of Dye–Protein Complexes

1. *Indigo Carmine–Fibrin.* Mix 75 gm blood fibrin with 1500 ml of 0.018 *M* hydrochloric acid containing 0.5% indigo carmine. Heat the mixture to 80°C and hold at this temperature with stirring for about one-half hour. Allow to cool and filter through folded cheesecloth (four layers). Wash the dyed fibrin by suspending in excess 0.018 *M* hydrochloric acid and stirring for about one-half hour. Filter as described above. Approximately four washings are required to produce a stable product (i.e., shows no further removal of dye upon standing in 0.018 *M* hydrochloric acid). The indigo carmine–fibrin is dried at 50°C (overnight) and then ground in a Micro Wiley mill using a 40-mesh screen. The ground fibrin is screened on a 100-mesh sieve, and the fine particles which pass through the sieve are discarded.

2. *Congo Red–Hide Powder.* Mix 75 gm of Hide-Powder[4] with 1500 ml of a warm (60°C) solution of 0.1 *M* potassium phosphate buffer pH 7.5 containing 0.25% Congo red dye. Stir and let stand for one-half hour. Wash two times by stirring for half-hour periods in an excess of distilled water. Filter through four thicknesses of cheesecloth between washings. Repeat the washing procedure first with excess of 0.1 *M* phosphate buffer pH 7.5, then four times with 0.1 *M* sodium borate buffer pH 8.5, and finally with 0.1 *M* phosphate buffer pH 7.5 until no further dye is removed (about 2–4 washings). After squeezing out the excess buffer, the Congo red–hide powder is spread upon trays and allowed to dry at room temperature. The dried product is ground through a Micro Wiley mill using a 40-mesh screen, and screened on a 100-mesh sieve. The fine particles passing through the sieve are discarded.

METHOD

The assay for proteolytic enzymes of either the pepsin or trypsin type is carried out in a similar manner. For pepsin the indigo carmine–fibrin is used as the substrate, and the pH of the enzyme solution is adjusted to 1.7–1.8 with hydrochloric acid. Crystalline pepsin was dissolved in 0.018 *M* hydrochloric acid for use as a standard. In the case of trypsin, the

[4] Standard Manufacturing Company, Ridgeway, Pennsylvania [see Ref. (5) for description].

Congo red–hide powder is used as the substrate, and the reaction is carried out in 0.1 *M* phosphate buffer pH 7.5.

The procedure is as follows: 100–150 mg substrate is placed in 13 × 100 mm test tubes incubated in a 35°C water bath, and 0.5 ml of enzyme solution is added. A blank tube is included which contains either 0.5 ml of the acid or the pH 7.5 buffer. After incubation for 30 min, the contents of the tubes are diluted with the appropriate acid or buffer (0.018 *M* HCl in the case of pepsin, 0.1 *M* phosphate buffer pH 7.5 in the case of trypsin), quantitatively transferred to graduated test tubes, and diluted to the 50-ml mark. The tubes are shaken vigorously for 10 sec (ten times), and the contents are filtered through *lens* paper into a test tube. The colored solution is read in a colorimeter at 620 mμ in the case of indigo carmine–fibrin, or 495 mμ in the case of Congo red–hide powder. We have allowed 2-min intervals between each tube to allow for the time of transferring, diluting, and filtering.

RESULTS

Standard curves were constructed using three-times recrystallized salt-free pepsin (Pentex) dissolved in 0.018 *M* hydrochloric acid and three-times recrystallized salt-free trypsin (Pentex) dissolved in 0.1 *M* phosphate buffer pH 7.5. A plot of the change in optical density at 620 mμ (Coleman Junior spectrophotometer) versus pepsin concentration is

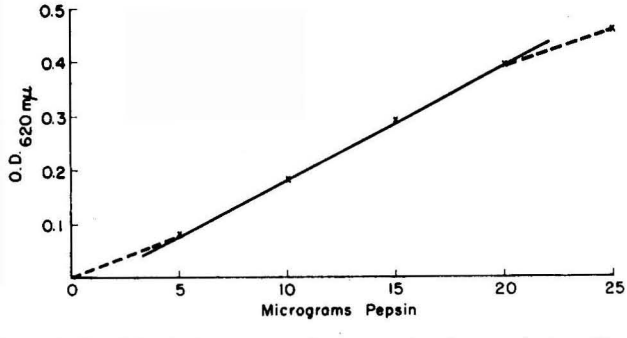

FIG. 1. The relationship between pepsin concentration and dye liberation from indigo carmine–fibrin. Reaction conditions: temp. 35° C, time 30 min, pepsin in 0.5 ml of 0.018 *M* HCl, 100 mg indigo carmine–fibrin, final dilution 50 ml.

shown in Fig. 1. A similar plot for trypsin is shown in Fig. 2. In each case, the relationship between dye liberation and enzyme concentration is essentially linear between concentrations of 5–20 μg in the case of pepsin and 0.4–1.6 μg for trypsin. One will also obtain a linear relationship between dye liberation and enzyme concentration when the reaction is

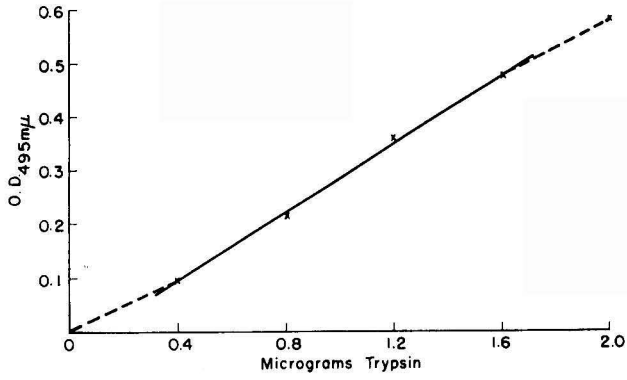

FIG. 2. The relationship between trypsin concentration and dye liberation from Congo red-hide powder. Reaction conditions: temp. 35° C, time 30 min, trypsin in 0.1 *M* phosphate buffer pH 7.5, 100 mg Congo red-hide powder, final dilution 50 ml.

run at 20, 25, or 30°C or at time intervals of 10 or 15 min. The lower temperatures reflect the difference in rate of reaction due to temperature. Under identical conditions of temperature, buffer, and substrate concentration, the increase in optical density is directly proportional to the time interval.

Figure 3 illustrates the relationship between dye liberation (optical

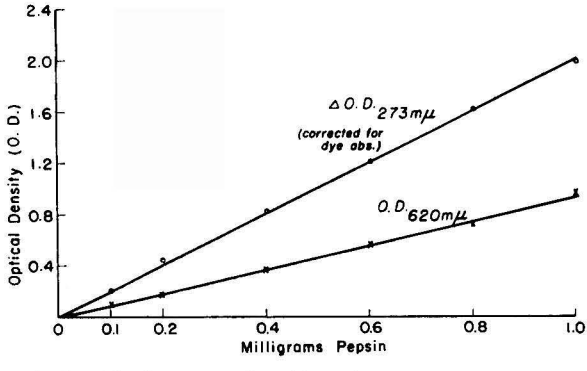

FIG. 3. The relationship between dye liberation (O.D. change at 620 mμ) when indigo carmine–fibrin is digested by pepsin (Merck, N.F. granular). Reaction conditions: temp. 35°C, time 30 min, pepsin in 0.5 ml of 0.018 *M* HCl, 100 mg indigo carmine–fibrin, final dilutions variable depending on product concentrations. Instrument, Beckman model DU spectrophotometer.

density change at 620 mμ) and concentration of the hydrolysis products (optical density change at 273 mμ) when indigo carmine–fibrin is digested

by graded concentrations of pepsin. In each instance, the relationship is essentially linear with respect to enzyme concentration.

DISCUSSION

Dye–protein complexes of indigo carmine–fibrin and Congo red–hide powder have been found to be suitable substrates for the quantitative assay of enzymes of the pepsin and trypsin type.

The rate of digestion of the insoluble dye-protein complexes varies within certain defined limits of enzyme concentration. This is indicated on the standard curve graphs in Figs. 1 and 2 by the dotted lines at both extremes of the experimental points, i.e., from 0 to 5 μg and from 20 to 25 μg in the case of pepsin, and 0–0.4 and 1.6–2.0 μg in the case of trypsin. Verification of this premise is illustrated by a graph of typical data (Fig. 4) using indigo carmine–fibrin as substrate and pepsin at concen-

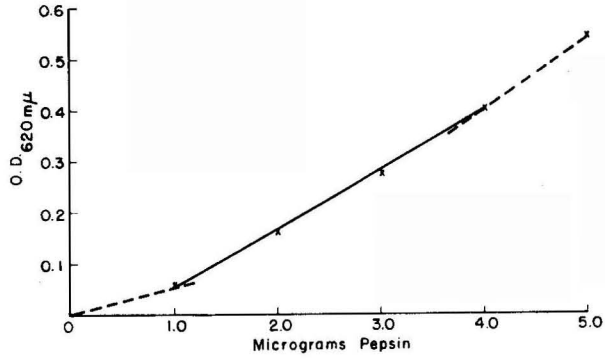

FIG. 4. The change in rate of dye–protein digestion at low enzyme concentration. Reaction conditions: temp. 35° C, time 30 min, pepsin in 0.018 *M* HCl, 100 mg indigo carmine–fibrin, final dilution 7 ml.

trations between 1 and 5 μg. It can be seen that the response is essentially linear between 1 and 4 μg with a marked change in rate occurring between 4- and 5-μg levels. This response appears to be typical of proteolytic enzymes inasmuch as a close inspection of the standard curves for pepsin and trypsin published by Anson (7) show a nonlinear rate change with increasing enzyme concentrations. Possible explanations for these observations have been summarized by Dixon and Webb (8).

The dye–protein complexes described above permit unique applications in studying various aspects of proteolytic enzyme activity. They have been used by Hess and Wainfan (9, 10) for continuous activity measurements in a study of the autolysis of trypsin. Since the concentration of dyed hydrolysis products can easily and rapidly be measured at various

time intervals, proteolytic activity can be measured by taking aliquots from the same experimental setup. Wootton and Hess (11) have used the dye–protein complexes in a study of the autolysis of acetyltrypsin taking advantage of the fact that the inactive autolysis products of the enzyme do not interfere with the assay. DiSanto (12) has used the method in an investigation of the salivary glands of the vampire bat.

SUMMARY

1. Methods for the preparation of stable dye–protein complexes for use in proteolytic enzymes are described.

2. Indigo carmine–fibrin provides a suitable substrate for the quantitative assay of proteolytic enzymes of the pepsin type, whereas, Congo red–hide powder provides a suitable substrate for the quantitative assay of proteolytic enzymes of the trypsin type.

3. The dye concentration, as measured spectrophotometrically at 620 mμ, parallels the concentration of the enzymically obtained hydrolysis products as measured at 273 mμ. This suggests that the dye concentration is directly proportional to the concentration of hydrolysis products.

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ANALYTICAL BIOCHEMISTRY 2, 45–49 (1961)

Sampling Technique for the Infrared Analysis of Volatile Organic Materials in Animal Tissues

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Stewart *et al.* have described methods for the analysis of trace organic compounds in body fluids by infrared spectroscopy (1, 2) and mass spectrometry (3). A gas chromatographic method has also been described (4). Attempts to apply these methods to the analysis of anesthetics in animal tissue during the course of anesthesia were not successful, however. The high volatility of ethyl ether led to evaporation of this material from the sample while preparing it for mass spectrometric analysis, and consistent results could not be obtained. The same difficulty would arise in the use of gas chromatography, and, in addition, solid samples would require special techniques to obtain representative chromatograms of the volatiles. The infrared method, wherein a CS_2 extraction of the sample was made, was unsuccessful because of large amounts of soluble lipoidal material in the tissue which masked the spectrum of the anesthetic. It was found that a simple distillation of the CS_2 extract effectively removed the interfering substances and accurate infrared results could be obtained by scanning the distillate.

METHOD

The apparatus used for sample preparation is shown in Fig. 1. Five milliliters of spectroscopic grade carbon disulfide is measured into distillation cup *A* which is then capped with stopper *D* and weighed. A 0.5–2.5-gm sample of tissue is quickly introduced, keeping the open time of cup *A* to a few seconds, and then the assembly is reweighed to give the sample weight. The tissue is permitted to soak for several hours before distillation to insure a quantitative extraction. The distillation apparatus is assembled as shown in Fig. 1, and the receiver is immersed in an ice–brine bath held at −10° to −15°C. The distillation cup is then warmed by immersion in a water bath at 75°C.[2] An insoluble grease such as

[1] Present Address: Arapahoe Chemicals, Inc., Boulder, Colorado.

[2] Extreme caution should be used in distilling carbon disulfide because of its low

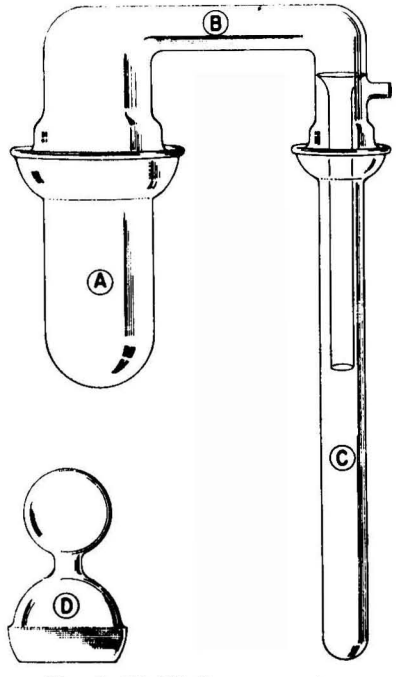

FIG. 1. Distillation apparatus.

Fisher Scientific "Nonaq" should be used on the joints, or some contamination of the distillate may occur. The distillation is continued until carbon disulfide no longer comes over (10–15 min). The distillate is then drawn out and an aliquot scanned in a double-beam infrared spectrometer (5). Absorption cells of 1.0 or 10 mm length (6) are used, depending on the sensitivity required. A certain amount of water may be carried over in the distillate but it will freeze out and remain in the receiver if the carbon disulfide is promptly removed.

The infrared absorbance is measured at the appropriate wavelength, and the concentration in mg/ml of anesthetic in the distillate is obtained from a standard curve. It has been found that for ethyl ether and chloroform more consistent results are obtained if this concentration is multiplied by the original volume of solvent (5 ml) to give the total weight of anesthetic, rather than the volume of the distillate. This indicates that for these two materials the losses of solvent and of anesthetic are approximately equal. The total weight of anesthetic is then divided by the weight of the tissue sample to give the concentration.

flash point (— 30° C); however, no difficulty has been encountered using the above conditions.

$$\text{concn. of anesthetic in tissue} = \frac{\text{concn. in distillate} \times \text{vol. of solvent}}{\text{wt. of tissue}}$$

STANDARDIZATION

A standard solution of ethyl ether in carbon disulfide was prepared, and three 5-ml aliquots were measured into separate distillation cups. To these were added three samples of rat tissue (liver or heart) of varying weights. These were allowed to soak for several hours, after which they were distilled and analyzed. The recoveries, shown in Table 1,

TABLE 1
TRANSFER OF ETHYL ETHER BY CODISTILLATION WITH CARBON DISULFIDE

Wt. of tissue (mg)	Ether added (mg)	Ether found (mg)
3230	2.85	2.78
400	2.85	2.75
770	2.85	2.80

indicate that sample size does not affect the quantitative transfer of ethyl ether.

Two rats were anesthetized with ethyl ether and chloroform, respectively. Sections of liver were obtained, soaked for varying lengths of time in CS_2, and distilled. The samples were then macerated and re-extracted with fresh carbon disulfide. The analytical results of both extractions in Table 2 indicate that the first extraction removes 85% or better of either

TABLE 2
EXTRACTION OF ETHYL ETHER AND CHLOROFORM FROM RAT LIVER BY CARBON DISULFIDE

			First extraction		Second extraction	
Sample	Weight (mg)	Anesthetic	Soak time (hr)	Found (ppm)	Found (ppm)	Per cent
1	920	Ethyl ether	3–4	650	11	1.7
2	1890	Ethyl ether	3–4	560	33	5.9
3	690	Ethyl ether	3–4	650	2	0.3
4	1360	Chloroform	4–4.5	380	55	12.6
5	980	Chloroform	4–4.5	440	35	7.5
6	1350	Chloroform	22.5	500	4	0.8
7	750	Chloroform	22.5	500	<0.5	<0.1

anesthetic when the samples are soaked for 3–4 hr, and better than 99% when soaked overnight. Larger samples apparently require longer soak times to insure quantitative extraction. In practice it was found con-

venient to soak all samples overnight in a refrigerated chamber and perform the distillations on the following day.

RESULTS

Table 3 shows the concentration of ethyl ether in various organs of

TABLE 3

ANALYSIS OF ETHYL ETHER IN VARIOUS ORGANS OF TWO ANESTHETIZED DOGS

Tissue	Ethyl ether (ppm)		Concn. ratio (Dog 2/dog 1)
	Dog 1 (Moderate anesthesia)	Dog 2 (Light anesthesia)	
Heart (rt. ventricle)	968, 950	756	.788
Liver	960, 918, 940	710	.756
Spleen	934, 933, 898	690	.748
Brain	1140	844, 800, 814	.718
Masseter muscle	853	638	.748
Pancreas	967	710	.733

two anesthetized dogs as determined by the method described above. Duplicate samples of the same organ agree within experimental error, although some variation might be expected in biological specimens of this type. The ratio of the concentrations in any particular organ between the two dogs is remarkably consistent as indicated by the third column of figures. Details of the anesthetic studies will be reported elsewhere.

The accuracy of the infrared determination of ethyl ether in the distillate is $\pm 3\%$ of the amount present. Distillation losses plus errors in measuring the sample weight and distillate volume bring the total error of the method to approximately $\pm 10\%$ of the amount present. This accuracy is satisfactory for biological systems where individual variations may be an order of magnitude greater than analytical precision.

DISCUSSION

The method presented herein should prove a valuable supplement to those in the literature (1–4) for the analysis of volatiles in animal tissue and other biological materials. The rapidity and specificity of the infrared method are, of course, advantageous. In addition, the proper choice of instrument parameters, cell length, and solvent should make the method adaptable to a wide variety of analytical problems.

SUMMARY

A method for the analysis of trace quantities of volatile organic materials in animal tissue has been developed. The tissue is extracted with

carbon disulfide, and the extract is distilled to remove soluble lipoidal material. The infrared spectrum of the distilled extract may then be used for both qualitative and quantitative analysis. Sampling and distillation procedures are described as well as an application of the technique to the analysis of various anesthetics in canine tissue.

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ANALYTICAL BIOCHEMISTRY 2, 50–58 (1961)

Identification of Hydroxamic Acids by Gas Chromatography of Isocyanate Derivatives

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Identification of acyl coenzyme A (CoA) compounds formed in biochemical reactions is often achieved by converting the thiolesters to hydroxamic acids and identifying the hydroxamates by paper chromatography (1–3). The reaction with neutral hydroxylamine using the conditions of Lipmann and Tuttle is quite specific for reactive acyls such as acyl phosphates, carboxylic acid anhydrides, acyl chlorides, and thiolesters (4), and is therefore very useful in identification of thiolesters in a crude enzymic reaction mixture which might also contain free fatty acids and esters. The lower molecular weight hydroxamates can be readily separated and identified by paper chromatography. Although some success has been reported with acetylated paper (5), the separation of the C_{10} to C_{18} hydroxamates by paper chromatography has not been very satisfactory. The recent interest in the biosynthesis of long-chain saturated (6–8), unsaturated (9), and branched-chain fatty acids (10) and the implication that long-chain acyl CoA compounds may be the direct products of the biosynthetic enzymes have stimulated the development of a gas chromatographic procedure for identification of long-chain hydroxamates.

The use of gas chromatography for the separation and identification of numerous classes of compounds (11–14) suggested that this technique might be applicable to the identification of hydroxamic acids or their derivatives. Preliminary attempts to separate the hydroxamic acids by gas chromatography failed because these compounds are susceptible to slow thermal decomposition. However, esters of hydroxamic acids readily undergo a Lossen type rearrangement to form the corresponding isocyanates when heated (15, 16). It was found that the hydroxamic acids could be quantitatively acetylated by treatement with excess acetic anhydride and pyridine, and this reaction mixture could be applied

directly to the gas chromatographic column without isolation or purification of the esters formed. The esters were converted to isocyanates quantitatively in the flash heater of the column, and the resulting compounds were readily separated by gas chromatography. Starting with an acyl CoA compound, the over-all reaction sequence was as follows:

$$R{-}CO{-}SCoA + NH_2OH \rightarrow R{-}CO{-}NHOH + CoASH \quad (1)$$

$$R{-}CO{-}NHOH + CH_3CO{-}O{-}COCH_3 \xrightarrow{\text{pyridine}} CH_3COOH + R{-}CO{-}NH{-}O{-}COCH_3 \quad (2)$$

$$R{-}CO{-}NH{-}O{-}COCH_3 \xrightarrow{\text{heat}} R{-}N{=}C{=}O + CH_3COOH \quad (3)$$

$$\text{Sum: } R{-}CO{-}SCoA + NH_2OH + CH_3CO{-}O{-}COCH_3 \xrightarrow[\text{heat}]{\text{pyridine}} R{-}N{=}C{=}O + CoASH + 2CH_3COOH \quad (4)$$

Reaction (1) is known to be quantitative (4). This paper will present evidence that reactions (2) and (3) proceed quantitatively under the experimental conditions used and that the isocyanate derivatives formed are readily identifiable by gas chromatography. An example will be given of the gas chromatographic analysis of a mixture of acyl CoA compounds that might be found in an enzymic reaction.

METHODS AND MATERIALS

Crystalline hydroxamic acid derivatives of capric, lauric, myristic, palmitic, stearic, and oleic acids were prepared from the respective methyl esters according to the method of Inone and Yukawa (17, 18). Capryl CoA, lauryl CoA, myristyl CoA, and palmityl CoA were synthesized by the method of Seubert (19) and assayed quantitatively by the method of Kornberg and Pricer (20).

The hydroxamic acid esters were prepared according to the following procedure given for acetyl myristhydroxamate. Myristhydroxamic acid, 166 mg, m.p. 99–99.5° [reported as 98–98.5° (17)] was dissolved in a minimum volume of reagent acetone, 0.15 ml acetic anhydride was added, and the solution was refluxed for 15 min. The acetone was removed under vacuum, and the product was recrystallized twice from hot *n*-hexane and twice from ether; m.p. 100–101°C. The infrared spectrum in chloroform is shown in Fig. 1.

Anal. Calcd. for $C_{16}H_{31}O_3N$: C 67.33, H 10.95, N 4.91. Found C 67.32, H 10.95, N 5.05.

Tridecyl isocyanate was prepared according to the directions of Allen and Bell from myristoyl chloride and sodium azide (21). The ester was

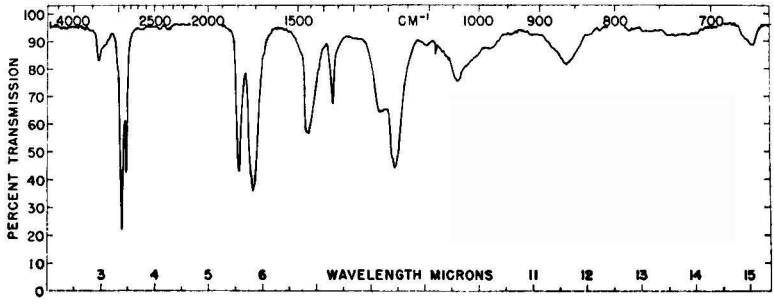

FIG. 1. Infrared spectrum of acetyl myristhydroxamate in chloroform.

purified by distillation at 101.1°C (0.15 mm). The infrared spectrum of this compound is shown in Fig. 2.

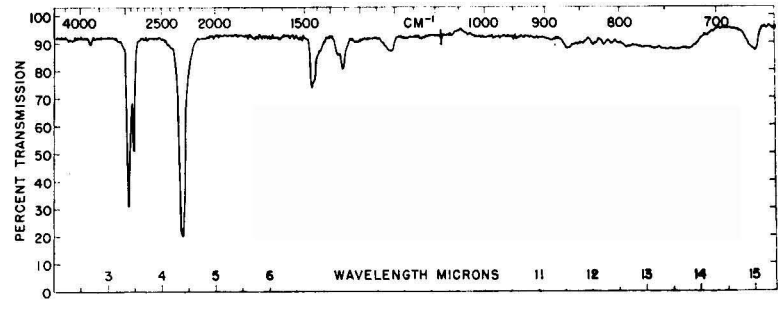

FIG. 2. Infrared spectrum of tridecyl isocyanate in chloroform.

Anal. Calcd. for $C_{14}H_{27}ON$: C 74.60, H 12.10, N 6.22. Found C 74.44, H 11.85, N 6.09.

Methyl caprate, methyl laurate, methyl myristate, methyl palmitate, methyl stearate, and methyl oleate were purchased from Applied Science Laboratories, Inc. Sodium azide and myristoyl chloride were purchased from Eastman Kodak Company and coenzyme A from Pabst Laboratories.

All melting points were taken on a Kofler hot stage and are uncorrected. The infrared absorption spectra were determined with a Perkin-Elmer Model 21 double-beam spectrophotometer. The gas chromatography was carried out on a Barber-Coleman, model 10 instrument, using a 6 ft, 4 mm i.d. U-tube at 138°C. The liquid phase was SE-30 (0.75%) on Gas Chrom P (100–140 mesh). The column was prepared according to the procedures developed for steroid separations (12). The elemental analyses were done by Joseph F. Alicino, Metuchen, New Jersey.

RESULTS

Acetylation of Hydroxamates and Gas Chromatography

The hydroxamates to be chromatographed were suspended in tetrahydrofuran (Eastman Organic Chemicals) up to a concentration of 10 mg in 1.0 ml (1% solution). The addition of 0.05 ml pyridine solubilized the hydroxamates that were not already dissolved. Then 0.05 ml acetic anhydride was added, and the solution was allowed to stand 10 min at room temperature. Aliquots of the tetrahydrofuran solution were chromatographed directly.

In order to ascertain that the product of the above reaction was an acetylated hydroxamate, 50 mg myristhydroxamate (m.p. 99–99.5°C and infrared spectrum shown in Fig. 3) were subjected to a similar procedure.

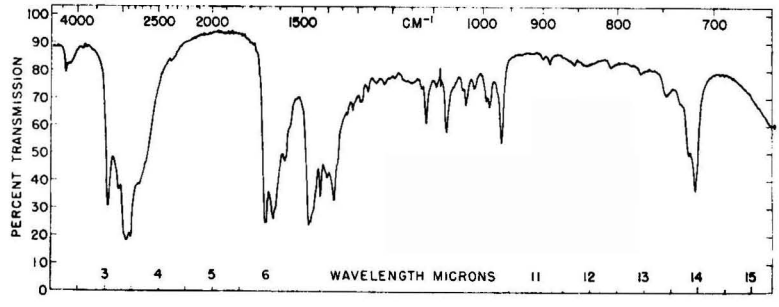

FIG. 3. Infrared spectrum of myristhydroxamate in Nujol.

After removing the solvent under vacuum, the product was recrystallized from hot *n*-hexane and ether. The product, acetyl myristhydroxamate, m.p. 100–101°, had an infrared spectrum identical with that of the authentic compound.

After determining the retention times of the individual hydroxamate derivatives, a mixture of the hydroxamic acids (caprate, laurate, myristate, palmitate, stearate, and oleate) was converted to the esters by the procedure described above, and the mixture was separated by gas chromatography on an SE-30 column (Fig. 4). It can be seen that all the saturated compounds were separated, and the olehydroxamate derivative was well separated from the stearhydroxamate derivative.

Lossen Rearrangement

Acylated hydroxamic acids will undergo an intramolecular rearrangement, upon heating, to form isocyanates (15, 16). Because the retention times obtained from gas chromatography of the acylated hydroxamic

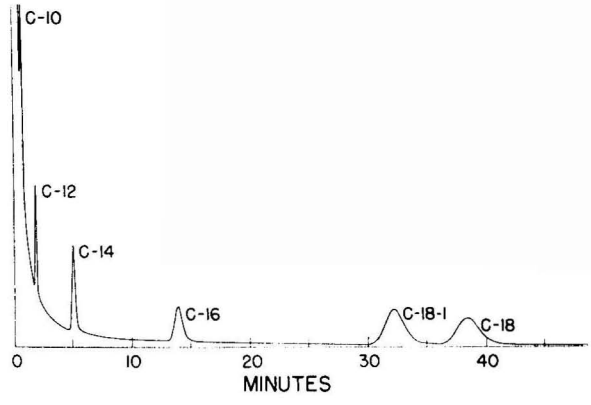

FIG. 4. Gas chromatographic analysis of a mixture of hydroxamate derivatives. A mixture containing 0.75 mg caprhydroxamate, 1.0 mg laurhydroxamate, 2.0 mg myristhydroxamate, 2.5 mg palmithydroxamate, 7.5 mg olehydroxamate, and 7.5 mg stearhydroxamate/ml tetrahydrofuran was treated with .075 ml pyridine and .075 ml acetic anhydride. One microliter of the resulting solution was put on the column. Gas pressure was 10 lb/sq in.

acids were less than those of the corresponding fatty acid methyl esters, it was suspected that the acetylated hydroxamates were being converted to the isocyanates by the flash heater (250°C) at the top of the column. In order to prove that the acetylated hydroxamic acid rearranged to the isocyanate, the acetyl myristhydroxamate and the expected rearrangement product, tridecyl isocyanate (see *Methods*), were chromatographed separately to determine their retention times. The retention time of the myristhydroxamic acid treated with acetic anhydride and pyridine and of acetyl myristhydroxamate proved to be identical with that of tridecyl isocyanate.

Fifteen milligram quantities of each of the compounds (acetyl myristhydroxamate, tridecyl isocyanate, and myristhydroxamic acid treated with acetic anhydride and pyridine) were chromatographed separately. The effluent products were collected in a length of Teflon tubing. They were washed out in chloroform, and the infrared spectrum of each was determined. The spectrum in each case was identical with that of authentic tridecyl isocyanate. Aliquots of the recovered samples were rechromatographed under the same conditions and were found to have the same retention times as the authentic isocyanate. There was no indication of decomposition products. These experiments show that myristhydroxamate treated with pyridine and acetic anhydride behaves on gas chromatography the same as acetyl myristhydroxamate and tridecyl isocyanate. They also show that the compound that emerged from the column

in every case was an isocyanate. Since it was shown earlier that the product of the reaction between myristhydroxamate, acetic anhydride, and pyridine was acetyl myristhydroxamate, it is clear that the intramolecular rearrangement occurred during gas chromatography.

Stoichiometry of Reactions

Since it was established that the compound ultimately being chromatographed in every case was an isocyanate, the amount of this product could be quantitated by measuring the area under the curve of the chromatogram. As shown in Table 1, chromatography of an equivalent

TABLE 1
STOICHIOMETRY OF REACTIONS

Experiment	Starting compound[a]	Area under curve (sq cm)
1	Myristhydroxamate	6.01
	Acetyl myristhydroxamate	5.9
	Tridecyl isocyanate	5.99
2	Myristhydroxamate	6.53
	Acetyl myristhydroxamate	7.4
	Tridecyl isocyanate	7.3

[a] In Expt. 1, 0.02 μmole myristhydroxamate, pretreated with pyridine and acetic anhydride, or 0.02 μmole of each of the other compounds was applied to the column. In Expt. 2,0.024 μmole of each of the starting compounds was applied. The area under the curve was calculated according to the formula: height × width at half height.

amount of myristhydroxamate treated with pyridine and acetic anhydride, acetyl myristhydroxamate, or tridecyl isocyanate gave essentially the same area under the curve, indicating that both reactions (2) and (3) went to completion.

Identification of Acyl CoA Compounds

An application of the above procedure for analysis of small amounts of acyl CoA compounds is demonstrated in Fig. 5. To a solution containing 0.46 μmole palmityl CoA, 0.3 μmole myristyl CoA and 0.5 μmole stearyl CoA in 2.0 ml (in a polypropylene centrifuge tube) was added 0.5 ml of 2.0 *M* neutral hydroxylamine. After standing for 10 min, 1.0 ml of 10% perchloric acid was added and the insoluble hydroxamates were recovered by centrifuging for 15 min at 25,000 × *g*. The supernatant solution was carefully removed from the precipitate with a Pasteur pipet and discarded. The precipitate was washed with 1.0 ml of 5% perchloric acid and centrifuged similarly for 15 min. The supernatant solution was care-

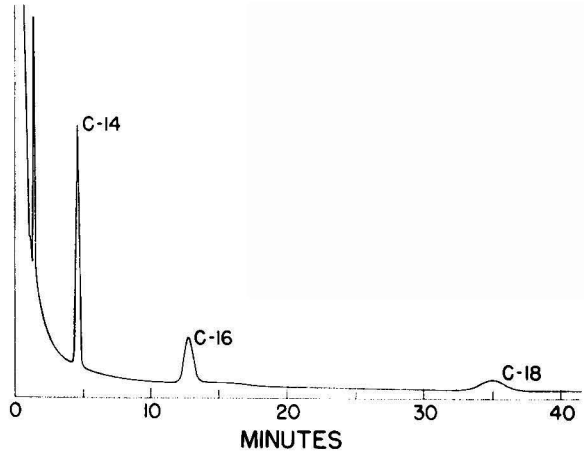

FIG. 5. Gas chromatographic analysis of acyl CoA derivatives. A solution containing 0.46 μmole palmityl CoA, 0.3 μmole myristyl CoA, and 0.5 μmole stearyl CoA was treated with neutral hydroxylamine. The insoluble hydroxamic acids were treated as described in the text and chromatographed. Gas pressure was 20 lb/sq in.

fully removed again and discarded. The precipitate was dried in the tube in a vacuum desiccator to remove all traces of water. It was then taken up in small aliquots of tetrahydrofuran and transferred to a 3-ml glass centrifuge tube. The volume was adjusted to 0.5 ml by evaporation of the solvent with a stream of nitrogen. Ten microliters of pyridine and 10 μl acetic anhydride were added. After standing at room temperature for 10 min, the insoluble material was removed by centrifugation and the volume further reduced to about 40 μl by blowing off the solvent with nitrogen. In the chromatographic record shown in Fig. 5, 1.0 μl was applied to the column. All the products were readily identified. The first peak after the solvent in the chromatogram represents an unidentified impurity, probably from the synthetic thiolesters.

DISCUSSION

The procedure outlined above can be used for both qualitative and quantitative analysis of mixtures of long-chain acyl CoA derivatives. As in the case of other compounds (22), when the logarithm of the retention times of the products being chromatographed are plotted against the number of carbon atoms in a homologous series, a straight line is obtained (Fig. 6). Using a limited number of standard hydroxamates with such a plot, one can identify other members of the series.

The procedure has been tested with mixtures of acyl CoA compounds containing, in addition, various free fatty acids. In such cases broad, flat

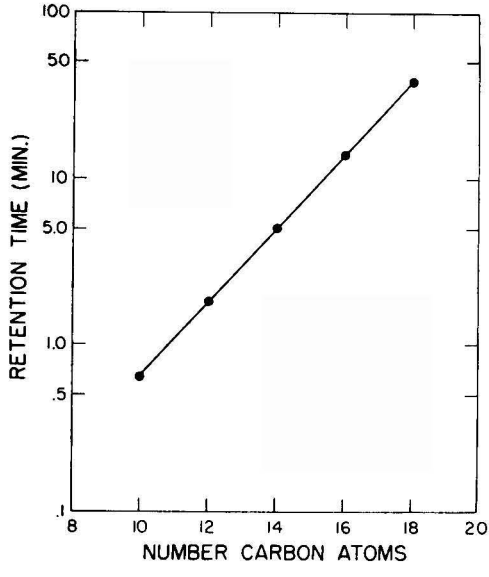

FIG. 6. Logarithm of the retention times of saturated hydroxamate derivatives plotted against the number of carbon atoms.

unidentified peaks were present on the chromatogram, but they did not interfere with the analyses since the retention times differed from those of the known compounds. In the event that free fatty acids or esters are present that would interfere with the hydroxamate identification, the acyl CoA derivatives can be freed from both free fatty acids and esters by extracting the mixture to be analyzed with ether–hexane (50:50) several times at pH 2.0. The acyl CoA derivatives are essentially insoluble in this organic solvent mixture which would extract most of the interfering compounds.

SUMMARY

A method is described for the analysis of hydroxamic acids. The hydroxamic acids are initially quantitatively acetylated by treatment with excess acetic anhydride and pyridine. This reaction mixture is applied directly to a gas chromatographic column. On the column the hydroxamate esters are quantitatively converted to isocyanates which are readily separated by gas chromatography. An example is given of the analysis of a mixture of acyl CoA compounds by converting them to hydroxamic acids and identifying the latter by gas chromatography of their isocyanate derivatives.

ACKNOWLEDGMENT

We are indebted to Mrs. Katherine S. Warren for the infrared analysis.

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Rapid, Preparative, Density-Gradient Electrophoresis[1, 2]

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INTRODUCTION

It has long been recognized that one of the needs of the biochemist is a preparative electrophoretic technique for separating proteins and other high molecular weight compounds rapidly and cleanly. The instrument described in this article was designed to accomplish this. It consists, essentially, of a stack of plastic plates which has a hole machined down through each plate to form a continuous cylindrical channel. The channel, in turn, serves to contain a liquid column in which the electrophoretic separation is accomplished. After the separation is completed, giving bands of separated proteins, the plates are slid apart, thus slicing the column into small segments without the use of a solid supporting medium. This instrument combines, then, the advantages of single liquid-phase electrophoresis with the sensitive sampling potential offered by starch or paper electrophoresis.

The apparatus is suitable for Kolin's (1–5) mobility spectrum technique in density-gradient columns. He makes the initial sample layer ion deficient and, due to the high mobility of the protein in this region, the sample is "focused" as it enters the adjoining ion-rich region. This band, owing to its thinness, can be resolved into its components in minimal time. It is evident, however, from the discussion of density-gradient stability conditions by Svensson, Hagdahl, and Lerner (6) that this technique cannot be applied when large amounts of protein are present in the sample. This is due to the fact that the density contribution of the protein must never override that of the gradient material to reverse the direction of the gradient at any point. There are two experimental approaches to prevent this. Either the protein concentration may be kept low or the initial gradient may be made steep. Kolin used the latter technique and reported no difficulty, but it should be pointed out that he operated on a

[1] This work was supported in part by Contract AT(30-1)-1822 between the Atomic Energy Commission and The Johns Hopkins University.

[2] This is contribution No. 310 from the McCollum-Pratt Institute.

very small scale. Svensson and Valmet (8) chose the former technique to obtain high yields per experiment (several hundred milligrams of starting material). The disadvantage in their system is that it requires 2 days of running time.

We have compromised between the two systems employed by Kolin and Svensson, respectively, by using a density gradient 5 cm long of intermediate intensity (0.02 gm/ml/cm). This permits the use of at least 5-mg samples without difficulty even when Kolin's focusing technique is employed and makes rapid separations possible with sufficient yield for a number of tests.

For making the gradients we have chosen to use the simple mixer of Britten and Roberts (9) which produces linear gradients. This linearity makes it possible to calculate the density of any part of the gradient and is of advantage in positioning the sample. De Duve, Berthet, and Beaufay (10) have described a more elaborate gradient mixer which may prove to be more versatile especially when highly viscous solutions are used. They have also reviewed the literature relating to gradient devices. The three-chambered mixing device analyzed by Svensson (7) and used by Svensson and Valmet (8) could also be employed with the described sampler although it forms nonlinear gradients.

Kolin's pH gradient technique does not appear to be promising with our apparatus until some means can be found for stabilizing the pH gradient against the charge transfer that occurs. However, Tuttle (11) reports the successful application of the pH gradient technique to hemoglobin separations.

APPARATUS[3]

The apparatus (Figs. 1 and 2) consists of a stack of circular (5 in. diameter $\times$ 0.125 in. thick) Plexiglas plates (P) mounted by center holes on a vertical steel rod (V). This rod in turn is fixed at its lower end to a $5\frac{1}{2} \times 7\frac{1}{2} \times 1\frac{1}{2}$ in. Plexiglas block (B) which together with plate (b) serves as a base for the instrument. A hole (H) is machined in each plate between the center axis and the outer edge to form a continuous channel down through the stack, and this then couples with a corresponding hole (H) and channel (C) in the base block. The holes in the plates were made $0.788 \pm .002$ in. in diameter so that each 0.125-in. thick plate would slice out exactly 1 ml of liquid when the plates were rotated. The base block channel makes a sharp 90° bend just below the block surface and extends back horizontally to the rear of the block where it connects to the center of two vertical concentric tubes (t' and T'). The inner tube is shorter

[3] Shop blueprints of this instrument may be obtained at cost from the McCollum-Pratt Institute of The Johns Hopkins University.

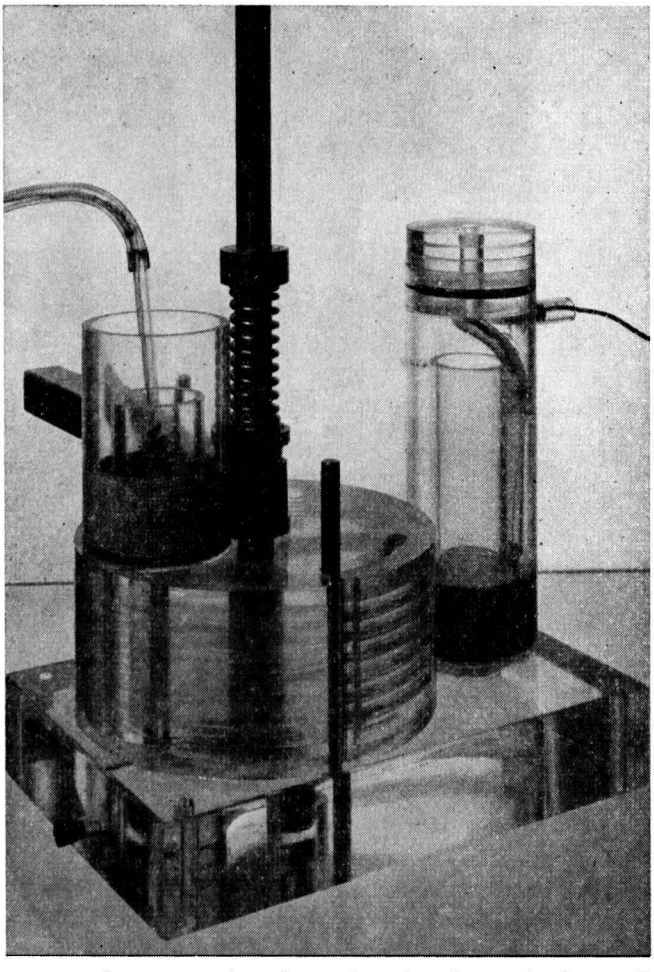

FIG. 1. Apparatus for preparative electrophoresis using a density-gradient stabilized liquid column. The photograph above shows the complete assembly with the electrodes in place.

than the outer so that liquid can extend above it, bridging over into the space between the tubes, the lower portion of which serves as an electrode chamber. A similar two-tube electrode chamber (t and T) is mounted on the top circular plate of the stack, which is 0.375 in. thick for greater strength. Thus a continuous U-tube is formed between the two electrodes (E).

The circular plates are designed to rotate alternately and simultaneously to cut off segments of the 0.788-in. channel. To accomplish this two

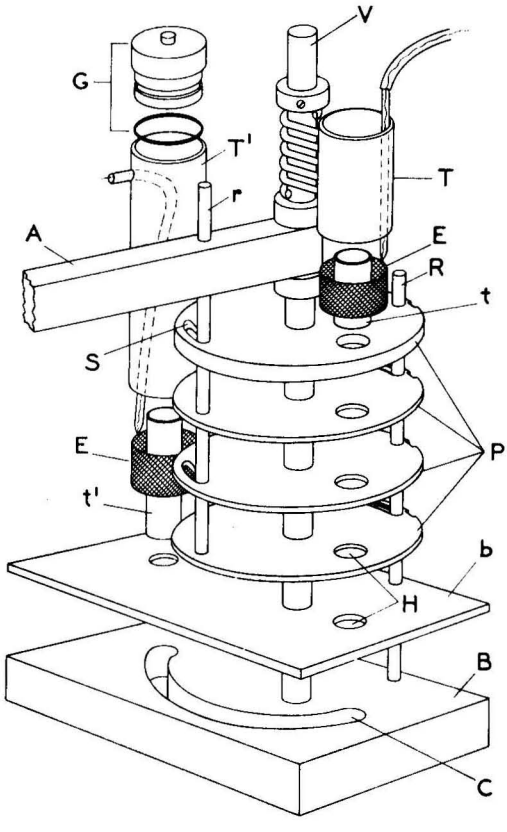

FIG. 2. Exploded drawing of the preparative electrophoresis apparatus. The circular plates (*P*) are coated with lithium or calcium grease before assembling them so that they rotate freely. Plate (*b*) is welded with solvent to block (*B*) to form a base for the instrument. Tubes (*T'*) and (*t'*) are in turn welded to the base block (*b* plus *B*). Tubes (*T*) and (*t*) are welded to the top plate of plates (*P*). All other elements are removable including the steel rod (*V*) which is held in place by a nut and lockwasher. (*E*), silver–silver chloride electrodes. (*R*) and (*r*), steel pin rods. (*H*), holes. (*C*), channel. (*G*), cap assembly with a rubber O ring. (*A*), brass lever arm for moving the plates, held in place by a spring and suitable washers. (*S*), slit. All of the parts listed above are made of Plexiglas except those otherwise noted.

¼-in. pin holes were drilled, one on each side of the center rod such that they were both on the same radius measured from the center rod, on the same axis with it, and perpendicular to the axis passing through the center rod and the main channel hole. One of these pin holes was milled back with a ¼-in. milling tool to form a slot (*S*) describing a 45° arc

about the center of the 5-in. plates. In assembling the apparatus the plates are alternately stacked so that pin holes alternate with slots on either side of the center rod. Two ¼-in. pins are then pushed down through the holes and slots. One pin (R) is held in place by a corresponding hole in the base block, and the other pin (r) is fixed at one end to a lever arm (A) attached to the center rod. The lever arm is simply pushed, and the plates rotate alternately. A spring on the center rod holds the assembly of plates together.

Close tolerances (within 0.001 in.) were observed in positioning the holes, so that the plates would mesh properly with one another and would be completely interchangeable. This was accomplished by using jigs to position the holes and precision reamers to size them. The 0.788-in. channel holes were machined on a conventional lathe by the use of a jig mounted on an eccentric. The plates were finally completed by cutting a notch in the side of each, to allow the plates to be moved either individually or in groups after the removal of the ¼-in. pins. This is required during filling and emptying operations.

Another essential feature is a removable plastic cap (G) for the rear electrode column. This cap makes it possible to seal the electrophoresis cell at one end, thus preventing oscillation of the contained liquid.

One of the major problems encountered in using the sampler was the need for a suitable lubricant for the plates. This has been solved to our satisfaction by use of lithium or calcium greases.[4] Rohm and Haas Co. has also suggested using thin sheets of Teflon between plates. It may also be possible in some applications to make either all or alternate plates of Teflon, but this would greatly hamper vision into the cell.

MATERIALS

Buffers

A stock solution of 0.04 M tris(hydroxymethyl)aminomethane (Tris) and 0.001 M ethylenediaminetetraacetic acid (EDTA) was made up, adjusted to pH 8.01 with dilute HCl. This was diluted 50% with water to make the light buffer (density at 20°C = 1.00 gm/ml) whose pH when measured was 8.00. A second volume of stock solution was diluted 25% by water, and 250 gm sucrose was added per 750 ml of diluted solution (density at 20°C = 1.10 gm/ml). The pH of the dense buffer was found to be 7.89, and it was used as such without adjustment.

[4] The lithium and calcium greases were contributed by the Battenfeld Grease and Oil Corporation, North Tonawanda, New York, and are designated by them as types "A" and "B."

Proteins

Crystalline aldolase from muscle suspended in ammonium sulfate solution and crystalline glyceraldehyde phosphate dehydrogenase (GAPD) also in ammonium sulfate were obtained from Sigma Chemical Co. Both suspensions contained 50 mg protein/ml. Dry crystalline horse cytochrome c, type II, was also obtained from Sigma.

Dialysis Medium

The buffer stock solution was diluted to 4×10^{-5} *M* Tris, and 110 gm sucrose was added to each 890 ml of this diluted buffer. The pH of the mixture was adjusted to about 8.0 with a small amount of KOH.

METHODS

For convenience, the following trial run was made at room temperature, which means that the sample was exposed to this temperature for about an hour. The proteins were otherwise kept at 0°C.

A sample mixture was made up with the ion-deficient dialysis medium containing 1 mg cytochrome c, 0.5 mg GAPD, and 0.5 mg aldolase per milliliter. This was dialyzed for 2 hr against more dialysis medium in preparation for the experiment.

The hole in the base block and the inner tube of the rear electrode chamber was filled with the dense buffer. The space between the inner and outer chamber tubes was filled with KCl solution of lower density to form a salt bridge.

When the instrument was half full of dense buffer, the stack of circular plates was rotated as a unit to cut off the connection to the channel in the block. This allows further filling without disruption of the liquid column. The rear electrode chamber was completely filled with liquid and then capped off at the top, air bubbles being carefully excluded to form a continuous liquid column. Heavy buffer remaining in the plates was withdrawn, and a gradient was formed between 9 ml of heavy buffer and 9.75 ml of light buffer. To introduce the sample, the gradient mixer was stopped after filling 12 plates, the eleventh and twelfth plates were cut off by rotating them, and the buffer in them was replaced by sample. The twelfth plate was then rotated back to its former position thus isolating the sample in the eleventh. Excess sample was then withdrawn and the formation of the gradient continued. The tube above the plates was filled with the light buffer, and 10 ml of 1/3 saturated KCl was pipetted into the bottom of the electrode chamber.

With the filling thus completed, the column was reassembled by rotating the groups of plates into position, and, after waiting 5 min to insure

gradient formation across the sample, the current was turned on. The run was made at 30 ma and 250–300 v for 52 min. The voltage requirement dropped during the experiment due to diffusion of buffer salts into the ion-deficient initial sample layer. At the end of the run the plates were alternately rotated by pushing the lever arm as described above. Following that, the pins were withdrawn from the plates, which were then rotated back into position one at a time, starting from the top. Each layer was pipetted out as it was uncovered.

RESULTS AND DISCUSSION

The curves shown in Fig. 3 demonstrate that the three electrophoretic

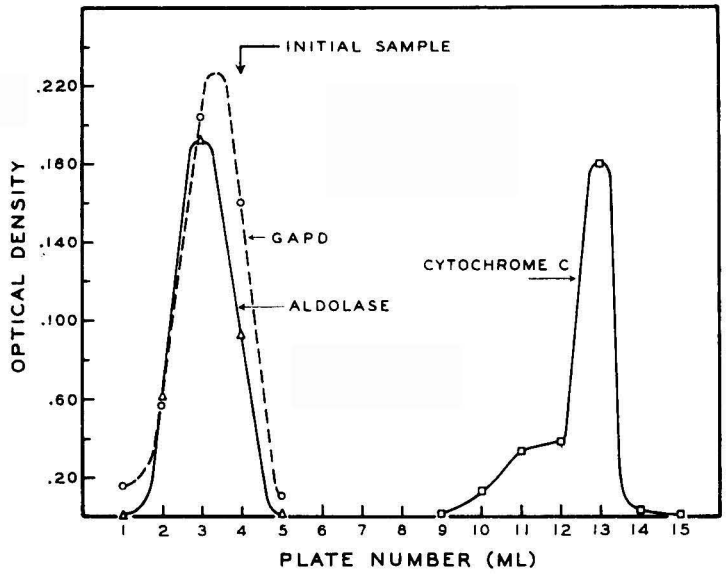

FIG. 3. Electrophoretic pattern after 52 min at 30 ma. The plates are numbered consecutively starting at the top plate. Each plate slices out a 1-ml segment of the column so that the plate numbers also represent volumes of column material. The plate numbers also increase in the direction of positive charge during the electrophoresis. The points on the GAPD and aldolase curves represent the optical densities at 340 mμ of the DPNH generated in 1 min under assay conditions. The points on the cytochrome curve represent the difference between the optical densities at 550 mμ and the corresponding optical densities at 490 mμ.

components can be distinguished after only 52 min of running (93 coulombs or about 0.001 mole of electrons was transferred). The cytochrome band is completely separated from the two enzymes. The two enzymes, in turn, are found to be partly separated from one another in

spite of the fact that little migration has occurred in this short period. This clearly illustrates the sensitivity of the sampler and also the desirability of actually testing for enzyme activity. On the basis of total protein alone, the two curves would be indistinguishable..

Another important factor in the sensitivity of this method is the initial focusing of the protein bands accomplished by making the sample layer ion-deficient. This is particularly evident with the cytochrome band which is little broader after 52 min than the original sample layer from which it stems. The relatively high diffusion rate of cytochrome c would ordinarily cause a certain amount of smearing in the electrophoresis pattern.

The GAPD (12) and the aldolase (13) were assayed spectrophotometrically to observe the amount of DPNH generated from suitable substrates. The points on the curve represent optical densities at 340 mμ obtained after 1 min of assaying time with a 1-cm light path. One-tenth milliliter of sample was required for each assay, making it possible to do nine assays on each fraction from the sampler. The maximum point on the aldolase curve represents 5.7×10^{-3} unit of activity (in 3 ml). (One unit equals that amount which transforms 1 mg P in 1 min.) The maximum point on the GAPD curve represents a second-order rate constant of 2.06×10^{3}. The cytochrome curve represents the difference between the absorption at 550 mμ after reduction with ascorbic acid, and the absorption of the cytochrome minimum at 490 mμ. This allowed us to correct for a small amount of turbidity present in the samples. The maximum cytochrome point represents a concentration of approximately 0.43 gm/ml. The absorption in plates 10 and 11 of the cytochrome curve may represent either tailing of the electrophoresis pattern or inhomogeneity in the cytochrome sample. We have insufficient data to exclude either hypothesis.

By adding the three points from each curve which show activity and comparing this with the activity of the original sample, the following recoveries were obtained: 84% of the aldolase activity, 100% of the GAPD activity, and 60% of the cytochrome optical density.

It is concluded from these data that the described apparatus, used in conjunction with Kolin's mobility spectrum technique, fulfills the objective of separating a protein mixture rapidly, sensitively, and with high recovery. Our next objective is to use this equipment to separate subcellular particles, since it is believed that the most unique value of this method lies in its potential ability to separate large colloidal particles (molecular weight over 100,000). Such particles have resisted other methods of separation because of their low migration rates, instability, and surface interactions.

SUMMARY

A density-gradient electrophoresis apparatus is described, which has a fractionator that can cut the liquid column of the apparatus into small segments. This permits the clean separation of electrophoretic components without the use of a solid supporting medium. A sample run is presented showing the efficiency of the fractionator and showing that high recoveries are possible even when the electrophoresis is conducted at room temperature. It is felt that the method will be most useful for separating large colloidal particles (molecular weight over 100,000) and unstable ones which require fast separation.

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Separation of Galactose 1-Phosphate from Other Hexose Phosphates by Ion Exchange

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INTRODUCTION

In connection with recent studies on the metabolism of galactose (1), the need arose for a procedure capable of separating galactose 1-phosphate from other hexose phosphates in amounts up to several hundred milligrams. A further requirement was that the separated hexose phosphates could be isolated as solid salts, uncontaminated by extraneous salts. In this situation, ion-exchange chromatographic methods seemed to be the logical recourse.

Several ion-exchange fractionations of hexose monophosphates have been described, and, in particular, Khym and Cohn (2) and Goodman, Benson, and Calvin (3) have given full details of methods which accomplish excellent separations. However, neither group of investigators included galactose 1-phosphate in the mixtures which they tested. A study of the behavior of this ester in both systems was therefore undertaken. The present communication describes the separation of galactose 1-phosphate from the other common hexose monophosphates in the system of Goodman, Benson, and Calvin, which uses Dowex-1 borate as the adsorbent and 0.1 *M* sodium tetraborate as the primary eluant. Mixtures containing galactose 1-phosphate are also resolved by the system of Khym and Cohn, but the system of Goodman *et al.* was chosen for detailed study because the eluants and elution procedure are simpler.

The isolation of sugar phosphates, after their elution from ion-exchange columns, was also studied. Essentially, the problem is one of separating the phosphates from large amounts of other anions, of which borate is the most troublesome. Two solutions to the problem were found, one generally useful and the other applicable in certain situations.

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The use of the Dowex-1 borate system for the fractionation of intestinal extracts containing galactose 1-phosphate is described in a separate publication (4).

MATERIALS AND METHODS[3]

Sugar Phosphates

Barium galactose-1-P was synthesized according to Hansen *et al.* (5). The method of Krahl and Cori (6) was used to prepare dipotassium glucose-1-P dihydrate. Dr. G. Jollès kindly assisted with these syntheses. Samples of barium galactose 6- and mannose 6-phosphates were generously provided by Dr. H. A. Lardy, while barium glucose 6- and fructose 6-phosphates were commercial preparations.

Ion-Exchange Resins

Dowex-1 X-8, 200–400 mesh unless otherwise specified, was used for the chromatography of the hexose phosphates. However, we eventually came to prefer the 50–100 mesh resin for this purpose. The swelling which accompanies the conversion of the resin to the borate form causes the smaller particles to pack very tightly and greatly reduces the flow rate through the columns. Dowex-50 X-8, 50–100 mesh, was employed to remove cations.

The resins were washed free of fines by decantation and then treated successively in the column with 1 *N* sodium hydroxide, 1 *N* hydrochloric acid, and distilled water. Dowex 1 was converted to either the borate or formate form by passing 0.15 *M* sodium tetraborate or 0.25 *M* sodium formate, respectively, through the column until a negative test for chloride was obtained. The resin was washed with 2–3 column volumes of distilled water before being used for the chromatography.

Hexose Analysis. Cysteine–Sulfuric Acid Assay for Hexoses

The anthrone method of Roe (7) was employed for quantitative determinations of the hexose content of both the pooled column eluates and the hexose phosphate preparations. It was found that when a hexose 1- or 6-phosphate is subjected to Roe's procedure, it gives the same molar color yield as the parent sugar. However, color yields differ considerably from one hexose to another (8). Values for each hexose phosphate were

[3] Abbreviations used: In the text, galactose-1-P, etc., for hexose phosphates. In the figures, G-1-P and G-6-P for glucose 1- and 6-phosphates, respectively; Gal-1-P and Gal-6-P for galactose 1- and 6-phosphates, respectively; F-6-P for fructose 6-phosphate; and M-6-P for mannose 6-phosphate. A_{440} and A_{620} are the absorbancies (optical densities) produced by the application of the cysteine–H_2SO_4 and anthrone procedures, respectively, to aliquots of chromatographic fractions.

therefore computed from the extinction coefficient determined for the parent sugar.

The anthrone analysis was initially used to monitor fractions eluted from ion-exchange columns, but was replaced by a more sensitive and more selective procedure developed during the course of the work. The new method is based on the observations of Dische, Shettles, and Osnos (9) on the reactions of cysteine with the acid-dehydration products of sugars. The procedure is as follows: One-milliliter aliquots of the samples are pipetted into colorimeter tubes, and to each tube is added, with swirling, 5 ml of concentrated sulfuric acid. After a few minutes, 0.1 ml of 3% cysteine hydrochloride is dispensed to each tube, and the contents are mixed again. The tubes are allowed to stand, without external heating or cooling, for 15–20 min, then read in a colorimeter at 440 mμ.

The method is extremely sensitive, and it serves well for the detection of hexose-containing peaks in the presence of pentose-containing substances. Apparently, the same chromogen is formed, in different yields, from all the common hexoses, and a different chromogen from the pentoses (arabinose and ribose). The respective absorption maxima are at 405 and 385 mμ, from which it results that the hexose chromogen has a yellow color, while the pentose chromogen appears colorless. The wavelength of maximal absorption was not employed for the hexose analysis because it was desired to use the procedure on pentose-containing fractions from intestinal tissue (4). In spite of its lack of visible color, the pentose chromogen shows considerable absorption at 405 mμ, whereas at 440 mμ the absorbancy produced by pentose is only 5–10% of that produced by the same amount of hexose. Under the conditions chosen, amounts of hexose or hexose derivative in the range of 0.01–0.1 μmole could be estimated with the colorimeter used (Evelyn). Beer's law is obeyed in the lower half of the range, but not in the upper.

Phosphorus Analysis

For the estimation of inorganic phosphate, we initially used the procedure of King (10), which differs from the original Fiske and SubbaRow procedure in that $HClO_4$, rather that H_2SO_4, is used as the acid. It was noticed, however, that this technique consistently indicated a small but significant amount of inorganic phosphate in chromatographically purified samples of hexose 1-phosphates. Since a number of investigators [(11–13) and others] have pointed out that the acid hydrolysis of labile organic phosphates is catalyzed by molybdate, it was suspected that the "inorganic phosphate" in these samples was an artifact. These suspicions were confirmed when it was found that, on a warm summer day (room temperature 30°), glucose-1-P subjected to the conditions of the King

procedure was hydrolyzed at the rate of 0.4%/min. Under the original conditions of Fiske and SubbaRow (H_2SO_4 as the acid), the rate was 0.1%/min. Nakamura and Mori (14) have reported similar findings.

We found that the hydrolysis of hexose 1-phosphates during the phosphorus analysis can be held to an acceptable minimum if the sample is first diluted to avoid exposing it to the high concentration of acid contained in the reagent, and the temperature is kept low during the period of color development. We did not find as great an effect of temperature as did Nakamura and Mori, who used slightly different reagents, but we did observe that the $Q_{10°}$ of the hydrolysis was somewhat over 2. Control of the temperature is therefore important unless the ambient temperature is below 25°C.

Our modification of the Fiske and SubbaRow procedure (15) for determining inorganic phosphate in the presence of hexose 1-phosphates was as follows: The sample was made up to 9 ml with water in a colorimeter tube and cooled to 20 ± 1°. One milliliter of the acid molybdate reagent [2.5% $(NH_4)_6Mo_7O_{24}.4H_2O$ in 5 *N* H_2SO_4] and 0.5 ml of the Fiske and SubbaRow reducing reagent were rapidly added with mixing. The color was allowed to develop at 20° for exactly 15 min, and the absorbancy was determined at 620 mμ. Under these conditions, inorganic phosphorus (2–20 μg) could be accurately determined in the presence of glucose-1-P with only 0.4% total hydrolysis of the ester.

Acid-labile phosphate and total phosphate were determined in the usual way (15). Samples for the total phosphate determination were first digested with nitric acid, then perchloric acid. After dilution with water and boiling to hydrolyze pyrophosphate formed during the digestion, the samples were neutralized with NaOH and analyzed.

An accurate analysis for phosphate could not be obtained on samples containing large quantities of borate ion. For example, 5 ml of 0.1 *M* sodium tetraborate generated a blue color with the phosphorus reagents equivalent to that produced by 5 μg phosphorus. Since the quantities of phosphate being determined were in this range, borate was removed prior to the analysis by one of the methods described in the *Experimental* section.

Borate Determination

The determination of boric acid in the presence of stronger acids (formic, hexosephosphoric) was accomplished by first titrating the stronger acids to a pH 7.0 end point (glass electrode) with standard alkali, then adding excess D-mannitol. The boric acid–mannitol complex, which behaves like a strong monobasic acid (16), was then titrated to a pH 9.0 end point.

EXPERIMENTAL

Separation on Dowex-1 Borate

Goodman, Benson, and Calvin (3) found that when a mixture of glucose 1-, fructose 6-, and glucose 6-phosphates was adsorbed on a column of Dowex 1 in the borate form and a solution of 0.1 *M* sodium tetraborate was allowed to percolate through the resin, glucose 1- and fructose 6-phosphates emerged, in that order, as well-separated peaks. Glucose-6-P was not removed by the borate eluant but was desorbed by 0.2 *M* sodium chloride.

In order to determine whether this system would effect the separation of galactose-1-P, the galactose ester was included in a mixture with the other hexose phosphates and the chromatography was performed according to the directions of Goodman and colleagues. The galactose-1-P was eluted in a sharp peak immediately after glucose-1-P but well separated from it (see Fig. 1). This separation has been repeated many times and

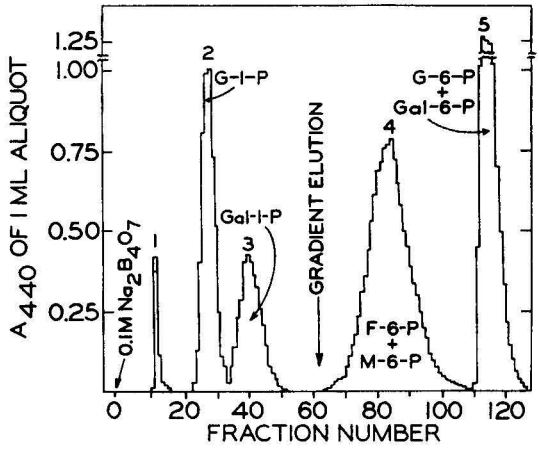

FIG. 1. Chromatography of a mixture of hexose monophosphates (G-1-P, 50 μmoles; Gal-1-P, 80 μmoles; F-6-P, 70 μmoles; M-6-P, 30 μmoles; G-6-P, 40 μmoles; Gal-6-P, 30 μmoles) on Dowex-1 borate. The resin used was 50–100 mesh. Column size, 1.3 cm (diameter) × 21 cm. Fraction volume, 20 ml. Carbohydrate assay, cysteine–H_2SO_4. The mixing chamber for the gradient elution contained 900 ml eluant. Peak 1 is free hexoses.

is always achieved if a column of adequate capacity and proper dimensions is used. Since the purpose of our work was to recover the separated sugar phosphates, sodium formate, which is more readily removed from the eluates than sodium chloride, was tried as an eluting agent for glu-

cose-6-P. It was found to be very effective for this purpose at a concentration of 0.25 *M*.

Attempts to further expand the scope of this system were made by including mannose 6- and galactose 6-phosphates in a mixture with the four hexose monophosphates mentioned above. Since Goodman and his co-workers had stated that mannose-6-P was eluted with fructose-6-P, efforts were made to accomplish finer resolution in this area of the chromatogram. After the glucose and galactose 1-phosphates were eluted with 0.1 *M* $Na_2B_4O_7$, the column was equipped with a gradient setup (17) and 0.1 *M* $Na_2B_4O_7$ adjusted to pH 8.5 with H_2SO_4 was introduced into the original eluant. By thus gradually decreasing the pH and introducing the strongly adsorbed sulfate anion into the eluant, it was hoped that a separation of the four remaining phosphates would be achieved. Nevertheless, only two additional peaks were obtained (Fig. 1). Quantitative analysis for hexose showed that each of these peaks contained two of the four hexose phosphates. The first (peak 4) contained fructose-6-P as judged by the absorption spectrum of the color which it gave with resorcinol–H_2SO_4 (18). Presumably, the other component was mannose-6-P, leaving glucose 6- and galactose 6-phosphates for the final peak. A number of additional attempts were made to effect the resolution of the 6-phosphates, but none proved to be effective.

Separation on Dowex-1 Chloride

In the system of Khym and Cohn (2), the mixture of sugar phosphates is put on a column of Dowex-1 chloride. After the column is washed with 0.001 *M* NH_4OH to elute free sugars, successive hexose phosphate peaks (glucose-1-P, glucose-6-P, fructose-6-P) are eluted with ammonium chloride buffers containing decreasing amounts of potassium tetraborate. The results of an experiment to test the ability of this system to separate glucose-6-P and galactose-1-P are shown in Fig. 2. It may be seen that galactose-1-P is held on the column by the concentration of borate which permits the elution of glucose-6-P, and thus would also be held by the stronger concentration used during the elution of glucose-1-P. The possibility of separating galactose-1-P from fructose-6-P by this system was not explored.

Removal of Borate from Chromatographic Eluates

Two considerations prompted us to study the problem of removing borate from chromatographic eluates containing sugar phosphates: (*a*) removal of borate facilitated the recovery of pure, crystalline salts of these compounds, and (*b*) borate interferes with the phosphate analysis and inhibits certain enzymes (acid phosphatase and glucose-6-P de-

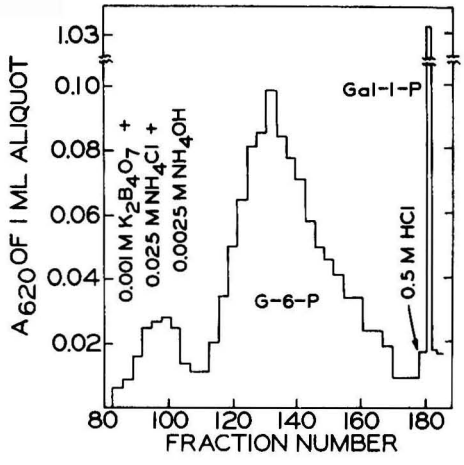

FIG. 2. Chromatography of a mixture of glucose-6-P (120 μmoles) and galactose-1-P (20 μmoles) on Dowex-1 chloride. Resin bed, 0.8 cm (diameter) × 11 cm. Fraction volume, 15 ml. Carbohydrate assay, anthrone. The first small peak is impurities, probably free hexoses.

hydrogenase) which were used in work (4) on the identification and assay of hexose phosphates.

Borate is commonly removed from carbohydrate materials by ion-exchange conversion to boric acid, evaporation to dryness, and repeated distillation of methanol from the residue (19). However, this technique is not applicable to sugar phosphates because it brings them into a very concentrated state in the free acid form, and excessive decomposition ensues. In the present work, two methods which obviate this difficulty were found useful, *viz.* an ion-exchange method and an extraction method. The former was employed whenever *complete* removal of borate was required (e.g., when the sugar phosphate was to be identified by enzymic means) or whenever an acid-labile ester (hexose-1-P) was to be isolated. The extraction procedure was used for the removal of the bulk of the borate from samples containing hexose 6-phosphates. The extraction method is more rapid and simpler than the ion-exchange method, but removal is not quantitative and the conditions are too severe for acid-sensitive phosphates.

Removal by Ion Exchange[4]

The solution containing the hexose phosphate and $Na_2B_4O_7$ is allowed to pass through a column of Dowex 50 (H^+) which has a capacity equal

[4] After our work was completed, it came to our attention that an ion-exchange procedure similar to the one described here had been used by Khym, Doherty, and

to 1.5 times the number of milliequivalents of Na^+ present. One column volume of distilled water is used to wash the resulting free acids directly into a column of Dowex-1 formate of adequate capacity to assure complete adsorption of the sugar phosphate (approximately 5–10 times the number of milliequivalents of phosphate present).

The boric acid, which is undissociated and thus has no affinity for a resin saturated with the anion of a stronger acid, emerges from the Dowex 1 column with the "front." Washing is continued with distilled water until the effluent is free of boric acid (see *Materials and Methods*). If the carbohydrate to be isolated is a hexose 1-phosphate, it is now eluted with 0.1 *M* ammonium formate buffer, pH 6.0, and recovered by evaporating the eluate to dryness and subliming off the ammonium formate at 0.005–0.01 mm Hg pressure at 55°C. Hexose 6-phosphates are desorbed with 0.1 *M* formic acid, and the resulting eluate is then concentrated to a small volume *in vacuo* at a temperature not exceeding 40°C to distill most of the formic acid. Dilute NH_4OH is added to pH 8, and the evaporation is continued until a dry residue is obtained. In either case, the residue is dissolved in a minimum of distilled water and obtained as the barium or potassium salt by the conventional methods of sugar phosphate isolation.

Removal by Solvent Extraction

In this procedure, boric acid is extracted from the aqueous sample with isoamyl alcohol. The apparatus used is a modification of the standard Kutscher-Steudel liquid-liquid extractor (Fig. 3). The solution containing sugar phosphate and $Na_2B_4O_7$ is first either adjusted to pH 4 with HCl, or passed over Dowex 50 (H^+) and adjusted to pH 4 with NaOH. It is then placed in the barrel of the extractor, which is constructed so that the hot solvent dripping from the reflux condenser is directed by a funnel through a water-jacketed external cooling tube. By cooling the solvent before it contacts the sample, its solubility in the aqueous phase is reduced, foaming is decreased, and decomposition of the sugar phosphate is lessened.

With this technique, the extraction of 60 mmoles H_3BO_3 from 40 ml solution is 95% complete in 2 hr. The aqueous solution containing the sugar phosphate is conveniently drained off at the end of the extraction period, and shaken once or twice with ether to remove dissolved isoamyl

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Cohn (20) for the isolation of the isomeric ribose phosphates from borate-containing eluants. Our use of free acid for the final elution of the sugar phosphates when their stability permits it, and of the volatile ammonium formate when a salt is required, makes our procedure simpler than that of the Oak Ridge workers.

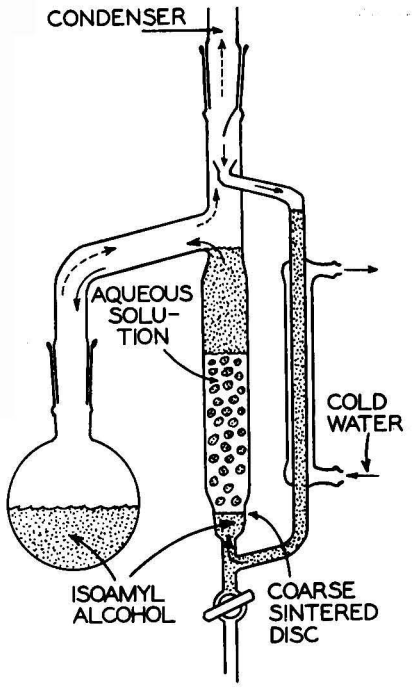

FIG. 3. Apparatus for the continuous extraction of boric acid. Arrows show the circulation of the extracting solvent, isoamyl alcohol (dotted arrows, vapor; solid arrows, liquid).

alcohol. If the amount of sugar phosphate is small (10 μmoles), some losses (10–15%) may be experienced.

Chromatography of Synthetic Hexose 1-Phosphates

It seemed of interest to apply the chromatographic procedure described above to some of the commonly available hexose phosphates. The compounds selected, glucose-1-P and galactose-1-P, were products of chemical synthesis as noted under *Materials and Methods*. The results show that when these two esters are obtained according to standard directions (*Biochemical Preparations*) they are of good over-all purity, but that each is contaminated with minor amounts of two or three other compounds.

Glucose 1-Phosphate

Crystalline dipotassium glucose 1-phosphate dihydrate was dissolved in 8 ml water and chromatographed on Dowex-1 borate as described above.

Sodium chloride, in increasing concentrations, was used to bring off the more strongly adsorbed impurities. The elutogram is shown in Fig. 4.

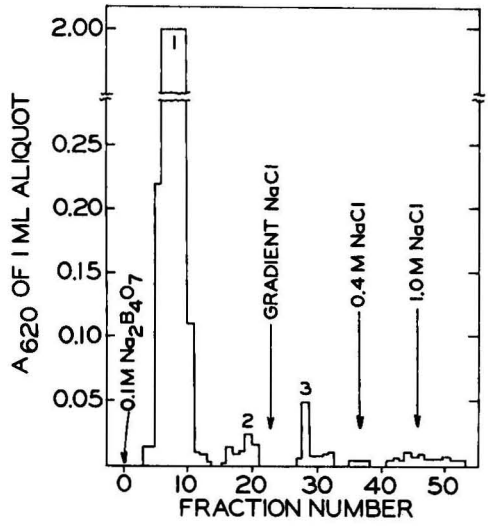

FIG. 4. Purification of synthetic glucose 1-phosphate on Dowex-1 borate. Column size, 0.8 cm (diameter) × 34 cm. Fraction volume, 40 ml. Carbohydrate assay, anthrone. For gradient elution, the mixing chamber contained 250 ml water, the reservoir 0.4 *M* NaCl.

Peak 1, which contained carbohydrate equivalent to 416 mg dipotassium glucose 1-phosphate dihydrate, was freed of borate by the ion-exchange method described in the preceding section. The brownish sirup which remained after sublimation of the ammonium formate was taken up in 15 ml water, brought to pH 9 with potassium hydroxide, and diluted with 3 vol of absolute methanol. Upon refrigeration, 311 mg (75%) of pure, white, spindle-shaped crystals formed.

Of the impurities, only peak 3 was further investigated. Analysis of the pooled fractions showed that the material of this peak contained sugar (calculated as glucose) and phosphorus in a molar ratio of 2:1, suggesting that it was a diglucose phosphate. Inasmuch as diglucose 1-phosphate is a possible intermediate in the hydrolytic process by which glucose 1-phosphate is obtained, its presence in the final product would not be surprising.

Galactose 1-Phosphate

The purity of the barium galactose 1-phosphate preparation used was 87% as determined by analyses for hexose and acid-labile phosphate.

Barium was removed prior to chromatography by passing the sample (9.5 mg) over a small column of Dowex 50 (H^+). The chromatography on Dowex-1 borate was modified in that a gradient (increasing concentration) of sodium tetraborate was applied instead of a uniform concentration. Sodium formate and sodium chloride were used to desorb the strongly held impurities.

The principal peak (Fig. 5) was notched, suggesting the presence of a

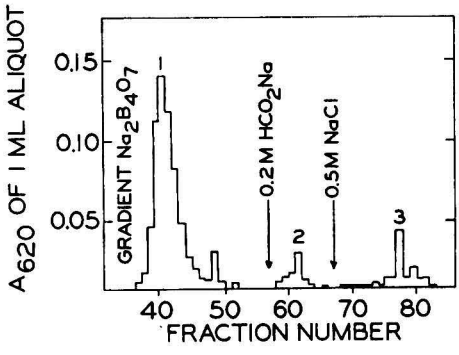

FIG. 5. Purification of synthetic galactose 1-phosphate on Dowex-1 borate. Column size, 0.8 cm (diameter) × 15 cm. Fraction volume, 20 ml. Carbohydrate assay, anthrone. For gradient elution, the mixing chamber contained 250 ml water, the reservoir 0.1 *M* $Na_2B_4O_7$.

small amount of material differing very slightly in structure from the main fraction of α-D-galactopyranose 1-phosphate. It seems possible that some β-D-galactopyranose 1-phosphate may have been present in the preparation, and that the gradient technique used may have partially resolved it from the α-isomer. An anthrone analysis of pooled fractions 37–50 indicated that all the galactose-1-P was recovered in peak 1.

The pooled fractions representing peak 3 were analyzed, and a high ratio of sugar (calculated as galactose) to phosphate was found, as in the case of the impurity from glucose-1-P.

SUMMARY

1. Galactose 1-phosphate is easily separated from the other common hexose phosphates in the chromatographic system of Goodman, Benson and Calvin (Dowex-1 borate column, borax eluant). It emerges from the column after glucose 1-phosphate and before fructose 6-phosphate. Mannose 6-phosphate is eluted with fructose 6-phosphate and galactose 6-phosphate with glucose 6-phosphate.

2. Procedures are described for the removal of borate from chro-

matographic fractions containing hexose phosphates, and for the subsequent isolation of the phosphates as salts.

3. A sensitive, convenient colorimetric method for monitoring chromatograms of sugars and their derivatives is described. The method discriminates well between hexoses and pentoses.

4. Instructions are given for the accurate determination of inorganic phosphate in the presence of hexose 1-phosphates.

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ANALYTICAL BIOCHEMISTRY 2, 80–82 (1961)

A New Technique for Direct Detection of Glycoproteins and Polysaccharides after Electrophoresis or Immunoelectrophoresis in Agar Gel

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The oxidation by periodic acid of polyhydroxylic compounds has been used successfully to characterize glycoproteins in zone electrophoresis. Many different reactions have been employed to enable the products of this periodic oxidation to be measured colorimetrically (1, 2). The reaction that we describe below is an adaptation for gel techniques of the procedures developed in the analytical chemistry of sugars (3). It is based on obtaining copper complexes of formazan derivatives from the aryl hydrazones of the glycoproteins or the polysaccharides. These insoluble and strongly colored complexes fill the necessary conditions for characterization reactions in zone electrophoresis.

METHODS

1. *Agar Electrophoresis and Agar Immunoelectrophoresis.* The apparatus and the technique of Grabar and Williams (4) were utilized.

2. *Characterization of the Glycoproteins and Polysaccharides.* The coupling of a diazonium salt with an aldehyde–aryl hydrazone is usually carried out at an alkaline pH in a solution of pyridine or of alcohol–pyridine. As these solvents are not suitable for the agar gel, we have looked for other conditions which would allow coupling to occur. The best results, as far as reproductibility and yield of the reaction are concerned, were obtained employing a concentrated solution of sodium acetate at a pH near neutrality.

The series of steps involved in this technique can be presented schematically as follows:

$$\text{glycoprotein} + \text{periodic acid} \rightarrow \text{oxidized glycoprotein (I)}$$

$$\text{oxidized glycoprotein} + \xrightarrow[\text{(acetate)}]{\text{phenylhydrazine}} \text{oxyglycoprotein–aldehyde–phenylhydrazone (II)}$$

[1] Chargé de Recherches of C.N.R.S. (Paris).

$$\text{oxyglycoprotein–aldehyde–phenylhydrazone} + \xrightarrow[\text{(acetate)}]{\text{o-dianisidine (tetrazotized)}}$$

o-dianisidine–4,4′-bis(3-oxidized glycoprotein-5-phenylformazan) (**III**)

III + Cu^{++} →

copper complex of the formazan derivative (stable and highly colored complex)

Reagents

A. Periodic Acid. One per cent solution in 0.2 *M* sodium acetate.

B. Phenylhydrazine. HCl. A 0.05% solution in an acetate acid buffer at pH 4 (equal parts of 2 *M* sodium acetate and 8 *M* acetic acid). The solution of phenylhydrazine should be made up fresh each 24 hr.

C. Buffer Solution. Sodium acetate–acetic acid, pH 4.0, $\mu = 0.1$.

D. Diazo Blue B (o-dianisidine tetrazotized). A 0.1% solution in the 1 *M* sodium acetate. Prepare the solution immediately before using.

E. Copper Acetate. A saturated solution in 1 *M* sodium acetate.

Method of Operation

1(*a*). *Electrophoresis in Agar.* After simple electrophoresis the agar plates were fixed during 4–16 hr in a solution of 50% ethanol containing 2% acetic acid.

1(*b*). *Immunoelectrophoresis in Agar.* Once the development of the specific precipitin lines had terminated, the plates were washed for 3 days in a solution of 0.15 *M* NaCl which was changed once per day.

1(*c*). In the two cases (1*a* and 1*b*) the plates were then dried under filter paper in an oven at 37°C (2). After drying they were treated in the following manner:

2. Immersed in reagent A for 10 min.[2]

3. The excess of reagent A was eliminated by washing the plates in running water for 10 min.

4. The hydrazone was formed by putting the plates in a bath of reagent B previously heated to 60–70°C. The temperature was maintained at 60–70°C and the incubation time was 5 min.

5. The excess phenylhydrazine was eliminated by washing in reagent C for 10 min.

6. The formazan derivative was obtained by immersing the plates in reagent D for 10 min.

7. The plates were then washed in running water for 10 min.

8. The copper complex of the formazan derivative was obtained by incubating the plates in reagent E for 5 min.

[2] Duration of the oxidation for serum glycoproteins. For dextrans oxidation in reagent A must be prolonged for 3 hr. In general, the time necessary for the oxidation of unknown constituents must be established experimentally.

9. The plates were finally washed in running water for 5 min.

The zones or the specific precipitin lines containing substances of a polyosidic character will be colored a blue–violet. The coloration is very stable. A description of the application of this method as well as its specificity in the study of serum glycoproteins will appear shortly (5).

The method as it has been described can also be applied for the detection of polyhydroxylic components separated by chromatography or electrophoresis on paper. The only modification necessary is that instead of immersion in reagents A, B, D, and E, these solutions may be applied by spraying.

SUMMARY

A technique for the detection of glycoproteins and polysaccharides after electrophoresis or immunoelectrophoresis in agar gels is described. The method is based on the formation of aldehyde–aryl hydrazones from the carbohydrates oxidized by periodic acid. The aldehyde–aryl hydrazones are subsequently converted to a formazan derivative by coupling with a diazonium salt.

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ANALYTICAL BIOCHEMISTRY 2, 83–88 (1961)

SHORT COMMUNICATIONS

Microanalytical Determination of Urinary Aromatic Acids by Gas Chromatography[1]

The metabolism of aromatic amines such as dopamine,[2] epinephrine, and serotonin is reflected in the urinary excretion of their acidic metabolites homovanillic acid, 3-methoxy-4-hydroxymandelic acid, and 5-hydroxyindoleacetic acid, respectively. The quantitative determination of these acids has been complicated by the necessity of separating them from a large number of acidic compounds in urine, many of which have not been identified. Armstrong *et al.* (1) developed a two-dimensional paper chromatographic method which has been used for the quantitative determination of homovanillic acid (2) and 3-methoxy-4-hydroxymandelic acid (3). We wish to report a method, using gas–liquid partition chromatography, for the simultaneous qualitative and quantitative estimation of urinary aromatic acids.

Aromatic acids were isolated from urine by the extraction procedure described by Armstrong *et al.* (1). The volume of urine corresponding to 20 mg creatinine was sufficient for the analysis; the yield of the acidic fraction was about 10 mg. Since the vapor pressures of the free aromatic acids were too low, it was necessary to convert them to methyl esters in order to apply the technique. For this purpose, the mixture of urinary acids was reacted with 4 ml of 1.2% diazomethane in anhydrous ethyl ether for 15 min at 25°. The diazomethane reagent, prepared from *N'*-nitro-*N*-methyl-*N*-nitrosoguanidine (4) (Aldrich Chemical Co.) was redistilled with ether and stored over anhydrous sodium sulfate at 4° for periods up to 2 weeks. After methylation of the acids with diazomethane, the ether solution was evaporated to dryness with a stream of nitrogen in a hood. The residue was dissolved in 200 μl of absolute ethanol and stored for short periods at 4° prior to gas chromatography. Diazomethane may react with phenolic groups in some cases; the small yield of *O*-methyl ethers by this process has not interfered with analytical determinations.

Two columns, *U*-shaped with a total length of 6 ft and internal diameter ¼ in., were used for the analysis by gas chromatography. The polar column, containing 10% (w/w) ethylene glycol–adipic acid polyester coated on 60–80 mesh siliconized Chromosorb W (5), was operated at 206° with an argon inlet pressure of 15 lb/sq in. (psi). The nonpolar

[1] Supported by a grant-in-aid from the U. S. Public Health Service.

[2] Abbreviation for 3,4-dihydroxyphenylethylamine.

column, containing 3% (w/w) silicone gum (General Electric, SE-30) on 80–100 mesh Chromosorb W, was operated at 191° with an argon inlet pressure of 18 psi. Using these conditions in an instrument equipped with argon ionization detectors similar in design to that of Lovelock (6), it was possible to obtain complete analyses with 50–100-μg loads (equivalent to a volume of urine that contained 0.2–0.5 mg creatinine).

Retention times were determined for several aromatic acids (as methyl esters) known to be constituents of urine; these retention times are given in Table 1. Methyl esters of the aromatic acids from normal human urine

TABLE 1

RETENTION TIMES IN MINUTES OF METHYL ESTERS OF STANDARD AROMATIC ACIDS

	Silicone, 191°C	Adipate, 206°C
p-Hydroxyphenylacetic acid	2.0	25.8
Homovanillic acid	2.9	17.8
Hippuric acid	4.4	42.0
3-Methoxy-4-hydroxymandelic acid	4.4	62.5
Indoleacetic acid	6.7	88.0
5-Hydroxyindoleacetic acid	18.3	unk (>120)

were prepared and analytical records were obtained by gas chromatography. Several component peaks were identified by comparing their retention times with those of the standards. Typical records obtained in the analysis of normal urinary constituents are shown in Figs. 1 and 2. Peaks corresponding to homovanillic acid (*A*), *p*-hydroxyphenylacetic acid (*B*), and hippuric acid (*C*) were easily recognized on the adipate column (Fig.

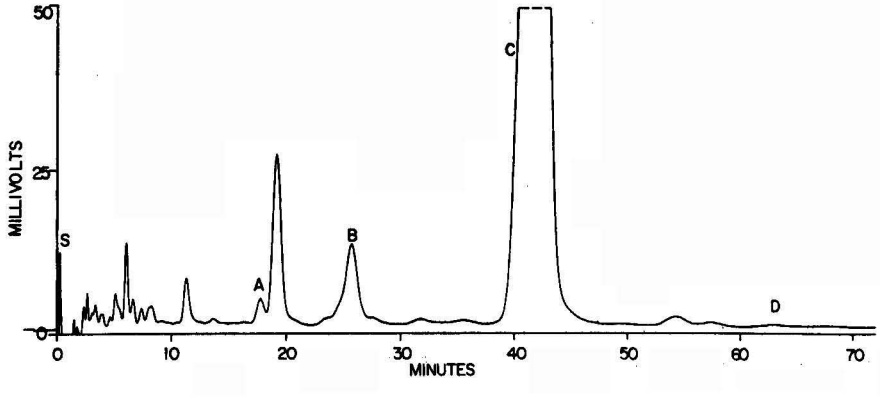

FIG. 1. Analytical record obtained in the analysis of normal urinary aromatic acids by gas chromatography on adipate polyester column. *S*, solvent; *A*, homovanillic acid; *B*, *p*-hydroxyphenylacetic acid; *C*, hippuric acid; *D*, 3-methoxy-4-hydroxymandelic acid.

1), while other more polar metabolites such as 3-methoxy-4-hydroxymandelic acid (*D*), indoleacetic acid, and 5-hydroxyindoleacetic acid, present in normal urine in very low concentrations, could not be determined with accuracy on the adipate column. These acids were more readily measured on the silicone column as shown in Fig. 2. The splitting of the major peak (*C*, *D*) in Fig. 2, representing the methyl esters of hippuric acid and 3-methoxy-4-hydroxymandelic acid, was an instrumental effect caused by temporary overloading of the detector. This effect was not observed with smaller loads of volatile esters. Normally, the peak corresponding to 5-hydroxyindoleacetic acid (*F*) was measured at a higher instrument sensitivity than that shown in Fig. 2.

A greatly increased excretion of 5-hydroxyindoleacetic acid above normal values has been established for patients with malignant carcinoid (7). In order to test the method, peak area per unit mass was calculated for methyl 5-hydroxyindoleacetate by multiple injections of pure standard

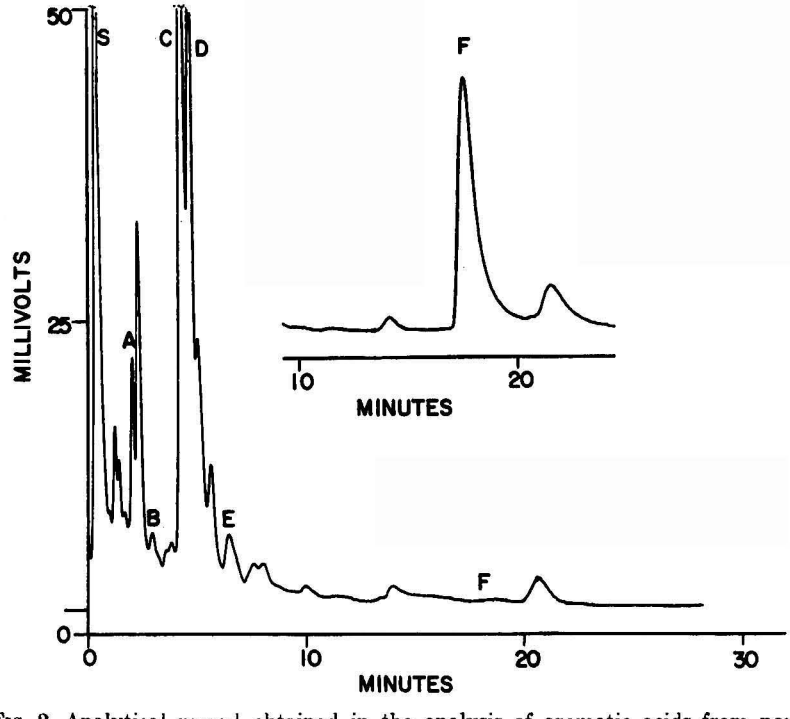

FIG. 2. Analytical record obtained in the analysis of aromatic acids from normal urine (80 μg creatinine equivalent) and urine from a patient with malignant carcinoid (insert, 80 μg creatinine equivalent), using a silicone column. *S*, solvent; *A*, *p*-hydroxyphenylacetic acid; *B*, homovanillic acid; *C*, hippuric acid; *D*, 3-methoxy-4-hydroxymandelic acid; *E*, indoleacetic acid; *F*, 5-hydroxyindoleacetic acid. Insert: *F*, 5-hydroxyindoleacetic acid.

in a concentration range from 0.2 to 5 μg. Analyses were then made of urine from a patient with malignant carcinoid. The results (Fig. 2, inset) indicated a level of 5-hydroxyindoleacetic acid in this patient which was more than 100 times that of normal urine, a difference of the same order of magnitude reported by others.

The argon ionization detector has been used successfully for the quantitative estimation of long-chain fatty acids (8). With compounds which contain varying proportions of oxygen in the form of hydroxyl and carbonyl groups, such as steroids, short-chain fatty acids, and urinary aromatic acids, the response of this detector is dependent on the oxygen content[3] (9). In microanalyses of these urinary metabolites by gas chromatography, it will be necessary, therefore, to relate the observed peak area of each component acid to a graph of area versus concentration for that component. By this method, calculations may be made of the urinary excretion levels of all of the known aromatic acids from a single gas chromatographic analysis.

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Application of the Paper-Strip Modification of Edman's Method to the Determination of Amino Acid Sequence in Small Peptides[1, 2]

The application of the Edman degradation to proteins is attended by difficulty because of the insolubility of the PTC-protein that must be formed as the first step in the procedure. Fraenkel-Conrat (1) has neatly circumvented this difficulty by using a paper strip as a carrier for the protein during all operations of the procedure. On the other hand, when the paper-strip procedure is applied to small peptides, the washing of the strips first with benzene and then with alcohol–ether to remove excess phenyl isothiocyanate is likely to result in the extraction and removal of the PTC-peptide from the paper strips. We have found that a very simple modification of procedure in most instances will prevent the extraction of the PTC-peptide: After the reaction with phenyl isothiocyanate, the strips are washed three times *with benzene only* (in which the PTC-peptides are insoluble) and not at all with alcohol–ether. After the degradation, the PTH-amino acid is extracted with acetone instead of alcohol–ether. The remainder of the procedure follows Fraenkel-Conrat (1) with certain modifications in the method (2) of exposing the strips to reagents. The PTH-amino acids are identified by paper chromatography by methods of Edman and Sjöquist as noted previously (2).

In applying the procedure to peptides, it has been convenient to use 0.5–2μ moles peptide distributed over six paper strips. Adequate material is thus obtained at each degradation to permit identification of the PTH-amino acid. Although the method has been used only qualitatively, it has been applied to peptides for which the amino acid composition has been determined. Thus, in any determination of sequence, one knows which residues have been accounted for and which have not. The yield of PTH-amino acid in consecutive steps has been as good or better than previously stated (2). In general, as the number of degradations on a given peptide increases, small amounts of PTH-amino acids from preceding degradations are apparent. How many degradations may be carried out before these small amounts begin to confuse the identification at a given degradation depends very much on the individual peptide.

The method has been successfully applied to numerous peptides from

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tryptic hydrolyzates of hemoglobins A and F. For example, it has shown the sequence of a decapeptide from the γ-chains of hemoglobin F to be val-leu-thr-ser-leu-gly-asp-ala-ileu-lys. The method does not distinguish between leucine and isoleucine so that the one isoleucyl residue that is known to be present from the amino acid analysis is placed only on the basis of somewhat uncertain indirect evidence. Further examples may be cited. A tridecapeptide from the γ-chains that is equivalent to "fingerprint" spot No. 26 (3) has the structure, val-aspNH_2-val-glu-asp-(thr, glu,ala,leu,gly_3)-arg. From the β-chains, a tryptic peptide equivalent to "fingerprint" spot No. 16 shows the sequence val-val-ala-gly-val-ala-aspNH_2-ala-leu-ala-his-lys. A tryptic peptide of the α-chains equivalent to "fingerprint" spot No. 23 on further treatment with chymotrypsin yields the heptapeptide, gly-ala-glu-ala-leu-glu-arg. The pentapeptide, phe-pro-thr-thr-lys, results when leucine aminopeptidase acts on a tryptic peptide from the α-chain equivalent to "fingerprint" spot No. 13. Certain sequences in the latter two peptides differ from those reported by Braunitzer *et al.* (4), who give them as gly-glu-ala-ala-leu-glu-arg and phe-thr-pro-thr-lys.

The modifications, of course, cannot eliminate problems, such as incomplete conversion to the PTH-amino acid, which are inherent in the method itself. The results may be less satisfactory if the peptide contains a repeating sequence or a succession of residues that give poor yields during the degradation. However, the degradation may often be carried successfully through more steps than is commonly possible with proteins themselves.

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Study of the Soret Band: A Screening Method for Urinary Porphyrins[1]

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INTRODUCTION

The errors in porphyrin biosynthesis found in the porphyrias result in the excessive excretion of urinary porphyrins and especially of uroporphyrins. The porphyrinurias found in certain anemias, in alcoholic cirrhosis of the liver, and in chemical poisoning (halogenated hydrocarbons, heavy metals) are characterized by excessive coproporphyrin but normal or only slightly elevated uroporphyrin excretion. The simultaneous measurement of urinary uroporphyrin and coproporphyrin is useful in the diagnosis of congenital porphyria and of acquired porphyrinuria.

Classical methods of urinary porphyrin analysis take advantage of the differential solubility of copro- and uroporphyrins. Coproporphyrin can be extracted by a mixture of ether and acetic acid (1), and uroporphyrin is then obtained from the aqueous phase by adsorption on alumina (2) or by extraction with ethyl acetate at pH 3.1 (3) or with cyclohexanone and petroleum ether at pH 1.5 (4). The fluorescence or absorbance of the porphyrins is measured after extraction or elution with hydrochloric acid.

The strong ultraviolet absorption band characteristic of the porphyrins is called the Soret band; it provides the basis for the spectrophotometric quantitation of porphyrins. A method for the direct estimation of uroporphyrin in the presence of coproporphyrin, based upon the behavior of the coproporphyrin and uroporphyrin Soret bands in the pH 5–7 range, is proposed in this study.

STUDY OF THE SORET BAND

1. Behavior of the Copro- and Uroporphyrin Soret Bands

The Soret band is known to be subject to changes in wavelength and intensity according to the state of ionization of the pyrrolic nitrogens of

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the porphin nucleus (5). The different spectra of porphyrins have been associated (6, 7) with the presence of a free base, a monocationic and a dicationic form.

In this study the coproporphyrin absorption spectrum shifted its maximum from 402 mμ in 1.5 *N* hydrochloric acid to about 370 mμ at pH 4 (acetate buffer) and from 370 to 394 mμ at pH 7 (phosphate or Tris buffer) corresponding to the 372 and 391 mμ maxima found by Neuberger and Scott (6) in different buffers and at different ionic strength. Neuberger and Scott assigned the 372-mμ maximum to monocationic coproporphyrin and the 391-mμ maximum to the free coproporphyrin base. The uroporphyrin absorption spectrum shifted its maximum only once: from 406 mμ in 1.5 *N* hydrochloric acid to 399 mμ at pH 5 (acetate buffer). The 399-mμ maximum was found also in alkaline buffers and in dilute sodium hydroxide: it corresponds to the 397.5-mμ maximum found by Rimington and Sveinsson (8) for uroporphyrin in basic solution; a spectrum corresponding to monocationic uroporphyrin has not been reported and was not found in this study.

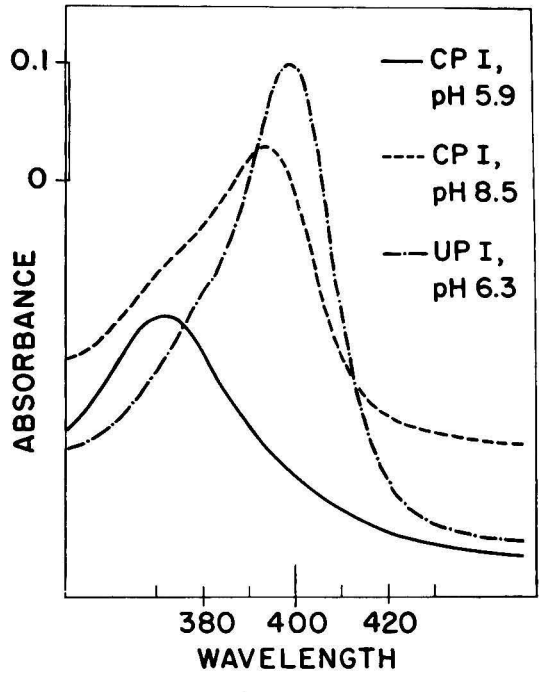

FIG. 1. Porphyrin spectra. Spectra of coproporphyrin I in the monocationic (pH 5.9) and free base form (pH 8.5); uroporphyrin I in the free base form (pH 6.3).

The absorption maximum of monocationic coproporphyrin (at about 370 mμ) is sufficiently remote from the absorption maximum of free uroporphyrin base (at 399 mμ) to permit measurement of the free base form of uroporphyrin in the presence of monocationic coproporphyrin. After a preliminary study indicated that this measurement was not possible above pH 7 (due to interference by the free base spectrum of coproporphyrin,

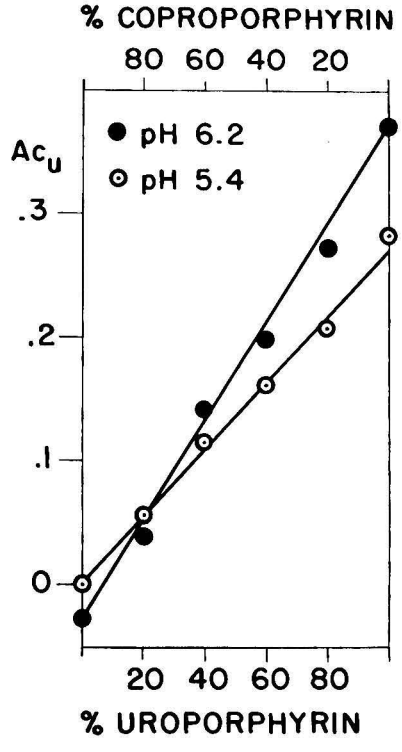

FIG. 2. Absorbance of uroporphyrin coproporphyrin mixtures. Mixtures containing an increasing proportion of uroporphyrin in a total porphyrin concentration of 1.5 μg/ml (pH 5.4) and 1.10 μg/ml (pH 6.2); $Ac_u = 2A_{399} - (A_{414} + A_{384})$.

max. 394 mμ) and below pH 5 (because of the appearance of the dicationic forms of copro- and uroporphyrin, respective maxima 402 and 406 mμ) the present studies were conducted at pH 5.4 (13 *M* acetate buffer) and at pH 6.2 (2 *M* phosphate buffer). The spectra of coproporphyrin I at pH 5.9 (1.0 *M* citrate buffer) and pH 8.5 (1.0 *M* Tris buffer) and of uroporphyrin I at pH 6.3 (1.0 *M* citrate buffer), given in Fig. 1, show that coproporphyrin in its monocationic form will interfere but little if uropor-

phyrin is estimated from a corrected absorbance Ac_u, calculated from absorbance measurements at 384, 399 and 414 mμ.

$$Ac_u = 2A_{399} - (A_{384} + A_{414})$$

The absorbance of coproporphyrin–uroporphyrin mixtures (Fig. 2) containing an increasing proportion of uroporphyrin in a total porphyrin concentration of 1.15 μg/ml (pH 5.4) and 1.10 μg/ml (pH 6.2) show that Ac_u can serve to quantitate uroporphyrin in the presence of coproporphyrin. Comparison of Ac_u at pH's 5.4 and 6.2 (Fig. 2) indicates that uroporphyrin measurements are more sensitive at pH 6.2 but suggests that small amounts of uroporphyrin are more likely to escape detection in the presence of large amounts of coproporphyrin at that pH.

2. Review of the Spectrophotometric Constants

The spectrophotometric quantitation of porphyrin solutions is based upon absorbance measurements in hydrochloric acid of a given normality. The absorbance A of pure porphyrin solutions is measured at the Soret band maximum while the corrected absorbance of impure solutions, Ac, is calculated from absorbance measurements at three wavelengths according to the formula:

$$Ac = 2A_{max} - (A_{430} + A_{380})$$

which was derived by Rimington and Sveinsson (8).

In order to quantitate uroporphyrin in buffered solutions and to establish new conditions for the estimation of porphyrin in acid solution, a brief study of the absorbance (A) values and of the "divisor constants" $k = Ac/A$ was made. The constants obtained were compared with those published by With (9). In the data summarized in Table 1, absorbance values for coproporphyrin in 1.5 N hydrochloric acid are based on the theoretical coproporphyrin concentration of hydrolyzed coproporphyrin tetramethyl ester solutions. Comparison of the A and Ac values obtained in 1.5 N hydrochloric acid with those measured by With (9) in 0.1–0.15 N hydrochloric acid reveals no significant difference.

The Ac_t values, which will be referred to in the second part of this study, were obtained from the same spectra as the Ac values.

Hydrolysis of uroporphyrin octamethyl esters yielded solutions whose uroporphyrin concentration was determined by absorbance measurements in 0.5 N hydrochloric acid and use of the published constants listed in Table 1. The absorbance constants reported for uroporphyrin I and uroporphyrin III (Table 1) were obtained by measuring the absorbance of these solutions in 1.5 N hydrochloric acid and in buffer.

TABLE 1
SPECTROPHOTOMETRIC FACTORS[a]

	A (μg/ml)	k (Ac/A)	Ac (μg/ml)	Ac_t (μg/ml)	Ac_u pH 5.4	Ac_u pH 6.2
CP						
Published						
(0.10–0.15 N HCl)	0.667	1.835	1.226	—	—	—
Found: CP I	0.675	1.835	1.239	1.154	−0.005	−0.021
CP III	0.667	1.834	1.223	1.156		
Adopted value	0.667	1.835	1.226	1.155	—	—
UP						
Published						
(0.5 N HCl)	0.649	1.844	1.198	—	—	—
Found: UP I	0.609	1.842	1.122	1.059	0.252 ± 0.024[b]	0.334 ± 0.013[b]
UP II	0.619	1.846	1.143	1.059		
Adopted value	0.614	1.844	1.132	1.059	0.252	0.334

[a] Absorbance and corrected absorbances of porphyrins measured in 1.5 N hydrochloric acid and in buffer. The published data are from With (9); k is the "divisor constant"; the corrected absorbances are:

$$Ac = 2A_{max} - (A_{380} + A_{430});$$
$$Ac_t = 2A_{max} - (A_{390} + A_{420}); \text{ and}$$
$$Ac_u = 2A_{399} - (A_{384} + A_{414}).$$

[b] Standard deviation.

SCREENING OF URINARY PORPHYRINS BY DIFFERENTIAL SPECTROPHOTOMETRY

1. Materials and Methods

Materials. (*a*) 3 N hydrochloric acid; (*b*) 3 N sodium hydroxide, adjusted to correspond to the acid; (*c*) 2 M disodium monopotassium phosphate buffer, pH 6.2; (*d*) equipment: ratio recording spectrophotometer.

Methods. (*a*) Screening of Urinary Uroporphyrin. Acidify a 5.0-ml aliquot of a 24-hr urine specimen with 1.0 ml of 3 N hydrochloric acid. Neutralize the acidified specimen with 1.0 ml of 3 N sodium hydroxide and buffer with 3.0 ml buffer. Scan against distilled water between 375 and 450 mμ. The corrected absorbance of the specimen is:

$$Ac_u = 2A_{399} - (A_{384} + A_{414})$$

and the uroporphyrin (UP) excretion in 24 hr is calculated from:

$$\mu g \text{ UP/24 hr} = 6 \times Ac_u \times \text{24-hr volume (ml)}$$

(*b*) Screening of Total Urinary Porphyrin. Acidify a 5.0-ml aliquot of a 24-hr urine specimen with 5.0 ml of 3 N hydrochloric acid. Scan the

acidified specimen between 375 and 450 mμ at an absorbance setting of −3+7, using distilled water as a blank if uroporphyrin is revealed by the screening test but using the buffered specimen [part (*a*)] if uroporphyrins are not detectable. The corrected absorbance of total urinary porphyrin is obtained from:

$$Ac_t = 2A_{max} - (A_{390} + A_{420})$$

and the total porphyrin (TP) excretion in 24 hr is calculated from:

$$\mu g \text{ TP/24 hr} = 1.81 \times Ac_t \times \text{24-hr volume (ml)}$$

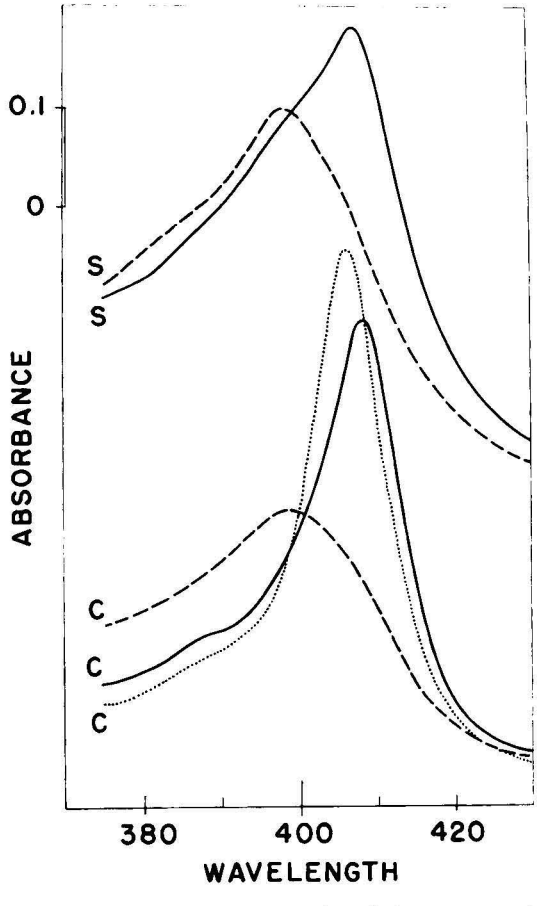

FIG. 3. Screening of uroporphyrin. Spectra of a chelate preparation (C) and of a specimen (S) buffered at pH 6.2 with (broken line) and without (plain line) previous acidification and neutralization. The dicationic spectrum of the chelate preparation is represented by a dotted line.

2. Screening of Urinary Uroporphyrin

When urine specimens from porphyria patients are buffered to pH 5.4 or pH 6.2 without previous acidification, a Soret band with a maximum at 408 mμ is seen in most cases (Fig. 3). A bathochromic shift from 399 to 408 mμ at that pH suggests the presence of a chelate. Since the occurrence of zinc uroporphyrin in porphyria has been reported by Watson and co-workers (10), a crude zinc uroporphyrin chelate was made (11) and its behavior compared to that of porphyria urine specimens. The chelate preparation showed the described 542- and 579-mμ bands (11); its Soret band maximum, 407.7 mμ at pH 6.2, was indistinguishable from that of porphyria urine specimens (Fig. 3). Porphyria urine specimens and the uroporphyrin chelate preparation behaved identically when acidified to quantitatively remove zinc from the complex (12) and when converted to the free uroporphyrin base form by subsequent neutralization and buffering (Fig. 3). This further suggests the 408-mμ maximum of porphyria urine specimens as representing a chelate.

Uroporphyrins are calculated using the constants of Table 1; at pH 6.2:

$$\mu g\ UP/ml = \frac{Ac_u}{0.334} \times \frac{10}{5} = 6.0 \times Ac_u$$

Comparative values obtained by this screening method and a conventional laboratory procedure are given in Table 2.

TABLE 2

PORPHYRIN MEASUREMENT IN PORPHYRIA URINE SPECIMENS[a]

	Extraction method				Present screening method			
					UP			
No.	CP	UP	Total	% UP	(pH 5.4)	(pH 6.2)	TP	% UP
1*	0.82	2.89	3.71	78	2.95	2.86	4.13	70
2	0.58	4.29	4.87	88	5.37	5.27	6.02	89
3	0.43	1.79**	2.22	80	2.32	2.43	3.80	63
4*	1.71	1.54	3.25	47	1.81	—	3.09	59
5	0.70	10.26	10.95	94	12.86	—	13.40	96
6	1.13	4.47	5.60	78	6.17	—	7.71	80
7	2.51	2.28	4.79	48	3.60	—	6.32	57
8	0.73	1.74**	2.47	70	—	1.52	2.10	72
9*	1.13	6.24	7.37	85	—	4.60	9.05	51
10*	0.48	1.86	2.34	80	—	1.62	1.93	84
11	0.21	0.34	0.56	62	—	0.56	0.76	74
12	0.58	3.97	4.55	87	—	2.75	3.17	87

[a] Comparison of values obtained with the present procedure and a conventional method involving ether extraction of CP (1), subsequent UP separation by ethyl acetate extraction (3) or by adsorption on alumina [(2) indicated by double asterisk] and spectrophotometric quantitation in HCl using published constants (9). The porphyrin excretion is expressed in mg/24 hr, or, if specimen number is followed by asterisk, in mg/l.

3. *Screening of Total Urinary Porphyrin*

Since Ac_t is 1.55 for 1 μg CP/ml and 1.059 for 1 μg UP/ml (Table 1), the porphyrin concentration of an aliquot diluted with 1 vol of 3 N hydrochloric acid and assumed to contain 50% uroporphyrin and 50% coproporphyrin is given by:

$$\mu g \text{ TP/ml} = Ac_t \times \frac{2}{1.11} = 1.81 \times Ac_t$$

When UP is present, an elevated TP can be expected and comparison of the acidified specimen with distilled water or acid (13) is possible. When UP is absent, the buffered specimen is used as a blank to assess TP in its dicationic form: this virtually eliminates background absorbance. The assumption that a specimen contains 50% UP and 50% CP is introduced to simplify calculations: it may lead to an error in TP of less than 5%, an amount compatible with the screening character of the method. A comparison of TP values obtained by conventional methods and by the present procedure is given in Table 2 for porphyria specimens

TABLE 3
PORPHYRIN SCREENING IN NON-PORPHYRIA URINE SPECIMENS[a]

No.	Extraction		Screening (Present)	No.	Extraction		Screening (Present)	Screening Schlenker (13)
	CP	TP[b]	TP		CP	TP[b]	TP	TP
1	52	77	102	15	540	630	510	212
2	283	308	336	16	47	47	51	—
3	91	91	115	17	162	178	153	—
4	6	7	14	18	98	106	144	131
5	88	115	104	19	61	61	83	0
6	8	9	9	20	56	60	92	0
7	92	102	123	21	45	53	72	0
8	23	31	25	22	11	14	16	0
9	150	160	163	23	27	34	41	0
10	28	28	29	24	42	45	51	—
11	272	288	238	25	34	34	81	—
12	122	127	141	26	58	67	97	—
13	78	83	101	27	129	129	119	—
14	21	34	42	28	68	74	101	—

[a] Comparison of the values obtained with the present screening test, Schlenker's method (13) and conventional extraction methods (see Table 2); all excretion values are expressed in μg/24 hr.

[b] CP + UP = TP.

and in Table 3 for non-porphyria specimens. Representative absorption spectra of non-porphyria specimens are given in Fig. 4.

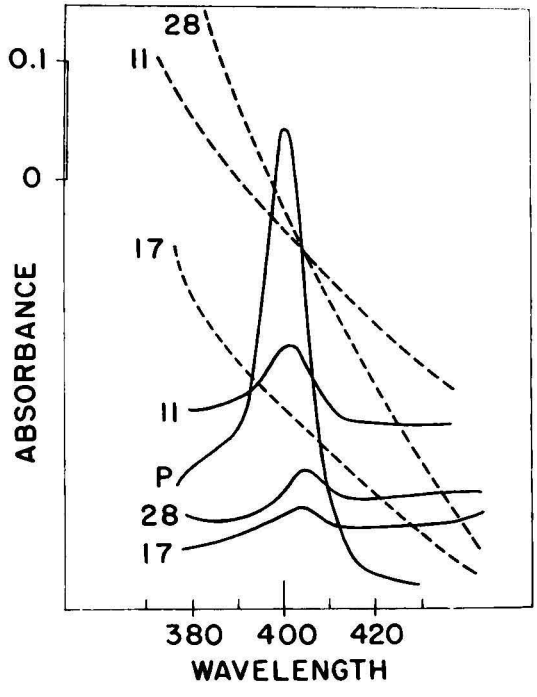

FIG. 4. Screening of total urinary porphyrin, representative spectra of non-porphyria specimens. Specimens read at pH 6.2 against distilled water (broken lines) and acidified specimens read against buffered specimens (plain lines). The numbers are those of Table 3 (No. column); P is a purified coproporphyrin extract.

DISCUSSION

The disappearance of the dicationic form (dihydrochloride in hydrochloric acid) of the Soret band has been used to estimate the pK of the pyrrolic nitrogens of the porphyrins (6, 7). The different pK values obtained for CP (tetramethyl tetrapropionic porphin) and UP (tetracetic tetrapropionic porphin) have been interpreted in terms of inductive and resonance effects by Neuberger and Scott (6). Phillips (7) found $pK_1 = 1.8$, $pK_2 = 2.2$ for UP octamethyl ester and $pK_1 = 1.7$, $pK_2 = 4.1$ for CP tetramethyl ester in detergent solution: these values suggest the existence of monocationic CP and explain the absence of monocationic UP.

The comparison between the screening test presented here and conventional methods reveals (Table 2 and 3) minor differences which may reflect lack of sensitivity and specificity in the screening technique as well as losses during the conventional extraction procedure. A major difference found occasionally is due to the oxidation of porphyrinogens to porphyrins

with iodine in routine laboratory procedures (1, 2). In porphyria specimens mere standing, irradiation with ultraviolet light, or oxidation with iodine markedly increase the quantity of measurable porphyrin (14); the timed action of iodine or ultraviolet light necessary for this conversion is outside the scope of a screening method and was therefore omitted.

Recently Schlenker and Kitchell (13) have proposed a rapid screening method in which total porphyrin is estimated by measuring the absorbance of urinary specimens acidified to 0.5 N with hydrochloric acid against 0.5 N hydrochloric acid at 380, 400, and 430 mμ. Their method was quoted (13) as being unreliable when the total porphyrin excretion was less than 300 μg/24 hr. A study of the Schlenker method revealed that in most cases (Table 3) its failure was due to the use of an Allen type correction (15) concomitant with a hyperbolic rather than linear background rise of nonspecific absorbance with decreasing wavelength (Fig. 4).

The estimation of total porphyrin by comparison of buffered and acidified specimens rests upon the assumption that the corrected absorbance Ac_t is due only to the appearance of a dicationic porphyrins. The total porphyrin content is calculated from Ac_t rather than Ac in order to decrease the interference due to the curvature of the background and to increase the specificity of the method. If, in the screening of total porphyrins in non-porphyria specimens, the background absorbance (the line joining the 390-mμ and the 420-mμ absorbances) deviates markedly from a horizontal line (in one out of five specimens in this study), extraction with conventional methods is desirable.

SUMMARY

A screening method for the estimation of uroporphyrin and total porphyrins has been presented and its merits and limitations evaluated.

The Soret band behavior of coproporphyrin, uroporphyrin, and zinc uroporphyrin has been reviewed and discussed.

ACKNOWLEDGMENTS

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ANALYTICAL BIOCHEMISTRY 2, 100–106 (1961)

A Micromethod for Fractionation of Lipids by Silicic Acid Chromatography[1]

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INTRODUCTION

Recently improved procedures for separation of complex mixtures of lipids by silicic acid chromatography (1–6) have made possible highly quantitative characterization of lipids of biological origin. However, since the available methods have required a minimum of 50 mg or more lipid, fractionation of the lipid extracts of small blood and tissue samples from a single animal has been virtually impossible.

The method reported here was developed in order to study the lipid components in plasma samples of the size obtainable from a single rat[3] (7). When followed by gas–liquid chromatography, it has proved satisfactory for characterization of the fatty acid moieties of the fractions composing 1–10-mg samples of mixed lipid. It is, essentially, a micro adaptation of a procedure (8) which has been used successfully in this laboratory for fractionation of 250-mg liver lipid samples, ten at a time, on larger silicic acid columns. The eluants are those of Fillerup and Mead (4). The fractions separated are: I, cholesterol ester; II, triglyceride; III, free cholesterol, mono- and diglycerides; and IV, phospholipids.

MATERIALS AND METHODS

The procedure selected was as follows: Bakers Analyzed reagent-grade silicic acid was washed two to four times with dry acetone and twice with absolute methanol, collected on a filter, and dried 12–15 hr at 110°C. One gram of the washed silicic acid was tamped tightly, little by little, with a glass rod, into columns made of glass tubing. These were 8 mm in

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[2] Postdoctoral Fellow in Nutrition, National Heart Institute.

[3] A more detailed description of the method may be found in the thesis submitted by Elaine Walker Lis in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Nutrition, University of California, Berkeley, June 1960.

diameter and 125 mm long, tapered to a tip at one end, and fused at the other to glass tubing 20 mm in diameter and 110 mm long. A small plug of glass wool was placed in the tip below the silicic acid and a filter-paper disk above it (Fig. 1).

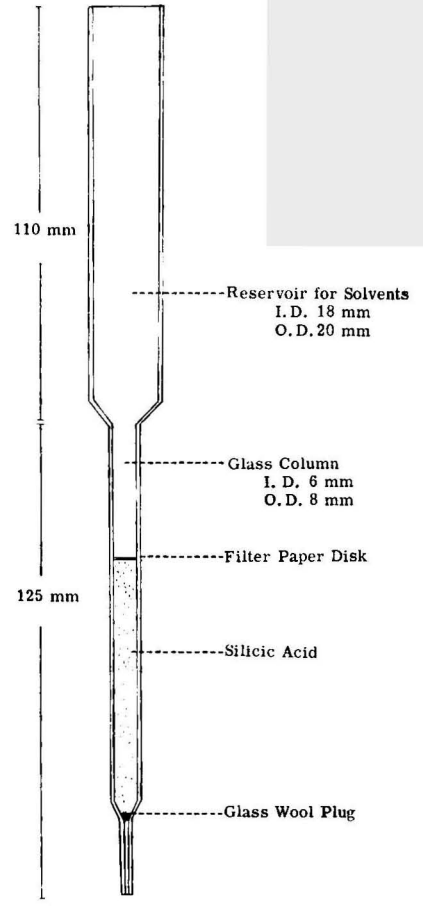

FIG. 1. Diagram of a single silicic acid microcolumn.

Ten packed columns were then placed in a wooden rack, with slotted corks as holders. The silicic acid was washed with successive 2-ml portions of absolute methanol, redistilled dry acetone, redistilled dry ethyl ether, and 6–10 ml of redistilled dry petroleum ether. The methanol was allowed to flow by gravity to make certain that the flow rate was satisfactory (10–15 min for 1 ml to wet the column), and the remaining washes were

forced through gently by means of nitrogen supplied through a glass manifold with outlets to each column. At no time was a column permitted to dry after the washing procedure was begun.

The glass manifold was constructed as follows: Ten ground-glass stopcocks were fused at right angles to a piece of glass tubing approximately 2 cm in diameter and 42 cm long which had been sealed at one end and tapered to about 1 cm at the other. Rubber tubing of appropriate length led from each stopcock to a No. 18 hypodermic needle pierced through a cork fitted to the top of a column (Fig. 2). To the open end of the mani-

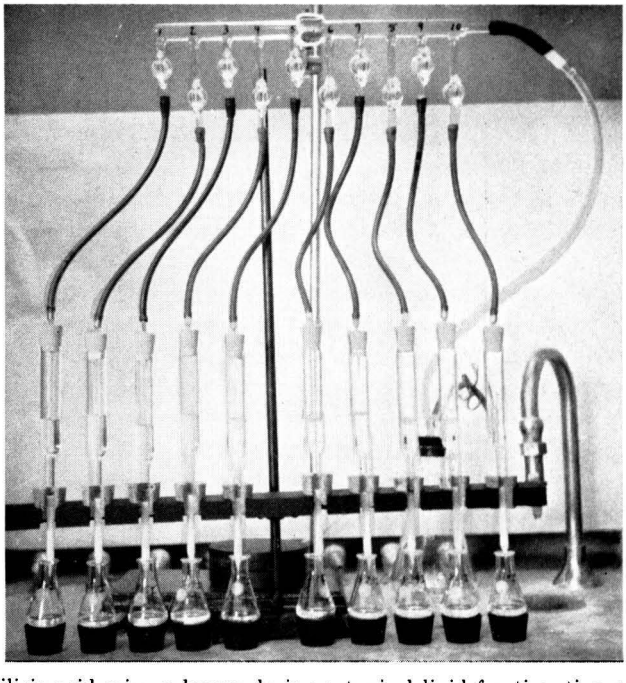

FIG. 2. Silicic acid microcolumns during a typical lipid fractionation sequence.

fold was attached a 500-ml filter flask which served as a reservoir for nitrogen, to absorb pressure fluctuations. A tank of pure nitrogen was then connected to this flask through a reducing valve.

Before the samples were loaded onto the columns, the petroleum ether was forced through until about 1 ml or less remained on each column. The extracts of lipid to be chromatographed were evaporated to less than 200 μl in 15-ml graduated centrifuge tubes. The 1 ml of petroleum ether on top of the first column to be loaded was then blown down just to the top of the silicic acid by means of a rubber bulb. The lipid extract was rapidly

taken up in a 200-μl Lang-Levy pipet, applied to the top of the column, and forced onto the silicic acid by means of the rubber bulb. The centrifuge tube and pipet were rinsed with the two 200-μl aliquots of petroleum ether, each of which was applied to the column as above. When the last wash was forced onto the silicic acid, 15 ml of the first eluant was applied, and the next column was prepared for loading. The ten columns could be loaded and ready with the first eluant in 15–30 min.

When all columns were loaded, they were attached to the manifold, and nitrogen pressure was gently applied. This was then adjusted so that the entire 15 ml would be eluted in approximately 30 min. The fractions were collected in 25-ml Erlenmeyer flasks. Figure 2 shows the columns during a typical lipid fractionation sequence.

Four fractions were collected, using 15-ml portions of each of four different eluants [modification of Fillerup and Mead (4)].

Fraction I, 1% ethyl ether (Bakers Analyzed reagent, anhydrous) in petroleum ether (cholesterol ester).

Fraction II, 4% ethyl ether in petroleum ether (triglycerides).

Fraction III, 50% ethyl ether in petroleum ether (free cholesterol, free fatty acids, and mono- and diglycerides).

Fraction IV, absolute methanol (phospholipids).

RESULTS

In preliminary analyses of lipid mixtures of known concentrations, each fraction was analyzed for cholesterol and phosphorus. Only fractions I and III were found to contain cholesterol, and phosphorus was found only in fraction IV. Each fraction was collected in four 5-ml portions, and in each case the entire lipid component was found in the first two. Since no lipid was found in either subsequent 5-ml portion in any case, 15 ml was arbitrarily chosen as ample for complete separation of the fraction.

Table 1 shows cholesterol recovery from ten different plasma lipid extracts fractionated by this method. An aliquot of each original extract was saponified, and the total cholesterol was determined by the method of Sperry and Webb (9), after which the remaining extract was chromatographed on the silicic acid microcolumns, and the cholesterol in fractions I and III was determined after saponification by the same method. As may be seen in the table, the recovery ranged from 94 to 106%, with an average of 99%.

Table 2 shows an example of recovery of individual fatty acids from all four fractions as compared with the data obtained by determining the total plasma fatty acids from one rat. Total fatty acid was obtained by saponification of an aliquot of the original extract, removal of the fatty acids, followed by their methylation and separation by gas–liquid chro-

TABLE 1

TYPICAL RECOVERIES OF PLASMA CHOLESTEROL FROM SILICIC ACID MICROCOLUMNS[a]

Animal No.	Cholesterol recovery from fractions (mg/100 ml plasma)			Cholesterol recovery from unfractionated extract (mg/100 ml plasma)	
	Ester	Free	Total = ester + free	Total as analyzed	Per cent recovery
25	34	11	45	48	94
55	124	19	143	148	97
58	206	36	242	241	100
73	63	10	73	76	96
79	75	12	87	92	94
85	45	14	59	56	105
106	37	15	52	54	96
127	33	10	43	44	98
133	136	26	162	161	101

[a] Aliquots of each extract were saponified and total cholesterol was determined by the method of Sperry and Webb (9). The remainder of each extract was chromatographed on the silicic acid microcolumns, and the cholesterol in fractions I (cholesterol ester) and III (free cholesterol) was also determined after saponification by the same method. The wide fluctuations in the ratio of ester to free cholesterol are due to dietary differences, the animals with high ester cholesterol being fed 1% cholesterol. These widely varying examples were chosen to illustrate the capacity of the system.

TABLE 2

RECOVERY OF PLASMA FATTY ACIDS FROM A SINGLE SILICIC ACID MICROCOLUMN

Fatty acid recovery from fractions	Fatty acid (mg/100 ml plasma)							
	C_{12}–C_{16}	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	Arachidonic	Total
Fraction I	1.0	2.8	4.7	0.2	7.2	4.1	3.5	23.5
Fraction II	1.7	8.6	4.4	1.8	11.8	3.9	—	32.2
Fraction III	1.0	3.0	1.7	0.4	3.0	1.1	—	10.2
Fraction IV	0.8	9.9	3.4	7.1	10.9	10.7	4.7	47.5
Total = sum of fractions	4.5	24.3	14.2	9.5	32.9	19.8	8.2	113.4
Total fatty acid recovery from unfractionated extract	4.8	22.9	15.7	8.5	34.2	19.4	9.0	114.5
Per cent recovery	94	106	90	111	96	102	91	99

matography.[4] The remaining lipid extract was chromatographed on the silicic acid microcolumn, the fractions were saponified where necessary, and the fatty acids were extracted, methylated, and determined by gas–liquid chromatography. The triglyceride (Fraction II) and phospholipid (Fraction IV) were methylated without previous saponification since it was found that fatty acid, and in the latter case, phosphorus, recoveries were quantitative with or without previous saponification. The estimates of fatty acid quantities were made using gas–liquid chromatography, by comparison of areas obtained with known amounts of known compounds.

Parallel separations were made on large columns (250 mg. capacity) and on the microcolumns (1–10 mg. capacity) on aliquots of liver lipid extracts. The separations and purities of the fractions obtained on the large columns by the method of Fillerup and Mead (4) were confirmed in our laboratory using known compounds. The fatty acid, cholesterol, and phosphorus analyses obtained on the microcolumns coincided with those obtained on the large columns. Also, quantities were sufficient on the large columns for the determination of lipid recoveries of all fractions by the method of Bloor (11) and were found to be quantitative. Therefore the recoveries and separations on the microcolumns can be confidently accepted.

DISCUSSION

The method has been found to be eminently satisfactory for convenient and rapid fractionation of a number of lipid samples at one time. The entire procedure can be handled conveniently by one person. The method has been described in detail, including sizes of glassware and pipets used, and transfer procedures which have been found to be of primary importance in achieving quantitative lipid recoveries.

Although other methods are available for separation of lipid extracts into more components, this method is considered to be of particular interest due to the suitability of the fractions for further analysis by gas–liquid chromatography. With 1–10 mg lipid, each fraction contains adequate lipid for determinations of the fatty acids by gas–liquid chromatography and the other components by chemical analysis (7).

SUMMARY

A micromethod has been presented for separating, by silicic acid chromatography, 1–10 mg of a complex mixture of lipid of biological

[4] Gas–liquid chromatography was done on a Wilkins Instrument Company Aerograph, Model A-110-C equipped with a thermal conductivity detector and polyester (diethylene glycol succinate on firebrick) packing, operated at 180–210°C., 100 ml He/min. Fatty acids were methylated by the method of Michaels (10).

origin into four fractions: I, cholesterol ester; II, triglycerides; III, free cholesterol, free fatty acids, and mono- and diglycerides; and IV, phospholipids.

The method was found to be rapid, convenient in handling, quantitative, and eminently suitable for further analysis of the fractions by gas–liquid chromatography.

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Microdetermination of Dihydroxyacetone in Biological Fluids

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INTRODUCTION

In spite of the numerous color reactions described for dihydroxyacetone and other trioses, there are only a few procedures reported for the determination of dihydroxyacetone in biological fluids. Among these procedures, the colorimetric or titrimetric methods seemed feasible for micro adaptation. However, Rabinowitch has shown the discrepancy of the early methods (1–3). Other colorimetric or titrimetric methods (4–6) based on the nonspecific reducing property of dihydroxyacetone also are unsatisfactory for the simple reason that an arbitrary blank is, or should be, included for the calculation. Since this triose cannot be quantitatively converted into pyruvaldehyde (7), the more sensitive and specific procedures for the latter cannot be utilized. A relatively specific method for the determination of low concentrations of dihydroxyacetone in biological fluids from small animals was needed for a metabolic study after administration of this substance. The procedure described in this paper has been developed to meet this need and has been satisfactory in our hands.

The blood is deproteinized with sulfosalicylic acid. The filtrate or a urine sample, after adjustment of pH, is incubated in a buffered glucose oxidase solution. The product is allowed to react with 2,4-dinitrophenylhydrazine. The excess reagent is removed by the addition of pyruvic acid and partition in benzene–sodium bicarbonate solution. The benzene layer containing the osazone of dihydroxyacetone is washed with sodium bicarbonate solution to remove the hydrazone of pyruvic acid. After benzene is removed by evaporation, the residue is taken up in alcoholic sodium hydroxide, and the resultant color is read. Since dihydroxyacetone is easily destroyed by strong alkali, interfering chromogens when present can be measured and subtracted from the results by running a sample or filtrate treated with alkali after the incubation with glucose oxidase.

REAGENTS

Sulfosalicylic Acid. A 3% solution prepared once every 2 weeks from AR grade (Mallinckrodt).

Glucose Oxidase Mixture. Prepared by mixing 0.5 gm glucose oxidase (Sigma Chemical Co.), in 5 ml water, 2.5 ml of 2.5 *M* phosphate buffer, pH 6.0, and 2.5 ml of 0.4% disodium ethylenediamine tetraacetate (Eastman Kodak) solution.

Metaphosphoric Acid. A 10% solution prepared fresh weekly.

2,4-Dinitrophenylhydrazine. 2,4-Dinitrophenylhydrazine (Eastman Kodak) is dissolved in hot 2 *N* alcoholic HCl. The crystals of the hydrochloride are recrystallized three times, dried in a desiccator, and kept refrigerated. A solution is prepared fresh weekly by shaking 100 mg of the hydrochloride in 20 ml of 2 *N* HCl and filtering.

Pyruvic Acid. Pyruvic acid (Paragon) is distilled under reduced pressure two or more times until crystalline material is obtained in the ice-chilled receiver. A 2% aqueous solution is prepared from the product kept at 0°.

Benzene. Thiophene-free reagent benzene (Merck) is allowed to stand over the 2,4-dinitrophenylhydrazine reagent for several days with occasional shaking in a separatory funnel. After removal of the acid solution, the solvent is then extracted with concentrated sulfuric acid until the acid is colorless. Then the benzene layer is washed with distilled water until the water is neutral. The washed benzene is distilled and the fraction boiling at 77–78° at 731 mm. Hg. is collected.

Sodium Bicarbonate. A 5% solution is prepared from AR grade sodium bicarbonate (Mallinckrodt or Baker & Adamson) and filtered.

Acetic Acid. A 0.2% solution is prepared from reagent-grade glacial acetic acid (Baker and Adamson).

Benzyl Alcohol. Benzyl alcohol (Eastman Kodak) is first distilled over 2,4-dinitrophenylhydrazine and then redistilled under reduced pressure.

Color Reagent. A mixture consisting of 1 part of 2.5 *N* sodium hydroxide, 2 parts of distilled water, and 7 parts of 95% ethanol is prepared just before use.

Sodium Hydroxide, 10 N. This is prepared by diluting saturated NaOH (18 *N*).

Lloyd's Reagent.

PROCEDURE

Blood

To 7.5 ml sulfosalicylic acid in a 15-ml centrifuge tube is added 1.5 ml of blood sample. The mixture is stirred immediately and thoroughly, using a glass rod. It is centrifuged at 375 $\times$ *g* for 15 min. Five milliliters of the

supernatant are pipetted into a 15-ml glass-stoppered centrifuge tube. The pH is adjusted to between 6.0 and 7.0 by using approximately 0.05 ml of 10 *N* sodium hydroxide. Then 0.2 ml of the glucose oxidase mixture is added, and the tube is shaken for an hour on a mechanical shaker at approximately 210 oscillations/min. The tube is then centrifuged for 5 min at 375 $\times$ *g*.

Into each of three 15-ml glass-stoppered centrifuge tubes is pipetted 0.3 ml of the supernatant fluid. The contents of the tubes are acidified with 0.1 ml of concentrated hydrochloric acid. To the remaining supernatant fluid is added sufficient 10 *N* sodium hydroxide (approximately 0.16 ml) to bring the pH to 13.0–13.5. The alkaline solution is warmed at 57° for 5 min, and 0.3 ml of the solution is pipetted into three more centrifuge tubes, each followed with 0.1 ml of concentrated hydrochloric acid. Then 0.3 ml of 2,4-dinitrophenylhydrazine hydrochloride solution is added to each of the six tubes. After mixing, the tubes are placed in a 57° water bath for 17 hr. At the end of the reaction, 0.5 ml pyruvic acid solution is added to all the tubes, and the contents of the tubes are mixed and allowed to react at 57° for 30 min. The reaction products are extracted by vigorous shaking with 5.5 ml benzene in each tube. The aqueous layer is subsequently removed by a 10-ml syringe with a 5-in. needle. The exterior of the needle is washed with a little benzene into the extract after each use. The upper layer is extracted three times with 5 ml of 5% sodium bicarbonate to remove the derivative of pyruvic acid. The benzene layer was extracted once with 3.6 ml of 0.2% acetic acid; 0.1 ml benzyl alcohol was added to the benzene layer, and the latter was evaporated to near dryness with an air stream.

To the residue in each tube is added 2 ml of color reagent; the color developed is read at 586 mμ. The difference between the filtrate without alkali and the filtrate after the alkali treatment gives the color due to dihydroxyacetone. The volume of the color reagent may be changed to yield a suitable absorbance reading. Blanks can be prepared by using water; or, the standard, after treatment with alkali, can be used as a blank. The results have been found to be the same.

A standard solution of dihydroxyacetone of the same concentration range as the samples is prepared and processed with the samples. The linear relationship between absorbance of the final solution and the concentration, as will be seen later, allows one to calculate the concentration of dihydroxyacetone in the samples either with a simple factor or from a straight-line standard curve.

Urine

The pH of a fresh urine sample is adjusted to 6.0–7.0 with 10 *N* sodium hydroxide, and treated with Lloyd's reagent. The treated urine is trans-

ferred into another stoppered centrifuge tube to be shaken with the glucose oxidase mixture as described for blood. One milliliter of the supernatant fluid is pipetted into 5 ml of 10% metaphosphoric acid in a centrifuge tube, allowed to stand for 30 min, and centrifuged at 375 $\times$ g for 15 min. The remaining supernatant fluid is alkalinized as described for blood filtrate, and then 1 ml is pipetted into metaphosphoric acid. The acidic supernatant fluids are processed exactly in the same manner as for acidified blood filtrate. A standard of same concentration range of the samples is run with the latter.

Tissue Homogenate

The procedure for blood may be applied without modification.

EXPERIMENTAL

It was observed at the beginning that two factors could render the principle of the proposed procedure inoperative, namely, the glucose present in most samples and an unknown chromogen that gives a red color to the final solution. The formation of an osazone by glucose under the experimental conditions contributes a considerable absorbance reading. However, if the concentration of glucose in the sample is less than 0.3 mg/ml the interference becomes insignificant. The problem then simplified itself to that of removal of glucose to below that limit. Attempts were made to correct the interference of the red chromogen by readings taken at different wavelengths without success. The easy destruction of dihydroxyacetone in alkali was then utilized to remove it, and the difference in absorbance readings before and after the alkali treatment would then give the absorbance corresponding to dihydroxyacetone. The red chromogen was apparently not affected by the alkali treatment.

Glucose Elimination by Oxidase

The conditions for the effective reduction of the concentration of glucose was established by testing various deproteinizing agents for the blood sample and observing the level of glucose after shaking with oxidase. Sulfosalicylic acid was found to be an agent that does not inactivate the enzyme while metaphosphoric acid or trace of barium hydroxide–zinc sulfate does. Assuming the glucose concentration in an average sample to be below 2.0 mg/ml, the amount of enzyme required to remove this amount of glucose after 30 min of shaking was established; then, the time of shaking with enzyme was extended to just before the triose level was affected. Since dihydroxyacetone is readily oxidized only in alkaline solution, the removal of glucose by oxidase in an acid medium minimizes the loss due to air oxidation during the shaking.

Correction for Red Chromogen by Alkali Treatment

Neutral carbonyl compounds other than dihydroxyacetone, glyceraldehyde, or pyruvaldehyde are obviously present in biological fluids and react with 2,4-dinitrophenylhydrazine to yield a red color (when present) in alcoholic alkali. Numerous attempts were made to correct this interference by reading the final color at various wavelengths. Factors and equations were empirically established to calculate the dihydroxyacetone value, but none were found satisfactory. The difficulty in eliminating this interference, either experimentally or by calculation, led to the removal of the triose with alkali as a solution of the problem. The pH of dihydroxyacetone solution was adjusted to 13, and aliquots were removed at 5-min intervals soon after the temperature was raised to, and maintained at 57°. The samples were neutralized immediately, and the remaining triose was determined. It was found that all the triose had been destroyed with 5 min. The amount of red chromogen in blood was generally found to be far less than that in urine.

Linearity of Standard Curve

The readings at 586 mμ in alcoholic alkali of various amounts of 2,4-dinitrophenylosazone of dihydroxyacetone equivalent to 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, and 2.0 μg/ml of the triose were obtained. These readings were found to bear a linear relationship to the concentration of the substance. Solutions containing dihydroxyacetone in 0.01, 0.05, 0.10, and 0.25 mg/ml amounts were analyzed by the complete procedure described; the absorbance readings were found to be proportional to the concentration of the triose.

Reproducibility of the Procedure

Three blood samples and four urine samples were each divided into six portions; each portion was then analyzed. The standard deviations were calculated by the equation:

$$\text{S.D.} = \sqrt{\frac{(a-\bar{a})^2}{N-2}}$$

For the blood samples the S.D. were: 0.6, 1.5, and 1.3 μg/ml. For the urine samples: 3.0, 2.2, 2.2, and 1.3 μg/ml.

Recovery of Added Dihydroxyacetone

Measured amounts of dihydroxyacetone were added to blood and urine samples. Triplicate analyses that were made of the samples before and after the addition of dihydroxyacetone are shown in Table 1.

TABLE 1
RECOVERY OF ADDED DIHYDROXYACETONE

Amount of DHA added (μg/ml)	Per cent recovered	
	Blood	Urine
10	105, 100, 100	100, 90, 95
20	101, 100, 88	96, 91, 84
30	103, 99, 100	94, 88, 87
100	101, 89, 97	95, 89

Interfering Substances

The partition of osazones between benzene and sodium bicarbonate solution serves to eliminate a large group of interfering substances, namely, the keto acids. Among the neutral carbonyl compounds, acetone does not contribute to the absorbance except in extremely high concentrations. Glyceraldehyde and pyruvaldehyde when present are not distinguishable from dihydroxyacetone. However, if necessary, pyruvaldehyde osazone may be separated from the osazone of dihydroxyacetone, which is identical with that of the glyceraldehyde, by paper chromatography (8). The red chromogen which is the major source of interference has not yet been identified in spite of a large number of neutral carbonyl compounds including methyl ethyl ketone, methyl *n*-propyl ketone, methyl *n*-butyl ketone, methyl *n*-pentyl ketone, formaldehyde, acetaldehyde, propionaldehyde, estrone, and dehydroisoandrosterone that were tested to reproduce the color and R_f on paper chromatography.

Monosaccharides tested gave color similar to that of dihydroxyacetone but most of them are removed by alkali treatment. At a concentration level of 200 μg/ml, fructose (recrystallized), xylose (Pfanstiehl), arabinose (Matheson), rhamnose (Fisher), galactose (Eastman), and mannose (Fisher) did not interfere. Ribose (Nutritional Biochemical) at 200 μg/ml gave the equivalent of 1.3 μg/ml dihydroxyacetone, and erythrose (California Corp. for Biochemical Research) gave the equivalent of 18.4 μg/ml.

Preservation of Samples

Since dihydroxyacetone is relatively labile even in neutral solution, no sample should be kept overnight even in the refrigerator. However, urine samples have been quickly frozen and kept at −10° for 24 hr without appreciable change in the results. The blood cells metabolize this substance fairly readily at room temperature; therefore, immediate processing of the sample should be started as soon as possible.

SUMMARY

Dihydroxyacetone is reacted with an excess of 2,4-dinitrophenylhydrazine. The excess reagent is removed by the addition of pyruvic acid and followed by partition between benzene and sodium bicarbonate solution. The benzene layer containing the 2,4-dinitrophenylosazone of dihydroxyacetone is evaporated, and the color developed with the addition of alcoholic sodium hydroxide is measured. Glucose, an interfering material in blood or urine, is removed by an oxidase. Other interference is corrected by the removal of dihydroxyacetone with alkali. Effective range of this method is from 5 μg dihydroxyacetone. The precision of the method has been measured.

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The Estimation of Urinary Metabolites of Administered Estradiol-17 β-16-C^{14} [1]

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INTRODUCTION

Although methods of high precision and accuracy are now available for the estimation of urinary estrone, estradiol-17β, and estriol (1–3), the isolation within the last few years of new phenolic steroids from the urine of pregnant women and subjects to whom labeled estrogens have been given has prompted a re-examination of the methods employed for the measurement of urinary estrogen metabolites. The new substances which have been added to the classical compounds estrone (4, 5), estradiol-17β (6), and estriol (7) include 16-epiestriol (8), 16α-hydroxyestrone (9), 16β-hydroxyestrone (10, 11), 2-methoxyestrone (12–14), 2-methoxyestradiol (15), 2-methoxyestriol (16), 18-hydroxyestrone (17), 16-ketoestrone (18), and 16-ketoestradiol (19). The conventional procedures for hydrolysis of conjugates which involve treatment with mineral acid at elevated temperatures and the subsequent treatment with strong base must be replaced by processes which are less damaging to sensitive compounds such as the α-diketone, the α-ketols, 18-hydroxyestrone, and the three catechol methyl ethers. Furthermore, the procedure should be so designed as to make it possible to accommodate new compounds as they are discovered. It is desirable also to reduce the number of steps involved in the preparation of extracts. This has the dual advantage of subjecting

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sensitive compounds to the minimum manipulation and of decreasing the mechanical losses which are inevitable in any complex procedure.

While the objectives outlined above have not been attained, progress has been made in this laboratory in the development of a procedure by means of which urinary metabolites of administered C^{14}-labeled estrogens may be studied in a convenient manner. The method as it is presently employed is designed exclusively for the measurement of metabolites of radioactive estrogens, but it may be anticipated that with suitable modifications, it will be applicable to the estimation of unlabeled estrogens.

The procedure has the following steps: (*a*) the measurement of urinary radioactivity, (*b*) the hydrolysis of the steroid conjugates, (*c*) purification of the lipid-soluble extract by an ion-change resin, and (*d*) the separation of five zones containing as principal constituents largely single compounds and two zones containing groups of compounds by means of a gradient-elution partition chromatogram. These procedures have been developed using urine of patients treated with radioactive estradiol.

METHODS

Reagents

All reagents were analytical grade and all solvents were distilled before use.

Measurement of Radioactivity

The samples were transferred to stainless steel cupped planchets, 1 in. in diameter, $\frac{5}{16}$ in. deep, in either water or ethanol, and the solvent was evaporated, either spontaneously or with the aid of an infrared lamp. Counting was done in a Nuclear windowless gas-flow counter operated in the Geiger region. Sufficient counts were collected to insure an accuracy of $\pm 5\%$.

Self-absorption was corrected by one of two methods. In the first, aliquots of decreasing volume were counted until a level was reached at which the count was proportional to the volume of the aliquot. This method was satisfactory only for relatively high levels of radioactivity.

In the second method an internal standard was used. For each urine sample three series of planchets were prepared in triplicate as follows: Three planchets received 1.0 ml urine. A further 3.5 ml urine was added to 0.7 ml of a standard leucine-1-C^{14} solution containing 10,000 counts/min/ml, and triplicate samples of 1.2 ml of this urine–leucine mixture were pipetted into the second three planchets. The remaining three planchets received 0.2 ml of standard leucine solution and 1.0 ml of distilled

water. All samples were dried under an infrared lamp at a distance of about 2 ft, and 10,000 counts were collected.

After subtracting background, the corrected counts/min/ml of urine were calculated from the relation given below.

$$\text{Urine counts/min/ml (corrected)} = \frac{\text{leucine counts/min} \times \text{urine counts/min (observed)}}{(\text{urine} + \text{leucine counts/min}) - \text{urine counts/min (observed)}}$$

Self-absorption of lipid-soluble extracts and more highly purified fractions was measured in a similar fashion using ethanol solutions and an ethanol solution of cholesterol-C^{14} as internal standard.

Hydrolysis of Conjugates and Preparation of Lipid Extract

A 100-ml portion of urine was adjusted to pH 6.5, and 20 Fishman units (20) of *Escherichia coli* β-glucuronidase (Sigma Chemical Co.) per milliliter was added; the solution was saturated with chloroform and incubated at 37° for 48 hr. After cooling, the urine was extracted with five 30-ml portions of ethyl acetate saturated with water. The ethyl acetate extract was then washed twice with saturated sodium bicarbonate solution (10 ml) and twice with water (5 ml), evaporated to dryness, and the residue was dissolved in 95% ethanol for counting.

Solvolysis was done by the method of Burstein and Lieberman (21).

Purification of Phenolic Fraction

A Dowex 2 column was prepared by the following method: 10 gm of moist Dowex 2-Cl^- (moisture content 39–45%, 40–80 mesh, 8% cross-linking, obtained as Nalcite SAR-8 from the National Aluminate Co.) were transferred to a column of the dimensions and form shown in Fig. 1 as a slurry in 4% sodium bicarbonate solution. The column was washed with about 350 ml of 4% sodium bicarbonate at a rate of 1–2 ml/min until the effluent was chloride-free. The column was then washed with distilled water until the effluent was neutral. This usually required about 200 ml at a flow rate of 1–2 ml/min. The column was then washed with 350 ml methanol at a rate of 1–2 ml/min.

The preparation of the resin column was begun in the morning, and the final methanol washing was allowed to flow through overnight. The extract was applied the next morning, as the bicarbonate form of the resin decomposed on longer standing. The resin bed was not allowed to dry; if it did, it was discarded.

The methanol level in the column was allowed to flow just to the top of the resin bed, and the sample in 1–2 ml methanol was applied to the column. When the sample had flowed into the column, it was rinsed in

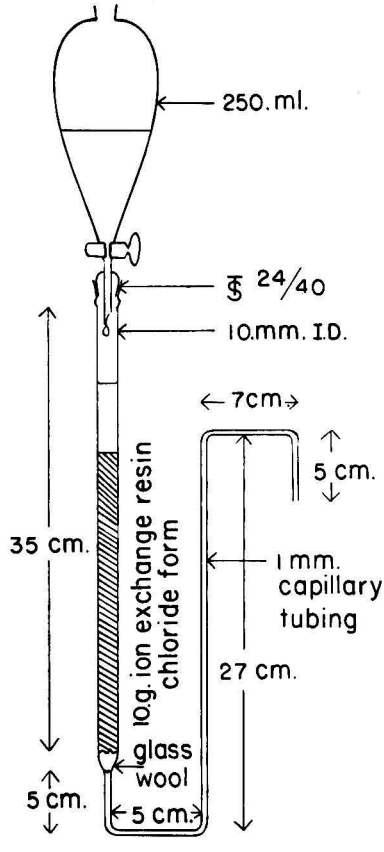

FIG. 1. Apparatus for ion-exchange separation of neutral from phenolic metabolites of estradiol-17β-16-C^{14}.

with five 1–ml portions of methanol which had been used to rinse the flask or tube in which the sample had been stored. The walls of the column were rinsed down with the last washing. Methanol (10 ml) was added to the top of the resin bed, and a reservoir containing 50 ml methanol was attached to the top of the column. The column was then washed with methanol at the rate of 1–2 ml/min to give a total volume of 70 ml. This effluent contained the neutral fraction. The reservoir was then loaded with 150 ml of 5 *N* acetic acid in methanol, and this solution was allowed to flow slowly into the column, the rate of flow being determined by the vigor with which CO_2 evolved, but it did not exceed 1 ml/min. When the CO_2 evolution ceased, the reservoir was attached to the column and the methanolic acetic acid was allowed to flow through at a rate of 1 ml/min.

The methanolic acetic acid (150 ml) which contained the phenolic fraction was evaporated to dryness under reduced pressure using methanol to remove the last traces of acetic acid.

Partition Chromatography

Gradient elution partition chromatography was performed in the apparatus shown in Fig. 2. The Hyflo-Supercel (Johns Manville Co.)

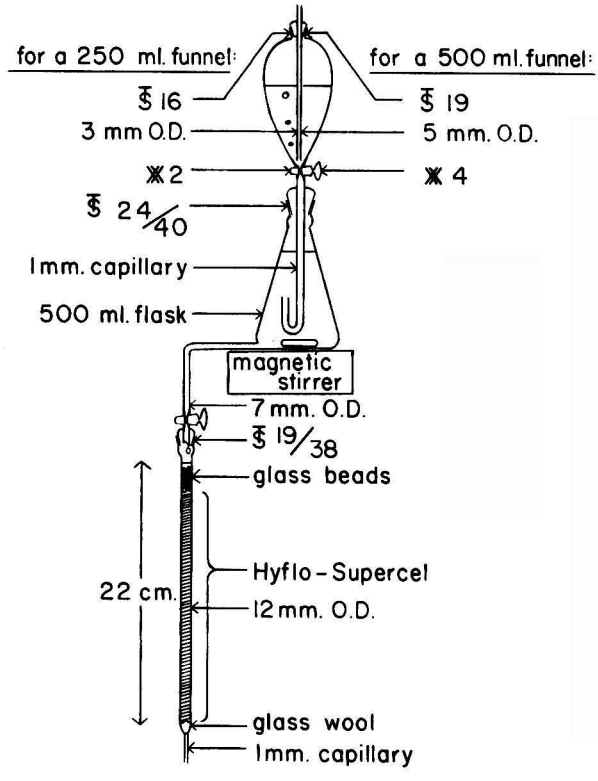

FIG. 2. Apparatus for gradient elution partition chromatography of urinary metabolites of estradiol-17β-16-C^{14}.

used in the preparation of the partition column was treated with concentrated HCl to remove metals, then washed by elutriation until chloride-free. In this process a large amount of extremely fine material was also removed. This improved the quality of the Supercel for chromatographic columns. Finally the Supercel was washed with absolute methanol, and with 2,2,4-trimethylpentane, and dried at 60°.

Solvent System. The solvent system employed was prepared by mixing 1 l of 90% methanol–10% water with 2 l 2,2,4-trimethylpentane. After agitation the mixture was allowed to equilibrate overnight at room temperature.

Preparation of Column. A 5-gm sample of washed Hyflo-Supercel was weighed into a 100-ml beaker and treated with 3 ml of the lower phase (90% aqueous methanol) prepared as above. The mixture was stirred until the solvent had been completely worked in. Upper phase (trimethylpentane) was then added to make a thin slurry. After inserting a small plug of glass wool into the bottom of the chromatographic column, a small amount of the slurry was added to the column and packed with a stainless steel perforated packer which fitted the column snugly. The packer was moved up and down slowly to remove air bubbles, and finally pressed down firmly. Successive portions of slurry were added and packed. Care should be taken to exert even pressure during the packing and to maintain a head of trimethylpentane above the column at all times. After the column had been packed, pre-equilibrated trimethylpentane was allowed to flow through by gravity overnight in order to condition it.

Determination of Column Volume and Testing of Column

The trimethylpentane above the packing was allowed to flow in just to the level of the column, and 0.2 ml of a solution of Sudan IV (0.05%) in trimethylpentane saturated with 90% methanol was applied to the top of the column. After this had flowed into the packing, 2–3 ml trimethylpentane was added to the column, and the reservoir was reconnected. The effluent was collected in a 10-ml cylinder, and the volume at which the dye began to come through was recorded. This was the column volume, and represented the volume of mobile phase contained in the column. The column was observed while the dye was flowing through to make certain that it moved down in a compact, discrete band. If the dye band spread excessively, or if it trailed or moved down unevenly, the column was discarded.

Application of Sample. The sample to be applied to the column was transferred to a small test tube and evaporated to dryness, and the residue was dissolved in 0.09 ml of pre-equilibrated 90% methanol. Then 150 mg of Hyflo-Supercel was added, and the mixture was stirred thoroughly to insure an even distribution of sample. If the sample did not dissolve in this quantity of 90% methanol, more solvent and correspondingly more Hyflo-Supercel were employed. The Supercel containing the sample was then packed into the column, a small amount of trimethylpentane being maintained above the packing at this time. The container which held the sample was rinsed with a small amount of trimethylpen-

tane, and the rinsings were added to the column. Glass beads were placed in the column above the packing to reduce the dead space.

The Chromatogram. Pre-equilibrated trimethylpentane, 500 ml, was placed in the lower reservoir, which was then attached to the top of the chromatographic column as shown in Fig. 2. The upper reservoir was filled with trimethylpentane, and the constant pressure device was attached. The column was started by opening the stopcocks between the two reservoirs and between the lower reservoir and the column, and about 40 fractions of 1 column volume each were collected. The flow rate was about 10–15 ml/hr.

After 40 fractions were collected, the column was stopped and the contents of the upper reservoir were replaced with a 1:1 mixture of 1,2-dichloroethane and trimethylpentane. Eighty additional fractions of one column volume each were collected. Finally, very polar metabolites were removed from the column by stripping it with 95% ethanol (30 ml).

Analysis of Eluate. The fractions were taken to dryness, the residues were dissolved in 95% ethanol, and the aliquots were transferred to planchets for counting.

RESULTS AND DISCUSSION

Self-Absorption

Corrections for self-absorption were always applied when the radioactivity of urine was assayed. The amount of self-absorption was very variable, both from one patient to another and for different specimens from the same patient. It did not appear to be related in any simple way to the total solids content of the urine.

Self-absorption was negligible in the residue from the ethyl acetate extracts of urine but was significant in the methanol fractions from the resin columns.

Hydrolysis of Urinary Conjugates

Using β-glucuronidase of bacterial origin, 74% of the radioactive compounds were rendered extractable by ethyl acetate (Table 1). This yield was very inconstant, not only from patient to patient, but also among

TABLE 1

HYDROLYSIS OF CONJUGATED METABOLITES OF ESTRADIOL-C^{14}

Procedure	Yield (% or urinary C^{14})	Range (%)	No. of samples
Bacterial β-glucuronidase	74	42–99	29
Solvolysis[a]	11	7–17	6

[a] Solvolysis was applied after treatment of the urine with glucuronidase and extraction of the steroids liberated.

urine samples from the same patient, and ranged from 40 to 100%. The great variability in the yield of radioactivity from these urine specimens probably reflects variation in the nature of the estradiol metabolites or their conjugation, rather than incomplete cleavage of glucosiduronates by the enzyme. In other experiments, retreatment of the urine with additional portions of enzyme or prolongation of the incubation period failed to improve the yield. Application of the solvolytic procedure of Burstein and Lieberman (21) yielded an additional 10% of the urinary radioactivity in an ethyl acetate-soluble form.

No combination of methods tried gave a quantitative yield of radioactive metabolites in a lipid-soluble form. The incomplete hydrolysis of conjugates and extraction clearly are important limitations to the usefulness of the present and of other existing methods designed to measure total estrogen metabolites.

Ion-Exchange Separation of Neutral from Phenolic Material[4]

The results obtained over a period of several years by three different operators using the ion-exchange procedure for the separation of neutral and phenolic material are shown in Table 2. It is evident that a uniformly

TABLE 2
RECOVERY OF C^{14} FROM RESIN COLUMNS

Operator	Mean recovery in acetic acid	S.D.[b]	No. of columns	Mean recovery in methanol	S.D.
A1	89.0	5.63	24		
A2	88.6	6.99	16		
B	89.8	3.18	11	1.3	0.91
C	84.9	8.38	17		
Total[a]	88.0	6.30	68		

[a] Analysis of variance shows that the between operator differences are not significantly different from the within operator differences.

[b] In this and subsequent tables S.D. is standard deviation.

high proportion of the radioactivity present in the crude extracts was phenolic in nature and could be separated effectively by this procedure. In carrying out this process it was important to use an extract which had been thoroughly washed with bicarbonate, since a large amount of carboxylic acid present in the extract overloaded the resin column and allowed phenolic material to pass into the methanol fraction. That this

[4] Since submission of this paper, the authors' attention was called to the publication of Sjöström and Nykänen [*Suomen Kemistilehti* **29B**, 23 (1956); *C. A.* **51**, 2104*i* (1957)] in which was reported the use of anion-exchange resins for the separation of phenolic from neutral steroids.

procedure did in fact separate phenolic from neutral material was demonstrated by model experiments carried out on extracts of the urine of patients treated with radioactive neutral steroids such as 11β-hydroxy-4-androstene-3,17-dione and cortisol. In these instances the overwhelming proportion of the radioactivity was eluted from the column in the neutral methanol fraction.

The form of the resin did not appear to be important, as both the hydroxyl and the bicarbonate forms performed equally well. However, in order to minimize exposure of the sensitive estradiol metabolites to strong base, the bicarbonate form was selected for the standard procedure.

Partition Chromatography

The solvent system employed in the partition columns is essentially that designed by Aitken and Preedy[5] (22), modified for the higher temperature prevailing in the laboratory.

Uniformity of Column Packing. The measured column volumes for 28 columns were analyzed to determine whether two different operators consistently packed columns with different characteristics. It was found that the two operators in question packed columns with significantly different column volumes (7.1 ± 0.66 and 8.7 ± 0.36 ml), but that each achieved a high degree of uniformity in both the packing and in the performance of the column.

Column Characteristics. The performance of the partition columns was then studied from the standpoint of the positions of the peaks and, where applicable, the percentage of dichloroethane in the eluting fluid at the time when a peak of a radioactive metabolite passed into the effluent. In view of the fact that different operators packed columns with different characteristics, it seemed inappropriate to compare the performance of columns packed by different persons. The data in Table 3 are therefore presented to indicate the uniformity with which this system worked in the hands of individual operators. The position of a compound in the effluent from the chromatogram is given in terms of two parameters: (*a*) the number of column volumes which passed through the column at the time of the appearance of the maximum concentration of the solute, and (*b*) the calculated percentage of dichloroethane, if any, in the moving phase. This quantity was calculated from the equation of Lakshmanan and Lieberman (23).

As can be seen from Fig. 3, which depicts a typical chromatogram, the separation of the peaks was satisfactory and, except in the region between

[5] The authors are grateful to Dr. J. R. K. Preedy for generously making available to them details of his method prior to full publication.

TABLE 3
CHARACTERISTICS OF PARTITION COLUMNS

Compound operator	Column volumes		No. of columns	$(CH_2Cl)_2$ (%) at peak	S.D.
	Mean peak	S.D.			
2-Methoxyestrone peak (1)[a]					
Operator A	5.5	0.88	9		
Operator B	7.3	0.53	14		
Operator C	5.2	1.5	14		
Estrone peak (2)					
Operator A	19	5.2	9		
Operator B	29	2.6	14		
Operator C	19	4.6	14		
Estradiol peak (3)					
Operator A	25	3.8	9	15	1.3
Operator B	27	2.9	12	15	1.4
Operator C	21	2.3	14	16	1.5
Middle peak (4)					
Operator C	27	2.4	12	20	1.5
Epiestriol peak (5)					
Operator A	36	3.4	8	19	1.0
Operator B	39	3.4	12	21	1.6
Operator C	32	2.9	13	23	1.5
Estriol peak (6)					
Operator A	57	7.8	7	27	4.4
Operator B	60	1.8	12	28	1.6
Operator C	50	3.6	12	32	4.4

[a] Numbers refer to peaks in Fig. 3.
[b] Renumbering of the eluate fractions is begun when the gradient is applied (see Fig. 3).

estradiol and epiestriol, the level of radioactivity between peaks approached zero.

Recovery of C^{14} from Partition Columns. In Table 4, the total recovery

TABLE 4
RECOVERY OF C^{14} FROM PARTITION COLUMNS

Operator	Total recovery (%)	S.D.	C^{14} between peaks (%)	S.D.	Range (%)	No.
B	88	4.8	5	2.0	1.7–9.9	17
C	74	9.2	6	4.3	3–16	14

of the radioactivity applied to the partition columns is presented. It will be noted that most of the C^{14} was accounted for by discrete peaks and the column stripping. The C^{14} constituting the pool of the tubes between the peaks contributed only about 5%.

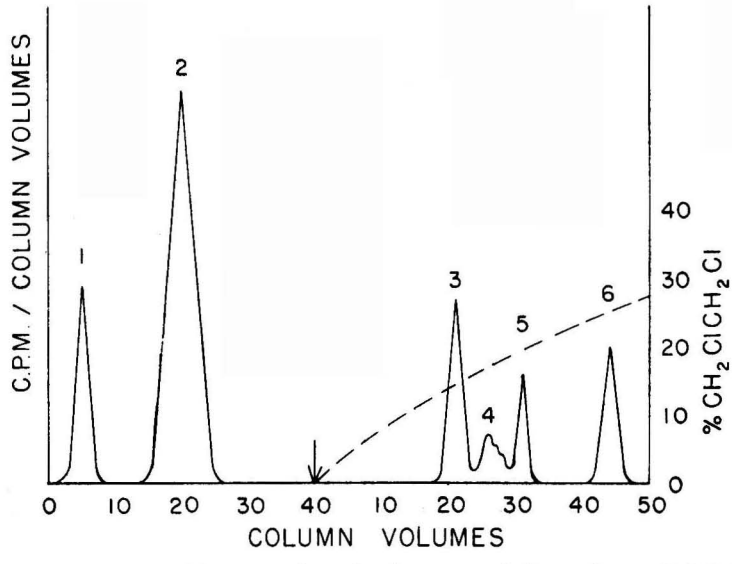

FIG. 3. Chromatographic separation of urinary metabolites of estradiol-17β-16-C^{14}. The left-hand ordinate is counts/min/column volume. The abscissa is column volumes, the arrow indicating the point at which the gradient is applied, and the dashed line the calculated percentage of dichloroethane in the eluting fluid referred to the right-hand ordinate. Peak 1 is 2-methoxyestrone, Peak 2 is estrone, Peak 3 is estradiol, Peak 4 is the "middle fraction," Peak 5 is 16-epiestriol, and Peak 6 is estriol.

Identification of the Components of the Individual Peaks. The major components of the individual peaks of radioactivity in the partition chromatograms were identified in two ways: (*a*) by the chromatography of known compounds and the establishment of their positions in the elution diagram, and (*b*) by the composition of the effluent at the time the peak was eluted from the column. In view of the reproducibility of the partition columns, this procedure provided a satisfactory identification in most instances. In order to confirm these identifications in the case of the urinary metabolites of estradiol-C^{14}, the method of isotope dilution was employed. Carrier amounts of unlabeled steroids were added to the pooled material under a radioactive peak, and the mixture was subjected to countercurrent distributions in suitable solvent systems. Radiochemical purity was examined using the statistical procedure of Baggett and Engel (24). In these ways 2-methoxyestrone, estrone, estradiol-17β, 16-epiestriol, and estriol were identified as the principal components of the corresponding peaks.

SUMMARY

A procedure for the quantitative estimation of five identified urinary metabolites and two groups of urinary metabolites of estradiol-C^{14} is described. It is based upon (*a*) hydrolysis of glucosiduronates with bacterial β-glucuronidase, (*b*) separation of neutral from phenolic substances with an anion-exchange resin, and (*c*) the separation by gradient-elution partition chromatography of 2-methoxyestrone, estrone, estradiol-17β, 16-epiestriol, and estriol. In addition, a group of metabolites eluted between estradiol-17β and 16-epiestriol and a group of metabolites more polar than estriol may be separated and estimated.

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The Determination of Ribonuclease Activity

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INTRODUCTION

The primary action of an RNase[2] consists in the depolymerization of its substrate, RNA. A very convenient method for assaying the RNase activity is based on the separation of the low-molecular-weight degradation products from the larger polynucleotides by precipitation of the latter, and the direct assay of the former by spectrophotometry at 260 mμ. The precipitating reagent employed, however, should precipitate the polynucleotides in a selective and reproducible way; it should not interact with one of the components of the system, and its own absorbancy at 260 mμ should be very low.

In an earlier work, we have developed a micromethod (20-μl scale), using a $Ba(ClO_4)_2$–water–2-ethoxyethanol solution as precipitating reagent (1). The latter proved so valuable, that we have worked out the method on a 400-μl scale. No special microapparatus is required for this modification.

MATERIALS

Instruments

Pipets. Constriction pipets of the "Carlsberg type" (2) are used.

Test Tubes. Ten-centimeter Pyrex test tubes, with 1 cm outer diameter.

Vibrator (3). This consists of a pentagon, made from a 3.5-cm hard-rubber stopper, and mounted vertically on the shaft of a small motor. The speed is regulated at 2200 rpm, resulting in a vibration frequency of 11,000 strokes/min.

Chemicals

Water. Twice-redistilled in a quartz apparatus.

Buffers. As the structure of RNA and the activity of many RNase's are

[1] "Chargé de recherches" of the National Foundation for Scientific Research (Belgium).

[2] The following abbreviations are used: RNase, ribonuclease; RNA, ribonucleic acid; DNase, deoxyribonuclease; Tris, tris(hydroxymethyl)aminomethane.

a function of the ionic strength, it is advisable to keep the latter constant in all assays.

Acetate (4), cacodylate (1), or Tris buffer (1) are suitable in their respective pH ranges. The final concentration of the latter two buffers is 0.1 *M*. The ionic strength is adjusted to 0.1 by addition of NaCl.

Other buffer systems can be used, provided their absorbancy is negligible at 260 mμ and they do not interact with the precipitating reagent (*vide infra*).

Substrate. (*a*) *Purification.* Commercial yeast RNA preparations usually contain large quantities of mono- and oligonucleotides. Hence purification is essential. All manipulations are carried out at 2–3°C. A 10–15% solution of the sodium salt is dialyzed in a rotating Visking cellulose tubing against water until the absorbancy at 260 mμ of the outer liquid remains almost constant (usually 5–6 days is required). The RNA can be further purified by precipitation, as described earlier (1). A simpler procedure, however, consists in lyophilizing the dialyzed RNA solution.

(*b*) *Preparation of the Stock Solution.* A 2% solution (e.g., 50 ml) of the purified sodium ribonucleate in water is made and equilibrated during 24 hr in the cold room. Subsequently 2-ml quantities are distributed out in a series of test tubes, which are stored at —14°C. The contents of each tube are thawed at room temperature just before use and serve for one series of RNase determinations.

Precipitating Reagents. (*a*) *Barium Perchlorate.* Once-recrystallized from water. The salt is dried first at 105°C, broken up, and further dehydrated at 260°C during 6 hr. The absorbancy of an aqueous solution is negligible.

(*b*) 2-Ethoxyethanol. A commercial preparation (Cellosolve) with a low absorbancy at 260 mμ is selected. The solvent is purified by fractional distillation under a pressure of 14 mm Hg. A 120-cm "Vigreux" column is used. A stream of nitrogen is led through the boiling solvent. The gas is freed from residual oxygen by passing through an alkaline hyposulfite solution and drying over KOH pellets; 20 gm stannous chloride ($SnCl_2 .2H_2O$) is added per liter 2-ethoxyethanol in order to destroy peroxides. The temperature at the top of the fractionation column is 47°C.

The absorbancy $E \frac{260m\mu}{1cm}$ of the best fractions amounts to 0.043. However all fractions with an absorbancy below 0.100 are suitable. The solvent is stored in the dark at —14°C.

(*c*) *Precipitating Reagent A.* Ten volumes of an 80% (w/v) $Ba(ClO_4)_2$ solution in water is added to 90 vol 2-ethoxyethanol. The reagent is used at pH values below 7.0.

(*d*) *Precipitating Reagent B.* Ten volumes of an 80% (w/v) $Ba(ClO_4)_2$ solution in water and 1 vol of a 1 *M* aqueous acetic acid solution are

added to 99 vol 2-ethoxyethanol. The reagent suits the whole physiological pH range.

METHOD

Assay Procedure

To 1.5 ml substrate solution are added 1.5 ml of 0.4 *M* buffer solution of appropriate pH and with an ionic strength of 0.4 and 1.5 ml water or activator or inhibitor. Then 300-μl quantities are pipetted out in the small test tubes. The enzymic reaction is started by adding 100 μl enzyme solution. A smaller but more concentrated amount of enzyme solution may be added, e.g., 10 or 50 μl provided the total quantity of enzymic reaction mixture amounts to 400 μl. The tubes are incubated in a thermostat.

At the end of the incubation the top of the tube is taken between thumb and forefinger and the bottom is held against the vibrator at an angle of 45°, thus giving the solution a swirling motion. The reaction is stopped by adding 2 ml of the precipitating reagent, contained in a constriction pipet. The latter is blown out in the middle of the test tube without interrupting the vibration; the remaining reagent, which collects afterwards in the top of the pipet, is also added. The mixture in the test tube creeps up in a thin film against the wall. No droplets are lost as the upper top is vibration free. The turbulent swirling is continued during 1 min.

The precipitation constitutes the crucial point of the method. An immediate and complete mixing is essential. After some training the described procedure can be carried out in a very reproducible way.

The tubes are closed with Parafilm (Marathon Corporation, Menasha, Wisconsin) and kept at room temperature. After 1 hr $\pm$ 10 min they are centrifuged for 8 min at 1200 RCF. The acceleration and deceleration should be slow. If an angle centrifuge is used, it is advisable to turn the tubes 180° on their own axis and to recentrifuge them for 8 min in order to collect the precipitate at the bottom. The supernatant fluid is transferred into an optical cell, and ΔE, the absorbancy at 260 mμ, is read against an appropriate control.

The controls are treated in the same way as described above. However, the enzyme solution is delivered as a drop on the wall of the horizontally placed tube right before the precipitation. By holding the tube against the vibrator, mixing is accomplished, immediately followed by the addition of the precipitating reagent. The absorbancy of the control is always checked against water. A normal value is about 0.150; this absorbancy is due to the precipitating reagent and the small amounts of nonprecipitable material present in the substrate. Unpurified enzyme solutions may result in slightly higher control values.

It is convenient to carry out a series of 12 determinations (controls included), spaced by 2½ min. The centrifugation of the first six tubes is started 70 min after the first precipitation. The last six tubes are centrifuged for the first time when the former six are recentrifuged.

Standard Curves

A stock solution containing 4.57 mg crystalline pancreatic RNase ("Armour Research Division," lot No. 381-059) in 0.1% gelatin was prepared. The measured activity (at 25.0°C, pH 5.0 and $\Gamma/2 = 0.1$) as a function of the enzyme concentration is shown in Fig. 1. A linear relation-

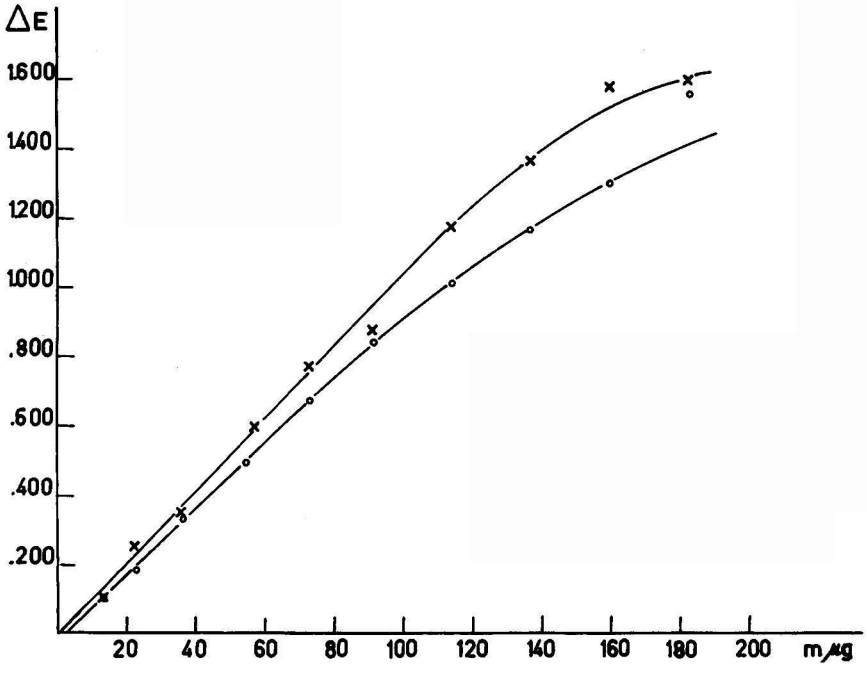

FIG. 1. Activity as a function of pancreatic RNase concentration. Diluted RNase stock solution, 200 μl, is added to 200 μl of a 1% RNA solution in acetate buffer pH 5.0 and $\Gamma/2 = 0.2$. Stock solution diluted in: 0——0 0.02% gelatin; x——x 0.1% gelatin.

ship is obtained up to ΔE values exceeding 0.800. With other RNase's, e.g., the one from barley (5), which is less inhibited by reaction products, a linear function is found in the whole measurable range.

High dilutions of pancreatic RNase have to be made in the presence of gelatin in order to protect it from surface adsorption (6). Larger activities

are obtained if the RNase stock solution is diluted in 0.1% gelatin, indicating insufficient protection in 0.02% gelatin (Fig. 1). However the reproducibility of the method suffers if the concentration of inert protein in the final reaction mixture exceeds 0.05%.

A ΔE value of 0.100 corresponds to 9.65×10^{-6} mg pancreatic RNase. Under more optimal conditions, i.e., at 37.0°C and pH 7.7, the same ΔE value is obtained with 7.6×10^{-8} mg RNase. The procedure here described is 20 times less sensitive than the micromethod (1).

One ribonuclease unit (Ru) has been defined as the amount of enzyme which results in a ΔE value equal to 1.000 when assayed under the following standard conditions: 1 hr incubation at 25.0°C, optimum pH, $\Gamma/2 = 0.1$, and the ΔE value measured in the linear range. One Ru is approximately equivalent to 2.1×10^{-6} mg crystalline pancreatic RNase.

ANALYSIS OF ERRORS

The Substrate

The properties of an RNA solution with respect to its precipitability are a function of the molecular dispersion. A freshly prepared solution changes considerably during 24 hr storage at 2°C. Further alterations are small. The same behavior is found at pH 4.0, 5.0, and 6.0. No changes could be observed in the equilibrated RNA solutions stored in the frozen state, provided they are only thawed once.

For high precision it is recommended that only results obtained in the same series of determinations be compared. Substrate–buffer mixtures, which are not immediately used, should be kept in melting ice.

The Enzyme

The method here proposed has already been successfully employed for the determination of several RNase's, both of animal and of vegetable origin, and with different specificities and pH-optima. When comparing the activities of different RNase's, it should be remembered that the mean molar absorbancy coefficient of the reaction products is a function of the enzyme specificity.

The Pipetting

The standard deviation due to the pipetting amounts to 0.4%.

The Incubation

The maximal error of the incubation time is 10 sec, resulting in a maximal deviation of 0.3% for an incubation time of 1 hr.

The Precipitation

It has already been stressed that inadequate mixing of the enzymic digest with the precipitating reagent is the main source of error. No true equilibrium exists between liquid and solid phase, once the precipitate has been formed. Furthermore, coprecipitation of low-molecular-weight reaction products with the precipitate is observed, and hence the physical nature of the latter is very important. Those remarks are probably applicable to most reagents which selectively precipitate the polynucleotides.

The influence of the time lapse between precipitation and centrifugation is shown in Fig. 2. The absorbancy is minimal after 60 min standing.

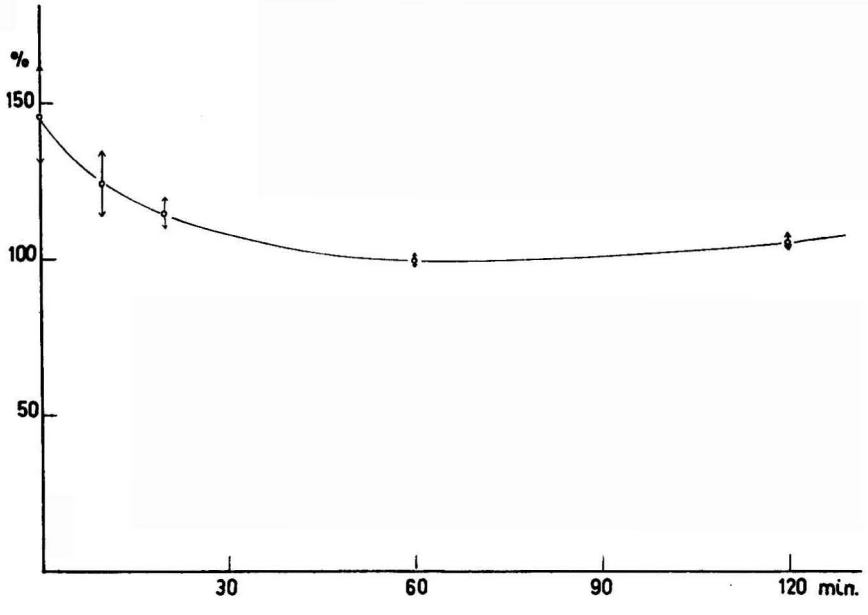

FIG. 2. Influence of time between precipitation and centrifugation. Reaction mixture, 400 μl, contains 0.5% RNA and RNA degradation products, dissolved in acetate buffer pH 5.0 and $\Gamma/2 = 0.1$. Precipitation is carried out as described in the text. After a given period (abcissa) the precipitate is spun down and the absorbancy of the supernatant fluid is measured against water. The results are expressed as per cent of an analogous determination, centrifuged after 60 min standing. 0——0 mean value; vertical arrow, standard deviation. Number of determinations, 28 (20 with reagent A and 8 with reagent B).

The error is less than 1% if the centrifugation is performed between 50 and 85 min after precipitation. This error is even smaller in an actual RNase assay, as a partial compensation is obtained by the control. The

results, shown in Fig. 2, indicate also that the reproducibility is much enhanced after an equilibration time of 1 hr. It may be noted that with the micromethod 10 min standing before centrifugation was found sufficient (1).

The Spectrophotometry

The time lapse between centrifugation and spectrophotometry is of no importance (Table 1). After a long period however, the precipitate may become loose and difficulties will arise.

TABLE 1
STABILITY OF THE SUPERNATANT[a]

Time (hr)	$E_{1\,cm}^{260m\mu}$ [b]
0	0.228
1	0.218
3	0.227
20	0.223

[a] Reaction mixture, 400 μl, contains 0.5% RNA and RNA degradation products, dissolved in acetate buffer pH 5.0 and $\Gamma/2 = 0.1$. Precipitation under standard conditions. After centrifugation the tubes are stored in the dark at room temperature. The absorbancy is measured after the time lapse indicated.

[b] Mean of two independent determinations.

The maximal relative error on one spectrophotometric reading is a function of the absorbancy ΔE. It is equal to 0.54% for $\Delta E = 0.434$ and to 0.74% for $\Delta E = 0.868$. At least three readings are noted for each determination, and hence the maximal error due to spectrophotometry is always lower than those given here.

Obedience to Beer–Law

With the micromethod it has been found that the absorbancy is a linear function of the concentration of RNA degradation products in the whole measurable range (1).

Composition of the Precipitating Reagent

The reasons for choosing 2-ethoxyethanol and $Ba(ClO_4)_2$ as components of the precipitating reagent have been discussed previously (1). Increasing the water concentration results in higher absorbancy values, as longer chain degradation products become soluble. However a concomitant rise of the controls is also observed. Conversely, exchange of part of the 2-ethoxyethanol by an equal volume of *n*-butanol results in smaller absorb-

ancy values. The very high concentration of Ba^{++} ions secures a reproducible precipitate, which is easily spun down.

REPRODUCIBILITY

No correlation is found between the deviation $\delta\Delta E$ and the corresponding ΔE value (1). This confirms that the error is mainly due to the precipitation. Hence, as a first approximation, the standard deviation may be considered constant at all ΔE values.

The standard deviation for the determination of the amount of soluble degradation products is given in Table 2. The result is almost identical for

TABLE 2
STANDARD DEVIATION[a]

	Control	Complete
No. of Experiments	11	12
$E_{1\text{ cm}}^{260\ \mu m}$ (mean)	0.1380	0.6855
Standard deviation $\sigma = \sqrt{\frac{\Sigma(\delta E)^2}{n-1}}$	0.0050	0.0068

[a] Reaction mixture, 400 µl, containing RNA, RNA degradation products and acetate buffer, is treated under standard conditions with the precipitating reagent. Controls are without RNA degradation products. All absorbancies are measured against water.

the control and for the determination. In an actual experiment, the standard deviation $\sigma_{\Delta E}$ is equal to the sum of both: $\sigma_{\Delta E} = 0.0050 + 0.0068 = 0.0118$.

In the experiments, described in Table 2, no errors of enzymological origin are considered. These have to be included if the standard deviation of the actual RNase determination is calculated. The deviation $\delta\Delta E$ between the experimental ΔE value and the statistical estimation of the real ΔE value may be computed either from standard curves, calculated by the method of the least squares, or from multiple assays under identical conditions. Four series of experiments, amounting in total to 21 RNase assays, carried out with enzyme either from pancreas or from barley, resulted in a standard deviation $\sigma_{\Delta E}$ equal to 0.0129. This value is slightly lower than the one obtained with the micro method, viz., 0.0164 (1).

The relative error decreases with increasing ΔE values. It is advisable to use enzyme concentrations resulting in a ΔE value of approximately 0.600. The relative standard deviation amounts then to 2%.

The constancy of the standard deviation holds only in a first approximation. At high ΔE values other sources of error besides the precipitation may become important, and no gain in relative standard deviation can be

obtained by increasing further the enzyme concentration or the incubation time.

INTERFERING COMPOUNDS

Interaction with the Substrate

Proteins form complexes with the polynucleotides. This interaction may seriously interfere with the RNase assay if the protein concentration in the enzymic reaction mixture exceeds 0.05%. Under such conditions a heat treatment has to be performed, as described previously (1). Generally, the specific activity of RNase solutions is so high that such interactions can easily be avoided.

Interaction with the Precipitating Reagent

Various cations and anions may form a precipitate with the barium or perchlorate ions. This results in lower absorbancy values, as some soluble degradation products are coprecipitated (1). The ΔE values, however, remain a linear function of the enzyme concentration, indicating that, with a given concentration of interfering compound, a constant proportion of the soluble degradation products is coprecipitated. Hence the method described is still valuable, but a correction factor has to be applied. This factor can easily be determined by adding the interfering compound right before the precipitation procedure (Table 3).

TABLE 3
INFLUENCE OF SALTS ON THE RNASE ASSAY[a]

Substance	Concn. (M)[b]	ΔE		Apparent activity (%)
		Complete	Blank	
KCl	0.025	0.579	0.594	97.4
$MgCl_2$	0.01	0.727	0.709	102.5
$MgCl_2$	0.025	0.511	0.486	105.1
$CaCl_2$	0.025	0.719	0.709	101.4
$CuCl_2$	0.001	0.651	0.709	91.8
NaF	0.025	0.314	0.594	52.8
Na phosph.[c]	0.001	0.539	0.594	90.8
Na phosph.[c]	0.01	0.406	0.594	68.4
EDTA[c]	0.01	0.561	0.709	79.1

[a] Enzymic digest, 300 μl, containing RNA, cacodylate buffer pH 6.1, and barley RNase is incubated for 1 hr. One hundred microliters of the salt solution tested is added before precipitation. Blanks are treated in the same way, but 100 μl water is added. Precipitating reagent A. All values are the mean of two determinations.

[b] In the 400-μl digest.

[c] pH adjusted to 6.1.

It is seen that some salts, e.g., $MgCl_2$, apparently enhance the activity measured; this may be due to the formation of soluble Mg oligonucleotide complexes.

The interference by some anions, e.g., CO_3^{--} and OH^- can be avoided by using the precipitating reagent B.

The correction factor may be slightly dissimilar for other RNase's which form a different mixture of RNA degradation products. Hence the factor should always be checked for the problem at hand. The apparent enzymic activity as a function of the phosphate concentration is shown in Fig. 3. The amount of soluble RNA degradation products coprecipitated

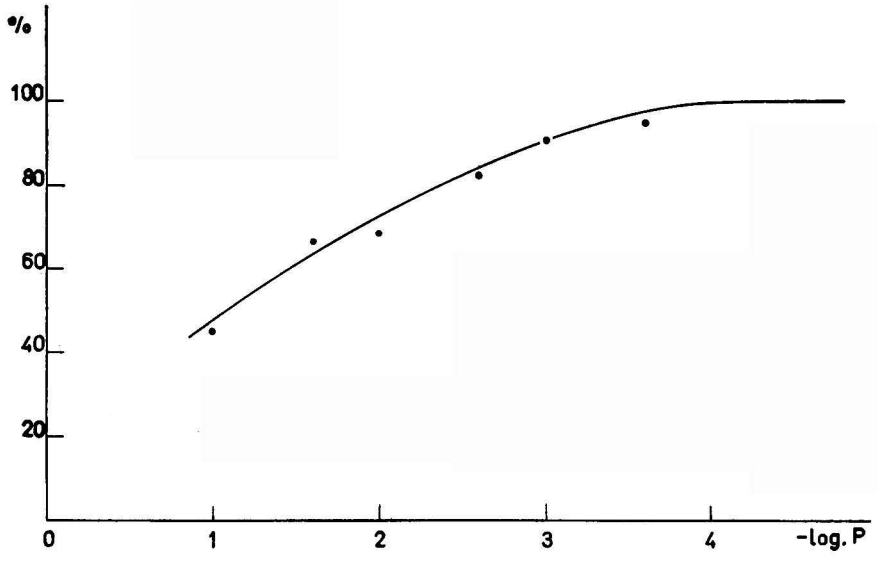

FIG. 3. Influence of phosphate on the RNase assay. Same experimental conditions as given in Table 4. Abcissa: negative logarithm of the phosphate concentration in the 400-μl enzymic digestion mixture. Ordinate: per cent apparent activity.

is not proportional to the phosphate concentration. These results indicate that some compounds, e.g., oligonucleotides, are more liable to be bound than others, e.g., mononucleotides.

COMPARISON WITH OTHER METHODS

Only methods, based on the precipitation of the polynucleotides and the subsequent measurement of the concentration of RNA degradation products by spectrophotometry at 260 mμ will be considered. RNA is usually precipitated by a combination of two of the following factors: acidification, addition of a heavy cation, or the lowering of the dielectric

constant. In Table 4 some characteristics of the $Ba(ClO_4)_2$–2-ethoxyethanol method are compared with those of methods employing either a uranyl–perchloric acid solution (8) or an ethanol–acetate buffer (9) as precipitating reagent.

TABLE 4
COMPARISON OF SOME METHODS FOR RNASE ASSAY

Method	Volume of enzymic digest (ml)	Concn. RNA (%)	Volume of precipitating reagent (ml)	Temperature at precipitation (°C)	Absorbancy of precipitating reagent ($E^{260m\mu}_{1\,cm}$)	Dilution factor of	
						Digest	Precipitating reagent
$(UO_2)^{++}$–$HClO_4$[a]	2.5	0.32	0.5	0	6.436[c]	37.2	186
EtOH–Hac[b]	1.0	0.50	7	0	0.022	8	1.14
$Ba(ClO_4)_2$–2-ethoxyethanol	0.4	0.50	2	22	0.064	6	1.20

[a] Uranyl acetate, 0.75%, in 25% perchloric acid (8).
[b] Contains 935 ml of 95% ethanol + 60 ml acetic acid + 4.5 ml of 2 *N* NaOH (9).
[c] Measured after a 186-fold dilution in water.

A prerequisite of these methods is a low absorbancy of the precipitating reagent at 260 mμ. Solutions containing uranyl ions are not too well suited in this respect as they require a high dilution and hence result in a decreased sensitivity.

Acid reagents may degrade the oligonucleotides. This makes it necessary to perform the operations at 0°C. However, even under these conditions a partial hydrolysis is not completely avoided (10).

The reproducibility of an analytical method is of the utmost importance. The standard deviation of the RNase assay methods, determined under strictly analogous conditions, is given in Table 5. It can be seen

TABLE 5
REPRODUCIBILITY OF SOME METHODS FOR RNASE ASSAY[a]

Method	No. of determinations	Mean ΔE	Standard deviation (σ)	Relative standard deviation (%)	ΔE/ml reaction mixture[b]
$(UO_2)^{++}$–$HClO_4$	12	0.596	0.0047	0.79	22.171
EtOH–Hac	12	1.586	0.0212	1.37	12.688
$Ba(ClO_4)_2$–2-ethoxyethanol	12	1.276	0.0074[c]	0.58	7.656

[a] The reaction mixture contains 0.5% RNA and RNA degradation products dissolved in acetate buffer pH 5.0 and $\Gamma/2 = 0.1$. Precipitation as described for each method (see Table 4). The absorbancies are measured against the correspondingly diluted precipitating reagents.
[b] The ΔE value measured is multiplied by the dilution factor (Table 4).
[c] This value is to be compared with the standard deviation 0.0068, given in Table 2.

that the reproducibility of a precipitation, based mainly on a lowering of the dielectric constant, is unsatisfactory. On the other hand, precipitation of polynucleotides as salts of heavy cations can be carried out in a very reproducible way.

Another important characteristic of the methods, here compared, is the chain length of the soluble oligonucleotides. If this is large, the method will be very sensitive. Indeed each effective splitting of a bond, i.e., the hydrolysis of an internucleotide bond in an insoluble polynucleotide chain with the formation of soluble degradation products, will result in the presence of several chromophores in the supernatant.

On the other hand it is known that RNase assay methods are only rectilinear in the range of high substrate concentrations (1, 11). We can define as effective substrate concentration the number of internucleotide bonds, the hydrolysis of which will result in an increase of the absorbancy of the supernatant fluid. It is evident that with a fixed amount of RNA, the effective substrate concentration and hence the rectilinear range will be larger in proportion as the chain length of the soluble oligonucleotides is smaller. In the $Ba(ClO_4)_2$–2-ethoxyethanol method some sensitivity is sacrificed in order to increase the effective substrate concentration. Indeed, it can be seen from the values given in the last column of Table 5, that in a given reaction mixture approximately three times more products are soluble under the conditions of the UO_2^{++}–$HClO_4$ method (8) than under those of the method here proposed. This is due to the smaller chain length of the soluble RNA degradation products, and not to a decreased solubility of the mononucleotides, as RNA digested by alkali is completely soluble in the $Ba(ClO_4)_2$–2-ethoxyethanol precipitating reagent. The high effective substrate concentration results in a large range of proportionality (Fig. 1), as has already been discussed earlier (1). The loss of sensitivity is more then compensated by the small dilution factor of the enzymic digest (Table 4).

DISCUSSION

The main virtues of the method here described are its relative simplicity, its good reproducibility, its sensitivity, its applicability to tissue extracts and homogenates, and its large range of linear proportionality between enzyme content and absorbancy measured. Furthermore, as the precipitating reagent is not acidic, no degradation of polynucleotides is to be feared, and all manipulations can be carried out at room temperature. Although the sensitivity of the method here proposed is 20 times less then the micromodification described earlier, it is still at least ten times better than most of the other procedures known (1).

The actual meaning of 1 Ru can only be estimated. If the enzyme studied liberates equimolar amounts of the four nucleotides and the chain

length of the soluble degradation products is 1, then 1 Ru results in the release of 0.219 μmole soluble reaction products. This may be considered as the effective action of the enzyme, in contradistinction to the hydrolysis of internucleotide bonds with production of two insoluble oligonucleotides.

The mean chain length of the soluble degradation products is actually greater than 1. On the other hand it is smaller than 4, because "core" obtained by extensive digestion of RNA with pancreatic RNase, and which has a mean chain length of 4 (7), is readily precipitated under the conditions outlined before. Hence 1 Ru results in the effective hydrolysis of about 0.073–0.108 μ equiv. of internucleotide bonds.

Several substances that may interfere have been tested. All those that form a precipitate either with the barium or with the perchlorate ions tend to lower the results due to coprecipitation of soluble RNA degradation products. However, the linearity of the method still holds true, and it is easy to apply a correction factor. Proteins in a concentration exceeding 0.05% may interfere by the formation of soluble protein–polynucleotide complexes (1).

The $Ba(ClO_4)_2$–2-ethoxyethanol method has also been successfully used for the determination of DNase activity.

SUMMARY

A method for the determination of RNase activity is presented. It is essentially a modification of the micromethod described previously. The assay is based on the precipitation of the polynucleotides with a $Ba(ClO_4)_2$–water–2-ethoxyethanol solution and the subsequent measurement of the absorbancy at 260 mμ of the supernatant. The method is suited for series analyses and requires no special microapparatus. An increase in absorbancy $\Delta E = 0.100$ is obtained with 7.6×10^{-8} mg crystalline pancreatic RNase after 1 hr incubation at 37°C and pH 7.7. It is rectilinear over a wide range of enzyme concentrations. The standard deviation equals 2% when ΔE amounts to 0.600. Possible sources of error have been analyzed. Substances which form a precipitate with one of the components of the precipitating reagent do interfere because of coprecipitation effects. Under such conditions, however, the method remains linear and a correction factor can be applied. The procedure proposed has been experimentally compared to some other, analogous methods.

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A Synthesis of δ-Aminolevulinic Acid Analogous to Its Biosynthesis[1]

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INTRODUCTION

δ-Aminolevulinic acid was first synthesized by Wynn and Corwin in 1950 (1) by reduction of methyl δ-isonitrosoacetylacrylate derived from levulinic acid. Since the discovery in 1953 of the biological significance of δ-aminolevulinic acid in porphyrin biosynthesis (2, 3), approximately a dozen different syntheses of the compound have been reported (2–10). Although in some of these syntheses carbons 1 to 4 of the final product are derived ultimately from succinic acid, and in others the carbon 5 of the final product is derived from the α-carbon of glycine, none of the published syntheses is completely analogous to the biosynthetic pathway; i.e., none of them involves condensation of a succinyl moiety with a glycine moiety, followed by a decarboxylation. In the actual biosynthetic pathway the "active" succinate moiety is apparently succinyl coenzyme A and the glycine moiety is activated apparently via formation of an imine with pyridoxal phosphate (11, 12).

Neuberger and Scott (4) attempted a Dakin-West reaction (13) between succinic anhydride and glycine under a variety of conditions, hoping to prepare δ-aminolevulinic acid directly, but their efforts were entirely without success. We have made similar attempts and also failed to isolate any crystalline products. This was disappointing in the light of the fact that the Dakin-West reaction has been used successfully for the synthesis of aminoketones from a number of aliphatic and aromatic anhydrides (14, 15). Glycine is admittedly the most difficult amino acid for this type of reaction (13–15).

The mechanism of the Dakin-West reaction has been discussed in a number of papers and reviews (13, 15–17). The *N*-acylated amino acid

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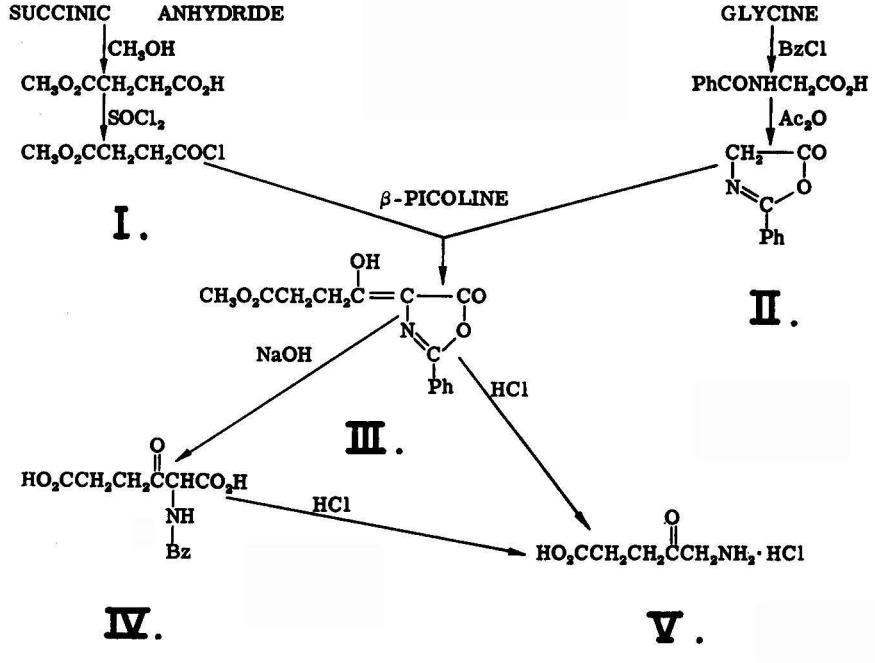
is cyclized to an oxazolone, which, in the presence of base, gives a resonance-stabilized carbanion capable of reacting with another molecule of the anhydride to form the oxazolone of an α-acylamido-β-keto acid; the latter oxazolone is then opened up and the α-acylamido-β-keto acid decarboxylated. It is possible to stop at several of these intermediate steps. Attenburrow, Elliott, and Penny (16) used this technique in a synthesis of DL-threonine: they condensed acetic anhydride with sodium hippurate (or the 2-phenyl-5-oxazolone formed from it in the reaction) in the presence of a pyridine base to give 2-phenyl-4-(1-hydroxyethylidene)-5-oxazolone, easily converted to ethyl α-benzamidoacetoacetate, which was hydrogenated to the corresponding threonine–allothreonine derivative.

More recently, Carter, Harrison, and Shapiro (18) obtained a desired intermediate for the synthesis of dihydrosphingosine by a similar condensation. In this case the palmitoyl anhydride did not condense readily, but palmitoyl chloride did condense with 2-phenyl-5-oxazolone in the presence of β-picoline to give a 40–60% yield of 2-phenyl-4-(1-hydroxypalmitylidene)-5-oxazolone.



We have now been able to synthesize δ-aminolevulinic acid in small yields via a similar condensation. Thus, β-carbomethoxypropionyl chloride (I) was condensed with 2-phenyl-5-oxazolone (II) in the presence

of β-picoline to give a low yield of 2-phenyl-4-(1-hydroxy-3-carbomethoxypropylidene)-5-oxazolone (**III**). This compound was converted by mild alkaline hydrolysis and subsequent acidification to α-benzamido-β-ketoadipic acid (**IV**), which on refluxing in 6 *N* hydrochloric acid gave the desired δ-aminolevulinic acid hydrochloride (**V**). Alternatively, **III** could be converted to **V** by refluxing 6 *N* hydrochloric acid.

EXPERIMENTAL

All melting points are uncorrected, taken in capillary tubes. β-Carbomethoxypropionyl chloride (**I**) was prepared from succinic anhydride via methyl hydrogen succinate according to Cason (19). 2-Phenyl-5-oxazolone (**II**) was prepared by treating hippuric acid with acetic anhydride according to Carter *et al.* (18). The instability of this compound has been pointed out in the literature (17), and we found that different runs, even under apparently identical conditions, gave widely varying yields of oxazolone varying in color and melting point. Removal of excess acetic anhydride as completely as possible after the reaction on a rotary evaporator led only to intractable tars; removal of too little of the excess acetic anhydride made isolation of the product difficult. Concentration of the reaction mixture to about one-third its original volume and addition of one volume of *tert*-butanol gave yields of 61–73% of crude product or 40–54% after recrystallization from absolute ethanol. The recrystallized oxazolone was usually pale yellow in color, m.p. 88–90°. It was noted that the product tended to sublime in the vacuum drying pistol at 78°, so a sublimation at 60–65° at water aspirator pressure was carried out. The sublimate collected on the cold finger as pure white crystals, m.p. 87–88°. [Literature melting point is reported variously from 86° (21) to 90–91° (18) or even 94–95° (20)].

*2-Phenyl-4-(1-hydroxy-3-carbomethoxypropylidene)-5-oxazolone (**III**)*

In a flask equipped with a magnetic stirrer and dropping funnel protected by a calcium chloride tube was placed 20 ml of β-picoline (dried over potassium hydroxide) and 8.0 g (0.05 mole) of the oxazolone (**II**), which dissolved with stirring. With cooling in an ice bath and continued stirring, 7.5 g (0.05 mole) of the acid chloride (**I**) was added over a 15-min period. Dense fumes were given off immediately, and a dark red solid separated but dissolved on further stirring. After an additional 20 min of stirring, the reaction mixture was treated with 25 ml of concentrated hydrochloric acid diluted with ice. The dark-red solid which separated was extracted into 400 ml chloroform. After washing with water until washings were no longer acid, the chloroform layer was dried over sodium sulfate and concentrated at reduced pressure, leaving a red

tarry residue. Addition of acetonitrile yielded almost immediately a suspension of red crystals, 1.35 g (9.8% yield), m.p. 178–180°. After three successive recrystallizations from ethyl acetate, the product melted at 180–182°.

Anal. Calcd. for $C_{14}H_{13}NO_5$: C, 61.09; H, 4.76; N, 5.09. Found: C, 60.43; H, 4.67; N, 4.86.

Attempts to decolorize the product with Norit A were unsuccessful, although the color varied in different runs from buff to red. Yields from 4 to 20% were obtained in various runs; changing the ratio of β-picoline to the other reactants did not affect the yield systematically. An infrared absorption spectrum of **III** in chloroform shows peaks at 6.03 μ (C = N), 5.76 μ (C = O), and 3.32 μ. The ultraviolet spectrum in dioxan shows two strong maxima at 310 and 325 mμ forming a doublet, with a shoulder at 340 mμ (Emol. = 1.2×10^4). The absorption at 340 mμ was used to follow the formation of **III** in several 0.01-mole runs; after addition of the acid chloride, 0.1-ml aliquots were taken at intervals, diluted 10,000-fold in dioxan, and read in the Beckman DU spectrophotometer. The data of a typical run are shown in Table 1.

TABLE 1

FORMATION OF 2-PHENYL-4-(1-HYDROXY-3-CARBOMETHOXYPROPYLIDENE)-5-OXAZOLONE (**III**) IN A 0.01-MOLE SCALE EXPERIMENT

Minutes after addition of **I**	Absorbancy at 340 mμ	Grams of **III** formed	Per cent yield
1	0.172	0.191	7.0
5	0.174	0.194	7.1
10	0.295	0.328	11.9
15	0.308	0.342	12.4
20	0.313	0.348	12.7
25	0.303	0.337	12.3
30	0.251	0.279	10.2

α-Benzamido-β-ketoadipic Acid (**IV**)

To 20 ml of 1 *N* sodium hydroxide was added 0.30 g of the oxazolone (**III**). The solid dissolved readily to give a pale yellow solution, which was allowed to stand at room temperature for several hours. Dropwise addition of concentrated hydrochloric acid gave a pale yellow crystalline precipitate. After washing with water, the crystals were dried *in vacuo* to give 0.275 g (90.3% yield) of product, m.p. 175–177°.

Anal. Calcd. for $C_{13}H_{13}NO_6$: N, 5.02; neut. equiv., 139.5. Found: N, 4.94; neut. equiv., 133.

δ-Aminolevulinic Acid Hydrochloride (**V**)

(*a*) *By Hydrolysis of α-Benzamido-β-ketoadipic Acid.* A 0.50-g sample of **IV** was refluxed in 50 ml of 6 *N* hydrochloric acid for 10 hr, the solid dissolving after about 1 hr of refluxing. Cooling the reaction mixture gave a precipitate of benzoic acid, identified by melting point (118–120°) and mixed melting point. After washing with ether to remove remaining benzoic acid, the aqueous acid layer was concentrated in a rotary evaporator to a small volume and placed in a vacuum desiccator containing solid potassium hydroxide and a desiccant. Crystals with a yellow-green cast were obtained in this way. Recrystallization of a portion of them from methanol–ether yielded about 50 mg of colorless product, m.p. 138–143°. (lit. m.p., 144–147°).

Anal. Calcd. for $C_5H_9NO_3HCl$: N, 8.35. Found: N, 8.19.

(*b*) *By Hydrolysis of the Oxazolone* (**III**). A suspension of 0.30 g of **III** in 3 ml of 6 *N* hydrochloric acid was refluxed for 10 hrs, the solid dissolving after several hours. The reaction mixture was worked up as in (*a*) to yield 53 mg of recrystallized product, m.p. 147–148°.

The products of the two procedures were identified as δ-aminolevulinic acid by comparison with an authentic sample prepared by an apparently unpublished procedure by Dr. D. Mauzerall and Dr. F. Sparatore of the Rockefeller Institute for Medical Research, and obtained through the courtesy of Professor Sam Aronoff of this department. The infrared spectra compared favorably but indicated possible contamination by trace impurities. Paper chromatographic behavior in two solvent systems was identical: In *n*-butanol–acetic acid–water (63:10:27 by vol.), R_f = 0.27 [lit. R_f, 0.23 (22)]; in phenol–water (4:1 by vol.), R_f = 0.56 [lit. R_f, 0.42–0.44 (6)]. The chromatograms were developed with ninhydrin, giving the characteristic yellow-brown color for δ-aminolevulinic acid (3).

DISCUSSION

The data of Table 1 indicate that the concentration of the desired condensation product (**III**) reaches a maximum about 20 min after addition of the acid chloride and then falls off. Carter observed similar behavior with palmitoyl chloride, but obtained higher yields at the maximum (18). It is possible that our acid chloride is being partially converted to succinic anhydride with the loss of methyl chloride; when succinic anhydride was used as the acylating agent, ultraviolet absorption measurements indicated formation of only traces of a condensation product.[2] Increasing the acid chloride to oxazolone ratio to 4:1 did not

[2] A manuscript referee points out that formation of δ-aminolevulinic acid via condensation of succinic anhydride and an oxazolone has previously been reported,

improve the yield. Slow self-condensation of the oxazolone (**II**) in β-picoline would be expected as a competing side reaction (17*b*, 18). Other bases were not investigated because β-picoline has been shown to be one of the most effective catalysts for similar condensations (16, 23).

The enolic structure of **III** is inferred from its ultraviolet (24) and infrared absorption spectra (17), and analogy with 2-phenyl-4-acyl-5-oxazolones prepared previously (16, 18). In comparing the infrared spectrum of **III** with that of **II**, it is noted that the C = N peak at 6.03 μ is present in both, but in **III** the C = O peak has been shifted to a higher wavelength (from 5.45 to 5.76μ). The new peak at 3.32 μ also offers additional evidence for an enolic structure for **III**.

Compound **IV** has apparently not been synthesized previously. Esters of α-amino-β-ketoadipic acid and of the *N*-carbobenzoxy derivative are known, but the parent α-amino-β-ketoadipic acid could not be prepared from them even under mild conditions (8). We are attempting the hydrolysis of **IV** by a renal acylase preparation.

We had hoped that the synthesis reported here might have some interest other than the novel analogy to the biosynthetic pathway. Glycine-2-C^{14} is easily available, and it was hoped that such a direct pathway to δ-aminolevulinic acid-5-C^{14} might be isotopically economical. The disappointingly low yield in the condensation reaction has made us abandon this scheme. However, we are still investigating other possibilities for acylation of the α-carbon of a glycine moiety by a succinyl moiety.

SUMMARY

δ-Aminolevulinic acid has been synthesized by a procedure in which C-1 to C-4 are derived from succinic acid and C-5 from the α-C of glycine, as in the biosynthetic pathway. β-Carbomethoxypropionyl chloride (from succinic anhydride) and 2-phenyl-5-oxazolone (from *N*-benzoylglycine) were condensed in β-picoline to give 2-phenyl-4-(1-hydroxy-3-carbomethoxypropylidene)-5-oxazolone. The condensation product, or the α-benzamido-β-ketoadipic acid derived from it by mild alkaline hydrolysis, was converted to δ-aminolevulinic acid hydrochloride by refluxing in 6 *N* HCl.

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ANALYTICAL BIOCHEMISTRY 2, 147–151 (1961)

A Method for Determining the Oxygen-18 Content of Orthophosphate or Water[1]

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Use of O^{18} as an isotopic tracer has given valuable information on the mechanism of various enzymic reactions in which an orthophosphate group participates. Such studies must employ suitable analytic procedures which determine the amount of O^{18} in orthophosphate. One general method involves the pyrolysis of orthophosphate to form metaphosphate and water followed by spontaneous equilibration of the oxygen atoms of water with those of a measured amount of CO_2 (1, 2), followed by determination of the 46/44 mass ratio of the CO_2 with the mass spectrometer. This procedure suffers from the disadvantages of a long period of time required for equilibration and the difficulty of handling small samples (water must be present as a liquid). Dostrovsky and Klein were able to reduce significantly the time required for isotopic equilibration by use of thermal catalysis provided by a heated platinum wire (3). This procedure has been used on a relatively small scale (4). However, the procedure is not highly reproducible with different chambers.[2]

This paper reports a rapid and reproducible means of reducing the time required for equilibration by use of a high voltage discharge.

EXPERIMENTAL PROCEDURE

The reaction between water and CO_2 is carried out in the chamber shown in Fig. 1. This is constructed of borosilicate glass and is provided with tungsten electrodes separated by a gap of 2 cm. The volume contained by the chamber between stopcocks 3 and 4 is approximately 76 ml. A high voltage discharge provided by a laboratory vacuum leak detector

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[2] M. B. Dempsey and P. D. Boyer, Department of Physiological Chemistry, University of Minnesota, unpublished findings.

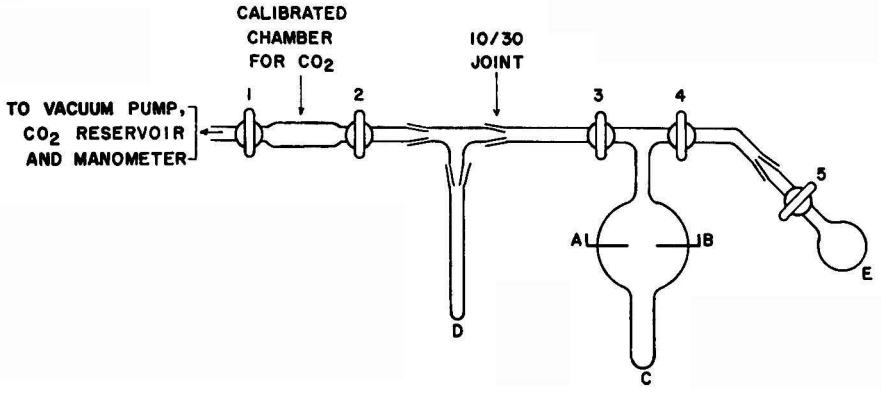

FIG. 1. Apparatus employed for introducing O^{18} into CO_2 and collecting CO_2.

of the Tesla coil type is applied at electrode *A*. Electrode *B* is grounded. The voltage delivered by the coil is regulated so that a spark gap of 2.5 cm between two points in air is obtained. A spark gap of this magnitude is equivalent to approximately 30,000 volts (5). Under the operating conditions outlined, an output of x-rays equivalent to 2 milliroentgens per hour (mr/hr) occurred during application of the induction coil.[3]

The procedure used is as follows: (*a*) A carefully weighed sample of KH_2PO_4 ranging from 2 to 10 mg is placed in tube *D*. The entire system is evacuated to a pressure of approximately 1 μ Hg. (*b*) Approximately 10 μmoles CO_2 is placed in a volume-calibrated chamber bounded by stopcocks 1 and 2. The amount of CO_2 contained is accurately determined by manometric means. (*c*) The KH_2PO_4 contained in tube *D* is pyrolyzed to water and potassium metaphosphate by a soft gas flame. (*d*) Stopcock 4 is closed; 2 and 3 are opened. The water and CO_2 are next collected in the reaction chamber by placing *C* in a liquid nitrogen trap for 3 min. (*e*) Stopcock 3 is closed, and the water and CO_2 in the chamber are converted to the gaseous phase by heating *C* in a water bath at room temperature. A high voltage discharge is applied at *A* for 2.5 min; *C* is then cooled by a liquid nitrogen trap and thawed. The voltage is applied once more for a 2.5-min period. (*f*) Stopcock 2 is closed and 3 is opened and water is removed by placing *D* in a Dry Ice–acetone trap. (*g*) This is followed by condensing out the CO_2 in a suitable gas-collecting bulb *E* by closing 3,

[3] The amount of radiation is quite small in relation to the recommendations of the National Bureau of Standards (6) concerning maximal permissible radiation exposures to man. The Bureau states that the maximum permissible dose accumulated at any age, shall not exceed 5 roentgen equivalents man(rems) multiplied by the number of years beyond age 18, and the dose in any 13 consecutive weeks shall not exceed 3 rems.

opening 4 and 5, and immersing the bulb in liquid nitrogen. (*h*) The system is evacuated by first opening stopcocks 1 and 2 (to evacuate CO_2 contained in the bore of 1), followed by opening 3 with *E* immersed in liquid nitrogen. This last step removes the extraneous gases formed by the reaction. (*i*) Finally, 5 is closed and the contents of *E* are subjected to mass spectrographic determination of the 46/44 mass ratio. In order to minimize memory effects the reaction vessel is carefully heated before the next determination with a soft gas flame for a period of 2 min with 1, 2, and 3 open.

RESULTS AND DISCUSSION

The atom per cent excess O^{18} in KH_2PO_4 is calculated by the following equation:

$$\left[\frac{R_e}{2+R_e} - \frac{R_0}{2+R_0}\right] \times \left[\frac{2a+b}{b}\right] \times (100)$$

where R_e = the observed mass ratio for the sample, R_0 = the mass ratio for tank CO_2 (0.00413), a = μmoles CO_2, and b = μmoles phosphate or water. This equation is similar to one developed by Dostrovsky and Klein (3) and Cohn (7) except that it ignores any possible fractionation effects as regards incorporation of O^{18} into CO_2 and other molecular species formed by the reaction. The validity of this assumption may be seen from the data in Table 1 where there is a close correspondence

TABLE 1

COMPARISON OF CURRENT METHOED WITH THAT OF COHN FOR DETERMINATION OF ATOM PER CNT EXCESS O^{18} IN KH_2PO_4

	Sample No.	KH_2PO_4 (mg)	CO_2 (μmoles)	46/44 mass ratio	Atom % excess
A. Method of Cohn	1	6.0	8.70	0.03180	1.85
	2	7.2	8.75	0.03333	1.86
	3	9.0	8.75	0.03499	1.86
B. Method described in text	1	2.8	7.84	0.02555	1.86
	2	5.7	7.84	0.03114	1.85
	3	6.2	7.73	0.03216	1.84

between values obtained by this method and that of Cohn (1) for the atom per cent excess O^{18} in a standard sample of KH_2PO_4. The values by the latter method were calculated by the equation developed by Dostrovsky and Klein (3) where the equilibrium constant for the equilibration reaction at 25°C is taken as 2.076.

The data in Table 2 reveal a small memory effect. This is most apparent

TABLE 2

EVALUATION OF MAGNITUDE OF MEMORY EFFECT WITH THE METHOD DESCRIBED

Consecutive sample No.	KH_2PO_4 (mg)	CO_2 (μmoles)	46/44 mass ratio	Atom % excess found	Theoretical atom % excess
1	7.4	11.34	0.00419	0.004	0
2	4.6	11.24	0.02632	1.82	1.85
3	6.3	11.13	0.02959	1.85	1.85
4	3.8	11.13	0.02497	1.85	1.85
5	6.2	11.03	0.00460	0.034	0
6	5.3	10.93	0.00425	0.009	0

when there occurs an abrupt change in the concentration of O^{18} in the samples. This small error may be almost completely abolished by performing duplicate determinations and arranging the sequence of sample analysis in increasing order of concentration of O^{18}.

In a series of seven consecutive determinations on samples ranging in magnitude from 2 to 10 mg KH_2PO_4 with identical amounts of O^{18}, a coefficient of variation[4] of $\pm 1.0\%$ was obtained. Similar results were obtained with samples of KH_2PO_4 containing 0–1.85 atom % excess O^{18}. Repeated use of the reaction chamber results in a small amount of carbon deposition in the vicinity of the junction of the tungsten electrodes with the inner wall of the vessel. This had no apparent effect on the reliability of the method after approximately 200 determinations.

Mass spectrographic scan from masses 1 to 50 revealed that samples taken for analysis were essentially identical to background scans except for masses 44, 46, and 48. On the other hand, a scan of the gas which was not condensable at liquid nitrogen temperatures (see step *h* above) revealed a pattern compatible with the formation of H_2, O_2, and CO and perhaps small amounts of methane. The total yield of these gases was less than 10% of the amount of CO_2 introduced.

SUMMARY

A method for determining the oxygen-18 content of orthophosphate or water is described. The method is based on the use of a high voltage discharge to bring about the rapid equilibration of oxygen-18 from water with carbon dioxide. Samples of KH_2PO_4 as low as 2 mg are readily analyzed.

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[4] Coefficient of variation $= \frac{\text{(standard deviation)}}{\text{mean}} \times 100.$

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Reversed Phase Partition Chromatography of Steroids[1]

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INTRODUCTION

The separation of steroids by paper chromatography has been described by Zaffaroni *et al.* (1–3) and by Bush (4). In these systems, which employ cellulose paper as the supporting medium, the more polar solvent serves as stationary phase and the less polar solvent as mobile phase. Under these conditions, relatively nonpolar steroids have the greatest mobilities and very polar steroids are resolved with difficulty by virtue of their failure to move appreciably from the origin. When a hydrophobic supporting medium is employed, the less polar solvent forms the stationary phase and the more polar solvent the mobile phase. Under these conditions, the relationship of polarity of solute and mobility is reversed as compared to conventional partition systems; the very polar steroids then have faster running rates and are rendered more amenable to chromatography.

Since the initial reversed phase chromatographic investigations of Boscott (5) in 1947, there have been several studies (6–10) dealing with reversed phase separation of steroids. The present report describes the use of cellulose acetate foil[3] as a supporting medium for the reversed phase chromatography of steroids. A distinct advantage of this material is that solvent extraction eliminates almost entirely any residue which interferes with spectrofluorometric and spectrophotometric analysis of submicrogram quantities of eluted steroids.

METHODS AND MATERIALS

1. Solvents

All solvents were reagent grade and were fractionally distilled prior to

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[3] Cellulose acetate foil is not acetylated paper and is produced by casting liquid cellulose acetate on glass.

use. The benzene used for purification of the foil and for the final elution of steroids from the foil was carefully purified by repeated shaking, 2 l with 150-ml portions of concentrated sulfuric acid (Du Pont, reagent grade), and then washed with distilled water until the pH of the wash water was neutral. The benzene was fractionally redistilled after removing traces of water with anhydrous sodium sulfate. Four milliliters of this solvent were taken to dryness, and the fluorescence of this residue was determined in the manner described in Sec. 8. Unless the fluorescent reading approximated the reagent (H_2SO_4) blank, the purification was repeated.

2. Steroid Standards

Steroids of known purity and homogeneity by virtue of their behavior in conventional chromatographic systems were used. The steroid glucosiduronates were synthesized by the method of Wotiz *et al.* (11) and were obtained from this investigator.

3. Cellulose Acetate Foil

Foil, No. 2500, 0.15 mm thickness, obtained from Carl Schleicher and Schuell Co., Keene, N. H., was cut into strips 2.5 and 5.0 cm wide and 37.6 cm long. In qualitative experiments, the foil was used without prior purification. In experiments designed to isolate and measure microgram or submicrogram quantities of steroids, the foil was purified by refluxing for 56–60 hr with purified benzene in a Soxhlet extractor. The strips were dried and stored between sheets of cellulose filter paper.

4. Chromatography Apparatus

Glass jars fitted with appropriate covers and lined with filter paper were employed. The foil strips were held between two microscope slides and placed without prior equilibration in Petri dishes, used as troughs, containing the mobile phase (descending technique).

5. Glassware

All glassware was cleaned with chromic acid, rinsed repeatedly in tap water, and finally in water distilled in an all-glass system.

6. Detection of Steroids on Chromatograms

This was carried out by using spot reactions previously described (12–14). All reagents were made up in methanol.

7. Elution of Steroids

After the development of the chromatograms, the steroids were eluted

from the appropriate area of the foil with 2-ml portions of purified benzene for two successive 24-hr periods. The eluents were combined and taken to dryness at 50°C under a stream of filtered nitrogen.

8. Spectrofluorometric Analyses

An Aminco-Bowman spectrofluorophotometer model No. 4-8100, equipped with an RCA IP28 photomultiplier tube, was used for these analyses. Two milliliters of 30 *N* sulfuric acid were added to the dry steroid residue. The reaction was allowed to progress at 26°C for 120 min, and the readings were promptly taken in the above instrument. Activation and fluorescent maxima were established for each sample.

RESULTS

The multiplicity of solvents (Table 1) applicable to reversed phase

TABLE 1
SOLVENTS APPLICABLE FOR CHROMATOGRAPHY ON CELLULOSE ACETATE FOIL

Mobile phase (In aqueous solution)	Stationary phase
Glacial acetic acid <30% (v:v)	*n*-Butanol
Formic acid <10% (v:v)	*n*-Octanol
Hydrochloric acid <10% (v:v)	Toluene
Phosphoric acid <10% (v:v)	Benzene
Trichloroacetic acid <15% (v:v)	Carbon tetrachloride
Acetate buffer pH 4.6	Cyclohexane
Na carbonate 1 *M*	2,2,4-Trimethylpentane
Na bicarbonate 1 *M*	*n*-Hexane
Water	Petroleum ether
Methanol <70%	Ligroin
Ethanol <70%	*n*-Decane
Propanol <70%	*n*-Dodecane
Isopropanol <70%	*n*-Tetradecane
tert-Butanol <40%	*n*-Hexadecane

chromatography on cellulose acetate foil contributes to the versatility of this technique for the study of a variety of organic substances. Specific systems and their resolving power for C_{18}, C_{19}, and C_{21} steroids, both as free alcohols and as glucosiduronates are presented in Table 2 with reference to the least mobile steroid in any given system. In relating these results to those obtained in conventional partition systems, these mobilities are reversed in order of descent. This is exemplified in the case of steroid glucosiduronates where unusually rapid running rates were observed.

The recovery of steroid from the foil was investigated by the chroma-

TABLE 2
MOBILITIES OF SOME FREE AND CONJUGATED STEROIDS

Chromatographic system	Running time (hr)[a]	Steroid (see Table 3 for key to designation)	Mobility
Water–MeOH–*n*-decane (4:6:10)	24	I	1.00
		II	1.50
		III	2.00
Water–MeOH–*n*-tetradecane (3:7:10)	36	I	1.00
		II	1.28
		III	1.77
Water–MeOH–cyclohexane (5:5:10)	48	IV	1.00
		V	1.70
		VI	2.32
		VII	2.62
		VIII	3.08
		IX	3.63
		X	4.40
		XI	5.00
Water–*tert*-but.–pet. ether (2:1:3)	72	XII	1.00
		XIII	1.38
		XIV	1.88
		XV	2.16
		XVI	2.44
		XVII	2.72
		XVIII	3.05
		XIX	4.11
		XX	4.61
		XXI	5.30
Water–*tert*-but.–benzene (2:1:3)	72	XII	1.00
		XIII	2.86
		XIV	2.50
		XV	3.00
		XVI	6.00
		XVII	8.00
		XVIII	9.60
		XIX	14.00
		XX	15.50
Water–*tert*-but.–benzene (2:1:3)	4	XXII	0.00
		XXIII	1.00
		XXIV	2.00
		XXV	8.33
		XXVI	22.33

[a] At 26°C.

TABLE 3
KEY TO STEROID DESIGNATION

	Chemical name[a]	Trivial name
I	3-Hydroxyestra-1,3,5,(10)-trien-17-one	Estrone
II	Estra-1,3,5,(10)-trien-3,17β-diol	Estradiol-17β
III	Estra-1,3,5,(10)-trien-3,16α,17β-triol	Estriol
IV	Pregn-4-ene-3,20-dione	Progesterone
V	3α-Hydroxy-5α-androstane-17-one	Androsterone
VI	Androst-4-ene-3,17-dione	Androstenedione
VII	3α-Hydroxy-5β-androstane-17-one	Etiocholanolone
VIII	17β-Hydroxyandrost-4-ene-3-one	Testosterone
IX	3β-Hydroxyandrost-5-ene-17-one	Dehydroepiandrosterone
X	3α-Hydroxy-5β-androstane-11,17-dione	11-Ketoetiocholanolone
XI	3α,11β-Dihydroxy-5β-androstan-17-one	11-Hydroxyetiocholanolone
XII	21-Hydroxypregn-4-ene-3,20-dione	Deoxycorticosterone
XIII	17α,21-Dihydroxypregn-4-ene-3,20-dione	11-Deoxy,17α hydroxycorticosterone
XIV	21-Hydroxypregn-4-ene-3,11,20-trione	11-Dehydrocorticosterone
XV	11β,21-Dihydroxypregn-4-ene-3,20-dione	Corticosterone
XVI	3α,17α,21-Trihydroxy-5β-pregnane-11,20-dione	Tetrahydrocortisone
XVII	17α,21-Dihydroxypregn-4-ene-3,11,20-trione	Cortisone
XVIII	11β,17α-21-Trihydroxypregn-4-ene-3,20-dione	Cortisol
XIX	3α,11β,17α,21-Tetrahydroxy-5β-pregnane-20-one	Tetrahydrocortisol
XX	11β,21-Dihydroxypregn-4-ene-18-al-3,20-dione	Aldosterone
XXI	3α,18,21-Trihydroxy-5β-pregnane-11,20-dione	Tetrahydroaldosterone
XXII	17-Oxo-5α-androstan-3α-yl-β-D-glucopyranosiduronic acid	Androsterone glucosiduronate
XXIII	3-Oxo-androst-4-en-17β-yl-β-D-glucopyranosiduronic acid	Testosterone glucosiduronate
XXIV	3-Hydroxy-estra-1,3,5,(10)-trien-17β-yl-β-D-glucopyranosiduronic acid	Estradiol-17-glucosiduronate
XXV	11,20-Dioxo-17α-hydroxy-5β-pregnanylene-3α,21-β-D-glucopyranosiduronic acid	Tetrahydrocortisone diglucosiduronate
XXVI	20-Oxo-11β,17α,21-trihydroxy-5β-pregnanyl-β-D-glucopyranosiduronic acid	Tetrahydrocortisol monoglucosiduronate

[a] International Union of Pure and Applied Chemistry "Nomenclature of Organic Chemistry." Butterworths, London, 1958.

tography of 20 μg of crystalline cortisol in the *tert*-butanol–water/petroleum ether system. The appropriate area was eluted with benzene and quantitated using the Porter-Silber reaction. In nine experiments, recoveries ranged between 53 and 62%.

In experiments designed to evaluate the absolute sensitivity of the chromatographic technique, 0.25, 0.5, 1.0, and 2.0 μg corticosterone and

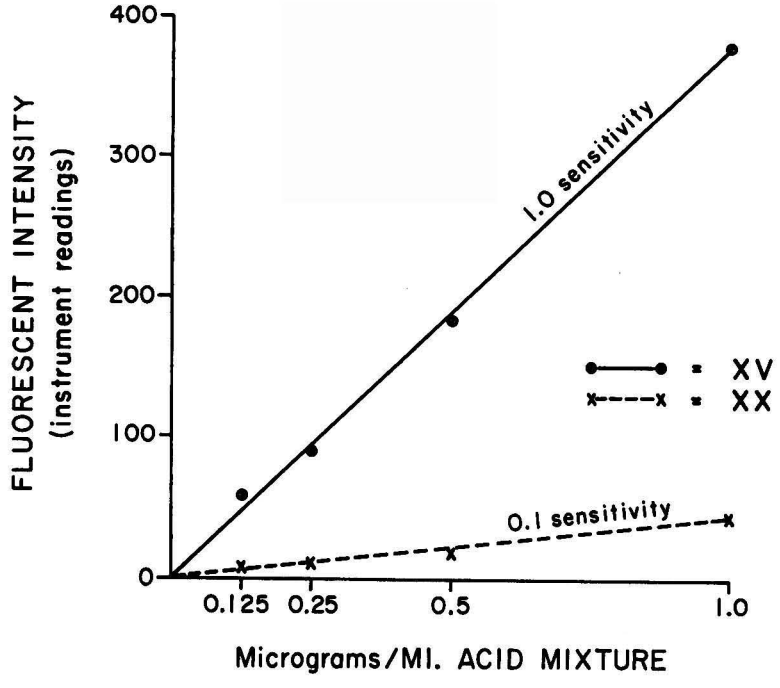

FIG. 1. Concentration–fluorescence relationship of eluted corticosterone (XV) and aldosterone (XX), applied as a mixture in increasing concentrations to separate strips of cellulose acetate foil and resolved chromatographically in the water–*tert*-butanol/pet. ether (2:1:3) system. Steroids measured by induced sulfuric acid fluorescence. Activation maximum 465 mμ for both steroids; fluorescence maximum 505 mμ for aldosterone (XX) and 525 mμ for corticosterone (XV).

aldosterone were applied as mixtures to strips of purified foil, and chromatographed in the *tert*-butanol–water/petroleum ether system. A reference strip was run using 20-μg amounts of the same steroid mixture to permit localization of the compounds. The steroids were eluted, and the sulfuric acid-induced fluorescence was measured. An activation maximum at 465 mμ was observed for both steroids. A fluorescent maximum of 525 mμ was noted for corticosterone and 505 mμ for aldosterone. The maxima

for corticosterone were in accord with those found by other investigators (16–18). In the course of the spectrofluorometric analysis of aldosterone, it was noted that the fluorescent peak of this steroid was represented by an inconspicuous projection taking off from the descending limb of the large resonance peak produced by the activating light. This fluorescent maximum was visualized only during careful inspection using a slit system permitting high resolution. The readings obtained in this experiment were plotted against concentration as micrograms per milliliter of final sulfuric acid reaction mixture. This relationship is shown in Fig. 1.

DISCUSSION

In the evaluation of possible solvents for this chromatographic technique the most significant limitation was the solubility of the foil itself. The solubilities for cellulose acetate are in agreement with those cited by Burrell (19) for cellulose acetate powder. In the form of a foil cellulose acetate was unable to tolerate 100% methanol, ethanol, dichloromethane, or 1,2-dichloroethane.

The relative mobilities of the different steroids are reciprocally related to those observed in partition chromatography on cellulose paper. Exceptions occurred in the cases of aldosterone and tetrahydroaldosterone, where the mobilities were greatly increased, and tetrahydrocortisone, where the mobility was less than expected. Corticosterone, 11-dehydrocorticosterone, and 11-deoxy-17α-hydroxycorticosterone are better resolved by increasing the time of chromatography in the *tert*-butanol–water/petroleum ether system.

The findings presented here are in accordance with the theoretical considerations of solvent interaction during conventional chromatography discussed by Cassidy (20); however, in the reverse phase system the direction of the forces is reversed. There is an additional difference in that adsorption phenomena are minimal when a hydrophobic supporting medium, such as cellulose acetate, is used, contributing to an enhancement of the partition effect of solute molecules between the solvent pair.

The following factors were found to be critical determinants of reproducible steroid mobilities: (*a*) constant ambient temperature; (*b*) velocity of solvent descent (controlled by the angle at which the strips are held in the trough: 8–10 degrees in these experiments; and constant height of solvent within the trough); (*c*) uniform thickness and degree of induced purity of the foil.

The application of cellulose acetate foil for quantitative chromatography, especially when dealing with submicrogram quantities of solute is predicated upon the ability of this material to withstand the extensive purification necessary to eliminate "background" residue. This purifica-

tion is in effect an exhaustive preliminary elution of the foil. Following chromatography in such a medium, eluted steroids are obtained in a relatively pure state, in which the highly sensitive fluorescence phenomena induced by concentrated sulfuric acid may be used for the measurement of submicrogram and microgram quantities of steroids. Experiments designed to test the sensitivity of this technique (Fig. 1), suggest that for the concentration of the two steroids chromatographed and eluted, good linearity exists between steroid concentration and fluorescent intensity. The slopes of these lines demonstrate the highly efficient fluorescent characteristics of corticosterone as compared to the weakly fluorescent behavior of aldosterone. The data for aldosterone, plotted at ten times the scale used for corticosterone, reveal a relative fluorescence of 1:100 as compared to the former steroid. The examination of these data suggests the absolute level of sensitivity for corticosterone chromatographed and eluted in the manner described was well below 0.125 μg/ml of final reaction mixture, while the amount of aldosterone detected by this technique was above this level with greater accuracy in the 0.25 and 1.0 μg/ml range. In terms of practical application it is felt that despite divergences in fluorescent efficiency of individual steroids, as exemplified in the two cases cited above, the degree of sensitivity was of the order of magnitude required for the microanalysis of steroids in biological fluids. The importance of steroid glucosiduronates as end products of steroid hormone metabolism in the mammalian species suggests the desirability of applying reverse phase chromatographic technique to the isolation of these substances.

SUMMARY

1. The reverse phase partition chromatography of several groups of steroids has been described using a cellulose acetate foil as the supporting medium.

2. The sulfuric acid-induced fluorescence of steroids may be used for quantitation after chromatography on this foil.

3. The recovery of cortisol from this foil averages 58%.

4. Evidence is presented that the recovery of corticosterone and aldosterone remains constant for submicrogram quantities.

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ANALYTICAL BIOCHEMISTRY 2, 161–168 (1961)

Ribonuclease Contamination of Crystalline Deoxyribonuclease, Trypsin, and of Partially Purified Foot-and-Mouth Disease Virus Preparations

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INTRODUCTION

The infectious nucleic acid moiety of foot-and-mouth disease virus (FMDV) has been postulated to be a ribonucleic acid (RNA) by virtue of its susceptibility to the action of ribonuclease (RNase) (1, 2). Bachrach (3) confirmed this finding but also observed that FMDV–RNA lost infectivity upon exposure to commercial preparations of crystalline deoxyribonuclease (DNase) and trypsin. It was presumed that this loss was due to the presence of RNase as a contaminant in the enzymes used. This is not improbable since the three enzymes are all derived from the pancreas. It is now possible to examine crystalline preparations of DNase and trypsin for contamination with extremely minute amounts of RNase by using an assay for RNase with 0.1-mμg sensitivity (4). The study was also extended to partially purified preparations of FMDV since they too, are known to contain RNase activity (3).

MATERIALS AND METHODS

Enzymes and Inhibitor

RNase, DNase (5, 6), trypsin, and soybean trypsin inhibitor (7) were obtained as the crystalline enzymes from Worthington Biochemical Corporation.

Virus

Partially purified preparations of FMDV, type A, strain 119, were made from virus produced in calf-kidney cell cultures (8). The harvested virus fluid was treated with 20% methanol at 0° to precipitate virus. The precipitate, after overnight storage at —12°, was resuspended in a 1:1 mixture of Hanks' balanced salt solution and isotonic Tris buffer, pH 7.5,

and shaken several times with cold butanol–chloroform mixtures. The aqueous phase was then dialyzed against the suspending fluid and stored at —60°.

Ion-Exchange Agents

Diethylaminoethyl cellulose (DEAE-cellulose) was thoroughly washed with 0.25 *M* phosphate buffer at pH 8.0 before use to remove color and low molecular weight dialyzable impurities which absorbed light at 280 mμ. Amberlite CG-50 (XE-64), 400–600 mesh, was obtained in a purified form from Fisher Chemicals.

Virus Assay

The plaque assay for FMDV in primary bovine kidney cell cultures were carried out as previously described (8). The inoculated cell layers were incubated 90 min at 37° before overlaying with agar. Plaques were counted after 72 hr additional incubation.

Enzyme Assays

Ribonuclease was assayed by a microbiological method with a sensitivity of 0.1 mμg RNase/ml based on the sensitivity of FMDV infectious RNA to RNase (4). Deoxyribonuclease was assayed by the spectrophotometric procedure of Kunitz (6).

Protein Determination

Protein was determined principally by absorption at 280 mμ and also with Folin-Ciocalteau reagent (9).

EXPERIMENTAL AND RESULTS

A DEAE-cellulose column was selected for use in separating RNase impurities from DNase because DNase, but not RNase, is adsorbed on DEAE-cellulose columns during the chromatography of pancreatic juice (10). Moreover, this resin has been used for the purification of many proteins and viruses (11–14).

Examination of Crystalline RNase

The chromatographic behavior of RNase alone was determined first, using the following general procedure: One-gram portions of adsorbent in 10-mm wide columns were equilibrated with 0.01 *M* potassium phosphate buffer, pH 8.0, at 4°. Column heights ranged from 85 to 92 mm. The sample charge was 10 mg RNase contained in 5 ml of the 0.01 *M* phosphate buffer. The sequence of eluting mediums was arranged to meet the needs in subsequent experiments for the chromatography of FMDV and

other enzymes. Selected specified volumes of pH 8.0 phosphate buffers of increasing ionic strength were used as follows: eluting medium A, 0.01 *M* potassium phosphate; B, 0.01 *M* potassium phosphate containing 0.025 *M* sodium chloride; C, 0.01 *M* potassium phosphate containing 0.05 *M* sodium chloride; D, 0.01 *M* potassium phosphate containing 0.075 *M* sodium chloride; E, 0.01 *M* potassium phosphate containing 0.10 *M* sodium chloride; F, 0.01 *M* potassium phosphate containing 0.15 *M* sodium chloride; G, 0.05 *M* potassium phosphate; and H, 0.25 *M* potassium phosphate. The rates of flow ranged from 20 to 30 ml/hr, and eluates were collected as 5-ml fractions. Table 1 shows the percentages of RNase

TABLE 1
RNase Chromatography on DEAE-Cellulose

Pooled eluates	Per cent of starting RNase activity
2– 4	93.
5–11	3.2
12–21	1.0
22–31	1.0
32–41	0.10
42–44	0.03
45–51	0.07

activities in eluates which were pooled on the basis of their absorption at 280 mμ.

In confirmation of a previous report (10), more than 96% of the starting RNase passed through the column without being adsorbed. However, beginning with the use of 0.01 *M* phosphate buffer containing 0.025 *M* sodium chloride, all fractions showed a low but definite level of RNase activity. Even with this tailing of activity, the RNase content of a protein or virus preparation could possibly be decreased by a factor of about 100, provided, of course, that the non-RNase material is adsorbed by DEAE-cellulose.

Examination of Crystalline DNase

At a 1:100 dilution of a 1 mg/ml DNase solution, RNase activity could still be detected. This corresponded to 0.01 μg RNase, or 1 part RNase in 100,000 of the original DNase, which represents an amount of contaminant far too small to be detected by the conventional methods of RNase assay (5, 15, 16).

An attempt was then made to separate the two different enzyme activities of the DNase by chromatography on DEAE-cellulose. A 5-ml sample charge containing 1 mg/ml DNase in 0.01 *M* potassium phosphate buffer,

pH 8.0, was passed through a 1-gm column of DEAE-cellulose as previously described. It was eluted consecutively with 20 ml of eluting medium A, 30 ml D, and 50 ml E. The optical density curve obtained is shown in Fig. 1; Table 2 compares RNase activity of the various fractions with their DNase content.

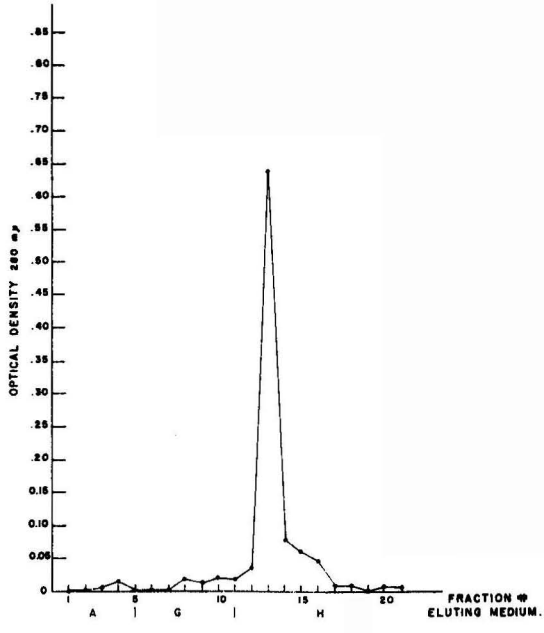

FIG. 1. DEAE-cellulose chromatography of DNase.

TABLE 2
DNASE CHROMATOGRAPHY ON DEAE-CELLULOSE, WITH ACCOMPANYING DISTRIBUTION OF RNASE CONTAMINANT

Eluate fractions	Per cent of starting DNase activity	Per cent of starting RNase activity
1–5 combined	1.6	50
6–11 combined	6.9	25
13	61.0	10

Apparently, the bulk of the RNase activity passed through the column in the initial stages of elution as previously shown for the RNase alone, whereas the DNase was eluted at a later stage. This observation confirmed the hypothesis (3) that the activity shown by DNase against infectious FMDV-RNA is due to the presence of RNase as a contaminant. The

amount of RNase still remaining in the DNase can be decreased by rechromatography, or by running the initial chromatography at a much lower flow rate.

Examination of Crystalline Trypsin

Using the method of assay for RNase described by Polatnick and Bachrach (4), it was first found that a dilution of 1:1000 of a 1 mg/ml trypsin solution containing 2 mg/ml of soybean–trypsin inhibitor represented the end point of the assay, in that RNase activity was just still present. The end point corresponded to 0.1 μg RNase; or 1 part in 10,000 of the original trypsin was RNase. The trypsin inhibitor by itself showed no activity toward FMDV–RNA. In view of the reported lack of adsorption of the cationic components of pancreatic juice by DEAE-cellulose (10), separation of the two enzyme activities was attempted by chromatography on Amberlite CG-50 columns. Following the procedure recommended by Hirs *et al.* (17), preliminary semiquantitative determinations were made of distribution coefficients in 0.2 *M* potassium phosphate buffer solutions of trypsin at different pH levels. There was very slight to no removal of the protein from the resin at pH levels less than 6.0, but use of the resin at pH 6.25 seemed more promising. However, an initial run at this pH with a known mixture of trypsin and RNase indicated extensive overlapping of activities, and the procedure was dropped.

Examination of Partially Purified FMDV

Bachrach (3) reported that virus preparations treated at pH 2.5 for 5 min and then neutralized no longer possessed infectivity but that such preparations even after 60-fold dilution were capable of inactivating FMDV–RNA. In the present study, a dilution of 1:125 of a purified virus preparation assaying 2.5×10^9 plaque-forming units/ml was the end point of the assay. This value corresponded to 0.013 μg RNase/ml virus and represented enzyme material that had passed through the deproteinization procedures used to purify the virus.

Since RNase had been successfully removed from partially purified virus by differential centrifugation (3), chromatographic treatment was tested for similar effectiveness. DEAE-cellulose columns were used since crude guinea pig vesicular fluid infected with FMDV had been fractionated on them with nearly quantitative recovery of infectivity (18). One-milliliter quantities of purified virus were diluted to 5 ml with 0.01 *M* potassium phosphate buffer, pH 8.0, and dialyzed against the same buffer system. The fully equilibrated sample was then passed through a DEAE-cellulose column as previously described. The low optical density readings

at 280 mμ for protein in the eluates necessitated that a more sensitive protein analysis be made (9). The protein analyses are shown in Fig. 2.

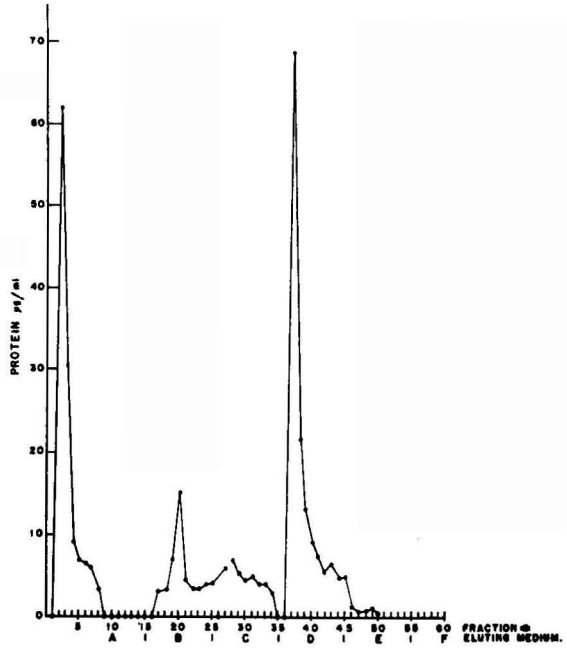

FIG. 2. DEAE-cellulose chromatography of partially purified FMDV.

Table 3 shows viral and RNase activities of various pooled eluates, together with protein distribution.

TABLE 3
FMDV CHROMATOGRAPHY ON DEAE-CELLULOSE

Pooled eluates	Per cent of starting viral infectivity	Per cent of starting protein	Per cent of starting RNase activity[a]
1–5	<0.01	31. (+4.6 for Nos. 6–15)	60.
16–20	0.7	8.3	0
21–25	0.6	5.6	0
26–30	69.	6.6	0
31–35	17.	4.6	0
36–40	5.4	32.	0
41–45	2.3	8.4	0
46–50	1.7	<1.	—
51–55	0.2	—	—
56–60	<0.05	—	—
	96.9	101.	

[a] Pooled eluates Nos. 6–15 not assayed for RNase activity.

The data shows an excellent recovery of virus activity with about tenfold decrease in protein content of the fraction containing the major portion of the virus activity. All the RNase activity passed through in the initial eluates, and the subsequent virus-containing eluates no longer had RNase activity. The peak of virus activity was eluted with 0.01 *M* phosphate buffer containing 0.05 *M* sodium chloride, whereas a higher ionic strength eluant, 0.01 *M* phosphate buffer (pH 7.6) containing 0.125 *M* sodium chloride, was needed to remove the major part of the infectivity starting with crude vesicular fluid (18).

DISCUSSION

The presence of RNase in crystalline preparations of DNase and trypsin has been able to be demonstrated by the use of an extremely sensitive microbiological method. The extent of this contamination, only 1 part in 100,000 in the case of DNase, is still sufficient to interfere with the activity of an infectious RNA, and the use of such a DNase preparation to identify a biologically active nucleic acid can thus lead to possible misinterpretations. Sensitivity to DNase is often used as the criterion for attributing biological activity to a DNA.

The minute amount of this RNase contaminant can be largely removed from the DNase preparation by chromatography on DEAE-cellulose. The cellulose column, equilibrated with 0.01 *M* potassium phosphate, pH 8.0, retains the DNase while allowing the RNase to pass through. The DNase can then be recovered with higher ionic strength eluants.

The 0.013 μg RNase found per milliliter of partially purified virus can be partially traced to the RNase present in the serum used in propagating the virus from calf-kidney cell cultures. The bovine serum used contained 0.08–0.10 μg/ml RNase (4), and was present in a 0.2% concentration in the growth medium used. This level of RNase (0.2 mμg/ml) coupled with the approximately 100-fold volume concentration achieved during virus purification yields 0.02 μg/ml as the amount of RNase which would be present if none of the enzyme content was removed during purification. Therefore, at least 35% of the RNase originally present was lost as a result of processing of the virus, since there would also be RNase contributed by the host cells. The presence of RNase as a contaminant in virus preparations should always be considered during purification procedures, since serum is a standard ingredient of growth media.

SUMMARY

Using a microbiological assay for RNase based on the inactivation of viral RNA and capable of detecting RNase at a concentration as low as 0.1 mμg/ml, commercially available crystalline preparations of the pancreatic enzymes DNase and trypsin were found to be contaminated with

1 part in 100,000 and 1 part in 10,000, respectively, of RNase. A previous hypothesis that the susceptibility of infectious FMDV–RNA to crystalline DNase and to trypsin was due to the presence of an exceedingly small amount of RNase was also confirmed. The RNase content of partially purified preparations of FMDV was found to be about 0.013 μg/ml, attributable to the bovine serum and host cells used for viral propagation. This RNase contamination could be removed by chromatography on DEAE-cellulose, which allowed most of the enzyme to pass through unadsorbed. Similar chromatographic treatment of DNase decreased the amount of RNase present. Amberlite XE-64 treatment of trypsin was unsatisfactory for the separation of RNase activity.

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ANALYTICAL BIOCHEMISTRY 2, 169–173 (1961)

A Method for Measuring Zinc in Leucocytes and Erythrocytes[1]

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INTRODUCTION

The study of zinc metabolism has been hampered by difficulties of methodology, so that results have not been reproducible in the past. Work by Vallee and Gibson (1), Hoch and Vallee (2), Berfenstam (3), Wolff (4), and Malmström (5) in recent years has resulted in the evolution of methods for the determination of zinc in biological materials which have made possible reliable and reproducible determinations of zinc in sera and erythrocytes, but no satisfactory modification and application of these methods to the measurement in leucocytes have been described previously. The problems which had to be solved in regard to leucocytes were those of (*A*) the selection of a sedimenting solution which would neither introduce contamination nor leach out zinc and which would yield a relatively pure white cell suspension in which the cells were intact and unclumped, so that the counting of cells and determination of their zinc content could be reliably performed, and (*B*) the selection of leucocyte suspensions and acid extraction solutions of such concentrations and volumes that (*a*) all the zinc could be extracted, (*b*) sufficient zinc could be recovered at levels allowing reproducible measurement, (*c*) no turbid suspensions would result, and (*d*) still permitted the assay of leucocytes obtainable from a practical amount of whole blood. Because of the increased interest in zinc metabolism and in the biochemistry of leucocytes, and also because of the many technical difficulties encountered in our studies of leucocyte zinc, we wish to record here the details of the method which has been found most simple and satisfactory for the determination of zinc in blood cells.

[1]This work was supported by grants from the U. S. Public Health Service and Parke, Davis & Company.

MATERIALS

Reagents

Chemicals. Use reagent-grade chemicals throughout.

Zinc-Free Water. Pass distilled water through a mixed-bed ion-exchange column (containing Rohm and Haas Co. resins IR-120 and IRA-410), thus obtaining water with a specific resistance of 10–15 million ohms. All water to be used in this method should be zinc-free water.

Cell Sedimenting Solution. Dissolve 25 gm polyvinylpyrrolidone, 4.25 gm sodium chloride, and 100 mg sodium heparin in water and dilute to volume of 500 ml.

Normal Saline. Dissolve 8.5 gm sodium chloride in water and dilute to 1 l.

Hydrochloric Acid. Wash anhydrous HCl gas through bubble towers of concentrated sulfuric acid and water, and then dissolve in chilled water; the resulting hydrochloric acid is to be titrated and then diluted with water to the desired concentrations.

Trichloroacetic Acid. Redistill trichloroacetic acid and then dissolve it in water to the desired concentrations.

Ammonium Hydroxide. Wash anhydrous ammonia gas through bubble towers of concentrated sodium hydroxide and water, and then dissolve in chilled water; the resulting ammonium hydroxide to be titrated and diluted with water to the desired concentration. Make up a new solution weekly.

Buffer-Complexing Solution. Dissolve 136 gm sodium acetate, 80 gm sodium thiosulfate, and 4.0 gm potassium cyanide in about 800 ml water, bring to pH 5.5 by the addition of glacial acetic acid, and then make up to a total volume of 1 l with water. On the day of use, an aliquot of the solution should be made "zinc-free" by repeated extractions with dithizone solution.

Indicator Solution. 0.04% bromocresol green in 96% ethanol.

Dithizone Solution. 1.0 mg dithizone/100 ml carbon tetrachloride; make up fresh daily and keep in a cool, dark place until used.

Zinc Standard Solution. Dissolve 100.0 mg of metallic zinc in hydrochloric acid and dilute with water to a volume of 1 l, producing a stock standard solution containing 100 μg zinc/ml. Working standard solution to be prepared every 1–3 days by diluting 5 ml of the stock standard to 500 ml with zinc-free water, so that the working solution contains 1 μg zinc/ml.

Glassware and Equipment

Polyethylene tubes, bottles, spatulas, etc., should be used whenever

possible. Glassware, when used, should be Pyrex. Stopcocks should be lubricated with silicone stopcock grease, or else Teflon stoppers and stopcocks may be used. After routine cleaning the plastic and glass equipment should be soaked in 2 *N* nitric acid for at least 8 hr, and finally rinsed 8–10 times with zinc-free water, dried and stored in a dust-proof plastic cabinet until used.

Rinse the silicone-coated needles and syringes with 2 *N* nitric acid and zinc-free water. Dry and store them in a dust-proof plastic cabinet. Seal needles in glass needle tubes and autoclave. Syringes, test tubes, etc., should be protected from dust during transportation and use by "Parafilm" coverings.

METHODS

Preparation of Blood Samples

Mix the sample of venous blood with about an equal volume of cell sedimenting solution and allow to stand for about 45 min, after which the leucocyte-rich supernatant should be pipetted off and transferred to a 50-ml polyethylene centrifuge tube. Transfer approximately 2 ml of the erythrocyte-rich sediment to a 50-ml polyethylene centrifuge tube containing about 40 ml of normal saline. Wash cells three times with normal saline and then dilute with saline so that the concentration of the erythrocyte suspension is about 500,000 red blood cells/cu mm and the leucocyte-rich suspension is approximately 40,000–50,000 white blood cells/cu mm. Quadruplicate counts of erythrocytes and leucocytes should be made on an aliquot of each suspension.

The method used to extract the zinc from the cells is that of Berfenstam (3) as modified and described below. Place a 3-ml sample of each cell suspension in a 15-ml polyethylene centrifuge tube and centrifuge at 2000 $\times$ *g* for 10 min; discard the saline supernatant and replace it with an equal volume of water, then treat each sample as follows:

1. Add 2 ml of 20% HCl and mix.
2. Incubate at 37° C for 1 hr with mixing.
3. Add 3 ml of 20% trichloroacetic acid and mix.
4. Centrifuge at 2000 $\times$ *g* for 10 min.
5. Decant the supernatant into a 125-ml Squibb separatory funnel.
6. Add 4 ml of 10% trichloroacetic acid and mix with the precipitate.
7. Centrifuge at 2000 $\times$ *g* for 10 min.
8. Decant the supernatant into separatory funnel containing sample collected in 5.
9. Add 4 ml of 10% trichloroacetic acid and mix with precipitate.

10. Centrifuge at 2000 $\times$ g for 10 min.

11. Decant supernatant into the separatory funnel containing samples of 5 and 8.

The dithizone method for the determination of zinc detailed below is that of Malmström with only slight modification. Each sample contained in a separatory funnel (see above) should be treated as follows:

1. Add 1 drop of indicator solution.
2. Titrate with 50% ammonium hydroxide to a blue color (about pH 5.5).
3. Add 50 ml buffer-complexing solution and mix.
4. Add 10 ml of dithizone solution.
5. Shake funnel vigorously for 3 min.
6. Draw off carbon tetrachloride–dithizone layer into a 1-cm absorption cell and determine absorbance (A) in a Beckman Model DU spectrophotometer at 520 mμ (with a zinc-free water blank, processed in exactly the same manner as and concurrently with the sample, as a reference).

In addition to duplicate zinc-free water blanks, include duplicate zinc standards (2 or 3 μg) with each set of cell samples.

Calculation of Zinc Content and Normal Values

The zinc content of the samples should be calculated on the basis of the calibration constant determined for each set of samples. Unit cellular zinc levels must be determined by simultaneous solution of the equations for erythrocyte and leucocyte zinc, since leucocyte-rich samples are contaminated with erythrocytes, and the erythrocyte-rich samples with some leucocytes.

This method has been employed in previously reported studies in which we have documented its reliability (6). Using this method we have found that in normal persons leucocytes contain 103 μg zinc/10^{10} cells (S.D. $\pm$25.5), and the erythrocytes contain 12.1 μg zinc/10^{10} cells (S.D. $\pm$1.51) (Table 1).

TABLE 1
NORMAL VALUES FOR ZINC IN HUMAN LEUCOCYTES AND ERYTHROCYTES (38 DETERMINATIONS)

	Range	Mean	Standard deviation
	Micrograms Zn/10^{10} cells		
Leucocytes	56.8–168.0	103.0	25.5
Erythrocytes	9.3–15.5	12.1	1.51

SUMMARY

A method for the determination of zinc in blood cells is described and values for erythrocytes and leucocytes of normal persons based on this method are reported.

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ANALYTICAL BIOCHEMISTRY 2, 174–177 (1961)

An Ultramicro Method for the Determination of Urea Nitrogen in Blood Serum[1, 2]

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INTRODUCTION

The published methods for the determination of blood urea do not employ samples small enough to permit serial determinations in small laboratory animals. Therefore, we investigated the possibility of modifying an existing method to an ultramicro scale in which no more than 5 μl plasma would be required. This size sample can be collected in a capillary tube from the tip of the tail of a rat, one tube supplying triplicate samples of serum. Difficulties were encountered with methods employing the enzyme urease because of turbidity development due either to the enzyme preparation or the subsequent nesslerization procedure. Stabilizing reagents such as those suggested by Connerty, Briggs, and Eaton (1) were employed without success. The colorimetric method developed by Archibald (2, 3) which requires a 2-ml sample permits direct determination of urea using α-isonitrosopropiophenone as the reagent for the development of color. The adaptation of this procedure for use with a 5-μl sample of plasma gives reproducible results and quantitative recovery of added urea and checks the analysis of synthetic control serum analyzed by a macro method.

METHOD

A calibration curve using standard solutions of urea was prepared as follows:

A standard solution containing 400 μg urea N/ml was prepared by dissolving 0.4287 gm of urea in 500 ml water. Aliquots of this standard were diluted to obtain standards containing 300, 200, and 100 μg urea N/ml.

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[2] Supported in part by a grant from The Nutrition Foundation, Inc.

[3] Submitted to The Graduate School of The Pennsylvania State University in partial fulfillment of the requirements for the Master of Science degree.

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1. 5.1 μl of urea N standard containing 100 μg urea N/ml was pipetted[5] in triplicate into 70 $\times$ 7 mm test tubes. This was repeated with standards containing the 200, 300, and 400 μg urea N/ml.

2. 10.5 μl of 5% trichloracetic acid was added to each tube and mixed.

3. Tubes were centrifuged for 15–20 min.

4. 5.1 μl of the supernatant was transferred to clean tubes.

5. 48.0 μl water was added to each tube.

6. 48.0 μl of a sulfuric–phosphoric acid–water mixture (1:3:1, v/v/v) was added to each tube and mixed.

7. 10.1 μl of the alcoholic color reagent, α-isonitrosopropiophenone (4 gm in 100 ml of 95% alcohol), was added and mixed thoroughly.

8. The tubes were stoppered with a vaccine cap through which was inserted a capillary tube approximately 30 mm long. The tubes were placed in a boiling water bath from which light was excluded for 1 hr.

9. After exactly 1 hr of heating, the racks were set for 15 min in a covered pan containing water at room temperature.

10. Absorption was read at 540 mμ in a Beckman DU spectrophotometer fitted with a micro adapter[6] and 2.5-mm quartz cuvettes.

11. Readings of standards were corrected for reagent blanks and a calibration curve of net readings was plotted against equivalent concentrations. From the formula

$$K = \frac{\text{net reading of standard}}{\text{equivalent } \mu\text{g urea N/ml}}$$

a K value for each standard was calculated and an average K computed.

The procedure employed for analyzing the serum and a commercial control serum[7] was identical to that used for the standards. Urea N in the serum was calculated as follows:

$$\mu\text{g urea N/ml} = \frac{\text{net reading of sample}}{\text{average } K}$$

As a further test of the accuracy of the method, recoveries were made of known quantities of urea N added to the water which was used to dilute the sample (step 5). Four solutions were prepared containing 1.562, 3.125, 6.250 and 12.50 μg urea N/ml. An average K value for these solutions was calculated and referred to as the recovery K.

To calculate urea N recovery, the average optical density of the plain serum, analyzed in the same run, was subtracted from the optical density

[5] Constriction pipets were used, and the indicated volumes are based on calibrations in this laboratory.

[6] Pyrocell.

[7] Lab-Trol, Dade Reagents.

of the serum plus the additional urea. This figure, which corresponds to the optical density of the added urea, was divided by the recovery K. Thus, the per cent of recovery was obtained:

$$\frac{\text{O.D. of added urea N}}{\text{recovery } K} = \mu\text{g urea N/ml recovered}$$

$$\%\text{ recovery} = \frac{\mu\text{g urea N/ml recovered} \times 100}{\mu\text{g urea N/ml added}}$$

RESULTS AND DISCUSSION

Calibration curves obtained with urea standards indicated that optical density was proportional to concentration between 100 and 400 μg urea N/ml. K values obtained at higher concentrations of urea N were slightly lower than the values at lower levels indicating a deviation from Beer's law. Comparable deviations were reported by Archibald (3) and Rosenthal (4). The average K value of the standards was 0.00140 with a standard error of $\pm$0.000038.

The synthetic serum, according to the manufacturer's analysis, contained 147 μg/ml of urea N with an allowable range of variation of $\pm$20 μg/ml. Forty-two sets of triplicate samples of this control were analyzed giving a range of values from 137 to 151 μg urea N/ml. The average concentration of urea N in these samples was 144 μg/ml with a standard error of $\pm$0.61.

Forty-three sets of triplicate samples of blood serum from one normal rat were analyzed. During each run, triplicate commercial control serum samples were included for the purpose of providing a control for each series. The urea N concentration of serum ranged from 235 to 250 μg/ml, averaging 243.4 μg/ml with a standard error of $\pm$0.60.

Table 1 gives the results of the recovery test at each of four levels of

TABLE 1
RECOVERY OF UREA N ADDED TO RAT SERUM SAMPLES

No. of triplicate samples	Urea N added (μg/ml)	Average urea N recovery	
		μg/ml	%
10	1.562	1.488 ± 0.069	95.3
11	3.125	3.139 ± 0.085	100.4
11	6.250	6.079 ± 0.061	97.1
11	12.50	12.00 ± 0.103	96.0

added urea N. In all, 43 sets of triplicate samples were analyzed. The recoveries ranged from 95.3 to 100.4% with an average of 97.5% and a standard error of $\pm$0.51. In a method employing a 1-ml sample and

nesslerization, Connerty, Briggs, and Eaton (1) reported urea recovery of 98.6%. However, calculation of their data indicated a standard error of ±3.0%. It appears, therefore, that more accurate results may be expected from the method presented in this paper.

SUMMARY AND CONCLUSIONS

An ultramicro adaptation of the Archibald method for the determination of serum urea N utilizing a 5-μl sample has been described. The data demonstrate that this adaptation provides an accurate and practical method for the determination of urea N in serum.

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ANALYTICAL BIOCHEMISTRY 2, 178–187 (1961)

SHORT COMMUNICATIONS

Chromatographically Pure Fluorescein and Tetramethylrhodamine Isothiocyanates

Since fluorescein isocyanate was first utilized in the tagging of antibodies by Coons and his co-workers (1, 2), its use and that of related compounds in the preparation of fluorescent antibodies has expanded rapidly. Fluorescein isocyanate is a rather unstable compound, and Riggs *et al.* (3) found that fluorescein isothiocyanate was more stable and more easily prepared. This compound (as isomer 1 or isomer 2, or as a mixture of the two) is commercially available and is now widely used.[1]

It was found that when many of the early samples of fluorescein isothiocyanate were assayed by paper chromatography, as described below, they were of low and variable purity. The fluorescein isothiocyanate content of the samples varied from 20 to 60%. This variation makes very difficult an accurate calculation of the amount of dye to be used in a conjugation. It was, therefore, felt that these compounds in their pure state should be prepared and characterized by elementary analysis, paper chromatography, and infrared absorption analysis. Although the rhodamines have not been as widely used as the fluoresceins, the two isomers of tetramethylrhodamine isothiocyanate [analogous to Pressman's tetramethylrhodamine isocyanate (4)] were included in the study.

Tetramethylrhodamine isothiocyanate was prepared conventionally. 4-Nitrophthalic anhydride and *m*-dimethylaminophenol, in 1:1 proportions, were fused at 180–190°C for 1 hr. The resulting isomers, "R" and "G," of nitrotetramethylrhodamine were separated chromatographically on a cellulose column, using 50% methanol as the partitioning liquid. The nitro compounds were then reduced and reacted with thiophosgene to give the desired compounds.

In the following tables are given analytical data on these four compounds. It should be noted that all the samples used were chromatographically homogeneous on paper, under the conditions used.

It was found that the information obtained by paper chromatography was a very significant criterion of purity of these isothiocyanates. Using

[1] Both isomers of fluorescein isothiocyanate and isomer "R" of tetramethylrhodamine isothiocyanate are available from the Baltimore Biological Laboratory, Inc., Baltimore 18, Maryland.

the developing media shown in the first table, it was possible easily to separate the two isomers of fluorescein isothiocyanate and of tetramethylrhodamine isothiocyanate, as well as to show decomposition and by-products occurring in their preparation. The R_f figures listed were obtained as ascending chromatograms using Whatman No. 1 paper, 1.5 × 21 in., and applying 4–6-μg quantities dissolved in acetone (Table 1).

TABLE 1
TABLE OF R_f VALUES

Compound	Developing medium	
	0.2 *M* Na_2HPO_4	20% dioxane
Fluorescein isothiocyanate, No. 1	0.29	
Fluorescein isothiocyanate, No. 2	0.37	
Tetramethylrhodamine isothiocyanate, isomer "G"		0.52
Tetramethylrhodamine isothiocyanate, isomer "R"		0.68

The microanalyses[2] show excellent agreement with theory in all cases (Table 2). The four compounds show an affinity for water and appropriate

TABLE 2
MICROANALYSES OF FLUORESCEIN AND TETRAMETHYLRHODAMINE ISOTHIOCYANATES

	Fluorescein isothiocyanate, No. 1	Theory (%)	Fluorescein isothiocyanate, No. 2	Tetramethylrhodamine isothiocyanate, isomer "R"	Theory (%)	Tetramethylrhodamine isothiocyanate, isomer "G"
Carbon	64.64	64.77	64.76	67.49	67.70	67.55
Hydrogen	2.92	2.85	3.10	4.75	4.75	4.81
Nitrogen	3.55	3.60	3.63	9.59	9.48	9.46
Sulfur	8.23	8.23	8.28	7.29	7.23	7.30

care must be taken in preparing samples for analysis. From the practical standpoint of storing these compounds, they should be protected from moisture to prolong their shelf life.

It is felt that the simple paper chromatographic analysis of these isomers can be used as a practical method for determining the purity of fluorescein or tetramethylrhodamine isothiocyanate.

Work on conjugation procedures, using these pure dyes, is now in progress and will be reported at a later date.[3]

[2] Microanalyses by Foster D. Snell, Inc.

[3] This work was in part supported by the National Cancer Institute, Field Investigations and Demonstrations Branch.

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A Convenient Technique for Dialysis

In the course of studying dialyzable components in digests of connective tissue,[1] a method of dialysis was sought which would offer the following advantages over the conventional procedure of equilibrating the filled dialysis sac with a large volume of fluid: (*a*) nearly complete removal of dialyzable material, (*b*) a relatively small volume of dialyzate, and (*c*) a rapid rate of dialysis.

The following procedure has been found to fulfill the foregoing requirements to a satisfactory degree.

The filled sac is suspended from a support, and the dialyzing fluid is permitted to flow on the sac at a rate of approximately 2 ml/min. A small diameter cord is then wrapped around the bag in a spiral with only a slight pitch and with successive windings approximately 3 mm apart (Fig. 1*a*). The cord must wet readily so that surface tension of the fluid will hold it to the bag.[2] When the sac has been wrapped, the flow of dialyzing fluid is adjusted to a slower rate to hold the dialyzate volume to a minimum.

The data presented in Table 1 will serve to illustrate the effectiveness of the method described. The results were obtained by suspending over a fraction collector a Visking 18/32 sac, wrapped as described, containing 50 ml of 0.9 *M* ammonium sulfate and allowing water to flow down the

[1] Connective tissue investigations aided by grants from Kansas Tuberculosis and Health Association, Jane Coffin Childs Memorial Fund for Medical Research, and U. S. Public Health Service (RG-6290).

[2] Clark's No. 8 Six Cord, not mercerized, is being used, but any similar cord would presumably be satisfactory.

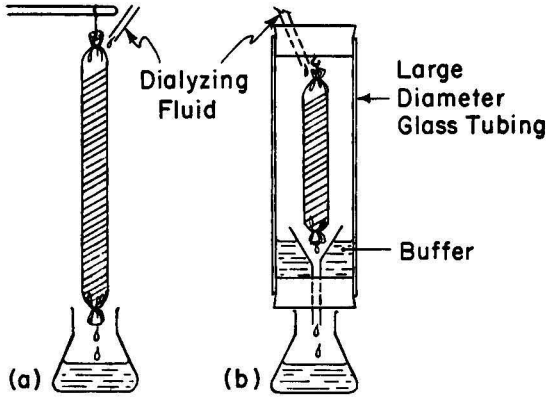

FIG. 1. Diagram of setup for dialysis. For dialysis against water the bag may be suspended in air (*a*); but for dialysis against buffers provision must be made to prevent changes in buffer concentration due to evaporation. This is accomplished by the arrangement shown in (*b*).

bag at a rate averaging 1.6 ml/min. The dialysis was carried out at room temperature. Fractions were collected at 10-min intervals. Aliquots of the dialyzate fractions were then analyzed for ammonia.

It can be seen that 99% of the ammonia is removed in slightly less than 7 hr with a dialyzate volume only 13 times that of the solution dialyzed. These results were obtained without stirring the contents of the bag. Stirring would be expected to increase the rate of dialysis (1) and would be particularly useful for large diameter tubing.

The conditions given for dialysis of ammonium sulfate are not necessarily the most favorable from the standpoint of rate of flow of dialyzing

TABLE 1

REMOVAL OF AMMONIA ON DIALYSIS OF 50 ML OF 0.9 *M* $(NH_4)_2SO_4$

Time (min)	NH_3 removed (mmoles)	NH_3 removed (%)
0	0[a]	0
10	9.2	10.3
20	17.7	19.8
30	25.2	28.2
40	32.2	36.0
50	38.4	42.9
60	43.9	49.1
400[b]	88.6	99.2

[a] Present in the bag: 89.4 mmoles.

[b] Total volume of dialyzate: 647 ml.

fluid and minimum volume required. The data given serve simply to show the advantages of this technique compared to the conventional equilibration procedure. We routinely dialyze 100-ml volumes for 17–20 hr at 4°C with a flow of dialyzing fluid over the bag of about 50 ml/hr. This has served to reduce 1 *M* NaCl and 0.1 *M* phosphate buffer to less than 10^{-4} *M* solutions with about 10 vol of dialyzing fluid. The equilibration procedure would require 3–5 changes of 10 vol each.

The procedure outlined permits almost complete recovery of a dialyzable substance in a minimum volume of solution.

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Separation of Urinary 17-Ketosteroids by Gas Chromatography

The principal 17-ketosteroids excreted by humans are androstan-3α-ol-17-one (androsterone), 5β-androstan-3α-ol-17-one (5β-androsterone, etiocholanolone) and Δ^5-androsten-3β-ol-17-one (dehydroepiandrosterone). These steroids are present in normal urine as sulfate and glucuronide conjugates, and their identification and estimation is of interest in studies of several endocrine abnormalities and in steroid metabolic studies. The free steroids may be obtained by solvent extraction after acid or enzymic hydrolysis of the conjugated excretion products,[1] and the determination of total 17-ketosteroids is usually carried out by a colorimetric method based on the Zimmermann reaction (2).

The recent development of gas chromatographic techniques for steroid

[1] Variations in the hydrolysis procedure may affect the yield of individual steroids; see, for example, the enzymic procedure of Givner *et al.* (1) for estrogen determinations. The hydrolysis problem is under investigation by Dr. B. G. Creech and Dr. J. A. Cooper, Methodist Hospital, Texas Medical Center, Houston, Texas.

separations (3–11) suggested that individual urinary steroids might be determined by using gas chromatographic procedures. It was known from earlier work that nonpolar phases[2] will separate ring A/B-*cis* and A/B-*trans* isomers, but a corresponding Δ^5 compound under these conditions usually has a retention time approximately the same as that of the 5α-H (allo) steroid. Retention times for 3-hydroxy epimers are usually different, but this effect is much smaller in magnitude for nonpolar columns than for polar columns. Androstan-3α-ol-17-one and Δ^5-androsten-3β-ol-17-one, when examined by gas chromatography with a 1.5% SE-30 silicone column, were found to have slightly different retention times, but when a mixture was chromatographed a single peak was obtained. The observed separation factor for the 5α/5β pair was 1.05, and for a three-component mixture the separation factor $(5\alpha + \Delta^5)/5\beta$ was 1.08.

A different separation pattern was seen with a 0.75% neopentyl glycol succinate (NGS) column. A small difference in retention times was observed for the 5α,5β pair of isomers, but a mixture of the two compounds gave a single peak. However, the retention time of the Δ^5 steroid was quite different; the separation factor $\Delta^5/(5\alpha + 5\beta)$ was 1.17.

These observations suggested that a quantitative determination might be made through the use of data obtained from two column separations; this is analogous to the use of two equations to solve for three unknowns. The only special requirement for the chromatographic separations was that posed by the low separation factor, 1.08, observed for the steroids on an SE-30 column. In order to obtain a separation with very little overlap, a column of better than 5000 theoretical plates was needed. This was prepared by using a 12 ft $\times$ 3 mm I.D. glass column with 1.5% SE-30 phase on 100–140 mesh Gas-Chrom P support. The column gave theoretical plate values of 6600–7300 (cholesterol and stigmasterol, respectively). The preferred theoretical plate value for an NGS column was about 2000–2500; an ordinary column, 6 ft $\times$ 5 mm I.D., was satisfactory for this purpose. This provided a good separation of the Δ^5 steroid from the 5α,5β pair, and it gave a single peak for the 5α,5β isomers. Table 1 contains relative retention time data for these compounds and for mixtures (1:1).

Synthetic mixtures of these 17-ketosteroids were prepared to correspond to three different situations given in the literature (12); these were a normal male, a male castrate, and a female with adrenal tumor. Figure 1 shows the gas chromatographic separation observed for these mixtures with an SE-30 column, and Fig. 2 shows the separation observed for the same mixtures with an NGS column. Table 2 gives the calculated and

[2] SE-30, SE-52, and SF-96 silicone polymers and Apiezons L and M have been used as nonpolar phases for steroid separations; currently we prefer an SE-30 phase.

TABLE 1

RELATIVE RETENTION TIMES FOR 17-KETOSTEROIDS

Compound	Relative retention time	
	SE-30[a]	NGS[b]
1. Androstan-3α-ol-17-one	0.38	3.96
2. 5β-Androstan-3α-ol-17-one	0.35	4.12
3. Δ^5-Androsten-3β-ol-17-one	0.37	4.74
4. Mixture of 1 and 2		4.05
5. Mixture of 1 and 3	0.38	
6. Cholestane	1.00[c]	1.00[d]

[a] Conditions: column, 12 ft × 3 mm I.D.; 1.5% SE-30 silicone polymer on 100–140 mesh Gas-Chrom P; 20 psi; 216°.

[b] Conditions: column, 6 ft × 5 mm I.D.; 0.75% neopentyl glycol succinate (NGS) on 100–140 mesh Gas-Chrom P; 24 psi; 209°.

[c] Time, 33.9 min.

[d] Time, 3.8 min.

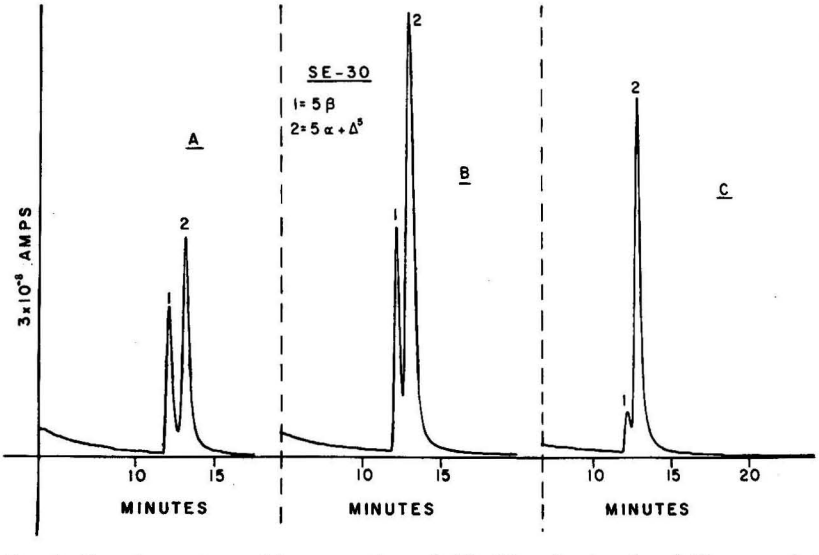

FIG. 1. Gas chromatographic separation of (*1*) 5β-androstan-3α-ol-17-one and (*2*) androstan-3α-ol-17-one and Δ^5-androsten-3β-ol-17-one. Composition data for mixtures A, B, and C are given in Table 2. Column conditions: 12 ft × 3 mm I.D. glass column; 1.5% SE-30 silicone polymer on 100–140 mesh Gas-Chrom P; 216°; 20 psi.

found values for the mixtures. These were calculated from area measurements using the conventional method of multiplying peak height by width

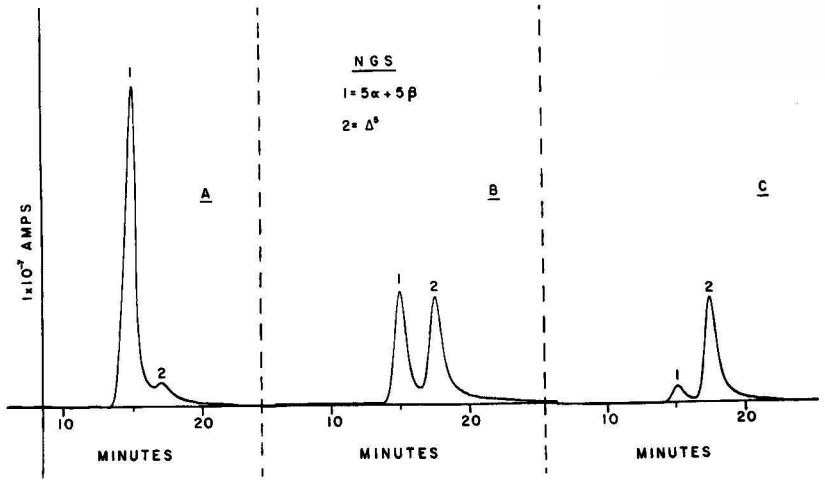

FIG. 2. Gas chromatographic separation of (*1*) 5β-androstan-3α-ol-17-one and androstan-3α-ol-17-one, and (*2*) Δ^5-androsten-3β-ol-17-one. Composition data for mixtures A, B, and C are given in Table 2. Column conditions: 6 ft × 5 mm I.D. glass column; 0.75% neopentyl glycol succinate (NGS) on 100–140 mesh Gas-Chrom P; 209°; 24 psi.

TABLE 2
COMPOSITION VALUES FOR 17-KETOSTEROID MIXTURES

Mixture	Component (% composition)		
	1[a]	2[b]	3[c]
A. Normal Male			
Weight composition	50	41	9
Found	49	37	14
B. Male castrate			
Weight composition	16	27	57
Found	21	28	51
C. Adrenal tumor (female)			
Weight composition	1	13	86
Found	1	12	87

[a] Androstan-3α-ol-17-one.
[b] 5β-Androstan-3α-ol-17-one.
[c] Δ^5-Androsten-3β-ol-17-one.

at half-height.[3] The determinations with reference compounds are accurate to about 1–5%, and this is sufficient for most biological work.

[3] When an ionization detector is used, a correction factor may be required in area calculations if the compounds have a differing oxygen content (C. C. Sweeley, private communication). In this instance, the steroids do not differ in oxygen content.

Mixtures of biological origin commonly contain compounds with a wide range in molecular weight or polarity, including many components that often contribute to analytical values obtained in spectroscopic or colorimetric determinations. When gas chromatographic procedures are used, minor constituents can be separated from the major constituents under study, and these appear as multiple small peaks in analytical charts. It is usually undesirable to assign quantitative values to these trace materials, although under some circumstances the presence or absence of specific compounds may be a matter of interest. In these cases a temperature programming technique is preferred in place of the usual isothermal technique, since this permits complex mixtures to be scanned with little alteration in peak width throughout the separation. With urinary steroids, an isothermal procedure is satisfactory if the 17-ketosteroids alone are under study; if C_{21} steroids are also of interest, it may be desirable to use temperature programming. Figure 3 shows an isothermal separation of

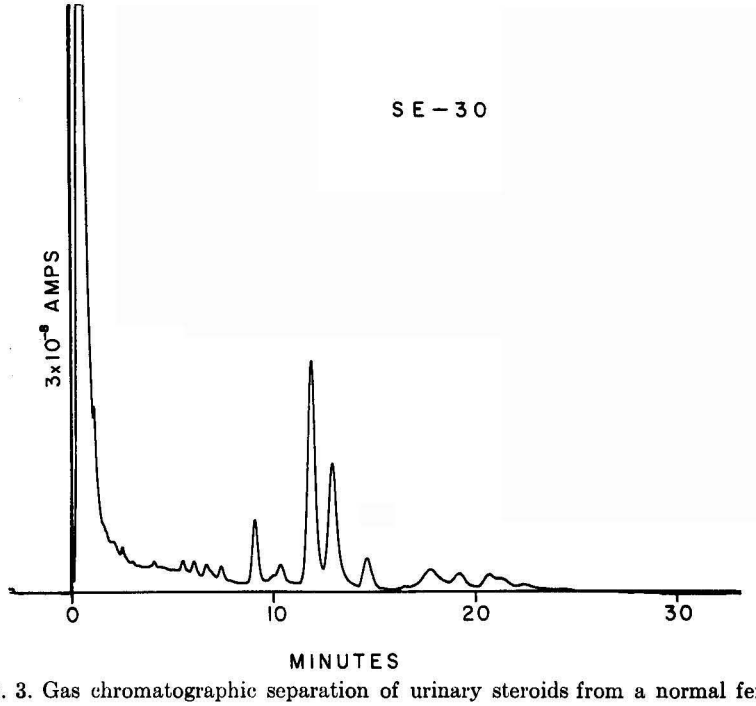

FIG. 3. Gas chromatographic separation of urinary steroids from a normal female. The steroid sample was obtained by an enzymic hydrolysis procedure and was not subjected to column or paper chromatographic purification. The two major peaks correspond to 5β-androstan-3α-ol-17-one and a mixture of androstan-3α-ol-17-one and Δ^5-androsten-3β-ol-17-one (see Fig. 1*A* for comparison). The identity of other components of the mixture has not been established. The column and column conditions are those used for Fig. 1.

urinary steroids of a normal female;[4] the two major peaks are due to 5β-androstan-3α-ol-17-one and a mixture of androstan-3α-ol-17-one and Δ^5-androsten-3β-ol-17-one. This was obtained with a 1.5% SE-30 column (support 100–140 mesh Gas-Chrom P) at 216°.

These separations suggest that gas chromatographic procedures may provide a new way of identifying and estimating urinary steroids, particularly those of the 17-keto group.

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[4] We are indebted to Dr. G. S. Duboff, Department of Obstetrics and Gynecology, University of Michigan, for the urinary steroid sample.

Erratum

H. J. McDonald, L. J. Banaszak, and J. Q. Kissane, "The Separation of Human Serum Lipoproteins by Conventional Descending and Centrifugally Accelerated Paper Chromatography." *Anal. Biochem.* **1**, 44–59 (1960).

PAGE *51*: The expression for the standard deviation (S.D.), in paragraph 4, should be raised to the "½ power":

$$\text{S.D.} = \left[\frac{\sum(x - m)^2}{n}\right]^{1/2}$$

The calculations based on the formula are correct.

New Approaches in CELL BIOLOGY

PROCEEDINGS OF A SYMPOSIUM HELD AT IMPERIAL COLLEGE, LONDON, JULY 1958

Edited by P. M. B. Walker

Department of Zoology, University of Edinburgh

1960, 208 pages, illus., $6.00

NEW APPROACHES IN CELL BIOLOGY emphasizes the application of these newer techniques to general biological problems. The contributions that electron microscopy and paper chromatography can make to the study of genetic problems are discussed. Also included are articles concerning the value of the analytical combination of electron and optical microscopy and cytochemistry in the study of chromosome structure at different stages of the cell cycle.

NUCLEAR TRANSFER OF EMBRYONIC CELLS OF THE AMPHIBIA

J. A. MOORE, *Columbia University, New York*

CELLULAR INHERITANCE AS STUDIED BY NUCLEAR TRANSFER IN AMOEBAE

J. F. DANIELLI, *King's College, London*

LAMPBRUSH CHROMOSOMES

H. G. CALLAN and L. LLOYD, *The University, St. Andrews, Scotland*

THE MORPHOLOGY OF DEVELOPING SYSTEMS AT THE ULTRAMICROSCOPICAL LEVEL

C. H. WADDINGTON, *Institute of Animal Genetics, Edinburgh*

THE ORIG'N OF THE NUCLEUS AFTER MITOTIC CELL DIVISION

J. BOSS, *University of Bristol*

LABELLED ANTIBODIES IN THE STUDY OF DIFFERENTIATION

R. M. CLAYTON, *Institute of Animal Genetics, Edinburgh*

A BIOCHEMICAL APPROACH TO CELL MORPHOLOGY

W. S. VINCENT, *Upstate Medical Center, State University of New York, Syracuse*

PAPER CHROMATOGRAPHY IN RELATION TO GENETICS AND TAXONOMY

A. A. BUZZATI-TRAVERSO, *University of California, La Jolla, California and Istituto di Genetica, Università di Pavia, Italy*

THE TRANSFER MECHANISMS IN ACTIVE TRANSPORT

W. D. STEIN, *Department of Colloid Science, Cambridge, England*

THE MATCHING OF DRUGS TO TUMOURS

P. HEBBORN, *King's College, London*

THE CYTOCHEMISTRY OF PROTEINS

E. A. BARNARD, *King's College, London*

CYTOCHEMISTY OF NUCLEIC ACIDS

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A Colorimetric Determination of Purine and Pyrimidine Deoxyribonucleosides and Their Phosphorylated Derivatives[1]

LAWRENCE GROSSMAN AND JANET GREENLEES

From the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts

Received October 31, 1960

INTRODUCTION

Colorimetric determinations of deoxy sugars have been described by Waravdekar and Saslaw (1, 2), Weissbach and Hurwitz (3), Pricer and Horecker (4), and Warren (5). The procedure for detecting 2-deoxyribose described by Waravdekar and Saslaw (1, 2) requires that the deoxypentose be in the free aldehyde form. 2-Deoxyribose glycosidically linked to purines or pyrimidines is, therefore, refractive to this colorimetric assay procedure, requiring chemical modifications to free the deoxyribose of its aglycone. In addition, phosphorylated derivatives of 2-deoxyribose fail to react in this chemical assay, and prior removal of the esterified phosphate is mandatory for color development.

Since the glycosidic linkage of the purine deoxyribonucleosides is relatively unstable, mild acid hydrolysis is sufficient to quantitatively liberate the 2-deoxyribose. This same linkage in the pyrimidine deoxyribonucleosides is considerably more stable and is resistant to mild acid hydrolysis. Reduction of the pyrimidine deoxyribonucleosides alters the stability of the *N*-deoxyribosyl linkage rendering it sensitive to mild acid treatment. Thus, chemical distinction between the purine and pyrimidine deoxyribonucleosides can be made. The scheme for separately determining purine and pyrimidine deoxyribonucleosides and their phosphorylated derivatives is depicted in Fig. 1.

The purine *N*-deoxyribosyl linkage is quantitatively cleaved by 5% trichloroacetic acid (TCA) at 100°. The pyrimidine deoxyribonucleosides are unaffected by this treatment. Thus acid hydrolysis of a solution con-

[1] Publication No. 95 from the Graduate Department of Biochemistry, Brandeis University. Work supported in part by grants from the National Institutes of Health (C-3572), National Science Foundation (G-4551) and the Research Corporation.

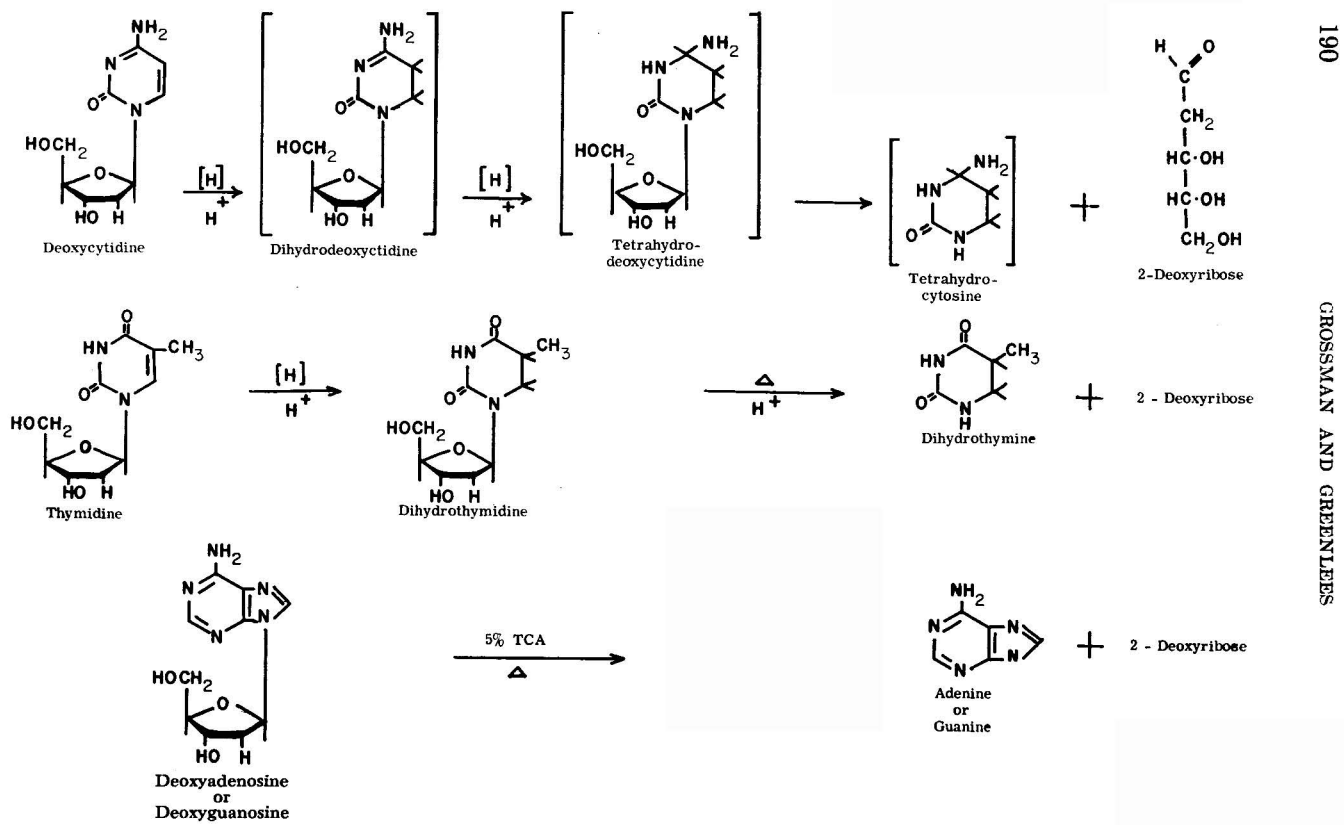

FIG. 1. A diagrammatic representation of the basis of discriminating between purine and pyrimidine deoxyribonucleosides and their phosphorylated derivatives.

taining both species of deoxyribonucleosides liberates 2-deoxyribose and the free purine. Phosphorylated derivatives of purine deoxyribonucleosides yield, under these conditions, the free purine and the colorimetrically inert deoxyribose phosphate esters. Pretreatment of the phosphorylated purine deoxyribonucleosides with specific enzymes is, therefore, necessary to render the liberated deoxyribose susceptible to this colorimetric assay procedure. The purine deoxyribonucleoside triphosphates are initially treated with potato apyrase and prostatic phosphomonoesterase to remove all phosphate groupings esterified to the sugar moiety. The deoxyribonucleoside monophosphates require only prostatic phosphomonoesterase treatment; thus it is possible to distinguish between the deoxyribonucleoside triphosphates, monophosphates, and deoxyribonucleosides.

The stable pyrimidine deoxyribonucleosides are made acid-sensitive by reducing the pyrimidine ring moiety. Thymidine is reduced by one mole of hydrogen gas in the presence of 5% rhodium-on-alumina (6, 7) to form dihydrothymidine. The latter has the same stability toward acid hydrolysis as purine deoxyribonucleosides, and can readily be hydrolyzed to free 2-deoxyribose. Deoxycytidine reacts with two moles of hydrogen gas in the presence of the same catalyst. Concomitant with complete reduction at pH 2, the reduced species of deoxycytidine is cleaved to yield 2-deoxyribose as one of the products. These facts make it possible to distinguish between thymidine which requires acid hydrolysis following catalytic reduction and deoxycytidine which is degraded spontaneously upon complete reduction. This forms the basis for the assay of purine and pyrimidine deoxyribonucleosides.

EXPERIMENTAL METHODS AND MATERIALS

The purine and pyrimidine deoxyribonucleosides and their monophosphorylated derivates were obtained from the California Corporation for Biochemical Research. The purine and pyrimidine deoxyribonucleoside diphosphates and triphosphates were obtained from the Sigma Chemical Company. The catalyst, 5% rhodium-on-alumina, was purchased from the Baker and Company Corporation, Newark, New Jersey.

(*a*) *Removal of Phosphate Esters by Potato Apyrase*

Potato apyrase was isolated and purified according to the directions of Krishnan (8). Solutions containing 1 μmole deoxyribonucleoside triphosphate were treated with sufficient quantities of purified enzyme to quantitatively release all of the labile phosphate in 15 min at 37°. The enzyme reaction mixtures contained 30 μmoles 3,3-dimethylglutaric acid (DMG) buffer, pH 7.0, and 10 μmoles Ca^{+2}. A standard enzyme reaction mixture contained 1 μmole adenosine triphosphate (ATP), enzyme, Ca^{+2} and

buffer in a final volume of 1.0 ml. The reaction was terminated with 0.30 ml of a 25% TCA solution. The sedimented protein was removed by centrifugation, and the level of inorganic phosphate was determined in the supernatant fluid by the method of Fiske and Subbarow (9).

(*b*) *Removal of Phosphate Esters by Prostatic Phosphomonoesterase*

Prostatic phosphomonoesterase was isolated and purified according to the procedure described by Schmidt (7). Sufficient quantities of enzyme were employed to quantitatively dephosphorylate 1 μmole nucleoside 5′-phosphate in 15 min at 37° at pH 7.0. The reaction mixtures contained 30 μmoles DMG buffer pH 7.0. Standard reaction mixtures included 1 μmole AMP-5′, enzyme, and buffer, in a volume of 1.0 ml. The enzymic reaction mixtures were terminated with 0.30 ml of a 25% TCA solution. The protein was removed by centrifugation, and the level of inorganic phosphate was determined in the supernatant liquid.

(*c*) *Assay of Purine Deoxyribonucleosides*

Solutions of either deoxyadenosine or deoxyguanosine containing not more than 0.10 μmole deoxyribose were heated at 100° in 5% TCA for 2 min. Following hydrolysis, the samples were immediately chilled, and the deoxyribose colorimetric determination was applied directly to the samples.

(*d*) *Preparation of Catalyst for Reduction*

Five milliliters of 0.01 *N* HCl was added to 1 gm of 5% rhodium-on-alumina in a glass homogenizer equipped with a Teflon pestle. The mixture was homogenized at room temperature for 5 min at 2500 rpm and centrifuged for 15 min at 1000 rpm (angle head IEC 824 International Centrifuge model PR-1 or PR-2). The supernatant fluid was removed and the precipitate was discarded. The concentration of catalyst dispersed in the 0.01 *N* HCl was estimated spectrophotometrically by determining its absorbance at 500 mμ in the Beckman spectrophotometer (model DU). The extinction coefficient, (in mg/ml) is proportional to the optical density at 500 mμ/mg catalyst/ml of 0.01 *N* HCl and is equal to 2.50. This E(mg/ml) at this particular pH and wavelength was corroborated by drying the samples of colloidal dispersion to a constant weight. Concentrations of 12–20 mg/ml catalyst have been obtained by this method. The catalytic and colloidal properties of such dispersions are stable in stoppered tubes for at least a week at room temperature. This dispersion is broken during catalytic hydrogenation, rendering the catalyst easily removable by centrifugation once reduction is complete.

(e) *Catalytic Reduction of Pyrimidine Deoxyribonucleosides*

The catalyst dispersion at a level of 1.5 mg was pipetted into a 13 × 100 mm test tube with a lip, and the volume was brought to 0.1 ml with 0.01 *N* HCl. A drawn-out capillary was placed in the bottom of the tube, and hydrogen gas was slowly bubbled through the dispersion. The hydrogenation of the catalyst was maintained for 15 min. Following prehydrogenation, 0.01–10 μmoles pyrimidine deoxyribonucleoside was added to the reduced catalyst. The mixture was flushed very rapidly with hydrogen gas for 30 sec by placing a 0.10-ml drawn pipet well above the solution. The tube was then immediately stoppered with a No. 00 rubber stopper and shaken for 20 min at room temperature in order to expose all parts of the solution to the atmosphere of hydrogen gas. It is critical that the shaking be maintained for at least 15 min and not more than 30 min, since the release of deoxyribose is dependent upon complete reduction of the pyrimidine ring. At the end of this time the stopper was removed, the tube centrifuged immediately, and the supernatant fluid decanted. In the reduction of thymidine solutions the reduced supernatant fluid was heated at 100° for 10 min. This heating period served to hydrolyze the dihydrothymidine liberating deoxyribose. It is critical that the reduction of deoxycytidine during the shaking period not exceed 30 min, since the liberated deoxyribose is also subject to reduction.

(f) *Removal of Proteins from Solutions of Pyrimidine Deoxyribonucleosides*

The determination of pyrimidine deoxyribonucleosides in the presence of proteins is complicated by the toxic effects of protein on the catalytic reduction. It is necessary, therefore, to completely remove proteins with a reagent which itself does not interfere with the reductive process. The most satisfactory procedure found involved a modification of the Somogyi barium–zinc procedure (11). The modification employs 6% $Zn(SO)_4$ $7H_2O$ and 0.3 *N* sodium hydroxide. The two reagents were titrated to an equivalent point at pH 7.0. The total volume required was dependent on the level of protein in solution. The advantage of this protein precipitation method is that there is essentially no introduction of extraneous ions into the solutions.

(g) *Preparation of Bacterial Extracts*

Escherichia coli strain B was grown in nutrient broth to a concentration of 1 × 10^9 cells/ml. The cells were harvested and washed with cold isotonic saline and resuspended in isotonic saline (5 ml/gm of packed cells). The cells were disrupted by treatment for 15 min in a Raytheon

10-kc sonic oscillator. The suspension was centrifuged for 15 min at 12,000 $\times$ g, and the supernatant fluid was collected.

(*h*) *Conditions of Transglycosidase Incubation*

E. coli extract, at a level of 0.1 ml, was incubated with 1 μmole each of thymidine and adenine and 5 μmoles of glycylglycine buffer, pH 7.0, in a final volume of 1.0 ml. Aliquots (0.10 ml) were removed at varying times and treated with 0.9 ml of cold 5% TCA. The mixture was held at 0° for 5 min and was then centrifuged at 4000 $\times$ g for 15 min. The supernatant fluid was decanted and heated at 100° for 2 min. An aliquot of 0.5 ml was removed and brought to 3.5 ml with water and assayed for deoxyribose. Control incubations consisted of an enzyme blank. In addition, nucleoside hydrolase activity was examined by incubation of thymidine or deoxyadenosine and enzyme in the absence of free purine or pyrimidine.

(*i*) *Paper Chromatography*

Transglycosidase reaction mixtures were incubated in the absence of buffer for chromatographic assay. The enzyme reactions were terminated by heating at 100° for 5 min. The denatured proteins were removed by centrifugation, and the supernatant fluid was evaporated to dryness. The dried reaction mixtures were dissolved in 0.10 ml water (pH 10), and 0.05 ml was applied to Whatman No. 1 chromatography paper. The dried paper was placed in a sealed vessel and developed by water adjusted to pH 10 with N NH_3 (12). The ascending chromatogram was terminated at the end of 10 hr and separation was judged by ultraviolet scanning of the dried paper.

(*j*) *Specificity of the Deoxyribose Assay*

The periodate oxidation of free 2-deoxyribose causes the splitting of deoxyribose at the *cis*-hydroxyl groups associated with carbon atoms 3 and 4. The product, malonaldehyde, reacts with 2 moles 2-thiobarbituric acid. The product of this reaction has an absorption maximum at 532 mμ (1, 2). Although ribose does not, on periodate oxidation, yield malonaldehyde, we have observed that a 1000-fold excess of ribose or ribonucleosides causes a diminution of the absorbance at this wavelength. This can be remedied, in part, by the addition of concentrated NaOH to a final concentration of 1 M. This causes a decrease in the nonspecific brown coloration due to ribose oxidation. The increased pH causes a shift in the spectrum of the product which is accompanied by an increase in extinction. This technique can be routinely employed for increased sensitivity of the deoxyribose assay.

EXPERIMENTAL RESULTS AND DISCUSSION

(a) *The Liberation of Deoxyribose in Glycosidic Linkage with Purines*

The *N*-glycosidic linkage between purines and deoxyribose was easily severed on heating in the presence of 5% TCA. Kinetic studies indicated that from 1 to 2 min of boiling was sufficient to quantitatively cleave this linkage and thereby liberate 2-deoxyribose. The results of a typical assay, in which deoxyadenosine was hydrolyzed in the presence of 5% TCA, is given in Fig. 2. The data show that there is a linear relationship between the concentration of deoxyadenosine and the release of deoxyribose.

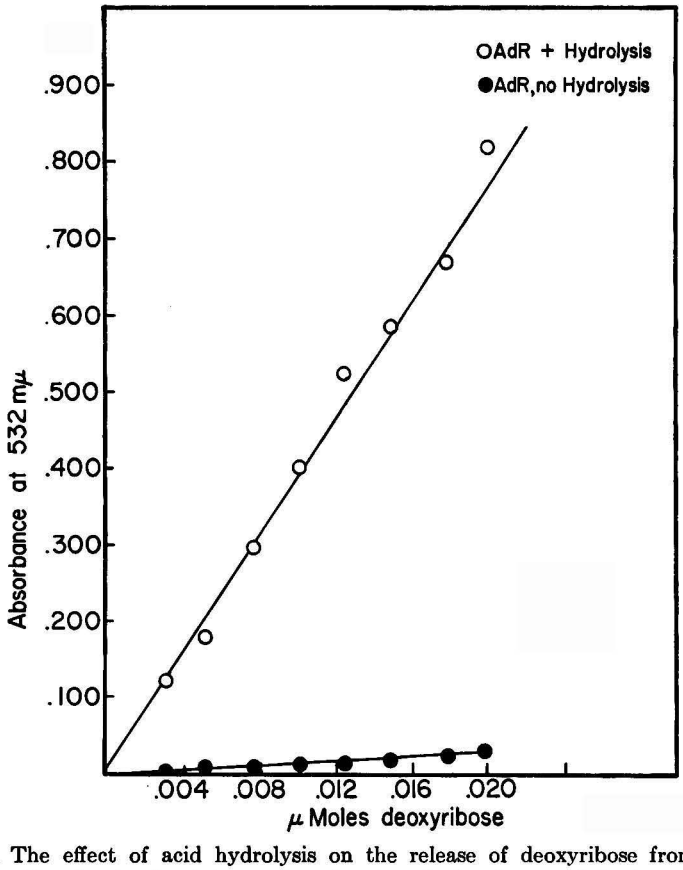

FIG. 2. The effect of acid hydrolysis on the release of deoxyribose from deoxyadenosine. Varying levels of deoxyadenosine, in a final volume of 1.0 ml, were treated with 0.3 ml of a cold 25% TCA solution. The samples were placed in a boiling water bath for 2 min and chilled immediately. Those samples which were not hydrolyzed were treated with cold TCA and not heated. The level of deoxyribose in the cooled samples was then determined.

Identical results were obtained when deoxyguanosine was examined by the same procedure. Deoxyadenosine monophosphate, when subjected to acid hydrolysis, did not yield colorimetrically reactive deoxyribose. However, when the deoxyribonucleotide was exposed to prostatic phosphomonoesterase, and then hydrolyzed, quantitative recovery of deoxyribose was obtained. The results of such an experiment, given in Fig. 3, show that

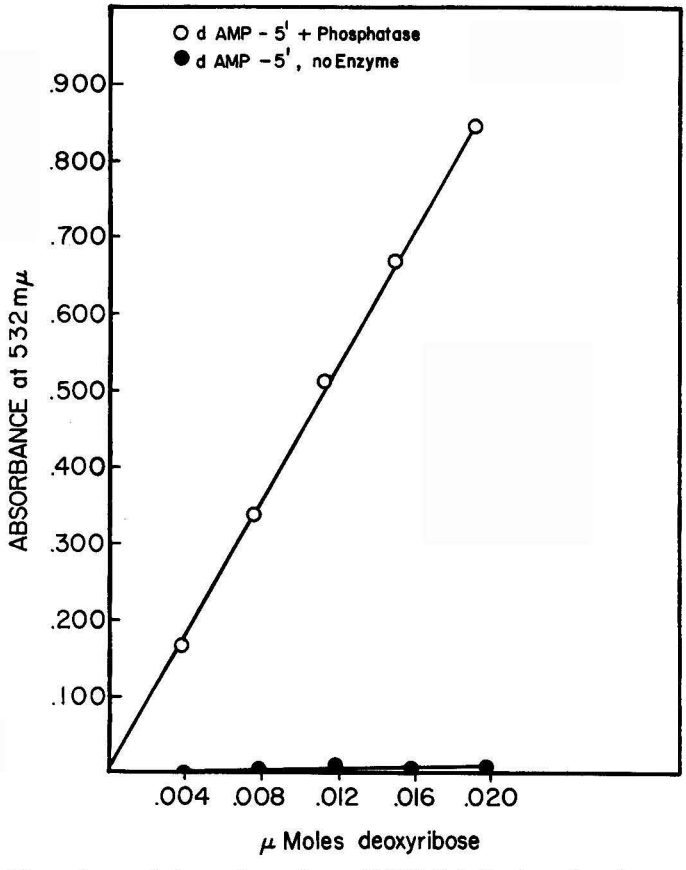

FIG. 3. The release of deoxyribose from dAMP-5′ following phosphomonoesterase treatment and acid hydrolysis. Varying concentrations of dAMP-5′ were treated with a constant amount of acid prostatic phosphomonoesterase and 30 μmoles DMG buffer, pH 7.0 in a final volume of 1.0 ml. The mixtures were incubated for 15 min at 37°, and the reaction was terminated with 0.3 ml of a cold 25% TCA solution. The samples were immediately chilled at 0° and centrifuged for 10 min at 3000 rpm (angle head IEC 324 in the model PR-2). The supernatant fractions were decanted and placed in a boiling water bath for 2 min and chilled. An aliquot was removed (0.1 ml) and brought to 3.5 ml with water. The deoxyribose colorimetric determination was applied to these samples.

deoxyribose is liberated quantitatively following prostatic phosphomonoesterase treatment and subsequent acid hydrolysis. The purine deoxyribonucleoside triphosphates were also analyzed by prior reaction with potato apyrase and prostatic phosphomonoesterase followed by acid hydrolysis. In Fig. 4 is shown the linear relationship between the concentra-

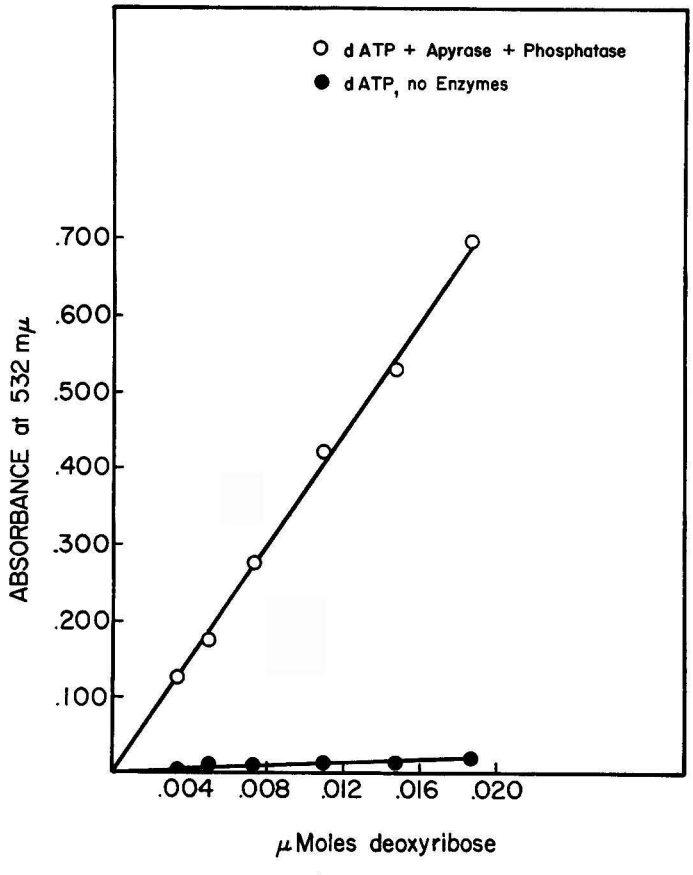

FIG. 4. The release of deoxyribose from dATP following treatment with potato apyrase, acid prostatic phosphomonoesterase and acid hydrolysis. Varying concentrations of dATP were treated with a constant amount of potato apyrase and prostatic phosphomonoesterase, 30 μmoles DMG buffer, pH 7.0, and 10 μmoles $CaCl_2$ in a final volume of 1.0 ml. The mixtures were incubated for 15 min at 37°, and the reaction was terminated with 0.3 ml of a cold 25% TCA solution. The samples were immediately chilled and maintained at 0° for 5 min and centrifuged at 0° for 10 min at 3000 rpm (angle head IEC #324 in the PR-2 centrifuge). The supernatant fluid was decanted and placed in a boiling water bath for 2 min and chilled. An aliquot was removed (0.1 ml) and brought to 3.5 ml with water. The deoxyribose colorimetric determination was applied directly to these samples.

tion of the phosphorylated deoxyribonucleosides and the deoxyribose released.

(*b*) *Analysis of Mixed Purine Deoxyribonucleosides and Phosphate Esters*

Mixtures of deoxyadenosine, its 5′-monophosphate, and the triphosphate were analyzed by selective enzymic treatment. The use of both enzymes in conjunction with acid hydrolysis liberated deoxyribose from

TABLE 1
RECOVERIES OF DEOXYADENOSINE AND ITS PHOSPHORYLATED DERIVATIVES IN MIXTURES[a]

Treatment (before hydrolysis)	Absorbance		2-Deoxyribose (μg)		Per cent recovery
	@ 532 mμ	Δ532	Added	Recovered	
Apyrase + phosphomonoesterase	.651	.217	0.740	0.724	98
Phosphomonoesterase	.434	.260	0.840	0.867	103
None	.174	.174	0.580	0.580	100

[a] Experimental details are provided in sec. 2 (*a*), (*b*), and (*c*).

the nucleoside, the nucleotide, and the nucleoside triphosphate. Treatment with the phosphomonoesterase and hydrolysis liberated the deoxyribose

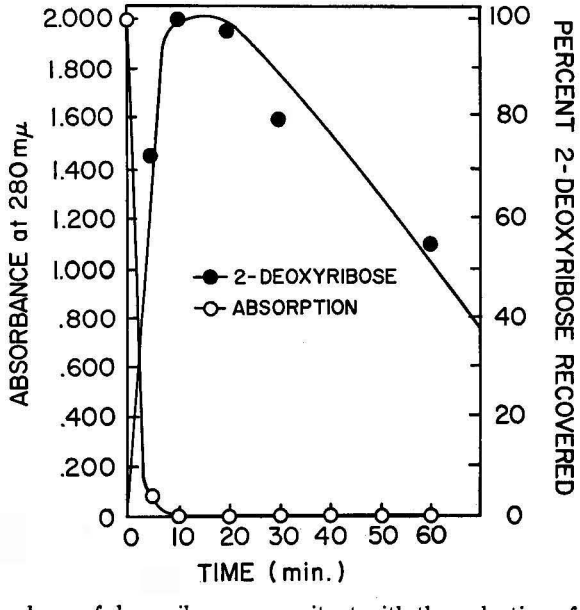

FIG. 5. The release of deoxyribose concomitant with the reduction of deoxycytidine at pH 2.0. The experimental details are provided in sec. 2 (*d*) and (*e*).

from the nucleoside and nucleotide. Acid hydrolysis without enzyme treatment yielded the deoxyribose from only the nucleoside. The data are summarized in Table 1.

(*c*) *Deoxycytidine Assay*

In order to determine the optimal conditions for the release of 2-deoxyribose from deoxycytidine following catalytic reduction, 1.5 μmoles deoxycytidine in 0.01 *N* HCl was exposed to hydrogen gas in the presence of 300 μg of 5% rhodium-on-alumina. Eight such reaction mixtures were exposed to hydrogen gas with shaking for varying times up to 60 min. An aliquot was removed for absorbance determination at 280 mμ, and the remainder was assayed for the deoxyribose. The results of such an experiment, given in Fig. 5, show that the absorbance at this wavelength was reduced rather quickly with a concomitant appearance of the free sugar. Exposure to hydrogen gas for extended periods resulted in the corresponding loss of deoxyribose. This is presumably due to further

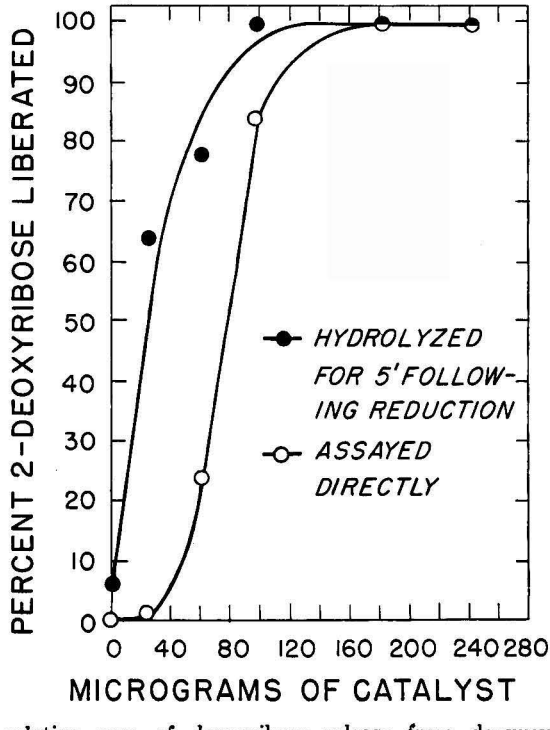

FIG. 6. The relative ease of deoxyribose release from deoxycytidine following catalytic hydrogenation as a function of catalyst concentration. The experimental details are provided in sec. 2 (*d*) and (*e*).

reduction of the aldehyde group of the deoxyribose to the corresponding alcoholic group to form 2-deoxyribitol.

In order to determine the optimal catalyst concentration for given concentration ranges of deoxycytidine, it was necessary to obtain a catalyst concentration profile as seen in Fig. 6. In this experiment 0.15 μmole deoxycytidine was exposed to varying concentrations of catalyst. At lower concentrations of catalyst there was a requirement for acid hydrolysis of the deoxycytidine reduction product. It is assumed that such a requirement may be due to the formation of some dihydrodeoxycytidine. It would be anticipated that such an intermediate would react to acid hydrolysis in a manner similar to that of the purine deoxyribonucleosides. Although it is not evident from Fig. 6, excess levels of catalyst resulted in subsequent decrease in the yield of 2-deoxyribose following catalytic reduction. It was subsequently shown that under these conditions deoxycytidine was adsorbed to the excess catalyst.

(*d*) *Assay of Thymidine*

Exposure of thymidine to the same analysis under identical conditions of pH and catalyst concentration resulted in a rapid loss of ultraviolet absorption at 260 mμ unaccompanied by the release of 2-deoxyribose. A comparison of the acid labilities of the catalytic hydrogenation products of thymidine and deoxycytidine is shown in Fig. 7. Deoxycytidine when

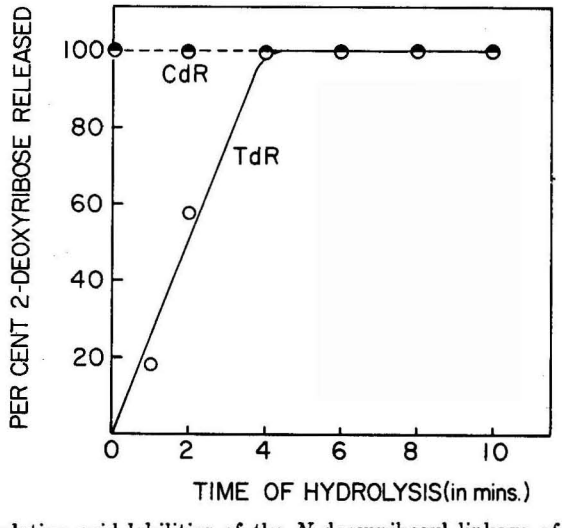

FIG. 7. The relative acid labilities of the *N*-deoxyribosyl linkage of deoxycytidine and thymidine following catalytic reduction. The details of the experimental techniques are provided in sec. 2 (*d*) and (*e*).

catalytically reduced at acid pH formed an unstable product, presumably tetrahydrodeoxycytidine, which was cleaved under the acid conditions of catalytic reduction. Thymidine, on the other hand, formed a relatively more stable reduction product which required elevated temperatures at this pH for splitting of the *N*-deoxyribosyl linkage.

(*e*) *Pyrimidine Deoxyribonucleoside Phosphorylated Derivatives*

The phosphate esters of pyrimidine deoxyribonucleosides were treated in the same manner as the purine derivatives with the exception that the phosphomonoesterase and apyrase activities were terminated by heating for 5 min. This was followed by removal of the denatured protein with sodium–zinc and centrifugation. The supernatant fluid was acidified with

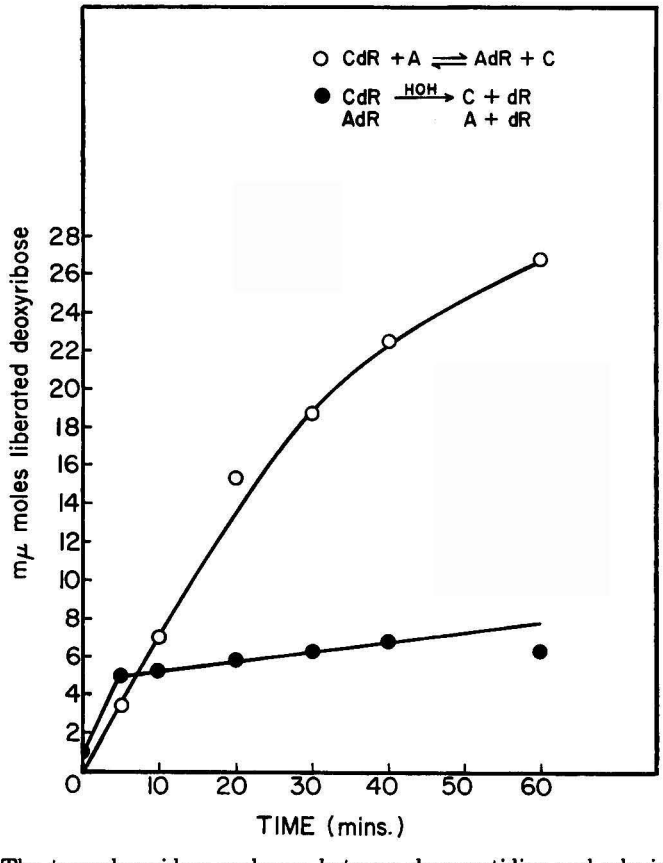

FIG. 8. The transglycosidase exchange between deoxycytidine and adenine and the deoxynucleoside hydrolase activity on deoxycytidine. The experimental conditions demonstrating these reactions are detailed in sec. 2 (*g*) and (*h*).

1 *M* HCl to pH 2.0. The catalytic reduction procedure was then applied to the acidified samples of pyrimidine deoxyribonucleosides formed from the enzymic hydrolysis.

(*f*) *Utilization of the Colorimetric Technique for Assaying Enzymic Transglycosidase*

The colorimetric technique described is applicable for determining enzymic transglycosidations occurring between a pyrimidine deoxyribonucleoside and a free purine (Fig. 8), or a purine deoxyribonucleoside and a free pyrimidine. Chromatographic corroboration of such an enzyme-induced exchange is seen in Fig. 9. This assay technique is not applicable

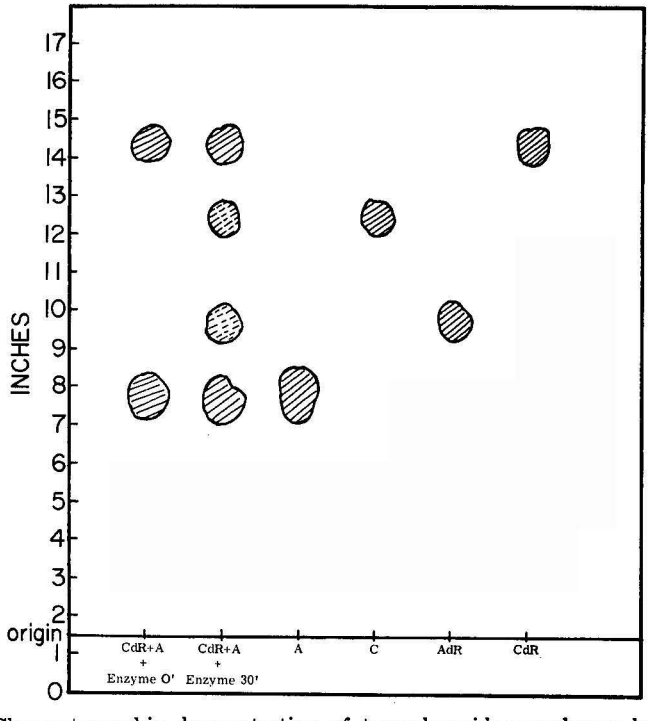

FIG. 9. Chromatographic demonstration of transglycosidase exchange between deoxycytidine and adenine. The details of this experiment are given in sec. 2 (*g*), (*h*), and (*i*).

for purine deoxyribonucleoside–purine systems and is only applicable for the pyrimidine deoxyribonucleoside–pyrimidine exchange in which the deoxyribose of thymidine or deoxyuridine, and free cytosine are involved. The reverse exchange can also be examined.

(g) *Inhibitors of Rhodium Catalytic Hydrogenation*

Protein, which consistently poisons most catalysts and does not spare rhodium-on-alumina, must be removed prior to catalytic reduction. The most efficient protein precipitant which is itself nontoxic to the 5% rhodium-on-alumina is the sodium–zinc reagent. Sodium is substituted for barium since the latter is toxic.

The presence of adenine compounds are also detrimental for the catalytic hydrogenation procedure (6). However, it is possible to overcome the influence of these compounds by increasing the concentration of catalyst for a given reduction.

It is usually advisable to obtain a number of different batches of catalyst since each varies in its hydrophilic properties, and as a result different batches exhibit varying colloidal properties when prepared by the method described.

SUMMARY

A colorimetric assay procedure has been described which, because of its strict requirement for the free aldehyde form of 2-deoxyribose, can be used to discriminate between purine and pyrimidine deoxyribonucleosides. The purine deoxyribonucleosides are quantitatively broken down to deoxyribose and free purine by acid hydrolysis. The corresponding phosphorylated derivatives require prior phosphomonoesterase and potato apyrase hydrolysis. The method is thus capable of distinguishing between deoxyribonucleosides, the monophosphate, and triphosphate esters.

Pyrimidine deoxyribonucleosides are resistant to cleavage by mild acid, and a routine catalytic hydrogenation procedure has been described which sufficiently modifies the pyrimidine ring and acid-labilizes the *N*-deoxyribosyl linkage. Accordingly, a method has been described which is applicable for assessing levels of pyrimidine deoxyribonucleosides. A differential acid hydrolysis procedure has been described which permits distinction between thymidine and deoxycytidine. The phosphate esters of the pyrimidine deoxyribonucleosides were also determined through the use of appropriate enzymic hydrolysis.

This assay technique has been used to measure transglycosidase and deoxyribonucleoside hydrolase activities in bacterial extracts.

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ANALYTICAL BIOCHEMISTRY, 2, 205–215 (1961)

An Ultrasensitive Assay of β-Galactosidase with C^{14}-Labeled o-Nitrophenyl-β-D-galactoside[1]

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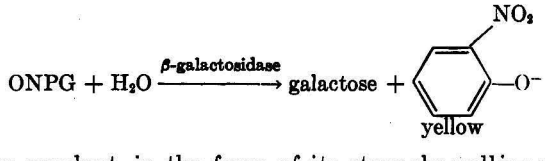
Received December 8, 1960

INTRODUCTION

Extensive studies involving the enzyme β-galactosidase have contributed significantly to the understanding of enzyme induction and of related aspects of the biochemical and genetic control of protein synthesis (1–3). An important factor determining the choice of β-galactosidase was undoubtedly the convenient and sensitive assay made possible by the introduction of *o*-nitrophenyl-β-D-galactoside as a substrate (4). It is obvious, therefore, that any increase in the sensitivity of this assay would even further extend the range of problems susceptible to analysis by this technique.

The present paper describes a modification of the original colorimetric assay which resulted in about 50-fold increased sensitivity. This method proved of value in the quantitative determination of the minimal β-galactosidase concentrations released from various host cells as a result of injury by animal viruses.[2]

The classical standard assay of β-galactosidase with *o*-nitrophenyl-β-D-galactoside (ONPG) (4) is based on the reaction



The reaction product, in the form of its strongly yellow phenolate ion, is assayed spectrophotometrically in alkaline solution. The sensitivity

[1] This investigation was supported by research grant No. E-1500 from the Division of Research Grants of the National Institutes of Health, U. S. Public Health Service and by Senior Research Fellowship No. SF-274 from the Public Health Service.

[2] H. Noll and J. Orlando, results to be published.

of the method is thus limited by the optically detectable concentration of the dye in the reaction mixture. An increase in sensitivity was achieved by a simple concentration procedure. *o*-Nitrophenol (ONP) was extracted quantitatively from the acidic reaction mixture with isooctane and re-extracted into a smaller volume of aqueous alkali for colorimetric determination. An additional advantage of this method is the elimination of the turbidity and yellow background color which often interferes with the direct assay. For greatest sensitivity, the extraction procedure was combined with the use of radioactive ONPG, labeled with C^{14} in the aglycon. The radioactivity extracted into isooctane was strictly proportional to the enzymic hydrolysis and could be measured with great precision and sensitivity.

MATERIALS AND METHODS

Reagents

Spectrograde isooctane (2,2,4-trimethylpentane) was purchased from the Fisher Scientific Company, the scintillation fluors 2,5-diphenyloxazole (PPO) and 1,4-bis-2-(5-phenyloxazolyl)benzene (POPOP) from the Packard Instrument Company, uniformly labeled phenol-C^{14} and a benzoic acid-C^{14} radiation standard from the Nuclear Chicago Corporation. The detector solution for liquid scintillation counting consisted of A.C.S.-grade toluene containing 4 gm PPO and 100 mg POPOP per liter. C^{14}-labeled ONPG was synthesized as described below.

Counting Equipment

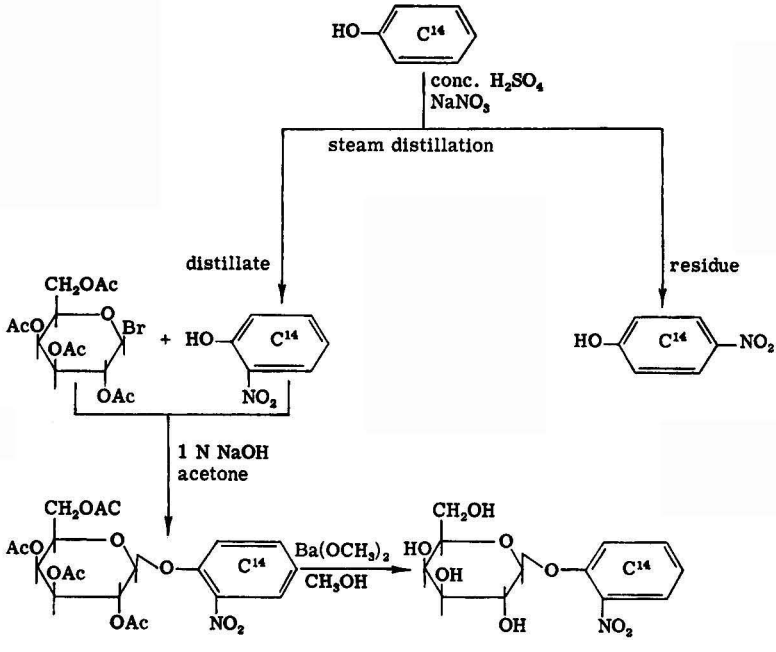
Radioassays were carried out either with a Nuclear Chicago D-47 windowless proportional gas-flow counter or with a Packard Tri-Carb liquid scintillation spectrometer equipped with automatic sample changer (model 314 X).

Spectrophotometric Assays

A Beckman model DU spectrophotometer with 1-cm cuvettes was used for ultraviolet absorption measurements; infrared spectra were recorded with a Beckman IR-4 double-beam instrument. The molar absorbancy index of ONP in 0.1 *N* NaOH at 415 mμ was $a_M = 4.72 \times 10^3$.

Synthesis of o-Nitro-UC^{14}-phenyl-β-D-galactoside (ONPG)*

The radioactive substrate was synthesized according to the following route:



ONPG*

A simple and reproducible small-scale synthesis is described in the following. To a solution of 160 mg $NaNO_3$ in 0.4 ml water in a small test tube was slowly added 0.11 ml of conc. H_2SO_4 ($d = 1.84$). The small glass ampoule containing 100 mg of radioactive phenol (approx. 0.3 mc) was warmed until the phenol melted, cut open with a glass file and dropped into a 4-ml glass screw-cap vial. After crushing the glass ampoule, the HNO_3 solution was added dropwise with agitation. The vial was then closed firmly with the screw cap and kept over steam with frequent shaking. At the end of 2 hr, the vial was cooled in ice and carefully opened. The reaction products consisting of a mixture of the ortho and para isomers of nitrophenol and separating as a drop of heavy oil at the bottom of the vial were washed four times with 1-ml portions of ice-cold 0.1 *N* HCl. The oil was then transferred with 4 ml water into a 100-ml steam-distilling flask fitted with a condenser in order to separate the volatile ortho isomer. The distillate was collected in two fractions, the initial 5 ml (I) into a graduated centrifuge tube, and additional 50 ml (II) into a round-bottom flask. The crystalline precipitate in fraction I was centrifuged at 2°, and the supernatant was added to fraction II. Fraction II was then acidified with 0.5 ml of 0.2 *N* HCl, and the remaining ONP* was extracted with two 3-ml portions of isooctane. The

isooctane extracts were placed over 1.4 ml of 1 *N* NaOH. The ONP* was extracted into the aqueous phase by mixing with a pipet, and the supernatant isooctane was discarded. To the centrifuge tube containing the precipitated ONP* from fraction I were now added, first, 100 mg of cold carrier ONP and, finally, the 1.4 ml of aqueous alkali containing the ONP* extracted from fraction II. The resulting alkaline solution of ONP* was used directly for the following condensation reaction. The yield of ONP*, based on phenol-C^{14}, was 49 mg (33%), as determined spectrophotometrically and by comparison of the specific activities of the starting material and final product (ONPG*).

The acetobromo-D-galactopyranose required for the subsequent condensation with ONP was prepared according to Haynes and Todd (5) and stored at −15° until used. The condensation and deacetylation were carried out in a small-scale modification of the procedures described by Seidman and Link (6). The alkaline solution (1.4 ml) of ONP (1.07 mmoles) prepared previously (containing 49 mg of labeled and 100 mg of carrier ONP) was mixed with a solution of 370 mg (0.9 mmoles) of acetobromogalactoside in 2.2 ml acetone and allowed to stand at room temperature overnight. The product, which had crystallized as needles, was separated by centrifugation in the cold and washed twice with 0.7 ml of cold ethanol. Traces of unreacted ONP were removed by washing a solution of the yellowish crystals in 4 ml $CHCl_3$ with 1 *M* Na_2CO_3 until all the yellow color was extracted. After further washing with water until the washes were neutral, the $CHCl_3$ solution was evaporated *in vacuo* and the residue was dried over P_2O_5. The yield of white crystalline tetraacetyl-ONPG of m.p. 172–173° was 211 mg (50%).

Catalytic deacetylation was carried out by suspension of 200 mg tetraacetyl-ONPG* in 10 ml of absolute methanol, followed by addition of 0.25 ml of 0.342 *N* $Ba(OCH_3)_2$. The mixture was agitated until a clear solution was obtained. The dense crop of hairlike crystals which formed during refrigeration overnight was dissolved by addition of 5 ml water. The solution was immediately adjusted to pH 5–6 with H_2SO_4. Traces of ONP* were extracted with three 1-ml portions of isooctane. The $BaSO_4$ precipitate was separated by centrifugation and washed twice with 1-ml portions of water. The pooled clear supernatants were lyophilized after most of the methanol had been removed *in vacuo*. The resulting white product was recrystallized from absolute ethanol, yielding 113 mg (88%) of pure ONPG*, m.p. 193–194°. Both ultraviolet and infrared spectra (Fig. 1) were identical with those of an authentic sample, and a mixed melting point showed no depression. The specific activity was 105 μc/mmole.

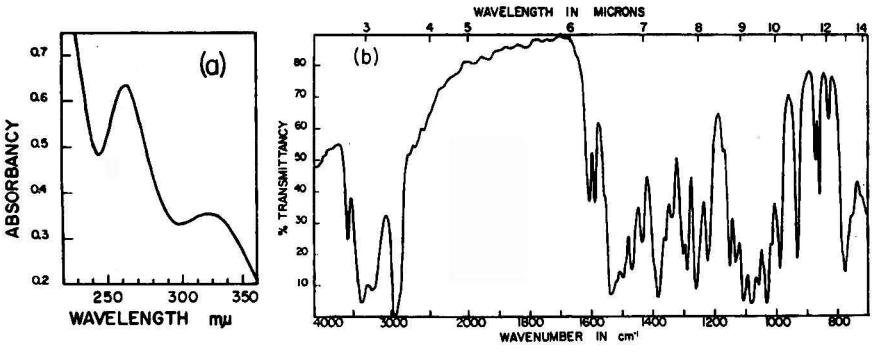


FIG. 1. (*a*) Ultraviolet and (*b*) infrared spectrum of ONPG. The ultraviolet spectrum[3] was recorded with an aqueous solution containing 0.0500 mg/ml in a 1.00-cm cuvette and shows two maxima: one at 263 mμ ($a_M = 3.81 \times 10^3$) and one at 318 mμ ($a_M = 2.13 \times 10^3$). The infrared spectrum was prepared from a Nujol mull.

RESULTS

Separation and Assay of Enzymic Reaction Product (ONP)

Extraction with Isooctane. The present method is based on the preferential solubility of the undissociated reaction product ONP at acid pH in water-immiscible organic solvents. Isooctane was found to be the most satisfactory. Below pH 1, the partitioning of ONP between isooctane and water was 3:1. This was determined by extracting 1.0 ml of an acidified aqueous solution of ONP (10 μg/ml) with 1.0 ml isooctane and measuring spectrophotometrically the ONP remaining in the aqueous phase. Two successive extractions with 1-ml portions of isooctane extracted 90–95% of the ONP originally present in the acidic aqueous phase. Re-extraction of ONP from isooctane into aqueous alkali is favored by an extremely one-sided distribution: after mixing 0.5 ml isooctane containing 3.1×10^4 counts/min ONP* (32 μg) with 2.0 ml of 0.1 *N* NaOH, only 5 counts/min could be detected in the isooctane phase by liquid scintillation counting.

Owing to its nonpolar nature, isooctane failed to extract detectable amounts of the glycoside from the aqueous phase. Thus, no radioactivity above background was detected in the organic phase after mixing 2 ml isooctane with 1 ml water, containing 4×10^5 counts/min ONPG*. Moreover, isooctane showed no quenching effect upon addition to the scintillating fluid.

Radioactivity Assay. Since isolation of the radioactive enzymic re-

[3] The authors wish to express their thanks to Dr. R. Basford for recording this spectrum on a Carey model 14 spectrophotometer.

action product involved extraction with a nonpolar solvent, the liquid scintillation technique offered at the same time the most convenient and sensitive assay method. However, since the required equipment is not generally at hand, a method for measuring ONP* by the conventional Geiger and proportional counting techniques was also worked out. Determination of the radioactivity of the water-soluble ONPG*, although not essential for the enzymic assay, was carried out by Geiger-counting only.

For liquid scintillation assay, a total volume of 2 ml isooctane, containing the extracted ONP*, was mixed in the standard vials with 8 ml of scintillating solvent and counted with an AA'-B-C discriminator setting corresponding to 10–50–100 v at a photomultiplier voltage of 900 v (tap 4). Under these conditions, the background was 10 counts/min and the absolute counting efficiency 58%. Addition of benzoic acid-C^{14} as an internal standard showed no quenching and was therefore omitted in routine assays.

Direct plating of ONP* in isooctane for assay in the gas-flow counter was not possible, since during the drying process almost all the ONP sublimed. It was therefore necessary to convert it into the salt form by re-extraction into a solution of Na_2CO_3. The following standard procedure was adopted. Two milliliters of isooctane extracts was added to 0.1 ml of 0.1 *N* Na_2CO_3 in a small test tube and mixed by vigorous shaking. Assay of the supernatant isooctane showed that 85–90% of the radioactivity was extracted into the aqueous phase.[4] Most of the isooctane was then removed with a capillary pipet and the rest evaporated *in vacuo*. The carbonate solution was transferred into the planchet with a Carlsberg micropipet, and the tube was washed repeatedly with a drop of water. The samples were placed under an infrared lamp and dried in the air draft of a hood. Even distribution after drying, resulting in negligible self-absorption, was achieved by the use of aluminum planchets which had been etched by short immersion in alcoholic NaOH. The solution was spread over the bottom within a circle drawn along the periphery with a wax pencil. When dried on polished metal surfaces, the high surface activity caused an uneven distribution of the material and erratic results, mainly because of variations in backscattering. For good reproducibility, it was also important to insure that the trapping solution always contained sufficient excess of carbonate (protection from CO_2).

[4] This result seems to contradict the extremely one-sided partitioning mentioned previously. However, the limiting factor in this case is the equilibration time which becomes slow with the small relative volume of aqueous phase employed. Hence, good reproducibility is dependent on a uniform mixing technique.

Standard Curves and Absolute Counting Efficiency

For the preparation of standard curves, a concentrated aqueous solution of enzymically hydrolyzed ONPG* was extracted with isooctane, and the ONP* concentration of the extract was determined spectrophotometrically. Dilutions of the isooctane solution were then assayed by both the liquid scintillation and windowless gas-flow techniques. With both methods, the counting rate was strictly proportional to the amount of ONP over the 1000-fold concentration range tested. The results obtained at the lower end of this range ($1–50 \times 10^{-10}$ mole) are plotted in Fig. 2. Radioactivity measurements by the liquid scintillation

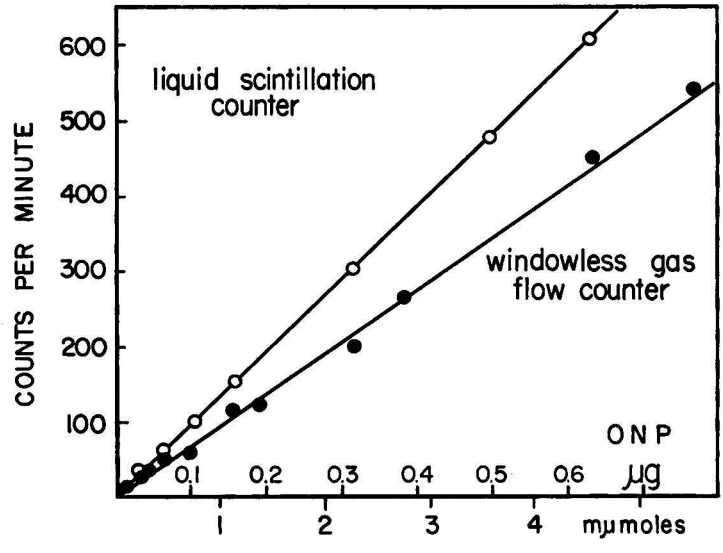


FIG. 2. Standard curves illustrating linearity between observed counting rates and ONP concentration in isooctane. Details of procedure are given in text.

technique were both more sensitive and accurate, the reproducibility corresponding to ±1%. Determinations by gas-flow counting, on the other hand, varied within about ±7%, mainly because of the additional manipulative and geometrical errors introduced during extraction and plating. The absolute counting efficiency, as determined with a benzoic acid-C^{14} standard, was 58% for the scintillation and 47% for the gas-flow counting under the conditions chosen. The slope of the standard curve for gas-flow counting indicates a lower apparent efficiency of 41%, since the plotted counting rates were not corrected for the loss of about 12% of ONP* during the extraction from isooctane into aqueous sodium carbonate.

Assay of β-Galactosidase

The reaction mixture is made up in small test tubes to 1.0–1.5 ml with the enzyme preparation, a suitable buffer, and substrate in a concentration of 100–500 μg ONPG*/ml (0.3–1.5 mmoles). A blank with heat-inactivated enzyme is included. At the end of the incubation period, the reaction is stopped by the addition of 0.1 ml HCl to about pH 1. The reaction mixture is then extracted twice with 1.0 ml isooctane. A clean separation of the phases is achieved by centrifugation. The combined extracts are assayed for radioactivity as described above.

The results of rate studies at two enzyme concentrations differing by a factor of 10 and measured with the gas-flow counter are illustrated in Fig. 3. It can be seen that the rates were linear with time and pro-

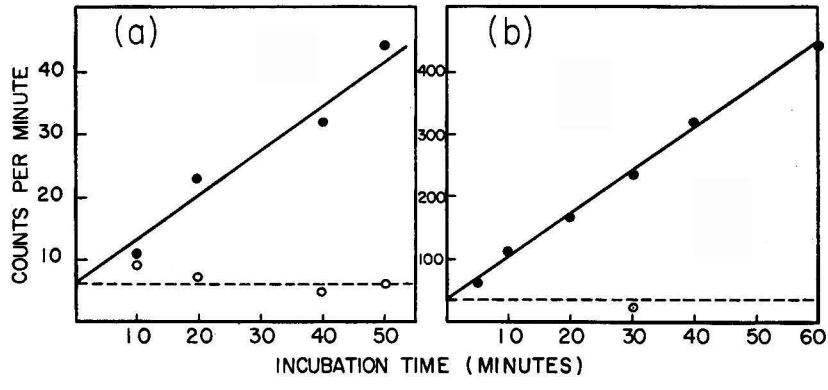


FIG. 3. Rates of enzymic hydrolysis of ONPG* at low enzyme concentrations determined with gas-flow counter. The reaction mixture (1.0 ml) contained 0.01 *M* acetate pH 5.0, 100 μg/ml ONPG* corresponding to 3.1×10^4 counts/min and 0.9 ml of enzyme solution consisting either of 1:10 diluted (curve *a*) or undiluted (curve *b*) allantoic fluid from embryonated eggs infected with influenza virus. Experimental values indicate radioactivity in isooctane extracts of reaction tubes (●—●—●), and of controls (○—○—○) containing heat-inactivated enzyme.

portional to the enzyme concentration. A high degree of reproducibility is indicated by the fact that at counting rates above 50 counts/min (corresponding to 2× background), the deviation of individual determinations from the straight line was less than ±5%, despite the two extraction steps involved in the sample preparation. The rate curve obtained at the lower enzyme concentration shows the method to be capable of measuring the formation of 2×10^{-10} mole ONP with an accuracy of ±10%.

The curves in Fig. 3 extrapolate to a small positive ordinal intercept

which corresponds to the isooctane-soluble radioactivity of the blanks containing heat-inactivated enzyme. These blank values were proportional to the substrate concentration and are probably due to a slow non-enzymic hydrolysis during extraction at low pH. This effect is illustrated in Fig. 4 which also shows the increase of the rate of enzymic hydrolysis

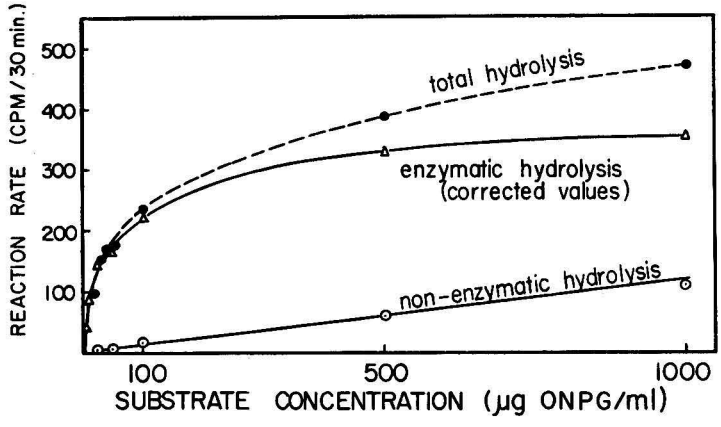


FIG. 4. Rates of enzymic and non-enzymic hydrolysis as a function of substrate concentration. The incubation time was 30 min and the enzyme concentration as in Fig. 3, curve *b*. Other conditions and symbols as in legend to Fig. 3. △-△-△ indicates radioactivity values after correction by subtracting the values of the corresponding non-enzymic controls.

as a function of the substrate concentration. It is obvious from these results that if this method is used for the determination of Michaelis constants at low enzyme concentrations, it is necessary to include blanks covering the range of substrate concentrations tested.

At the low substrate concentration of 100 μg/ml chosen for our routine enzymic assays, the background due to non-enzymic hydrolysis was in the order of only 10 counts/min even though no special precautions were taken to control the extent of non-enzymic hydrolysis. A background of this magnitude did not interfere with the determination of enzymic activities above the level of 10^{-10} mole ONP formed/30 min. The assay of lower enzyme activities was difficult because the specific activity of the substrate became limiting.

DISCUSSION

The sensitivity of the conventional assay of β-galactosidase with ONPG is limited by the absorbancy of the reaction product ONP at 415 mμ in alkaline solution. This corresponds to a concentration of 2×10^{-9} mole/ml if a reading of 0.01 absorbancy units is accepted as the

lower limit. An about fivefold increase in sensitivity is achieved by concentration of the dye after extraction with isooctane and by the use of ultramicro cells for spectrophotometric assay. A considerable increase in sensitivity was made possible by the use of C^{14}-labeled ONPG with a specific activity of 2.33 disintegrations/min/10^{-11} mole. From the results obtained with this preparation, the measurement of 5×10^{-11} mole is estimated to represent the lower limit in an optimally adjusted liquid scintillation counter. The sensitivity of the method may be further increased by the use of ONPG with a higher specific activity. This conclusion is supported by the fact that the preparation used in these experiments was synthesized with starting material of relatively low specific activity which, in addition, was diluted threefold with inactive carrier. Direct labeling of ONPG with tritium by the Wilzbach method (7) may offer a less expensive and extremely simple way of obtaining ONPG of high specific activity. It should be pointed out, however, that the limit of sensitivity attainable with substrate of higher specific activity will be determined by the increasingly unfavorable ratio of counts released by non-enzymic and enzymic hydrolysis. It seems possible to reduce the extent of non-enzymic hydrolysis by careful adjustment of the conditions (pH, temperature, time) of extraction.

The practical application of this method to problems requiring a highly sensitive assay is facilitated by its simplicity. The synthesis of the radioactive substrate offers no difficulties, and the entire assay, when carried out with a liquid scintillation counter, consists of a simple extraction procedure.

A wide range of applications is indicated by the observation that all organs or cell cultures of warm-blooded animals so far tested contained β-galactosidase (8).[2] Therefore, the sensitivity of the method may be well suited for the quantitative study of cell permeability and for the study of specific protein synthesis. In our laboratory, the method has been successfully applied to the quantitative study of viral cytopathogenicity as reflected by the extent of enzyme release from infected cells. The method proved of particular value when applied to cell cultures infected with viruses which failed to cause overt cytopathogenicity.[2]

Finally, it is interesting to consider the application of this method to certain problems concerning the induced synthesis of β-galactosidase in *Escherichia coli*. Calculations based on data published by Wallenfels *et al.* (9) on crystalline β-galactosidase from *E. coli* indicate that one bacterial cell of the strain ML 309 contains about 10^4 β-galactosidase molecules capable of catalyzing the hydrolysis of 0.5×10^{-14} mole ONPG/min. This means that about 200 cells would be required to obtain a measurable rate with our assay during 1 hr of incubation. It

follows that, unless other factors such as non-enzymic hydrolysis become limiting, the direct measurement of the induced synthesis of β-galactosidase in a single bacterial cell with ONPG of about 1000-fold greater specific activity might not be entirely impossible.[5]

SUMMARY

An ultrasensitive assay of β-galactosidase capable of measuring hydrolysis rates of less than 1×10^{-10} mole *o*-nitrophenyl-β-D-galactoside/20 min with an accuracy of ±10% is described. Radioactive *o*-nitrophenyl-β-D-galactoside labeled with C^{14} in the benzene ring was used as substrate, and the extent of hydrolysis was measured by radioassay of isooctane-extractable *o*-nitrophenol. Detailed descriptions of a small-scale synthesis of *o*-nitro-UC^{14}-phenyl-β-D-galactoside and of the radioassay procedures are given, and the application of the method to some biological problems is discussed.

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[5] The use of a sensitive fluorometric assay of β-galactosidase with 4-methylumbelliferone-β-D-galactoside as substrate (10) has recently been referred to by McCaman and Robins (11). Although details of this assay have not yet been published, it appears to be of great value because of the extremely high sensitivity of fluorometric methods.

ANALYTICAL BIOCHEMISTRY, 2, 216–220 (1961)

A Simple Method for Measuring Nitrogen Fixation by Cell-Free Enzyme Preparations of *Clostridium pasteurianum*[1]

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Received January 5, 1951

Recently cell-free preparations of *Clostridium pasteurianum* were reported which carry out the following set of reactions (1–3):

$$N_2^{15} \rightarrow N^{15}H_4^+ \rightarrow \text{glutamate-}N^{15} \rightarrow \text{amino acids}$$

Since the conversion of N_2 to ammonia is the most rapid step in this reaction sequence, a simple method for measuring ammonia production rather than N^{15} incorporation has been developed.

METHODS

Reaction Mixture for Nitrogen Fixation

The cell-free enzymes were prepared from dry cells of *Clostridium pasteurianum* by methods previously described (3). The nitrogen-fixing reaction mixture in a 25-ml flask consisted of 10–15 mg protein N (biuret) as cell-free enzyme preparation and 100 mg sodium pyruvate in 8 ml of 0.05 *M* potassium phosphate buffer at pH 6.5. The remaining space in the flask contained 0.5 atm N_2, 61.5 atom % N_2^{15}, or argon. The flask containing the reaction mixture was incubated at 30°C for 1 hr on a Brunswick rotary shaker, and then the contents were analyzed for nitrogen fixed.

Measurement of the Nitrogen Fixed

A modification of the micro-Conway technique (4) was used as a means of measuring the ammonia produced during nitrogen fixation. Two flasks were assembled, each containing the reaction mixture described above. One flask contained 0.5 atm of 61.5 atom % N_2^{15}; the other contained 0.5 atm argon. After incubation, the flasks were opened

[1] Contribution No. 652.

and 4 ml of the final reaction mixture from the flask containing N_2^{15} was analyzed for N^{15} excess (5). In addition, two-ml samples of the reaction mixture in the flask containing the N_2^{15} and the flask containing argon were analyzed for ammonia. In the modification of the Conway technique used, 1 ml of 2% boric acid and one drop of Tashiro's indicator (4) were added to the center chamber of the dishes; and, after adding the sample to the outside chamber, 1 ml of saturated K_2CO_3 was added to the reaction mixture and the dishes were covered. A 400-gm weight was placed on top of each dish to assure a tight seal. The progress of the distillation was followed by the change in indicator color. After distillation, the trapped ammonia in the center chamber was titrated to the violet–gray end point of the indicator by addition of 0.010 *N* HCl. The difference in titration of ammonia between the argon control and the experimental flask containing N_2^{15} was the measure of nitrogen fixed by the cell-free preparation.

In some experiments no distillable ammonia was present in the preparation incubated under argon. This meant that either the preparation contained no free ammonia or that the ammonia originally present had been incorporated into amino acids or amides. Because the blank had no ammonia, the titration value of the experimental flask and, consequently, the nitrogen fixed could be only a minimum result. To eliminate the possibility of a zero blank in these experiments, enough ammonia was added to the control (and experimental) flask to produce a control with a slightly positive titration. This amounted to an addition of 50–150 μg N as ammonia to each 8 ml of reaction mixture. The addition of this amount of ammonia to a reaction vessel had no effect on nitrogen fixation as measured by N^{15} incorporation.

The reproducibility of this method for measuring nitrogen fixation was established in several experiments by determining the deviation from the mean of six ammonia determinations from each flask. The sensitivity of the method was estimated from this deviation.

RESULTS

A comparison of the nitrogen fixed as measured by the N^{15} technique and by the technique of ammonia distillation followed by titration is presented in Table 1. In two separate experiments the nitrogen fixed measured by the N^{15} technique was 208 and 181 μg compared to 210 and 182 μg, respectively, found by the distillation–titration technique. These figures differ by only 0.6–0.9% which indicates that at this level of fixation the method is comparable to the N^{15} technique.

The reproducibility of this method for determining the amount of nitrogen fixed was checked in several separate experiments, two of which

TABLE 1
MEASUREMENT OF NITROGEN FIXED BY CELL-FREE PREPARATIONS
COMPARISON OF THE TITRATION AND N^{15} METHODS

Experiment	Flask[a]	Nitrogen fixed: By N^{15} determination: N^{15}/N^{14}	Atom % excess N^{15}	Total N fixed (μg)	By ammonia production: HCl required[b] (ml 0.010 N)	Total N fixed (μg)
1	Under A	—	—	—	3.3	—
	Under N_2^{15}	.0100	0.64	208	4.8	210
2	Under A	—	—	—	3.3	—
	Under N_2^{15}	.0092	0.56	181	4.6	182

[a] Each flask contained 20 mg protein N as cell-free preparation and 100 mg sodium pyruvate in 8 ml of 0.05 M potassium phosphate buffer at pH 6.5. One-half atmosphere of the gases indicated was present in the flasks. See *Methods* for other details.

[b] Milliliters of 0.010 N HCl required to neutralize the ammonia distilled from the final reaction mixture upon addition of saturated K_2CO_3 (see *Methods* for further details).

are given in Table 2. Six separate ammonia determinations were made on the contents of each flask. The argon control flasks contained means of 90.0 and 90.5 μg N as NH_3 with standard deviations from the mean of ± 1.28 and 1.79 μg N, respectively. The experimental flasks contained means of 266.7 and 299.6 μg N as NH_3 with standard deviations from the

TABLE 2
REPRODUCIBILITY OF DISTILLATION–TITRATION METHODS
FOR MEASURING NITROGEN FIXATION

Experiment	Flask[a]	Mean (μg N as NH_3 per flask)	Variation from mean (μg N as ammonia[b])	Standard deviation from mean (σ)[c]
1	Under A	90.0	0.2, 0.8 1.9, 0.2 1.4, 1.4	1.28
	Under N_2	266.7	3.5, 1.3 1.3, 3.5 2.1, 2.1	2.71
2	Under A	90.5	3.2, 1.9 0.3, 0.3 1.4, 0.3	1.79
	Under N_2	299.6	1.6, 1.6 0.6, 2.8 3.9, 3.1	2.77

[a] Each flask contained 12 mg protein N as cell-free enzyme preparation. For other contents of incubation mixture, see *Methods*.

[b] Six 1-ml samples from each flask were assayed for ammonia content by the distillation–titration technique.

[c] Ref. (6).

mean of ±2.71 and 2.77 μg N, respectively. The standard deviation for the difference between two samples [$\sqrt{2}\ X\ \sigma$ (standard deviation)] is 1.81 and 2.53 μg N for the experimental flasks. For significance the amount of ammonia in the experimental flask must exceed that in the control by twice the standard deviation (probability level = .05) or 3.62 and 5.06 μg, respectively (6). Since the mean of the control flasks was approximately 90 μg N as ammonia, a difference of 4–5.5% would be significant. It is obvious. therefore. that the preparation in the experimental flasks has fixed nitrogen.

The amount of ammonia required for a significant difference from means of 266–300 μg N (experimental flasks) is 7.68–7.84 μg N or about 3% of the mean (Table 2). From these data and the data from the control flasks, one can see that the sensitivity of the method is greatest when the control ammonia is lowest. In addition, since the difference (in μg N) necessary for significance decreases as the number of replicate flasks increases (equals $\sigma/\sqrt{\text{no. replicates}}$), the sensitivity of the method is potentially greater than in the experiments described here.

Further study of the technique to determine whether strong alkali or saturated K_2CO_3 should be used to distill the ammonia showed that either could be used (Table 3). The addition of K_2CO_3 is preferred

TABLE 3

MEASUREMENT OF NITROGEN FIXED BY CELL-FREE PREPARATIONS DISTILLATION OF AMMONIA FROM NaOH COMPARED WITH K_2CO_3[a]

Experiment	Flask	HCl required (ml .010 *N*)		Total N fixed (μg)	
		Distilled from 3 *N* NaOH	Distilled from K_2CO_3	NaOH method	K_2CO_3 method
1	Under A	3.12	0.36	—	—
	Under N_2	3.82	1.11	—	—
	N_2-A	0.70	0.75	98	104
2	Under A	3.28	.24	—	—
	Under N_2	5.00	1.84	—	—
	N_2-A	1.72	1.60	240	224

[a] See Footnote *a* in Table 1.

however, since the amide nitrogen hydrolyzed and distilled by strong alkali decreased the sensitivity of the method by increasing the blank.

DISCUSSION

With this titration technique as little as 3–5 μg nitrogen fixed can be detected. This is about one-twentieth the sensitivity of the N^{15} technique which can detect in the range of 0.2 μg N^{15} (.015 atom % excess N^{15} with about 1 mg total nitrogen needed for assay). Since, in most experi-

ments the total nitrogen fixed was about 50 μg, the error is less than ± 3 μg. The advantages this method has over the N^{15} technique are that it requires little time, positive fixation can be detected qualitatively within 5 min after addition of K_2CO_3 by comparing the change in indicator color of the control dish with that of the experimental one, and quantitative knowledge of the amount of nitrogen fixed can be obtained 1 hr after running the experiment.

SUMMARY

A simple, rapid method for measuring nitrogen fixation by cell-free enzyme preparations has been devised. The method comprises the measurement of the ammonia produced during nitrogen fixation and can detect from 3 to 5 μg nitrogen fixed. The validity of using this technique as a means of measuring nitrogen fixation has been verified by comparing its results with those of similar ones measuring N^{15} incorporation.

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The Loss of Sodium and Potassium during the Dry Ashing of Animal Tissue[1]

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INTRODUCTION

The need to determine the sodium and potassium content of composite samples of dry ashed human tissue for the Internal Dose program of the Health Physics Division, Oak Ridge National Laboratory (9) raised the question as to whether a fraction of these elements had been lost in the process of direct dry ashing. The literature indicated this question of loss was an old problem; that it had been considered only with small samples, chiefly with blood, plasma, urine, etc.; and that it had been studied to only a limited extent with larger samples of plant tissue, foodstuffs, and animal feeds.

Kramer, in 1920 (7), ashed small samples of blood and serum in a platinum crucible which was placed on several pieces of porcelain in a quartz dish and was heated with a Meeker burner. His studies with synthetic samples indicated no loss of sodium or potassium. The literature of that period reported ashing conditions as "heated to redness, dull redness, at incipient red heat," etc. Due to this problem of reproducing "red heat" under different conditions, Stewart and Arthur, in 1935 (10), in their studies on ashing of plant tissue indicated the need of precisely stating the conditions in place of "ashing at low heat not exceeding dull redness." Cullen and Wilkins, in 1933 (3), ashed various "pretreated" tissue samples overnight at 500°C, then briefly raised the temperature to 600°C before cooling. Hald, in 1933 (4), ashed small samples at 580–600°C for 3 hr. No references were made to specific

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studies for sodium or potassium loss. Due to poor results with ashing at 600°C, Strauss, in 1937 (13), added thorium nitrate to 1–5-ml samples of serum and urine. The dried samples were ashed at 600°C for 20 min, or 750°C for 10 min, with no loss of potassium, since thorium forms double salts with sodium and potassium that volatilize at much higher temperatures than the individual salts. In 1944, Van Slyke and Reiben (15) treated 1-ml samples of serum and plasma with 4 N H_2SO_4, evaporated them to dryness, then ashed the samples overnight between 460 and 480°C. These workers stated that there may be a loss of potassium above 480°C.

Studies on possible losses due to direct ashing of larger samples were done chiefly on foods and agricultural products, and in most cases the interest was in terms of the quantity and quality of the ash. Adams (1), studying time and temperature requirements for removing all the carbon in the ashing of dried peas, found a reduction in the weights of the ash between 500 and 900°C and a loss in potassium oxide. St. John (11, 12), in the dry ashing of feeds at 500–700°C, found that the loss of potassium increased as the temperature was increased, but this loss of potassium was much less than the loss of sulfur and chlorine. No loss of calcium was reported.

There is a lack of information concerning the loss of sodium and potassium during dry ashing of larger samples of animal and human tissue. Therefore, this work was undertaken to determine a suitable temperature range for dry ashing animal tissue to secure a reasonably clean ash without loss of sodium and potassium.

EXPERIMENTAL PROCEDURE

The concentration of sodium and potassium in tissue was determined from the ash of five different tissues and dry milk. The samples were weighed in groups of six, i.e. six samples of each material were used for each temperature and ashing time. Several additional groups of samples were weighed in order to have extra samples in case of accident or for rechecking data when necessary.

Weighing

All weighing was performed with a single-pan type balance, in previously tared crucibles, and under constant temperature and humidity conditions, since preliminary experiments indicated the necessity of weighing rapidly at one time period all the samples of a tissue needed for ashing throughout the complete temperature range. The samples were ashed from the low to high temperatures in the same sequence as they were weighed. For certain tissues as lung or liver, the rate of mois-

ture loss as a function of time was determined for different sized samples so it was possible to estimate more nearly the true sample weight when this was necessary.

Drying

The samples were dried in a 100°C oven and stored in a refrigerator until ashed. This was a convenient means of storing 140–150 dried samples in crucibles until ready for ashing.

Ashing

The samples were carbonized and ashed within the temperature range from 300 to 900°C at 50° intervals. The dried samples were started in a cold furnace, and the temperature was increased in 50° increments every 30 min until the desired ashing temperature was reached, then remained for 24 hr at this temperature. At certain temperatures additional samples were ashed for 72 hr in order to determine the effect of increased ashing time. The muffle furnaces were equipped with quartz sleeves, and Pt:Pt-13%Rh thermocouples were enclosed in ceramic tubes.

Dissolving

The carbonized and ashed samples were dissolved by heating with concentrated nitric acid on a low-temperature hot plate, then perchloric acid was added, and the solutions were fumed until they became colorless. One or several additions of both acids or of perchloric acid only were necessary depending on the degree of ashing and the color of the ash–acid mixture. The samples were fumed to near dryness, i.e., until only moist crystals remained, then dissolved in deionized water. The solutions were transferred quantitatively to volumetric flasks, buffered with enough 1.0 *M* citric acid–ammonium citrate solution to make the solution 0.1 *M* (6),[5] and then diluted volumetrically. A trace of mercuric iodide was used to prevent the growth of mold in the buffer and sample solutions.

The sample size and volume were predetermined with exploratory samples so the concentration of sodium and potassium would be in the proper concentration range for the flame photometer. The sample solutions, as well as all stock solutions and the deionized water were stored in clean polyethylene bottles. The nitric acid was redistilled in a quartz still, while the perchloric acid was vacuum redistilled by means of a Vycor still and stored in Vycor ampules.

[5] Smart, T. R., Grove, E. L., and Kassner, J. L., unpublished work.

Spectrophotometry

The sample solutions were atomized into the flame, and the per cent transmittance readings from the spectrophotometer were converted to parts per million (ppm) from a new standardization curve prepared at the same time. The solutions for the standardization curves were prepared from a stock solution of "specpure" sodium chloride and potassium chloride which had been treated in the same manner as the ashed samples. A Beckman spectrophotometer, model DU with the 9200 flame attachment and the hydrogen–oxygen burner, was used in this work.

SOURCES OF TISSUE AND MILK

Milk

Commercial dry milk was placed in an evaporating dish until its moisture content was in equilibrium with the air. Then all the samples, approximately 1 gm each, were weighed in rapid succession. Dry milk was selected as the first material because of its homogeneity and ease of handling.

Cattle Serum

Fresh blood was obtained directly from a packing plant with no specific precautions for purity or sterility. The blood was centrifuged and the resulting decantate recentrifuged. However, due to hemolysis of the red blood cells before centrifuging, this serum was not perfectly clear. Approximately 4-gm samples were transferred to tared quartz crucibles and weighed immediately.

Blood

The blood was obtained by cardiac punctures from healthy young rabbits that had not been used for other experimental work. Samples of 15 ml were taken from each rabbit at 2-week or longer intervals in order to avoid the decrease in sodium and potassium concentrations resulting from the loss of blood at too frequent intervals. Aliquots of 2–3 ml were ejected immediately from the syringe into tared crucibles before clotting occurred. The crucibles were covered and weighed immediately.

Brain

Packaged frozen pork brains were allowed to thaw and then were blended in a Waring blendor. Samples were approximately 2 gm each.

Lung

A section of a lower lobe of beef lung was sliced and then mixed by passing it twice through a meat grinder. The samples weighed approximately 3 gm each.

Liver

A section of calf liver was mixed by the same procedure as for lung tissue, and divided into samples weighing approximately 3 gm each.

EXPERIMENTAL RESULTS AND DISCUSSION

The concentrations of sodium and potassium found in six different materials by wet ashing, nitric–perchloric acid method, and by dry ashing at different temperatures are shown in Table 1. An examination of this table indicates that for a 24-hr period 550°C is the maximum temperature that can be maintained without the loss of sodium and potassium becoming appreciable. The loss of these two elements with respect to temperature is illustrated in Figs. 1 and 2. In the 800–900°C range approximately 90% of the potassium and 40–70% of the sodium are lost from the blood and serum samples. The percentage loss from the other tissues does not appear to be as great.

The length of ashing time is a significant factor at the temperature at which sodium and potassium losses begin to occur. However, in the 400–500°C range the longer ashing period caused no apparent loss of these elements.

No attempt was made to discover how the losses took place, i.e., if the compound was swept out on colloidal particles or by the volatilization of an alkali compound. However, the loss is probably not entirely due to the volatility of the alkali chloride since sodium chloride does not begin to volatilize until 780°C (5) and the incipient temperature for potassium chloride is slightly below this.

The samples subjected to 300 and 350°C were only carbonized and the proteins were only partially destroyed. These samples were much more difficult to put into solution by oxidation with nitric and perchloric acids than the tissue itself. The long oxidation time, the extra additions of acid, and the tendency of these sample solutions to creep resulted in rather poor precision and probably unreliable results in several series of samples. At 400°C the ashing was more complete for the 72-hr period than for the 24-hr period. Between 450 and 500°C most of the 1–5-gm samples were free from carbon. However, serum was not completely ashed at 500°C and some carbon was still present at 550°C. A layer of carbonized material was on the bottom of the crucible indicating the

TABLE 1

SODIUM AND POTASSIUM FOUND IN THE TISSUE ASH FOLLOWING DIFFERENT ASHING TEMPERATURES AND TIMES

	Dry milk		Cattle serum[a]		Rabbit blood		Pork brain		Cattle lung	Calf liver[b]
Sodium (mg/gm of tissue)										
Wet ash	6.05		3.31		2.15		1.34		1.72	0.700
Dry ash (temp. °C)	24 hr	72 hr	24 hr	72 hr	24 hr	72 hr	24 hr	72 hr	24 hr	24 hr
300	6.11	6.06	3.34	3.30	2.08	2.15	1.24	1.28	1.68	0.725
350	6.04		3.37		2.21		1.31		1.68	0.715
400	6.09	6.08	3.35	3.36	2.19	2.16	1.32	1.29	1.67	0.715
450	6.08		3.35		2.18		1.31		1.66	0.720
500	6.05	6.06	3.36	3.28	2.08	2.12	1.31	1.30	1.68	0.720
550	6.02	5.79	3.38	3.38	2.13	2.09	1.29		1.67	0.710
600	5.85	5.66	3.20	3.20	1.91	1.74	1.25	1.20	1.64	0.685
650	5.76		3.13		1.76		1.13		1.61	0.680
700	5.35	5.08	2.14	1.31	1.23	1.17	1.14	1.09	1.60	0.680
750	5.06		1.34		1.33		1.11		1.50	0.655
800	4.84	4.65	1.16	1.04	1.23	1.07	1.08	1.04	1.49	0.675
850	4.73		0.80		1.20		0.94		1.57	0.660
900	4.69	4.55	0.77	0.60	1.12	0.78	1.01	0.97	1.51	0.650
Average std. dev.[c]	0.2		0.007		0.04		0.04		0.03	0.02
Potassium (mg/gm of tissue)										
Wet ash	15.4		0.376		1.63		3.48		2.55	3.13
Dry ash (temp. °C)	24 hr	72 hr	24 hr	72 hr	24 hr	72 hr	24 hr	72 hr	24 hr	24 hr
300	15.1	14.9	0.375	0.389	1.70	1.60	3.26	3.47	2.55	3.22
350	15.6		0.372		1.58		3.33		2.53	3.17
400	15.9	15.4	0.383	0.370	1.53	1.69	3.45	3.38	2.58	3.19
450	15.4		0.388		1.57		3.49		2.56	3.17
500	15.5	15.6	0.390	0.373	1.66	1.71	3.46	3.45	2.53	3.17
550	15.3	14.9	0.380	0.367	1.66	1.54	3.39		2.54	3.15
600	15.0	14.5	0.364	0.305	1.62	1.45	3.31	3.15	2.50	3.06
650	13.4		0.261		1.36		3.08		2.38	2.97
700	11.8	8.40	0.113	0.075	0.31	0.29	2.91	2.86	2.33	2.90
750	6.96		0.057		0.27		2.87		2.19	2.70
800	4.85	4.54	0.047	0.050	0.25	0.18	2.66	2.61	2.23	2.72
850	4.66		0.038		0.08		2.41		2.22	2.66
900	4.61	4.33	0.032	0.020	0.10	0.04	2.44	2.31	2.12	2.66
Average std. dev.[c]	0.4		0.07		0.04		0.05		0.05	0.04

[a] Source, packing plant. Potassium values expected to be high due to hemolysis, sodium values possibly high due to contamination.

[b] Source, meat market. Sodium values possibly low due to loss of body fluid.

[c] Average of standard deviation of samples at each temperature.

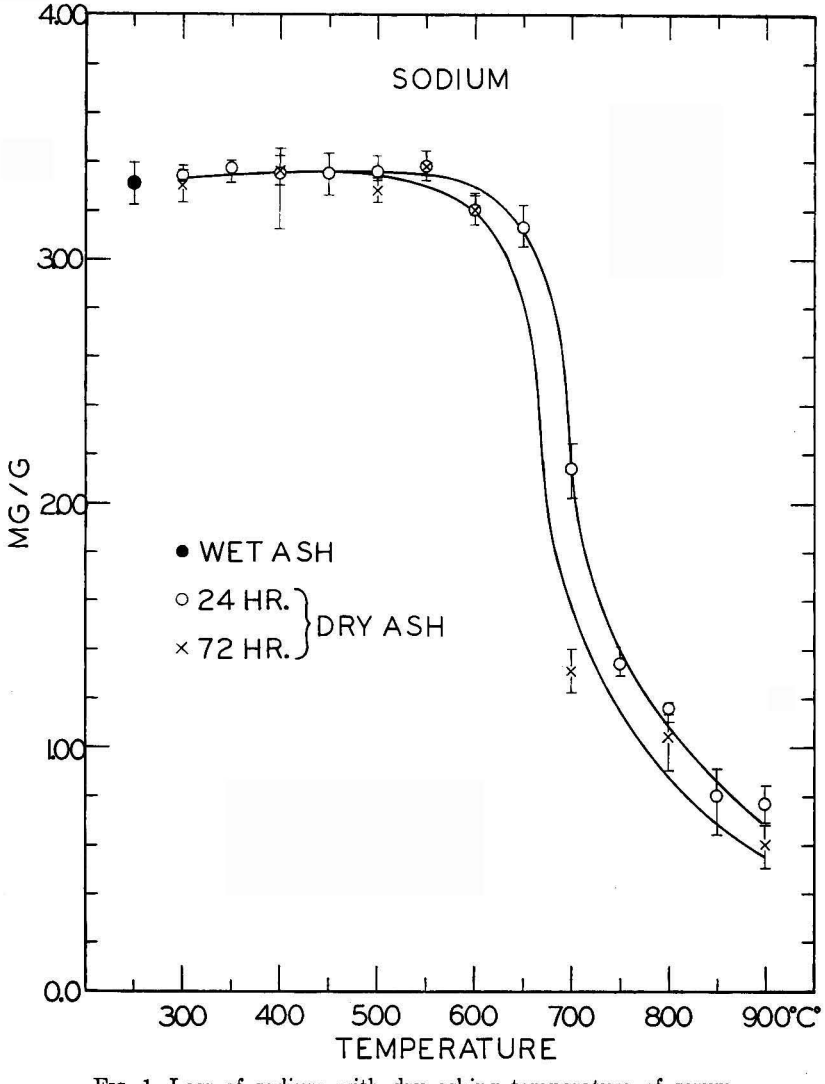

FIG. 1. Loss of sodium with dry ashing temperature of serum.

ashed material apparently had acted as a protective blanket to keep air away. This was in contrast to blood which was almost completely ashed at 400°C, and within the temperature range of 400–550°C it appeared as a reddish and very porous sponge or a bundle of very fine lacework.

The quartz crucibles were attacked by the samples at the higher temperatures, probably due to the alkali compounds being converted to the alkali oxides in the temperature range of 400–700°C (5). Etching

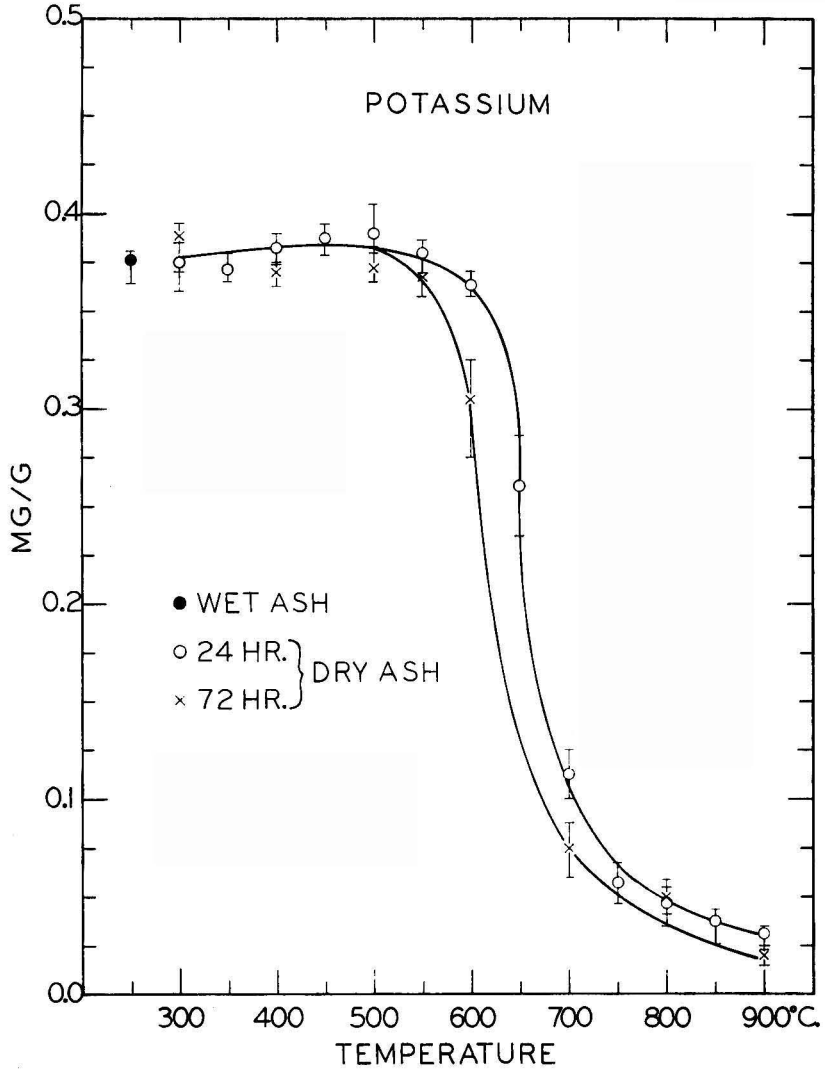

FIG. 2. Loss of potassium with dry ashing temperature of serum.

of the crucibles was observed at about 700°C and became more noticeable as temperature and time were increased. Since the alkali oxides attacked the quartz, some of the sodium and potassium may have been trapped, which would account for the data obtained in the 700–900°C range (Figs. 1 and 2).

When 12 samples were in the furnace instead of six, within the 400–

500°C range, more carbonaceous material was observed to remain in the samples. This suggested that the addition of oxygen into the furnace would be beneficial, as observed by other workers (2, 8, 10), for different ashing situations. Samples of 25, 50, and 100 gm of lean ground beef were ashed at 450°C and other conditions similar to those reported by Tipton *et al.* (14), without and with a small flow of oxygen into the furnace for a 24-hr period. The samples receiving oxygen while ashing were much more completely ashed than those not receiving oxygen.

Rabbit blood was centrifuged carefully and the serum analyzed for sodium and potassium. The values listed in Table 2 are based on 12

TABLE 2
VALUES FOR SODIUM AND POTASSIUM IN RABBIT BLOOD AND SERUM

	Element	μg/gm	Deviation (%)
Blood	Sodium	2140	3.0
	Potassium	1640	3.1
Serum	Sodium	3280	2.2
	Potassium	176	1.6

samples of rabbit serum ashed at 450° for a 24-hr period, and the blood samples ashed at 400, 450, 500, and 550°C.

SUMMARY

The maximum temperature at which animal tissue may be dry ashed for a 24-hr ashing period without loss of sodium and potassium is 550°C. for the sample sizes which range from 1 to 5 gm most of the carbon is burned from the ash at 450–500°C in a 24-hr period.

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Determination of N′,N′-Anhydrobis(β-hydroxyethyl)-biguanide Hydrochloride in Urine

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INTRODUCTION

The compound, N′,N′-anhydrobis(β-hydroxyethyl)biguanide hydrochloride (ABOB), a new antiviral drug, can be assayed by titration with perchloric acid in an acetic acid medium (1, 2), by a colorimetric determination with potassium ferricyanide and sodium nitroprusside in an alkaline medium (3), by precipitation of the base with Reinecke's salt, and by ultraviolet spectroscopy in aqueous medium.

Previous investigators (3–5) working with guanidine-like bases in biological media adsorbed the bases on charcoal, eluted with acid alcohol, and determined the bases colorimetrically using a potassium ferricyanide–sodium nitroprusside mixture for color development. Zappacosta (6) used a modified Sakaguchi (7) reagent for his color development. In biological fluids ABOB is generally determined by the colorimetric method of Andes and Myers (3) or by a spectrophotometric procedure. The inherent background in the latter procedure often interferes with the absorption peak of ABOB at 237 mμ.

A spectrophotometric method, developed for the determination of ABOB in urine in which the background problem was overcome by using a portion of the urinary filtrate as a blank, is the subject of this report.

APPARATUS AND REAGENTS

Recording spectrophotometer with 1.00-cm square cells (Process and Instrument Co.).

Chromatography columns with Teflon stopcocks, 14 mm O.D. and 15 cm long having a 25-ml reservoir and 12 mm O.D. and 15 cm long having a 25-ml reservoir.

Barium hydroxide, 0.3 *N* and zinc sulfate, 5%. Prepare as described by Van Pilsum *et al.*, but omitting the biphthalate buffer (8).

Dowex 1 resin (Cl^-). Prepare as described by Price (9).

Bromine water, approximately 5%.

Sodium thiosulfate, 0.1 *N*.

Standard *N'*,*N'*-anhydrobis(β-hydroxyethyl)biguanide hydrochloride solution containing 1 mg/ml. Prepare a series of standards for a standard curve to cover the range of 0–300 μg/ml (30, 60, 120, 180, 240, and 300 μg/ml).

PROCEDURE

Prepare two resin columns, 8 cm and 2–2.5 cm in length, for each determination. Wash the columns with 25 and 15 ml water, respectively. Operate the columns without added pressure and discard after use.

Pipet 1.0 ml of the urine sample into a 40-ml centrifuge tube containing 9 ml water. Add 5 ml of 0.3 *N* barium hydroxide followed by 5 ml of 5% zinc sulfate to remove urinary pigments. Stopper the tube, shake vigorously for a few seconds and centrifuge at 3000 rpm for 3 min. Filter the supernatant liquid through a Whatman No. 1 (7 cm) filter paper directly onto the 8-cm packed resin column. Collect the eluate in a 50-ml glass-stoppered graduated cylinder. Wash the precipitate twice with 4 ml water, centrifuging after each wash. Pour each supernatant onto the column. As the last portion of the second wash solution reaches the resin surface, add 5 ml of 0.1 *N* sodium hydroxide and collect the alkaline eluate in the same cylinder. Bring the eluate volume to 30 ml by adding water to the column. Stopper the cylinder and mix.

For the sample blank, transfer 10 ml of the eluate to a test tube. Add 0.2 ml of the bromine water and shake. After 10 min add 0.1 ml of the 0.1 *N* sodium thiosulfate solution and shake. Set the solution aside for a few minutes. Pour the solution onto the 2–2.5-cm packed resin column to remove the bromide ions. Discard the first 3 ml eluate.

Transfer a portion of the brominated eluate to a blank cuvette and a portion of the nonbrominated eluate to the sample cuvette. Determine the ultraviolet absorption spectrum from 220 to 285 mμ on a recording spectrophotometer. Subtract the absorbance at 285 mμ (minimum) from the absorbance at 237 mμ (maximum). The difference in absorbance at these two wavelengths is a function of the concentration of ABOB in the urine specimen. The absorbance value is then converted to concentration by means of a standard curve.

STANDARD CURVE

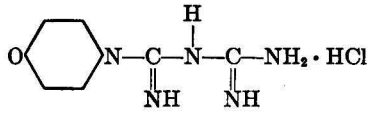
Add 1.0 ml of each diluted standard to 1 ml of normal urine in a 40-ml centrifuge tube. Add 8.0 ml water and take each standard through the procedure as previously described. Plot the absorbance (absorbance

at 237 mμ minus absorbance at 285 mμ) against concentration on linear coordinate paper to obtain the standard curve.

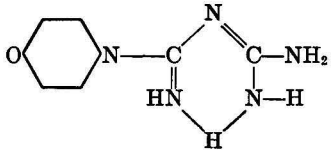
DISCUSSION

The addition of barium hydroxide and zinc sulfate to the urine sample removes urinary pigments and produces a protein-free filtrate (8). The passage of the filtrate through the Dowex 1 column removes background substances in the urine which interfere with the ultraviolet absorption curve of ABOB in the 237-mμ region of the spectrum.

The chemical structure of ABOB is:



It is theorized that the ultraviolet absorption is produced by a cyclization of the biguanide portion of the molecule in the form of an intramolecular hydrogen-bonded cation as suggested by Shapiro, Parrino, and Freedman (10).



Treatment with bromine alters the ABOB molecule and destroys its property to absorb in the ultraviolet region of the spectrum.

When added to a dilute solution of alkaline hydroxide, bromine forms the bromide and hypobromite salts (11). Aryl biguanides can be oxidized with aqueous sodium hypobromite at room temperature to give one mole of nitrogen per mole of aryl biguanide (12). Stehle (13) has described a gasometric determination for urea based on the liberation of nitrogen by treatment with hypobromite. Therefore it is postulated that the hypobromite ion, formed by the addition of bromine to the alkaline eluate from the resin column, breaks the proposed cyclized moiety with the liberation of nitrogen and the resulting product does not absorb in the ultraviolet region of the spectrum.

The bromide and the hypobromite ions must be removed from the blank sample as they absorb in the 237-mμ region of the spectrum. In our early studies, following bromination of the blank sample, the solution was passed through the resin column without the addition of the thiosulfate. At times erratic ultraviolet scans were obtained and it was difficult to maintain a linear relationship based upon concentration.

When thiosulfate was added to the brominated blank sample and the sample was subsequently passed through the resin column, a constant linear relationship was obtained throughout the range investigated (0–300 μg/ml urine) (Fig. 1).

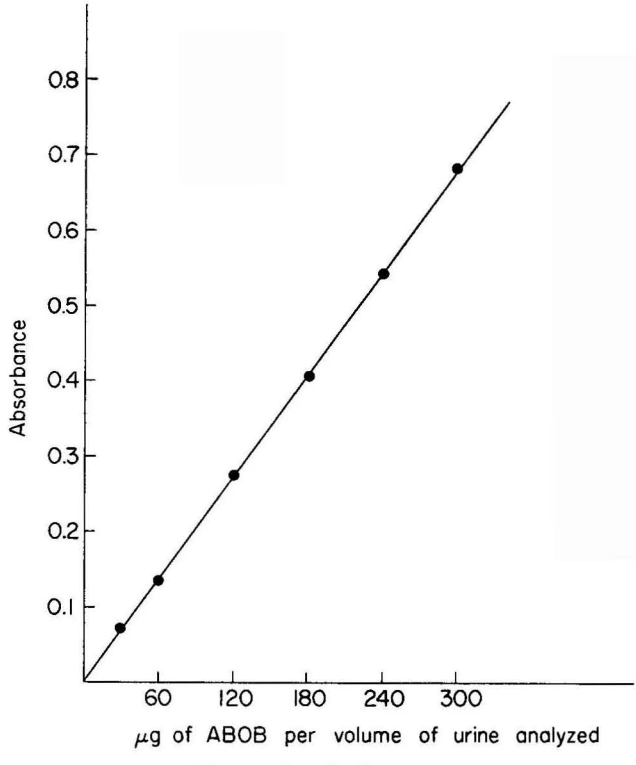


FIG. 1. Standard curve.

Urine was used in the preparation of the standard curve in order to simulate test specimens. Data obtained for the preparation of the standard curve are summarized in Table 1.

Initially water was used to rinse the resin after passage of the urine filtrate. However, higher recoveries of ABOB and more reproducible results were obtained when alkali was passed through the column.

If the concentration of ABOB in the test specimens is low, larger amounts of urine can be analyzed. When the volume of urine is increased, the amount of water added to the specimen prior to the addition of the barium hydroxide is proportionally decreased. Over 250 test specimens from 15 human subjects have been analyzed satisfactorily by this procedure using aliquots ranging in volume from 1 to 3 ml.

In an attempt to simulate pathological urine, several drops of bile, blood and bile, and blood were added to 5-ml portions of normal urine and mixed. Known concentrations of ABOB were added to 1-ml aliquots of these urines, and then the urines were analyzed by the described procedure. The results obtained on these urines were the same as those obtained on normal urines which contained the same concentrations of ABOB. No interference was observed from these simulated pathological specimens.

TABLE 1
STANDARD CURVE DATA

Micrograms of ABOB added to 1 ml urine	Number of determinations	Absorbance (mean value)	Range	Std. dev. (%
30	5	.072	.068–.079	.43
60	4	.134	.128–.141	.55
120	6	.275	.268–.284	.72
180	5	.407	.402–.414	.47
240	5	.543	.523–.558	1.66
300	12	.683	.670–.691	.85

In previous studies it was found that the ultraviolet absorption spectrum of ABOB could be destroyed by alkaline hydrolysis. However, on some urine specimens this technique would affect the inherent background, causing the ultraviolet curve to fall below the base line in the 260–285-mμ region of the spectrum. This inconsistency caused the technique to be abandoned.

Good results were obtained in a Beckman DU spectrophotometer when the maximum and minimum were measured at 237 and 285 mμ, respectively. However, since a recording spectrophotometer was available, all of the experimental data were obtained on the recording instrument.

Preliminary evidence indicates that this method may be used to determine the concentration of ABOB in plasma specimens. Other biological media have not been investigated.

SUMMARY

A differential spectrophotometric method for the urinary determination of *N'*,*N'*-anhydrobis(β-hydroxyethyl)biguanide hydrochloride (ABOB) is described. Following preliminary purification by precipitation of contaminating material with barium hydroxide plus zinc sulfate and ion-exchange chromatography, a portion of the sample is treated with bromine water to alter the ABOB molecule so that it does not absorb in

the ultraviolet region of the spectrum. The concentration of ABOB is then determined by reading a second portion of the sample against the bromine-treated sample. Each urine specimen therefore serves as its own blank.

ACKNOWLEDGMENT

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ANALYTICAL BIOCHEMISTRY, 2, 237-241 (1961)

A Microfluorometric Method for the Determination of Agmatine

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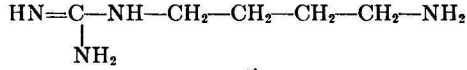
Agmatine, the decarboxylation product of arginine, has not been the subject of extensive investigation. This may be due, in part, to the lack of a sensitive chemical method for its determination.



agmatine

This paper describes a simple, sensitive method for the fluorometric determination of agmatine based on the condensation of agmatine and *o*-phthalaldehyde (OPT) to yield a highly fluorescent reaction product. Arginine also yields a fluorophore upon treatment with OPT, but agmatine is readily separated from the amino acid by selective extraction. Agmatine is one of the classical substrates of diamine oxidase, and the fluorometric method has been utilized in this laboratory as the basis of a sensitive method for estimation of diamine oxidase activity (1). The method should be applicable in studies of the decarboxylation of arginine (2) or in other systems in which agmatine is formed or metabolized.

MATERIALS AND PROCEDURE

Solvents and Reagents

n-Butanol, reagent grade, is washed with one-fifth volume of 1 *N* HCl and then twice with distilled water. *n*-Heptane, Phillips 99 mole %. *o*-Phthalaldehyde reagent, 10 mg, is dissolved in 1 ml of reagent-grade absolute methanol; prepared fresh daily. Salt-saturated 0.1 *N* NaOH is prepared by adding sufficient solid NaCl to 0.1 *N* NaOH so that an excess of solid NaCl remains.

Extraction of Agmatine

Proteins are precipitated from tissue homogenates or biological fluids by addition of perchloric acid to a final concentration of 0.4 *N*. After removal of the protein precipitate by centrifugation, an aliquot of the supernatant fluid (up to 4 ml) is transferred to a 25-ml glass-stoppered shaking tube containing 0.5 ml of 5 *N* NaOH, 10 ml of washed *n*-butanol, and about 1.5 gm of solid NaCl. The tube is shaken for 5 min to extract the agmatine into the butanol and, after centrifugation, the aqueous phase is removed by aspiration. The organic phase is then shaken for about 1 min with 3 ml of salt-saturated 0.1 *N* NaOH. This wash removes any residual amounts of arginine that may be present. In systems in which little arginine is present, this wash may be omitted. The tube is then centrifuged and an 8-ml aliquot of the butanol is transferred to a 40-ml shaking tube containing 15 ml *n*-heptane and 3 ml of 0.1 *N* HCl. After shaking for 1–2 min the tube is centrifuged and the agmatine in the aqueous phase is assayed fluorometrically as described below. Standards, prepared by adding known amounts of agmatine to 0.4 *N* perchloric acid, are carried through the entire procedure. Under conditions described above, about 85% of agmatine is carried through the extraction procedure. As little as 0.25 μg agmatine added to tissue homogenates (kidney, liver, and intestine) are recovered with high precision.

Fluorometric Assay

After extraction, the agmatine is condensed with *o*-phthalaldehyde in strongly alkaline solution to form a fluorescent product. A 2-ml aliquot containing 0.05–5 μg/ml agmatine is transferred to a test tube, and 0.4 ml. of 1 *N* NaOH is added, followed by 0.1 ml of OPT reagent. The contents of the tube are thoroughly mixed after each addition. After 10 min reaction time the solution is transferred to a quartz cuvette, and the fluorescence at 470 mμ resulting from activation of 355 mμ (wavelengths uncalibrated) is measured in a spectrophotofluorometer.[1] The fluorescence is stable for approximately 15 min after completion of the reaction. The fluorescence intensity is proportional to the agmatine concentration over the range of 0.05–5 μg/ml (Fig. 1). Excitation and fluorescence spectra of the agmatine–OPT fluorophore are illustrated in Fig. 2.

DISCUSSION

The method described in this paper is in many respects similar to the one described previously for the estimation of histamine (3). In both

[1] American Instrument Co. or Farrand Optical Co.

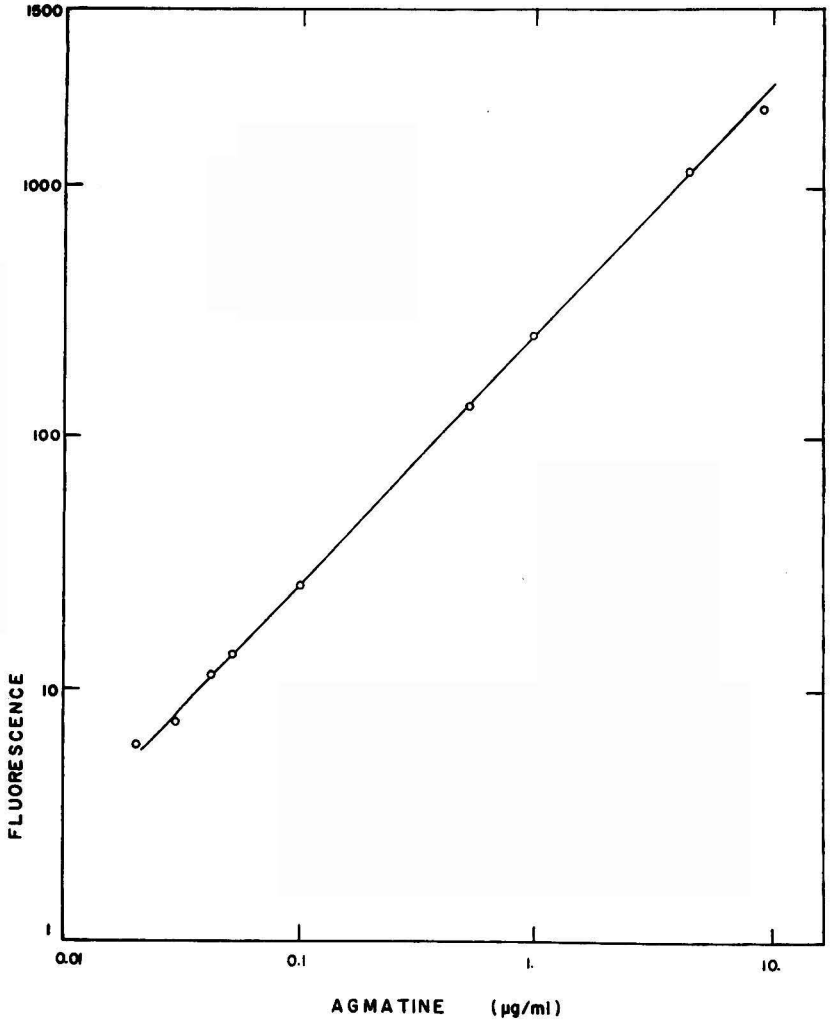


FIG. 1. Linearity of fluorescence and agmatine concentration. The ordinate represents fluorometric readings.

cases, condensation of OPT with the amine in a strongly basic medium leads to production of a highly fluorescent reaction product. However, a marked difference is seen upon acidification of the fluorophores. In the case of histamine, acidification stabilizes and intensifies the fluorescence, while in the case of agmatine, acidification leads to complete loss of fluorescence. This difference suggests that the agmatine–OPT

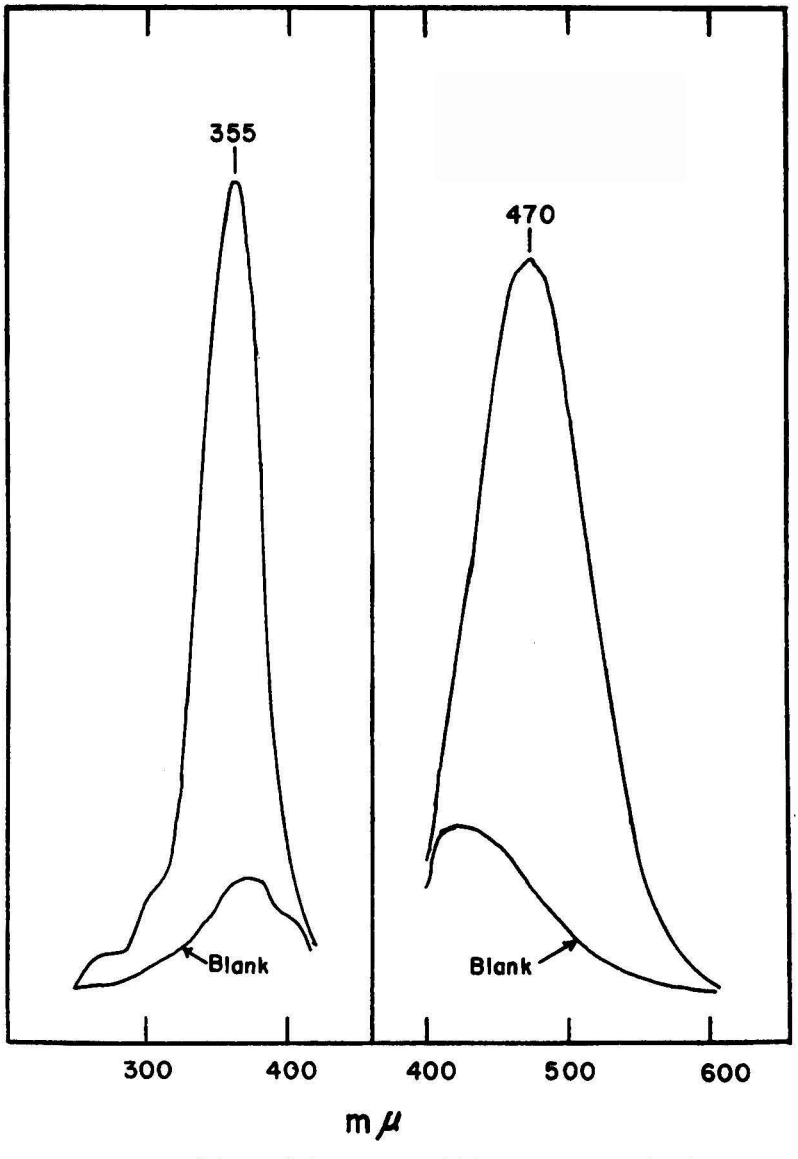


FIG. 2. Excitation (left) and fluorescence (right) spectra of the fluorescent reaction product of agmatine and OPT. To obtain the excitation spectrum, the fluorescence monochromator of a recording spectrofluorometer was set at 470 mμ and the spectrum from the excitation monochromator was scanned. To obtain the fluorescence spectrum, the excitation monochromator was set at 355 mμ and the spectrum from the fluorescence monochromator was scanned. Wavelengths are uncalibrated. The blank curves shown are from reagent blanks.

reaction does not proceed beyond a Schiff-base type compound, while the histamine–OPT reaction product undergoes further reaction (3).

SUMMARY

Agmatine reacts with *o*-phthalaldehyde in an alkaline medium to form a highly fluorescent reaction product. The technique may be utilized for estimation of agmatine in systems in which the amine is formed or metabolized.

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ANALYTICAL BIOCHEMISTRY, 2, 242–247 (1961)

Variation of the Respiration of Protozoan Cells with Length of Centrifuging

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INTRODUCTION

A wide variety of manometric techniques have been developed in the study of biochemical reactions. One of the most commonly used instruments in this study is the Warburg constant-volume respirometer. One of the applications of this respirometer involves the determination of oxygen uptake in intact cells such as bacteria, algae, molds, and protozoa (1). As part of the process of preparing these cells for manometric measurements, centrifugation is commonly employed to concentrate them to densities sufficient to measure oxygen uptake in the respirometer. In general, such cells do not appear to be damaged during concentration by centrifugation. As a result, a lack of effect of centrifugation on the oxygen uptake of such cells has been implied, but not tested. However, during a current experimental study, it was observed that the oxygen uptake of intact protozoan cells varied with the amount of centrifuging they had undergone before introduction into the respirometer. The present paper is concerned with the effect of different centrifuging times on the respiration rate of the flagellate protozoan *Euglena gracilis*.

MATERIALS AND METHODS

Experimental and stock cultures of a streptomycin-bleached strain, SM-L1, of the free-living flagellate protozoan, *Euglena gracilis* var. *bacillaris*, were grown axenically in the dark on Cramer-Myers medium (2) at pH 6.8 and 25°C. Sodium acetate (0.61 *M*) was the sole carbon source for growth. The cells were grown in 300 ml medium in 1 l Erlenmeyer flasks. Cultures were inoculated to begin growth at a density of $5–10 \times 10^3$ cells/ml and allowed to grow to densities of approximately 2×10^5 cells/ml. Five 15-ml samples were taken, pipetted into 15-ml

centrifuge tubes and centrifuged individually at 3000 rpm ($1470 \times g$) in an International clinical centrifuge (International Equipment Co., Boston) for 1, 2, 3, 5, or 10 min, respectively. The supernatant was siphoned off and, in each case, the cells were resuspended in 1.3 ml basal Cramer-Myers medium (minus acetate and vitamins B_1 and B_{12}). Cell counts were made on each sample after centrifuging and resuspending in basal medium. One milliliter of cells from each centrifuging condition was added to single-arm Warburg respiration vessels. One milliliter of Cramer-Myers medium minus the vitamins was added to the sidearm of each vessel. The carbon source again was sodium acetate (0.061 *M*). Vitamins B_1 and B_{12} were not added to the respiration flasks since "carry over" amounts of these vitamins were considered sufficient for the duration of the respiration experiments (3, 4). The substrate was tipped in after an equilibration time of 20 min, and readings were taken at 10-min intervals up to 80 min. Respiration rates, i.e., μl $O_2/10^6$ cells/hr, were determined by the direct method of Warburg (1) at 26.5°. These values may be converted to μl O_2/mg dry weight/hr by dividing by 1.06, the dry weight in milligrams of a million *Euglena* cells grown to a density of $1\text{–}2 \times 10^5$ cells/ml of growth medium (5). Both ways of expressing the oxygen uptake of protozoa have been used (6, 7).

In another series of experiments, two 15-ml samples were taken: one was centrifuged for 10 min at 2000 rpm in an Electrifuge (Labline, Inc., Chicago), and the other, as already described, in a clinical centrifuge. The supernatant was siphoned off, and the cells in each case were resuspended in 15 ml of basal medium and centrifuged again for 10 min. The process was repeated again and resulted in a total centrifuging time of 30 min. All other conditions were as outlined above. All experiments were performed at least three times.

RESULTS

Figures 1 and 2 and Table 1 give the results of these experiments. When centrifuged for only 1 min, *Euglena* respired at a rate of 54.1 μl $O_2/10^6$ cells/hr (Fig. 1). When centrifuged for 10 min, *Euglena* respired at a rate of 42.4 μl $O_2/10^6$ cells/hr, a rate which is only 78% of the rate obtained with 1 min of centrifuging. Intermediate centrifuging times resulted in intermediate respiration rates for *Euglena*. In these experiments, the respiration rate in each case was linear throughout the time of measurement (80 min). Figure 2 shows the result of a 2-min centrifuging experiment. In contrast, when the cells were centrifuged for a total of 30 min, two distinct respiration rates for *Euglena* were observed during the time of measurement (Fig. 2). In this latter case,

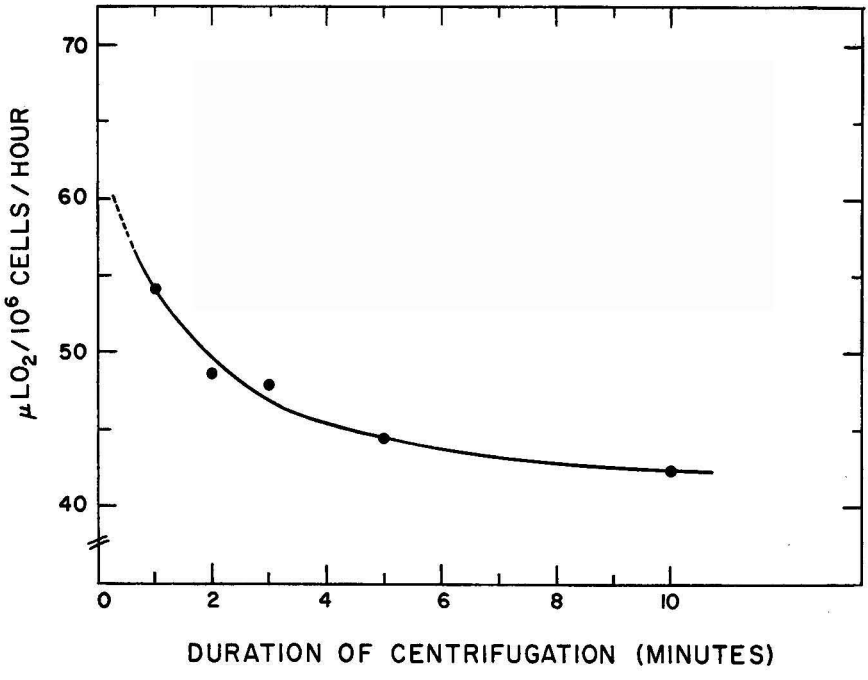

FIG. 1. The effect of length of centrifuging on the oxygen uptake of *Euglena*.

Euglena respired at a rate of only 22–23 μl $O_2/10^6$ cells/hr (Fig. 2 and Table 1) for the first 30 min. After this time, there was an increase in the oxygen uptake of these cells to 36.6 μl $O_2/10^6$ cells/hr in the case

TABLE 1

EFFECT OF PROLONGED CENTRIFUGING[a] ON O_2 UPTAKE[b] IN *Euglena*

rpm	μl $O_2/10^6$ cells/hr	
	0–30 min[c]	30–60 min[c]
2000[d]	23.2	41.1
3000[e]	21.7	36.6

[a] 30 min.
[b] Gas phase, air; temp., 26.5°C; pH, 6.8; equilibration, 20 min.
[c] Zero minutes represents the time of tipping-in of substrate.
[d] In Electrifuge.
[e] In clinical centrifuge.

of those centrifuged at 3000 rpm and to 41.1 μl $O_2/10^6$ cells/hr in the case of those centrifuged at 2000 rpm.

Routine microscopic examination showed the cells to be viable at the conclusion of all respiration experiments reported herein.

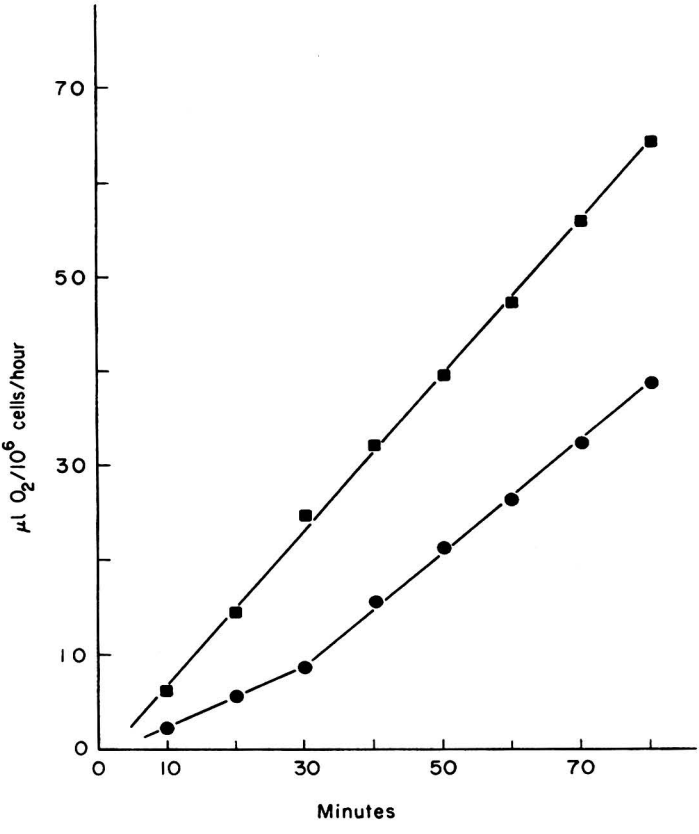

FIG. 2. Respiration of *Euglena* centrifuged for 2 min (■——■) and for 30 min (●——●). Zero time represents the time of tipping-in of substrate.

DISCUSSION

Varying the length of centrifuging time affects the respiration rate of *Euglena* cells. The longer the cells are centrifuged prior to introduction into respiration vessels, the lower is their respiration rate, at least under conditions reported in this paper. Cells centrifuged for a total of 30 min respired initially at a rate only 40% of the rate of cells centrifuged for only 1 min, i.e., 21.7 μl $O_2/10^6$ cells/hr as compared to 54.1 μl $O_2/10^6$ cells/hr. However, after about 1 hr (including an equilibration time of 20 min), these "low respiring" cells sharply increased their oxygen uptake (Table 1). The data thus indicate that both length of centrifuging and length of measurements are important in determining the oxygen uptake of cells.

The present results do not seem explainable on the basis of increased

temperature effects on metabolic activity as a result of centrifugation. The temperature of the supernatant fluid, checked before and after centrifugation, rose only 1.5° over ambient room temperature (even after 10 min of centrifugation) in the clinical centrifuge. The temperature of the supernatant did not change in the larger Electrifuge. Also, any exposure of the cells to a small temperature change was of short duration. Immediately after centrifugation, the cells were resuspended in fresh, room-temperature basal medium in all cases. No sample of cells was centrifuged for more than 10 min at a time (see *Methods*).

Further, the present results cannot be explained on the basis of a gradual decrease in enzymic activity with time. *Euglena* has a generation time of about 20 hr, a time long compared to the duration of the present respiration measurements (8). In addition, it has recently been shown that the endogenous respiratory rate of nonphotosynthetic *Euglena* can be maintained for at least 26 hr during prolonged respiration measurements (9).

Attention to Fig. 1 reveals that the "normal" respiration rate of noncentrifuged cells would be higher than the values reported for centrifuged cells usually employed in respiration experiments. Extrapolation of the curve in Fig. 1 to zero centrifuging time indicates a value of approximately 63 μl $O_2/10^6$ cells/hr. To check this latter point, further experimentation with highly sensitive respiration methods such as the polarographic technique of Davies and Brink (10) would be useful. An oxygen uptake of approximately 23 μl $O_2/10^6$ cells/hr for centrifuged *Euglena* cells has been reported previously (7). This value correlates only with the initial respiration rate of cells centrifuged for 30 min in the present experiments. As a whole, the present data show that a lack of effect of centrifugation on the respiration of cells must not be assumed in the course of manometry experiments.

SUMMARY

The respiration rate of the chlorotic, free-living flagellate protozoan, *Euglena gracilis* var. *bacillaris*, depends, at least in part, upon the amount of centrifuging the cells undergo before the measurements are made. Centrifugation is necessary to concentrate these and other cells to densities sufficient to measure oxygen uptake in a Warburg-type respirometer. The longer the cells are centrifuged prior to introduction into respiration vessels, the lower is their respiration rate. Cells centrifuged for a total of 30 min initially respire at a rate only 40% of the rate of cells centrifuged for only 1 min. The data indicate that both length of centrifuging and length of measurements are important in determining the oxygen uptake of cells.

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Determination of Dehydroepiandrosterone and Total Neutral 17-Ketosteroids in Human Plasma[1]

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Several methods have been described for the determination of individual and total neutral 17-ketosteroids (17-KS) in human plasma (1–10). Commonly, the number of manipulations and time taken for analysis preclude their general use in the clinical laboratory.

The purpose of this paper is to describe a method for the separate determination of androst-5-ene-3β-ol-17-one (dehydroepiandrosterone, DHEA) and other neutral 17-ketosteroids in plasma. After purification by the method described by Kornel (11), the extract is submitted to solvolysis (12) and acid hydrolysis for cleavage of steroid conjugates. The plasma extract is then further purified and divided into two portions, one for the determination of DHEA by an ethanol–sulfuric acid reaction (13), and the other for the estimation of total 17-ketosteroids by the Zimmermann reaction (14).

MATERIALS

Chemicals, unless specified, are reagent grade and solvents are distilled freshly before use.

Carbon tetrachloride: (CCl_4).

Methanol (MeOH): distilled over 2,4-dinitrophenylhydrazine and redistilled twice.

Acetate buffer, 2.5 *M*, pH 4.3, prepared with 70% aqueous methanol by mixing 70 ml absolute MeOH, 29.2 ml of 2.5 *M* acetic acid, and 0.8 ml of 2.5 *M* sodium acetate.

Petroleum ether (b.p. 60–80°) redistilled, after washing three times

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with concentrated sulfuric acid on separate days, and then with 5% aqueous sodium carbonate and water.

Barium acetate: 40% (w/v) aqueous solution.

Ethanol (EtOH): U.S.P.

Sulfuric acid, 50%.

Sulfuric acid, 5%.

Ethyl acetate (EtOac): redistilled from anhydrous potassium carbonate.

Sodium hydroxide, 5 *N*.

Methylene chloride ($MeCl_2$): purified by running 10–12 l down silica gel (15), storing in the cold, and distilling over anhydrous K_2CO_3 immediately before use.

Sodium hydroxide, 0.1 *N*.

EtOH for color reactions: U.S.P. distilled from silver oxide.

Sulfuric acid–EtOH reagent: one part EtOH, two parts concentrated sulfuric acid, prepared immediately before use.

m-Dinitrobenzene: purified according to the method of Sheath (16); 1% EtOH solution, prepared freshly each day.

Potassium hydroxide in EtOH, 2.5 *N*. This is prepared according to the method of Wilson and Carter (17).

Standard steroid solution: sodium DHEA sulfate (Schering):[4] 15 μg/ml in absolute MeOH.

METHODS

Preparation of Plasma Extracts

Ten milliliters of plasma is diluted with 20 ml absolute MeOH and partitioned with 30 ml CCl_4. After centrifugation for 15–20 min at $750 \times g$, 20 ml of the supernatant methanolic layer is transferred to 80-ml centrifuge tubes. Usually, the protein precipitate forms a compact layer so that the upper phase is easily decanted. To the aqueous MeOH in the centrifuge tube, 5 ml of 2.5 *M* acetate buffer is added in order to adjust the pH to between 4 and 5, as measured by pH paper. The sample is then extracted once with an equal volume of petroleum ether, the petroleum ether layer being removed and discarded following short centrifugation of the mixture.

To the samples, 0.5 ml. of 40% barium acetate and 0.4 vol of absolute EtOH are added. After 1 hr at 5°C, the precipitate formed is removed by centrifugation and decantation. EtOH and MeOH are

[4] We are indebted to Professor Dr. K. Junkmann, Schering AG, Berlin, Germany, for his generous gifts of these steroids.

evaporated under air on a water bath at 45–50°C. About 8 ml water remains.

The addition of 0.9 ml of 50% sulfuric acid to the aqueous extract results in precipitation of $BaSO_4$, which is removed by centrifugation. The precipitate is washed with 5 ml of 5% sulfuric acid and centrifuged, and the acid wash is combined with the original supernatant. The pH of the acid solution should be less than 1 as measured by pH paper. This solution is now extracted twice with equal volumes of EtOAc and centrifuged, and the combined EtOAc extracts are solvolyzed for 15–18 hr (overnight) in a water bath at 38–40°C (12). The remaining aqueous-acid phase is submitted to hydrolysis by heating in a boiling water bath for 20 min. The hydrolyzate is cooled and extracted once with an equal volume of EtOAc, the extract being combined with the EtOAc layer from solvolysis. The combined extracts are washed once with 7–8 ml of 5 *N* NaOH and twice with 5 ml water. After evaporation of the organic solvent under nitrogen the dry residue is dissolved by the addition of 10 ml water. The aqueous phase is extracted with 40 ml $MeCl_2$; the $MeCl_2$ extract is then washed once with 2 ml of 0.1 *N* NaOH and then with 5 ml water. After each extraction, the supernatant is removed by aspiration, the sample being centrifuged for about 2 min on the last occasion. One quarter of the $MeCl_2$ (10 ml) is used for estimation of DHEA while one half is used for assay of total 17-ketosteroids. Both samples are evaporated to dryness under nitrogen.

Blanks and Standards

The final colors of plasma extracts and standards are read against an extract of a water blank taken through the entire method. The plasma level of DHEA and 17-ketosteroids is estimated by comparison with the color developed by authentic DHEA sulfate that has been submitted to the same procedure as the plasma samples.

Color Reactions

1. *DHEA.* Depending on the anticipated level of DHEA in the plasma extract, the residue is dissolved in 0.5–1.0 ml of freshly prepared EtOH–sulfuric acid color reagent. After mixing and allowing the sample to stand for approximately 5 min at room temperature, the absorbance of the chromogen is read in a spectrophotometer at 380, 405, and 430 mμ. Maximum absorbance at 405 mμ is corrected by a formula similar to that of Allen (18) as modified by Brown (19) since the absorption curves of extracts from water or 5% aqueous serum albumin in this reaction follow a straight line between 380 and 430 mμ.

2. *Total 17-KS.* To the dry residue of the plasma extract 0.2 ml of 1% *m*-dinitrobenzene in 95% EtOH and 0.1 ml of 2.5 *N* KOH in EtOH are added. After mixing, the samples are incubated in an ice bath for 3 hr in the dark. Thereafter they are diluted with 0.3 ml of 85% aqueous EtOH and read at 450, 520, and 590 mμ. The correction of maximum absorbance at 520 mμ seems justified in view of the fact that extracts from 5% serum albumin give rise to absorption curves which between 450 and 590 mμ are practically linear.

Calculation of Plasma Values

DHEA (μg/100 ml plasma: as free DHEA) =

$$\frac{\text{Ac (sample)}}{\text{Ac (standard)}} \times \mu\text{g DHEA } SO_4 \text{ (standard)} \times 0.74 \times \frac{100}{10}$$

Total Zimmermann Chromogens (μg/100 ml plasma: as free DHEA) =

$$\frac{\text{Ac (sample)}}{\text{Ac (standard)}} \times \mu\text{g DHEA } SO_4 \text{ (standard)} \times 0.74 \times \frac{100}{10}$$

where Ac = corrected absorbance,

0.74 = factor for conversion to μg/100 ml free steroid based on molecular weights of free DHEA and sodium DHEA sulfate.

RESULTS

Hydrolysis of Steroid Conjugates

The effect of hot acid hydrolysis on free 17-KS as well as on 17-KS sulfates are recorded in Table 1. While the recovery of androsterone or DHEA as measured by the Zimmermann reaction was adequate, irregular losses of DHEA were observed as measured by the EtOH–sulfuric acid reaction. Following hot acid hydrolysis of 400 μg DHEA and subsequent paper chromatography of the extracted 17-KS, less than 60% of the original DHEA could be recovered as such, based on R_f value and Zimmermann and sulfuric acid–EtOH reactions.

Solvolysis

Experiments with solvent systems most favorable for solvolysis indicated that of organic solvents such as ether, EtOAc, and mixtures of both, and of aqueous solutions such as 5% sulfuric acid and 3 *M* NaCl and sulfuric acid (pH 1), the combination of 5% sulfuric acid and EtOAc gave the highest recoveries of 17-KS. In all experiments 15–45 μg of steroid conjugates were used (Table 2). Recoveries of DHEA or androsterone by this method exceeded 85%, using the EtOH–sulfuric

TABLE 1
RECOVERIES OF ANDROSTERONE AND DHEA AFTER ACID HYDROLYSIS

Conditions of experiment	No. of expts.	Per cent recovery: By Zimmermann reaction, Mean	By Zimmermann reaction, Range	By sulfuric acid–EtOH reaction, Mean	By sulfuric acid–EtOH reaction, Range
DHEA: free steroid					
Recovery from water	6	101.7	95–108	67.7	56–81
DHEA: free steroid					
Recovery from artificial plasma	6	77.4	61–96	61.3	50–75
DHEA sulfate					
Recovery from water	6	73.2	66–85	62.8	49.72
DHEA sulfate					
Recovery from artificial plasma	6	70.1	64–76	59.8	44.69
DHEA phosphate					
Recovery from water	4	84.2	78–88	84.0	58–94
Androsterone sulfate					
Recovery from water	8	88.1	72–105	—	—

[a] In each experiment between 15 and 45 μg steroid was used. The acid concentration was 10%; time of boiling varied from 15 to 20 min.

TABLE 2
RECOVERIES OF STEROID CONJUGATES USING THE SOLVOLYTIC SYSTEM: 5% H_2SO_4–EtOAc
(Incubation at 38–41°C for 15–18 hr)

Steroid	No. of expts.	Per cent recovery: Zimmermann, Mean	Zimmermann, Range	EtOH–H_2SO_4, Mean	EtOH–H_2SO_4, Range
DHEA sulfate	15	89.3	84–104	88.6	82–106
DHEA phosphate	9	4.2	0–14	3.8	0–13
Androsterone sulfate	11	90.3	75–100	—	—

acid and/or the Zimmermann reactions for quantitation. Apparently, synthetic DHEA phosphate is not cleaved under conditions optimal for solvolysis of 17-KS sulfates.

Recoveries

Results of recovery experiments are listed in Table 3. In these experiments 12.5–50 μg sodium DHEA sulfate or sodium androsterone

TABLE 3
RECOVERIES OF DHEA AND ANDROSTERONE SULFATES WHEN TAKEN THROUGH THE PROCEDURE FOR PLASMA ANDROGENS

Recovery from:	Steroid added: (% recovery)		Number of experiments
	DHEA sulfate	Androsterone sulfate	
Water	61.8–85.1 (69.5)[a]	68.2–88.7 (74.2)[a]	27 (DHEA) 20 (Androsterone)
Plasma	66.4–81.2 (70.1)[a]	69.1–86.5 (73.8)[a]	8 (DHEA) 6 (Androsterone)

[a] Mean values.

sulfate were added to water or plasma and subjected to the method described. Although recoveries in excess of 85% were sometimes obtained, mean recoveries ranged from 70 to 74% (Table 3).

Plasma Values

Individual plasma levels of DHEA and total Zimmermann chromogens for 26 normal males and 18 normal female subjects are given in Table 4.

TABLE 4
PLASMA LEVELS OF DHEA AND TOTAL ZIMMERMANN CHROMOGENS (μg/100 ML PLASMA) AS DETERMINED BY THE METHOD CURRENTLY DESCRIBED

Age	Total 17-KS	DHEA
	Normal males	
20	249	163
22	222	202
23	252	234
24	130	71
25	325	230
25	203	110
25	116	110
28	193	165
29	133	70
29	210	113
30	120	85
32	195	153
32	215	160
34	95	54
34	185	148
34	294	253
36	135	109
39	207	200
41	220	179

TABLE 4 (*continued*)

Age	Total 17-KS	DHEA
42	174	118
42	172	63
42	250	155
47	164	95
57	102	69
61	103	68
73	43	20
Mean values	181	130
	Normal females	
19	242	197
20	293	184
24	118	111
28	115	99
29	151	125
38	127	99
38	151	95
38	126	103
39	114	103
47	179	155
47	191	161
50	75	67
54	80	49
54	67	39
57	84	57
61	62	38
67	59	37
75	42	26
Mean values	126	97

In all but two persons blood was drawn after breakfast; cells and plasma were separated immediately, and the plasma was stored at −15°C until processed. Typical absorption curves obtained from plasma extracts of two normal subjects and a patient with Addison's disease are shown in Fig. 1.

Precision

Results of replicate analyses on five different plasma pools are listed in Table 5. Applying analysis of variance, the standard deviation for Zimmermann chromogens was found to be ±7.1 μg/100 ml and for the EtOH–sulfuric acid reaction ±7.3 μg/100 ml. However, it remains to be seen whether similar precision exists at all levels of plasma values.

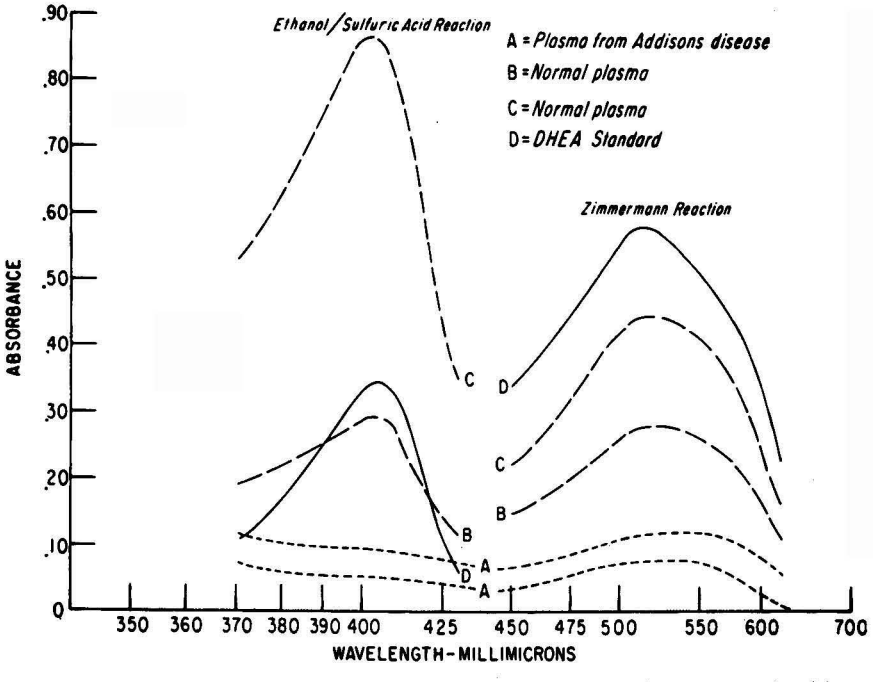

FIG. 1. Absorption curves obtained from plasma extracts of two normal subjects and duplicate samples from a patient with Addison's disease.

Additional Release of 17-KS by Acid Hydrolysis

In several experiments on pooled plasma, levels of 17-KS as determined after combined use of solvolysis and hot acid hydrolysis were compared with values obtained after solvolysis only. From Table 6 it can be seen that combined use of solvolysis and hydrolysis gave consistently higher values than mere solvolysis. This may be due to the presence of more stable conjugates.

Paper Chromatography

In order to determine the composition of total 17-KS measured by this method, plasma extracts were submitted to paper chromatography for 28 hr in the solvent system propylene glycol–methylcyclohexane (20). Zimmermann-positive spots were eluted and quantitatively assayed for 17-KS. Besides DHEA and androsterone two additional 17-KS were detected, one of which exhibited an R_f value similar to that of etiocholan-3α-ol-17-one. The second unidentified 17-KS was more polar

TABLE 5
REPLICATION OF PLASMA SAMPLES

Plasma sample	Zimmermann reaction		Ethanol–sulfuric acid reaction (DHEA)	
	Corrected absorbance	μg/100 ml plasma	Corrected absorbance	μg/100 ml plasma
CI-0811	0.320	118	0.165	55
	0.301	116	0.155	52
CJ-7283	0.238	96	0.202	69
	0.225	91	0.193	66
	0.223	90	0.246	85
CM-0112	0.334	132	0.324	105
	0.322	127	0.277	90
	0.318	125	(lost)	—
	0.350	138	0.333	108
	0.374	147	0.281	92
	0.327	129	0.309	101
CP-1316	0.286	132	0.172	64
	0.267	124	0.161	60
B.G.	0.456	202	0.314	98
	0.488	217	0.329	103

TABLE 6
PLASMA LEVELS OF TOTAL 17-KETOSTEROIDS FOLLOWING CLEAVAGE BY SOLVOLYSIS (ALONE) AND SOLVOLYSIS AND ACID HYDROLYSIS

Plasma Sample	Total 17-ketosteroids (μg/100 ml plasma)	
	Solvolysis alone	Solvolysis and acid hydrolysis
CK 1 215	135	152
CM 3235	91	111
CO 7174	163	176
CJ 0508	71	106
PP 0561	169	210

than testosterone. Because it was close to the origin, quantitation of this fraction was only possible after a second paper chromatography for 30 hr. The results of these experiments are shown in Table 7.

TABLE 7
COMPARISON OF VALUES FOR DHEA AND OTHER 17-KETOSTEROIDS BETWEEN CURRENT METHOD AND PAPER CHROMATOGRAPHIC SEPARATION OF DIFFERENT STEROIDS

Sample	Vol. of plasma (ml)	Current method (μg/100 ml)		Chromatography (μg/100 ml)			
		Total	DHEA	DHEA	Androsterone	Etiocholanolone	Polar fraction
G.P.	20	176	96	84	31	—	—
S.W.	20	121	78	68	24	8	—
M.K.	150	198	104	91	31	19	*ca.* 27
B.G.	200	210	100	90	60	41	*ca.* 12

Comparison of Different Methods used for Determination of Plasma 17-KS

Table 8 contains values for plasma 17-KS as measured by more recent methods. Plasma levels of DHEA and total 17-KS obtained by the present method seem to be higher than those previously reported.

DISCUSSION

In the method described, the separate determination of DHEA and total Zimmermann chromogens has been made possible by the combined use of procedures that have only recently been reported (11–13). Estimation of DHEA as a Δ^5-3β-hydroxysteroid by the ethanol–sulfuric acid reaction was described originally by Oertel and Eik-Nes (13). Concerning the specificity of this reaction, it was shown that a number of steroids possibly present in the final extract of plasma neither possessed absorption maxima in the 400–410 mμ region nor, when mixed with DHEA interfered with the reaction. It seems unlikely, therefore, that steroids other than DHEA or those metabolites possessing the Δ^5-3β-hydroxy configuration are measured by the EtOH–sulfuric acid reaction. It is, however, conceivable that pregn-5-ene-3β-ol-20-one or pregn-5-ene-3β,17α-diol-20-one and their metabolites (21) may contribute to the reaction, should either of these steroids be present in peripheral plasma in measurable quantities.

Unless mild methods of hydrolysis such as continuous extraction at pH 1 or solvolysis are employed, formation of artifacts from DHEA or its sulfuric acid ester cannot be excluded. The various transformations that may occur in Rings A and B of Δ^5-3β-substituted C_{19} steroids have been described in detail by Lieberman (22).

Since additional 17-KS are released by hot acid hydrolysis following solvolysis of the 17-KS sulfates, the presence of 17-KS conjugates other

TABLE 8
VALUES OBTAINED BY OTHER METHODS FOR PLASMA 17-KETOSTEROID ESTIMATIONS

Method	Year	Reference	μg/100 ml plasma		
			Total 17-KS	DHEA	Androsterone
Zimmermann	1946	(1)	1,500	—	—
Dumazert and Valensi	1952	(2)	870–970	—	—
Gardner	1953	(3)	25–130	—	—
Migeon and Plager	1954	(5, 6)	—	29–69 (40.5)[a]	3–37 (18.0)[a]
Clayton *et al.*	1955	(4)	171	117	12.3
Tamm *et al.*	1957	(7)	101.1	46.9	30.1
Oertel and Eik-Nes	1958	(8)	—	43–81 (57.5)[a]	—
Ceresa and Cravetto	1958	(9)	57–144 (106)[a]	—	—
Saier *et al.*	1959	(10)	19–191 (94.5)[a] f[b] (79.0)[a] m	4–116 (50.5[a] f (38.2)[a] m	—
This method	1960		42–325 (126.5)[a] f (181.0)[a] m	20–253 (97.0)[a] f (130.6)[a] m	—

[a] Mean values.
[b] f, females; m, males.

than sulfates is suggested. As shown by Migeon and Plager, incubation of plasma extracts with β-glucuronidase did not result in liberation of measurable amounts of 17-KS. Hence, hot acid hydrolysis was selected for cleavage of nonsolvolyzable 17-KS conjugates. In the method described by Saier *et al.* (10), omission of acid hydrolysis may result in underestimation of plasma 17-KS. Furthermore, their assumption that DHEA phosphate is cleaved by solvolysis does not seem justified in view of the data presented here.

In order to eliminate contaminating material which might interfere with the color reactions used, a rather extensive purification of plasma extracts must be performed. Of the various methods tried, such as

column chromatography on aluminum oxide or silica gel and solvent partitioning, only the procedure developed by Kornel (11) provided adequate initial purification. The nature of the spectral curves for the EtOH–sulfuric acid and the Zimmermann reactions (Fig. 1) indicates the degree of purification achieved. Unless similar curves are obtained, no valid conclusion may be drawn about DHEA or 17-KS concentrations in individual plasma samples. Should free 17-KS exist in normal peripheral plasma as has been suggested by Tamm *et al.* (7), these would be lost during partitioning between aqueous MeOH and CCl_4 as well as between buffered 70% MeOH and petroleum ether. Since up to 10% of total 17-KS present in peripheral plasma may be retained with the protein precipitate, a measured amount of the supernatant (two-thirds) is taken in the first step of the method; and the aqueous methanol layer from blank and standards is treated in a similar fashion.

The only two neutral 17-KS as yet identified in human peripheral plasma have been DHEA and androsterone (23, 24), although androst-4-ene-3,17-dione, androst-4-ene-11β-ol-3,17-dione, and DHEA have been detected in adrenal vein plasma (25, 26) and androst-4-ene-3,17-dione and testosterone in spermatic vein blood (27). There is, however, good reason to believe that other neutral 17-KS are present in human peripheral blood. In plasma samples from this series of investigations, two Zimmermann-positive spots were observed besides those of DHEA and androsterone. By such criteria as mobility of free, acetylated, and oxidized material in different solvent systems, and sulfuric acid spectra, the less polar material has been identified as etiocholane-3β-ol-17-one, while the more polar material appears to be ethiocholane-3α,11β-diol-17-one. The latter compound has already been tentatively characterized by Sandberg *et al.* (28). Chromatograms have not been examined for steroids less polar than androsterone.

Chromatography of the final plasma extracts has also served to verify results obtained by the ethanol-sulfuric acid and the Zimmermann reactions. There was reasonable agreement between the levels of DHEA found by the two procedures: The total amount of Zimmermann-positive material from the chromatogram approximates values of total Zimmermann chromogens obtained with final extracts not subjected to chromatography.

Although the number of individual plasma samples assayed in this series is relatively small, the method developed yields higher values for DHEA and total 17-KS than those methods previously reported. Excluded from consideration are the results of Zimmermann (1) and of Dumazert and Valensi (2) whose methods almost certainly yielded rather impure extracts and measured nonspecific chromogens. It is

important to analyze the possible reasons for the discrepancies between the high values for DHEA as determined by this method and those previously described—in particular the method of Oertel and Eik-Nes (8). It must be pointed out that there are a number of differences between this and other methods. Thus, acid hydrolysis in the presence of plasma protein may lead to inadequate cleavage of conjugates due to the buffering capacity of the proteins present. Further, the precipitation of proteins by ethanol leads to the formation of a bulky precipitate in which steroid conjugates are readily trapped.[5] In the method described by Oertel and Eik-Nes (8) the plasma proteins are precipitated with ethanol and the steroid conjugates hydrolyzed with acid in the presence of the protein precipitate. Ethanol is also used to extract steroid conjugates and precipitate plasma proteins in the method described by Migeon and Plager (6). It would appear that no method previously described takes into account steroid losses when calculating plasma values. If allowance is made for between 66 and 75% recovery of steroid conjugates when submitted to all the stages of the plasma estimation, plasma levels would be increased by a factor of from 33 to 50%. An additional factor that may increase the discrepancy is the absence of a chromatographic step which may further increase losses and falsely lower plasma values. It seems probable therefore that a combination of these factors serve to explain why DHEA and total 17-KS as determined by this method are significantly higher than have been previously reported. Precision of individual determinations can be determined from variability observed in replicates of plasma samples. In the present investigation insufficient plasma samples have been analyzed to permit any more rigid statistical appraisal of the precision of an individual estimate.

Although there is a difference between the mean levels of both DHEA and total 17-KS in this group of normal males and females, it is felt that the groups are too small for any comment with reference to the possible biological significance of this finding. Among the normal female group are a greater number of older persons than in the male group; and it would seem that with advancing age both levels tend to fall. Furthermore, the comparatively small number of individual plasma samples assayed does not warrant any comment on the surprisingly wide range of DHEA levels in normal subjects.

SUMMARY

A method for separate estimation of dehydroepiandrosterone (DHEA) and total 17-ketosteroids (17-KS) in human blood plasma is described.

[5] G. W. Oertel, unpublished observations.

The plasma is partly purified before hydrolysis of conjugated steroids. Cleavage of conjugates is achieved by combined use of solvolysis and acid hydrolysis. After additional purification the final extract is divided to permit estimation of DHEA by an ethanol–sulfuric acid reaction and of neutral 17-KS by the Zimmermann reaction.

Mean corrected levels of DHEA by this method are 130.6 μg/100 ml plasma for normal male subjects and 97.0 μg/100 ml for normal female subjects. The mean total neutral 17-KS levels for males is 181.0 μg/100 ml plasma and for females 126.5 μg/100 ml plasma, respectively.

Average recoveries of DHEA and androsterone sulfate are in excess of 70% when either of these steroid conjugates is taken through the entire method.

ADDENDUM

As a further verification of the plasma values for DHEA obtained by this method, approximately 40,000 cpm 7α-H^3-DHEA (specific activity 2.6 mc/mg) was added to two separate plasma pools that had been processed to the stage of solvolysis. Thereafter, aliquots were withdrawn from these samples and processed separately in triplicate, one series of samples being treated in the manner described in the method for plasma, the other being submitted to paper chromatography in a cyclohexane: methanol:water (100:100:25) system and run for 16 hr. The areas corresponding to DHEA were eluted and the paper eluates purified by partition between methylene chloride and water. Half of every sample was used for the determination of DHEA by the ethanol sulfuric acid reaction and the other half for the measurement of radioactivity, which was determined in a Packard Tri-Carb Liquid Scintillation spectrometer. The following values of specific activity were obtained:

Plasma pool	Specific activity (cpm/μg)	
	Chromatographed	Not chromatographed
1	907	905
	891	921
	888	911
2	1505	1350
	1336	1493
	1335	1356

Since the difference between the mean specific activities was found not to be significant ($t = 0.8$; $p > 0.3$), it is felt that this further validates the levels of plasma DHEA as determined by this method.

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ANALYTICAL BIOCHEMISTRY, 2, 263–266 (1961)

Paper Chromatography of Diethyl Dithioacetals of 2-Amino-2-deoxyhexoses, Uronic Acids, and Neutral Monosaccharides

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INTRODUCTION

The sugar dithioacetals(mercaptals) have usefully been applied for the identification and characterization of sugars (1) and of the sugar components of antibiotics (2). Mercaptolysis (simultaneous hydrolysis and mercaptal formation) has also been proved to be helpful for the investigation of the structure of polysaccharides (3).

Diethyl dithioacetals of 2-amino-2-deoxyhexoses (4–7) and of uronic acids (7, 8) have been reported, and these are of potential significance in relation to the structure of mucopolysaccharide (7).

The paper chromatography of diethyl dithioacetals of 2-amino-2-deoxyhexoses was reported by Whitehouse, Kent, and Pasternak (4) who employed iodine vapor for detecting the dithioacetals on the paper chromatogram. Weygand, Bestmann, and Ziemann (9) used as the spraying reagent bromine water followed by 0.1% solution of methyl orange which left a red spot on paper chromatogram.

The iodine method (10) is not specific for dithioacetals but is common for other compounds such as amino acids, steroids, glycerides, polysaccharides, and *N*-acetylamino sugars. The detection procedures therein used so far were tedious, and, therefore, it was desired to find a convenient procedure for the detection of sugar dithioacetals.

The present paper describes a procedure of detecting diethyl dithioacetals of 2-amino-2-deoxyhexoses, uronic acids, and neutral sugars by paper chromatography with the use of 1% solution of potassium permanganate as the spraying reagent which was found useful in our laboratory. After spraying, the paper chromatograms were washed with water to remove potassium permanganate whereupon brown spots were left, indicating the presence of the sugar dithioacetals. It should be noted that ethanethiol is estimated colorimetrically by *bis*(*p*-nitro-

phenyl) disulfide (11). It was found that this reagent did not react with sugar dithioacetals.

PROCEDURES

Preparation of Diethyl Dithioacetals of Sugars

Diethyl dithioacetals of 2-amino-2-deoxy-D-glucose(D-glucosamine) and 2-acetamido-2-deoxy-D-glucose(*N*-acetyl-D-glucosamine) were prepared according to the procedures reported by Wolfrom and Anno (5), Whitehouse, Kent, and Pasternak (4), and Hough and Taha (6). 2-Amino-2-deoxy-D-galactose(D-galactosamine) and 2-acetamido-2-deoxy-D-galactose(*N*-acetyl-D-galactosamine) diethyl dithioacetals were prepared by the procedures of Wolfrom and Onodera (7). Sodium D-glucuronate diethyl dithioacetal (7) and D-galacturonic acid lactone diethyl dithioacetal (8) were also prepared by the reported procedures. It is mentioned that mannuronic acid diethyl dithioacetal was prepared by mercaptolysis of alginic acid which was reported to produce on acid hydrolysis no appreciable amount of free mannuronic acid (12). Diethyl dithioacetals of D-glucose, D-galactose, D-mannose, L-arabinose, and L-rhamnose were prepared by the usual procedures (1, 13, 14).

Developing Solvents

A series of solvent systems was investigated as follows: (*a*) isopropyl alcohol–pyridine–acetic acid–water (8:8:1:4) (15); (*b*) ethanol–acetic acid–water (10:3:2) (16); (*c*) pyridine–ethyl acetate–water–acetic acid (5:5:3:1) (17, 18); (*d*) pyridine–*n*-butanol–water (5:1:3); (*e*) ethyl acetate–acetic acid–water (9:2:2) (16, 19); (*f*) ethyl acetate–acetic acid–water–pyridine (9:2:2:1); (*h*) *n*-butanol–pyridine–water (10:3:3) (19); (*i*) *n*-butanol–acetic acid–water (4:1:5) (20).

The paper chromatography was run with Toyoroshi No. 51 filter paper: (I) impregnated with borate buffer (pH 8, 0.2 *M* borax 5.5 parts–0.1 *N* HCl 4.5 parts) and air-dried; (II) untreated. All were run by ascending procedures at 25 ± 2°.

RESULTS

The results are listed in Table 1. As can be seen, with the solvent (*a*) and the treated papers (I) it was possible to separate uronic acid diethyl dithioacetals from *N*-acetylhexosamine diethyl dithioacetals. With untreated papers (II), hexosamine diethyl dithioacetals were separated from neutral sugar diethyl dithioacetals. It is noted that no tailing was observed in either case. With the solvents (*b*), (*c*), (*d*), and (*e*), poor separation and large mobilities were observed. The results in these cases

TABLE 1
R_F VALUES OF SUGAR DIETHYL DITHIOACETALS

Sugar diethyl dithioacetals	m.p. (°C)	R_f					
		(a)[a]		(f)[a]	(g)[a]	(h)[a]	(i)[a]
		I[b]	II[b]	II	II	I	II
D-Glucose	126–127	0.84	0.92	0.66	0.83	0.50	0.80
D-Galactose	141–142	0.87	0.93	0.66	0.83	0.50	0.82
D-Mannose	132–133	0.90	0.94	0.70	0.81	0.54	0.82
L-Arabinose	124–125	0.91	0.95	0.89	0.95	0.63	0.88
L-Rhamnose	137–138	0.91	0.95	0.92	0.98	0.85	0.86
D-Xylose	62–64	0.90	—	—	0.90	0.75	0.80
2-Amino-2-deoxy-D-glucose	105–107	0.86	0.87	0.26	0.70	0.67	0.68
2-Amino-2-deoxy-D-galactose	152–153	0.87	0.87	0.29	0.58	0.61	0.68
2-Acetamido-2-deoxy-D-glucose	129	0.94	0.93	0.76	0.79	0.61	0.85
2-Acetamido-2-deoxy-D-galactose	160–162	0.96	0.95	0.81	0.89	0.73	0.88
Sodium D-glucuronate	114–116	0.84	0.96	0.69	0.59	0.10	0.83
D-Galacturonic acid lactone	80–81	0.86	—	—	—	—	—
D-Mannuronic acid[c]	—	0.88	—	—	—	—	0.88

[a] (a), (f), (g), (h), and (i) represent the solvent systems which were found useful.
[b] I and II represent the pretreated filter paper and untreated filter paper, respectively.
[c] This compound was prepared by the mercaptolysis of alginic acid.

are not shown in the table. With solvents (f) and (g), the separation of diethyl dithioacetals on paper chromatograms was made effectively. The addition of butyl acetate or pyridine to the solvent systems was effective in preventing tailing. With the solvent (h) uronic acid diethyl dithioacetals were able to be separated from other diethyl dithioacetals, and the separation of 2-amino-2-deoxy-D-glucose diethyl dithioacetal from 2-amino-2-deoxy-D-galactose diethyl dithioacetal and of 2-acetamido-2-deoxy-D-glucose diethyl dithioacetal from 2-acetamido-2-deoxy-D-galactose diethyl dithioacetals was effected.

SUMMARY

A paper chromatographic procedure is described for detecting dithioacetals of sugars. A 1% aqueous solution of potassium permanganate was used as the spraying reagent, by which the sugar dithioacetals were detected as the brown spots on paper chromatogram. A series of developing solvents was investigated, and some of them were found useful for separating 2-amino-2-deoxy-D-glucose diethyl dithioacetal from 2-amino-2-deoxy-D-galactose diethyl dithioacetal, and both the diethyl dithioacetals from uronic acid diethyl dithioacetals.

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ANALYTICAL BIOCHEMISTRY, 2, 267–273 (1961)

Determination of C^{14} and H^3 in Biological Samples by Schöniger Combustion and Liquid Scintillation Techniques[1,2]

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The development of liquid scintillation over the past several years has led quite naturally to the application of this technique to the determination of radioactivity where isotopes are employed in biological systems. The method represents a particularly efficient and sensitive way of measuring the weak β particle of carbon-14 and hydrogen-3, two of the most important isotopes used in biological research. Inherent in liquid scintillation technique, however, are two difficulties which make simple application to biological samples impractical. First, organic solvents are employed for liquid scintillation counting. These are often immiscible with aqueous biological systems and are poor solvents for the polar constituents of biological samples. Second, liquid scintillation technique requires a relatively colorless sample so that the light resulting from the interaction of the β particle with the phosphor in the solution may reach the phototube. Biological samples are almost invariably colored and any absorption of light manifests itself as a quenching, resulting in lowered counting efficiency.

These difficulties have been dealt with in a number of ways. Where sufficient radioactivity is present, a very small sample may be dissolved directly in the phosphor, sometimes with the aid of a solvent such as methanol, ethanol, or dioxane. Passmann *et al.* (1) and Frederickson and Ono (2) have described the use of Hyamine hydroxide, a lipophilic quaternary nitrogen base, for the preparation of an organic solvent that will dissolve small samples from biological sources, including those which contain high concentration of protein. Herberg (3) has suggested the

[1] Presented in part before the Division of Biological Chemistry, American Chemical Society, 138th Meeting, New York, September, 1960.

[2] This work was performed in the Department of Pharmacological Research, Earl H. Dearborn, Head.

peroxide oxidation of sample for removal of color. Funt (4) in 1956 suggested counting suspensions in a thixotropic phosphor. Where possible, residues have been extracted from the sample into a phosphor-soluble solvent. Sealed tube combustion methods for the conversion of samples to water or carbon dioxide before the determination of the radioactivity have been described (5, 6). Although this latter technique can apparently be adapted to the routine determination of tritium and carbon-14 in biological samples, the practical limit of the sample size is about 25 mg dry weight. Several oxygen-train combustion procedures have been reported (7), and an oven has been developed in our laboratories that will simultaneously combust three samples of up to 2 gm dry weight (8).

Even with these developments, there was a need for a more rapid and simpler method for the determination of tritium and carbon-14 in biological samples. The lack of color in products resulting from the combustion of biological samples and the fact that these products could be made soluble in the solvents used in liquid scintillation spectrometry encouraged us to re-examine combustion techniques. Our attention turned to the oxygen-flask method of combustion as developed by Schöniger (9) in the determination of organic halogens and employed by Götte *et al.* (10) for the measurement of carbon-14 by conversion to barium carbonate.

Measurement of radioactivity in biological samples by a procedure combining combustion in a modified Schöniger flask and liquid scintillation counting is reported herein.

METHODS AND MATERIALS

Counting is done in a Packard Tri-Carb liquid scintillation spectrometer. The phosphor solution is made up of 4.0 gm 2,5-diphenyloxazole (PPO) and 50 mg 1,4-bis(5-phenyl-2-oxazolyl)benzene (POPOP) per liter of toluene. Hyamine hydroxide is used as a 1 *M* solution in methanol (Packard Instrument Co.)

Combustions are carried out in a heavy-walled 2-l filter flask which is affixed at the side arm with a stopcock and a 15–20-ml reservoir. A commercially available electric ignition head (F. and M. Scientific Co. Standard Head) is fitted snugly into a rubber stopper to adapt it to the flask and electrical jacks soldered to the contacts. Alternatively, 18-gage platinum leads are sealed in glass tubing and the tubing inserted into a two-hole stopper. A platinum ignition filament and basket similar to those described by Martin *et al.* (11) are welded to the leads (Fig. 1*A*). Both the filament and the basket are made from 23-gage platinum. A Cenco laboratory transformer (similar to Cenco Catalog No. 80295), delivering 8–9 v, is used to trigger the ignition.

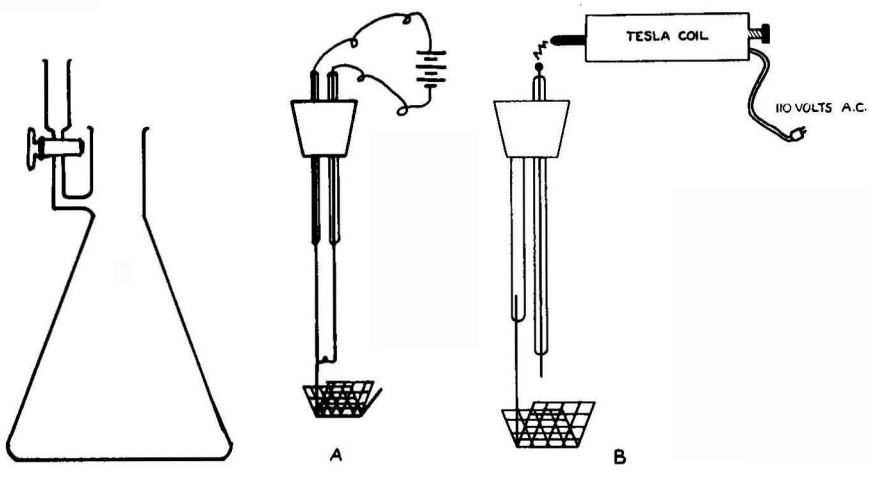

FIG. 1. Modified Schöniger flask and ignition heads.

Where a laboratory transformer is not available, ignition may be triggered by means of a high-frequency induction coil (Tesla coil) (12) using the ignition head shown in Fig. 1*B*. The platinum basket and electrodes are similar to those described, but a filament is not required between the electrodes. The sample must be placed in the path of the spark which is induced between the two electrodes, and the coil applied as shown in Fig. 1*B*.

For a sample holder, a bag is made from a 5-cm. piece of ¾-in. diameter Visking seamless cellulose sausage casing by folding one of the ends and sealing with a drop of Duco cement. The bag thus formed is opened by the use of a hemostat wrapped with adhesive tape, gum side up, on the ends. The open bag is then cut into the shape of a ladle with a 2-cm "cup" as a sample holder. The "handle" serves the double purpose of fuse for the ignition and tab by which to hang a wet sample for drying. A bag prepared in this manner weighs slightly less than 100 mg and is able to hold up to 2 ml of liquid sample. It permits efficient drying of the sample by evaporation through the dialysis tubing without loss of solid material. Furthermore, since it is made of cellulose, it aids the combustion.

For samples such as urine, blood, bile, plasma, and tissue homogenates, an amount of the liquid known to contain 200 mg or less of dry weight material is pipetted into the bag and permitted to air-dry overnight. More rapid drying may be accomplished with the aid of a heat lamp. Samples of intact tissues are handled by simply placing a weighed slice

in the prepared bag. Powders derived from lyophilized and crushed samples (e.g., feces, feed, and tissues) are weighed into the bag, 0.2 ml of 10% sucrose is added, followed by water such that the entire sample is wet. These samples are then treated as are the homogenates. The added sucrose serves both to bind the sample into a solid mass and to act as a flux for the combustion.

When dry, the sample, containing either carbon-14 or tritium, is placed in the platinum basket and the cellulose fuse is folded over the ignition filament. The flask is filled with oxygen and immediately stoppered tightly with the ignition head assembly containing the sample. Current is allowed to flow through the wire until the combustion becomes self-sustained.

For carbon-14 analysis, the flask is cooled either by a stream of cold water or by placing it in a pan of Dry Ice in order to reduce the pressure within. Ten milliliters of 1 *M* Hyamine hydroxide in methanol is then delivered into the side arm reservoir, and the stopcock is opened to permit the solution to go into the flask. When all of the Hyamine solution is in the flask, the stopcock is closed and the flask is permitted to stand at room temperature for ½ hr. A 3-ml aliquot of the solution is then removed for counting and added to 10 ml of the toluene–phosphor solution.

After a sample containing tritium is combusted, the flask is placed on a Dry Ice–chloroform–carbon tetrachloride bath so that only the bottom surface of the flask is in contact with the freezing mixture. The water vapor within is permitted to condense and freeze on this bottom surface. After ½ hr in the freezing mixture, a 20-ml aliquot of toluene–phosphor solution containing 20% ethanol is added and swirled around the bottom of the flask to dissolve the ice. The flask is then placed in ice water for 10–15 min to attain a constant temperature. A 15-ml aliquot is used for liquid scintillation counting.

TABLE 1

RECOVERY OF ADDED CARBON-14 ACTIVITY FROM BIOLOGICAL SAMPLES BY THE SCHÖNIGER COMBUSTION AND LIQUID SCINTILLATION TECHNIQUES

Sample	Amount	Per cent recovery
Feces	20 mg (dry)	99.7 ± 2.5
Feces	80 mg (dry)	98.3 ± 8.8
Liver	50 mg (wet)	89.2 ± 2.9
Liver	200 mg (wet)	96.1 ± 8.5
Blood	0.2 ml	97.7 ± 4.6
	Over-all	96.2 ± 6.0

RESULTS

Table 1 shows the results obtained on various samples to which were added known amounts of carbon-14 in the form of one of the tetracycline antibiotics. Each figure represents the mean of five determinations with the standard deviation for individual determinations. Different weights of sample were employed in order to assess the effect of this variable on the method. The over-all recovery was 96 ± 6%. Variation of sample size seemed to have little effect on recovery within the weight limits specified. It is to be recognized, however, that samples, including the cellophane bag, greater than 300 mg cannot be determined by this method because of incomplete combustion of sample in the size flask used. As reported elsewhere (8), our laboratory employs an oven of special design for combustion of samples up to 2 gm dry weight.

TABLE 2

RECOVERY OF ADDED TRITIUM ACTIVITY FROM BIOLOGICAL SAMPLES BY THE SCHÖNIGER COMBUSTION AND LIQUID SCINTILLATION TECHNIQUES

Sample	Amount	Per cent recovery
Liver	20 mg (dry)	99.1 ± 6.0
Liver	70 mg (dry)	98.3 ± 5.0
Urine	0.1 ml	95.2 ± 1.4
Urine	0.2 ml	93.4 ± 2.0
Paper	200 mg	95.9 ± 5.3
Blood	0.2 ml	92.6 ± 2.9
		Over-all 95.8 ± 5.4

Table 2 shows similar data as derived from tritium added to samples. The tritium added was also in the form of labeled tetracycline. The over-all recovery of added radioactivity was 96 ± 5%.

In the experiments discussed above, Hyamine hydroxide was used for the absorption of carbon dioxide. Since the cost of this material is high, several other absorbents were tested as potential replacements. Primene 81R, suggested by Oppermann *et al.* (13) did not quantitatively absorb carbon dioxide under our conditions. Ethanolamine has been used by the U. S. Navy for the absorption of carbon dioxide in submarines (14) and was reported by Jeffay and Alvarez (15) to be an effective trapping agent when dissolved in methyl cellosolve. We confirm the Jeffay and Alvarez results with ethanolamine, using 10 ml of the 20% ethanolamine solution suggested by these authors in place of the 10 ml Hyamine hydroxide.

SUMMARY

The Schöniger combustion technique has been adapted for the preparation of biological samples containing tritium and carbon-14 for liquid scintillation counting. A combustible sample holder made of dialysis tubing permits the efficient drying of biological tissues and homogenates prior to combustion. Following combustion, carbon dioxide is absorbed in a Hyamine hydroxide solution which is then dissolved in a toluene phosphor. Tritiated water is collected as ice which is dissolved in a toluene–ethanol phosphor. A recovery of $96 \pm 6\%$ was obtained for a series of 25 determinations of carbon-14 added to various biological samples. A similar experiment involving 30 determinations of added tritium gave results of $96 \pm 5\%$ recovery.

The method is simple and employs inexpensive and readily available apparatus (Fig. 2). In our experience 50 determinations can be carried out by one individual in an 8-hr period.

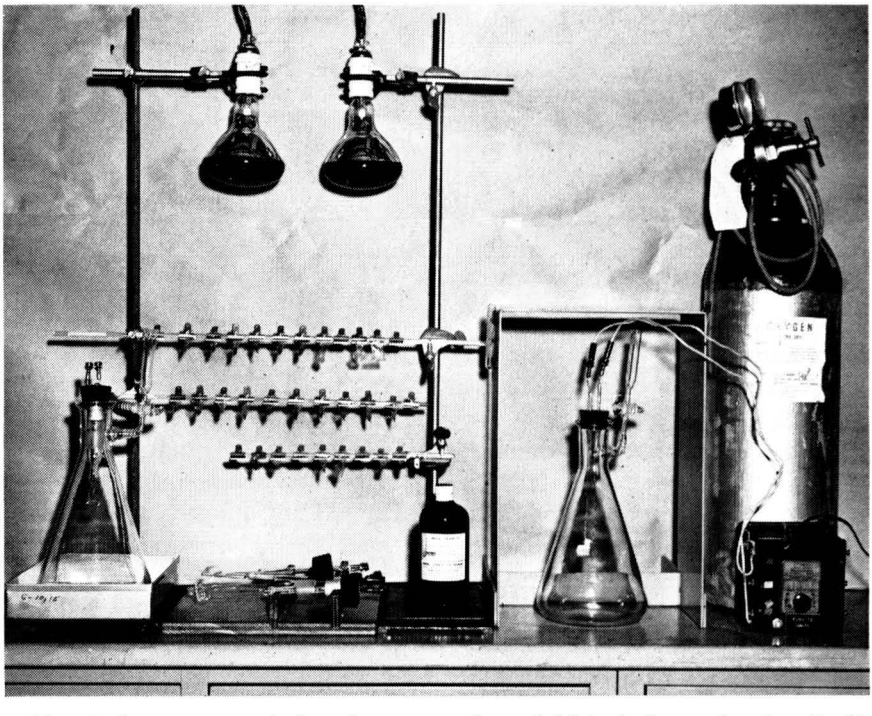

FIG. 2. Apparatus used for the preparation of biological samples for liquid scintillation counting.

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The Determination of Deuterium in Biological Fluids[1]

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A simple, spectrophotometric method for the determination of deuterium in micro amounts of biological fluids has been developed. On the basis of available data on the near-infrared absorption characteristics of light and heavy water (1–3), cells were constructed that make it possible to analyze aqueous mixtures ranging from 0 to 100 atom % deuterium. This analytical method has been an invaluable adjunct to recent studies of the chemical and biological consequences of the extensive replacement of hydrogen by deuterium (4–6). Previously published methods for the analysis of deuterium are many and varied, but are generally not applicable to the rapid, routine analysis of large numbers of small samples of biological fluids. Methods involving the use of the mass spectrometer or of density differences (7–9) require a high degree of sample purity and are not suited to rapid, routine measurements. Published spectrophotometric methods (1–3, 10, 11) also do not lend themselves readily to rapid analysis of micro amounts of urine or blood serum. Techniques utilizing nuclear resonance (12), measurements of freezing point (13), and effusiometry (14) have disadvantages with respect to sample volume, purity, range, and procedural complexity. Refractive index (15, 16) and thermal conductivity (17, 18) methods were also not applicable to our requirements. Consequently, a spectrophotometric method using the 1.44- and 1.66-μ absorption peaks of liquid water was developed. The method has the great advantage that a good estimate of the deuterium level is possible even in the presence of a variety of solutes.

EXPERIMENTAL

Construction and Loading of the Cells

The cells are 1 in. (2.54 cm) long and 0.875 in. (2.22 cm) in diameter. Those in use in our laboratory have been machined from gray or black

[1] Based on work performed under the auspices of the U. S. Atomic Energy Commission.

Teflon (polytetrafluoroethylene) or from Bakelite. As illustrated in Fig. 1, they are fitted with 0.375 in (9.52 mm) by 0.1 cm quartz windows

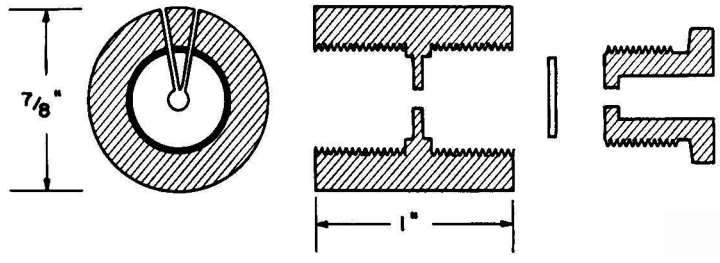

FIG. 1. Details of the construction of the cells for deuterium determinations From left to right we have an end view, side view, window, and screw plug. See text.

and with blackened aluminum screw plugs. The center hollow of all cells is 0.125 in. (3.175 mm) in diameter, but of two thicknesses so that optical paths of 0.08 cm (Type I cells) and 0.2 cm (Type II cells) were available. Two filling holes are drilled in each cell: 0.018 in. (0.457 mm) in diameter in the cells of short optical path; 0.035 in. (0.889 mm) in diameter in the cells of longer optical path.

Syringes with 27-gage needles with rounded points have been used successfully for filling the cells. If clean and free of grease, the cells generally fill effortlessly when the sample is introduced slowly at the bottom of the cell. If a small bubble persists, as may be the case with serum or plasma samples, the bubble can be tapped to the top and forced out by a sharp introduction of a little more sample. Cells of Type I can be filled with as little as 11–12 μ sample. Type II cells require 25–30 μ.

Spectrophotometric Analysis

The construction of the cells is such that they may be used directly with the Beckman DK recording spectrophotometer. Table 1 indicates the modus operandi used in making analysis over the range of 0–100

TABLE 1

A SUMMARY OF THE PROCEDURES USED IN THE ANALYSIS OF H_2O–D_2O MIXTURES

Range (atom % D)	Cell type	Reference liquid	Sensitivity setting	Sample standard deviation (atom % D)
10–70	I	CCl_4	0.30	0.6
0–20	I	H_2O	1.0	0.22
70–100	II	CCl_4	0.30	0.11

atom % D_2O (atom %D). With cells of Type I, concentrations are estimated from the difference in absorbancy between the HOH peak at 1.44 μ and the HOD peak at 1.66 μ. With H_2O as a reference liquid, the spectrophotometer is "100% adjusted" to read 0.50 on the absorbancy scale in order to be able to include the negative HOH absorption. In the 70–100 atom %D range, concentrations are estimated from the absorbancy of the HOD peak at 1.66 μ. Typical calibration curves are shown in Fig. 2.

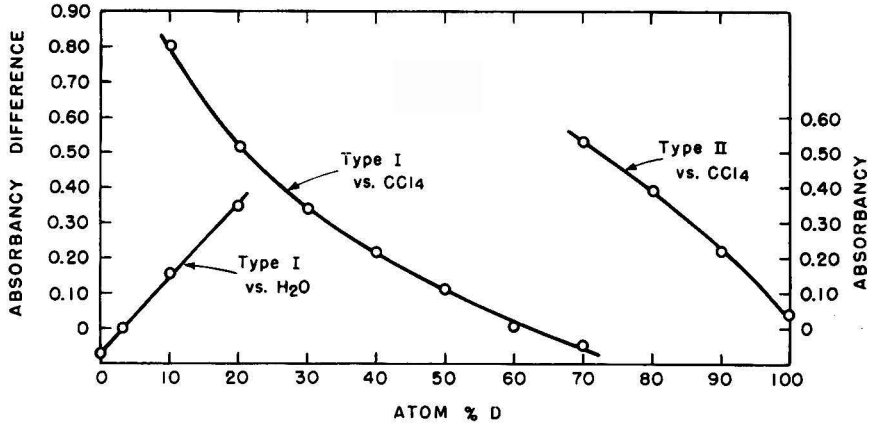

FIG. 2. Typical calibration curves obtained with cells and procedures outlined in Table 1.

The calibration curve obtained with a cell of Type I and with H_2O in the reference cell is linear to 20 atom %D, after which it curves and becomes poorly sensitive to changes in the D_2O level. This behavior is to be expected; because of the equilibrium point of the reaction HOH + DOD $\rightleftharpoons$ 2HOD, the sum of the increments in the HOH and HOD concentrations becomes less sensitive to changes in deuterium content. Calibration of Type I cells with CCl_4 in the reference cell gives a curved plot, particularly in the region of 10–20 atom %D. The sample standard deviation in this lower region is 0.9 atom %D, while at higher deuterium levels replicate analyses showed a sample standard deviation of 0.4 atom %D. Below 10 atom %D the peak at 1.66 μ becomes a progressively disappearing shoulder and above 70 atom %D the method is insensitive. For analyses in the 70–100 atom %D range, cells of Type II with CCl_4 as reference liquid give good results. Calibration curves show a moderate curvature.

RESULTS AND DISCUSSION

Cells of Type II have been found to be most useful in the study of the growth of various microorganisms in highly deuterated media. Nutrient media, intracellular water, and water of combustion are quickly and directly determined. After filling these cells it is desirable to analyze the contents without undue delay or to cover the filling holes with Apiezon "Q" wax. Samples in uncovered cells are diluted on standing by exchange with moisture in the air at the rate of about 0.5%/hr.

Type I cells make it possible to follow the deuterium hydrogen proportions in the aqueous and solid phases of tissues of animals such as the mouse, rat, and dog. Serum and clear urine samples may be analyzed directly. Turbid urine must be clarified by centrifugation or, if very dark in color, by adding activated charcoal and then centrifuging the sample. Cells of Type I may stand uncapped for at least 30 min after filling with no detectable isotopic dilution.

In order to examine the effect of such factors as the presence of solute or slight turbidity, the deuterium content of water distilled from urine and serum samples was determined and compared with the deuterium values obtained by direct analysis. The results of these analyses, presented in Table 2, indicate that direct analysis of urine and serum gives

TABLE 2
RESULTS OF DIRECT ANALYSIS COMPARED TO ANALYSIS OF DISTILLATE
Atom % D

Urine	Dist.	Serum	Dist.	30% D	Dist.
29.6	25.2	30.7	30.0	30.4	30.3
22.4	20.4	32.1	29.3	30.4	30.3
30.8	27.2	33.7	33.7	30.4	30.2
20.4	20.0	30.1	29.2		
13.0	13.9	17.6	14.9		
12.5	9.0	18.1	15.1		
26.6	19.6	19.9	18.1		
26.5	24.3	21.2	21.0		
27.8	27.3	31.3	29.7		
32.4	30.8	22.5	20.6		
32.3	30.1	19.0	16.6		

deuterium values that average 2.3 and 1.7 atom % higher in deuterium, respectively, then the urine and serum water distillates. Distillation and analysis of similar quantities of known mixtures of H_2O and D_2O indicated negligible isotopic dilution during manipulation.

These analytical methods have also proved useful in the analysis of the water of combustion of fully deuterated, purified compounds obtained by biosynthesis (19). It has been found possible to obtain quantitative yields of water of combustion from sugars and amino acids by combustion on a vacuum line with partially powdered CuO wire. A 20–40-mg sample of the organic compound is weighed into a quartz test tube, 5 in. (12.7 cm) long and fitted with a standard taper joint, and is then mixed with about 1 gm of CuO wire that has been ground to a mixture of powder and short wire. The reaction tube is then filled to a depth of 3.5–4 cm with CuO so prepared, fitted with a plug of glass wool, and attached to a vacuum line by means of an *h*-shaped holder. A small sample-receiving vessel is also attached to the holder. The upper part of the quartz tube is fitted with a polyethylene, asbestos-wrapped cup that is to be used as a condenser.

With the entire system evacuated, the plastic cup is filled with powdered solid carbon dioxide and the receiving vessel is immersed in liquid nitrogen. The top 1 cm of CuO is slowly heated to dull red heat with a hand torch, and the heating is gradually extended to the bottom of the tube. When the reaction is complete, the liquid nitrogen is removed from the receiving vessel, and the CO_2 and other volatile products of combustion are pumped off. The water is then collected in the receiving vessel and removed for analysis. Table 3 shows the results of a number of such analyses.

TABLE 3

COMBUSTION ANALYSES OF DEUTERIO HEXOSES AND DEUTERIO AMINO ACIDS

Substance	Weight of sample (mg)	Yield of water (% of calcd.)	% D calcd.[a]	% D found	% of calcd.
Glucose-d_7[b]	23.7	96	58.1	57.2	98.5
Mannose-d_7[b]	16.1	100	58.1	56.7	97.6
Glycine-d_2[c]	29.1	102	39.8	39.2	98.6
Glycine-d_5[c]	36	96	99.6	97.8	98.2
Aspartic acid-d_3[b]	36.5	91	42.9	42.3	98.7
Deuterated algae	77.9	—	99.6	99.3	99.7

[a] Assumed isotopic purity, 99.6 atom %.

[b] By biosynthesis.

[c] From Volk Radiochemical Company, Chicago, Ill.

Admittedly, this combustion method has rather limited application and unless the yield of water of combustion can be ascertained to be 100%, a method such as that of Jones and Mackenzie (20) must be used. The compounds analyzed have given good results, however, and the

method described here offers the advantages of speed, simplicity, and directness.

SUMMARY

Simple spectrophotometric methods for the analysis of deuterium in biological fluids are described. The methods cover the entire range from 0 to 100 atom % D_2O, and micro amounts of sample may be analyzed directly. Results obtained with urine, serum, distillate, and water of combustion samples are presented.

ACKNOWLEDGMENTS

We are indebted to Dr. H. Hyman for his help in the development of this work and to Mrs. Dolores Symon for her technical assistance.

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ANALYTICAL BIOCHEMISTRY, 2, 280–286 (1961)

SHORT COMMUNICATIONS

A Note on the Determination of Tryptophan

The method of Searcy (1) based on "a new color reaction for tryptophan or tryptophan-containing proteins" is not suitable for the determination of free tryptophan. It was recently reported that this method is merely a modification of the Adamkiewicz reaction and therefore it depends upon the glyoxylic acid content of the glacial acetic acid used in preparing the reagent (2).

Since satisfactory assays in the range of about 2–100 μg tryptophan are inferred (1), the method is apparently very sensitive for protein tryptophan. An attempt was made to use this method for the determination of free tryptophan in deproteinized blood plasma, which contains about 1 mg of free tryptophan/100 ml according to microbiological assays (3). Known amounts of tryptophan, dissolved in glacial acetic acid, gave very low color yields when tested in the expected concentration range. Initially it seemed probable that the lability of tryptophan in glacial acetic acid solution might account for this anomaly. However, such solutions were relatively stable for several days at 4°C. Two milliliters of a fresh solution of DL-tryptophan (0.5 mmole/l) heated for 15 min in a boiling water bath with 10 ml of 12% (w/v) *p*-toluenesulfonic acid in laboratory-grade glacial acetic acid, initially gave an optical density of 0.009 at 560 mμ; and after 4 days at 4°C, 0.005.

N-Acetyl-DL-tryptophan was expected to be more stable in acetic acid solution than tryptophan, and experiments with this derivative gave surprising results. The color yields for *N*-acetyl-DL-tryptophan and *N*-benzoyl-DL-tryptophan were relatively much greater than that for tryptophan itself, when compared on a molar basis. Thus at a concentration of 0.5 mmole/l, the following optical densities were obtained at 560 mμ: tryptophan 0.009; *N*-acetyl-DL-tryptophan, 0.167; *N*-benzoyl-DL-tryptophan, 0.590. A likely explanation for this phenomenon is that blocking the α-amino group of tryptophan, by acylation or by peptide-link formation as in a protein, may prevent loss of tryptophan during quantitative analysis by preventing some side reaction.

A practical outcome of these experiments is that colorimetric assays for tryptophan which rely upon such methods as the Adamkiewicz reaction may be expected to give very different results if protein hydrolyzates are compared with the protein before hydrolysis. Any acetylation procedure before or during the tryptophan determination may also produce erratic results.

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Received February 17, 1961

A Simple Procedure for the Preparation of Uridine Diphosphogalactose[1]

The galactose-negative mutant *Escherichia coli* C7M, isolated by Fukasawa and Nikaido (1), was shown to lyse when grown in the presence of galactose (1, 2). The genetic lesion produced in this bacterial strain was demonstrated (3, 4) to be involved in the synthesis of the enzyme uridine diphosphogalactose-4-epimerase, responsible for the interconversion of uridine diphosphogalactose (UDPGal) and uridine diphosphoglucose (UDPG). The addition of galactose to a logarithmically growing culture of C7M results in the accumulation of approximately 1 μmole UDPGal/gm wet weight cells (4). The addition of galactose to stationary phase cells does not induce subsequent lysis of the culture, yet these cells are capable of enzyme induction, and can accumulate as much as 20 μmoles uridine nucleotide/gm wet weight.

RESULTS

Strain C7M was grown in 1% tryptone + 0.01 *M* Mg^{++} broth at 37° with aeration. The growth of the culture was followed spectrophotometrically using a Beckman DU spectrophotometer. At an optical

[1] Contribution No. 322 of the McCollum-Pratt Institute, Johns Hopkins University.

density (at 650 mμ) of 1.0, essentially glucose-free Sigma grade galactose (Sigma Chem. Co., St. Louis, Mo.) was added to a final concentration of 0.01 *M*. The cells were allowed to remain in the presence of the sugar for approximately 90 min, after which time they were harvested by centrifugation and either stored as a frozen pellet, or extracted immediately.

To extract the uridine nucleotides, approximately 15–20 vol of 70% ethanol, heated to 70°, was used to suspend the cells. The hot ethanol suspension was then mechanically stirred at room temperature for 10–15 min. The sediment was removed by centrifugation and discarded. The

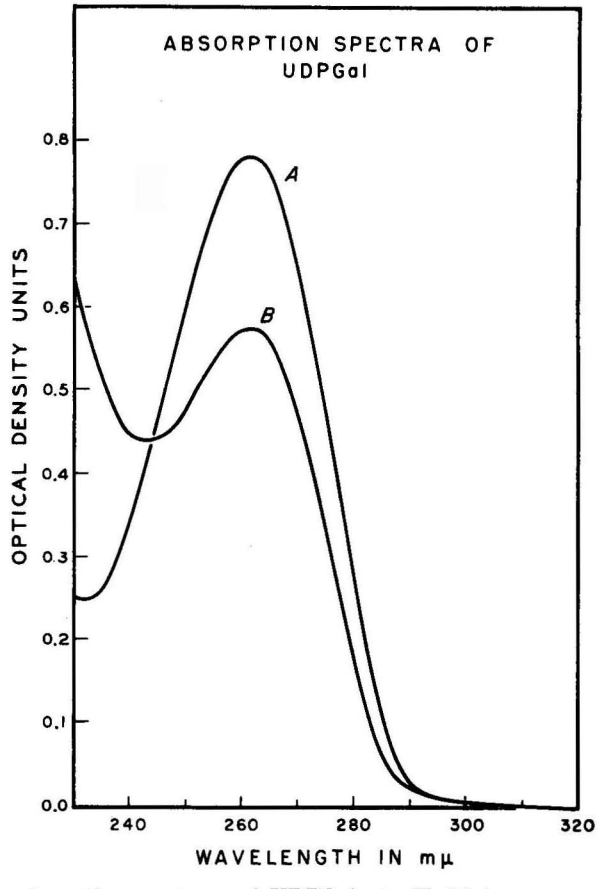

FIG. 1. The absorption spectrum of UDPGal at pH 5.5 is represented by curve *A*. Curve *B* represents the absorption spectrum of this material at pH 12. Both spectra were obtained by means of the Cary model 14 spectrophotometer.

approximate concentration of the uridine nucleotide present in the supernatant fluid was estimated from the absorption at 262 mμ using a molar extinction coefficient of 10,000 (5). The ethanol extract was then concentrated by lyophilization to approximately half its original volume.

Acid-washed Norit was added to the concentrated supernatant to a point where the liquid, after removal of the charcoal, appeared water clear (1 gm Norit/200 ml concentrated ethanol extract). The use of Norit in this case was for the removal of material other than the uridine nucleotides (the supernatant of this treatment retains 90% of the 262 absorbing material). Following the Norit treatment, the extract was again concentrated by lyophilization. The concentrated material was applied to Whatman 3MM paper and chromatographed overnight, in descending fashion, using the neutral ammonium acetate–ethanol solvent system of Paladini and Leloir (6). The band corresponding to UDPGal was located by its quenching effect when examined under a source of ultraviolet light. The band was excised from the paper and eluted with water. The eluate had the spectral properties of the uridine nucleotide and served as substrate for the enzyme uridine diphosphogalactose-4-epimerase. The spectrum of this material in water and dilute alkali is shown in Fig. 1.

A typical preparatory run is shown in Table 1. Here the cells from 10 l

TABLE 1

Fraction	Total vol. (ml)	Total μmoles 262 absorbing material
70% EtOH extract	500	190
Concn. 70% EtOH extract	200	180
Norit supernatant	200	160
Concn. Norit supernatant	7.5	130
Chromatographed concn. Norit supernatant		110[a]

[a] Equal or greater than 56 μmoles as determined enzymically.

culture, approximately 15 gm wet weight, served as the starting material. Since the stationary phase C7M culture does not lyse, the amount of UDPGal that can be prepared is dependent only upon the volume of culture grown.

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A Procedure for the Isolation of the Epithelial Brush Border Membrane of Hamster Small Intestine[1]

In the course of work on the mechanism of intestinal active transport of sugars, it became of interest to determine the locus of action of several hydrolytic enzymes of the small intestine. Experiments to be reported elsewhere show clearly that the enzymic hydrolyses of sucrose, maltose, and glucose 1-phosphate carried out by hamster small intestine take place inside the mucosal epithelial cells rather than on their surface or within the intestinal lumen. Some of the data which suggested that the responsible enzymes are not randomly distributed throughout the cell but are largely confined to its most superficial region led us to attempt the isolation of the brush border portion. Described below is a procedure by means of which the brush border portion of hamster intestinal epithelial cells may be preserved as a distinct particulate entity during the preparation of homogenates and recovered in large yield substantially free of other portions of the cell. Experiments with the material obtained by this procedure show that the enzymes, invertase and maltase, are predominantly if not exclusively localized within the brush border. Additional experiments are in progress to determine other possible enzymic components of this subcellular structure.

[1] Supported by grants from the National Science Foundation.

PROCEDURE

Adult golden hamsters of either sex were killed by a blow on the head. The small intestine was removed, everted over a metal probe, and washed in 0.15 *M* NaCl. Using the edge of a glass slide, the mucosa from a single animal was scraped into 100 ml of cold 0.005 *M* ethylenediaminetetraacetic acid (EDTA), pH 8.0, and homogenized in a Waring blendor at 15,000 rpm for approximately 30 sec. The homogenate was passed through fine bolting silk to remove coarse particles and was centrifuged at 1500 rpm for 10 min in an International PR-2 refrigerated centrifuge set at 4°. The supernatant liquid was aspirated and the small amount of sediment was resuspended in 0.005 *M* EDTA to the original volume. The cycle of centrifugation and washing was repeated several times, and aliquots of the resuspended sediment were taken for microscopic examination.

This procedure resulted in the complete disruption of the epithelial cells except for the brush borders which were easily recognizable under the phase microscope (Fig. 1). Electron micrographs of particles washed

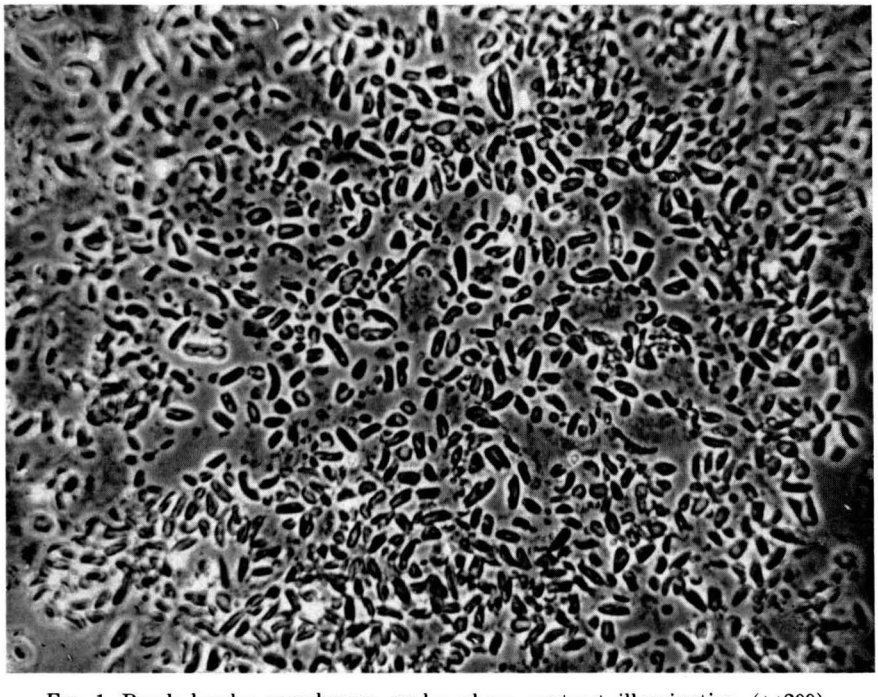

FIG. 1. Brush border membranes under phase contrast illumination (×200).

several times in EDTA showed good preservation of the ultrastructure of the brush borders, particularly of the microvilli. As seen by the electron microscope, these structures included varying but small amounts of subjacent cytoplasm which could not be completely removed by repeated washing.

Successful isolation of brush borders by this procedure appears to depend upon the relative resistance of these structures to both osmotic and mechanical forces. At higher salt concentrations, homogenates contained many intact cells and naked nuclei which sedimented with the brush borders. In the absence of EDTA, very few brush borders were obtained and these were fragmented and poorly preserved.

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Biological Approaches to Cancer Chemotherapy

A Symposium held at Louvain, June 1960, under the auspices of the UNESCO Cell Biology Programme with the assistance of the World Health Organisation

Edited by R. J. C. HARRIS, *Imperial Cancer Research Fund, London*

May 1961, 431 pp., illus., $14.00

J. F. DANIELLI, General Cell Theory and Drug Design

RUDOLPH ALBERT PETERS, Lethal Synthesis

R. T. WILLIAMS, Detoxication Mechanisms and the Design of Drugs

BORIS MAGASANIK, Feedback Mechanisms in Biological Synthesis

CHARLES HEIDELBERGER, Nucleic Acid Synthesis and Mechanism of Action of Flouropyrimidines

PAUL TALALAY, Enzymatic Interactions between Steroid Hormones and Pyridine Nucleotides

H. HOLTER, The Induction of Pinosytosis

HANS H. USSING, Ion Transport Mechanisms

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PIERRE DEMERSEMAN, Oxydation de Substrats Organiques par les Peroxydes Organiques

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F. BERGEL, J. A. STOCK *and* ROY WADE, Peptides and Macromolecules as Carriers of Cytotoxic Groups

L. F. LARIONOV, An Approach to the Creation of Antitumour Drugs with Diverse Action Spectra

W. JACOBSON, Biological Aspects of Chemotherapy with Folic Acid Antagonists

S. GARATTINI *and* V. PALMA, A Procedure for the Evaluation of Specific Effects of Antitumour Drugs

J. MAISIN, Protection and Restoration of Bone Marrow in Laboratory Animals Following Administration of Radiomimetic Drugs

GEORGE KLEIN, Population Changes and Drug Resistance in Tumours

P. A. GORER, The Isoantigens of Malignant Cells

L. A. ZILBER, An Immunological Approach to Tumour Growth Control

P. DE SOMER *and* A. PRINZIE, Modifications Antigeniques en Culture Tissulaire

JACOB FURTH *and* UNTAE KIM, Biological Foundation of Cancer Control by Hormones

O. MÜHLBOCK *and* R. VAN NIE, Hormone Dependence and Autonomy

H. LETTRE, Cytotoxic Hormones

FRANCOISE HAGUENAU, The Possible Role of Electron Microscopy in the Study of the Biological Aspects of Cancer Chemotherapy

ALBERT J. DALTON *and* MORRIS BELKIN, Electron Microscopic Studies on the Process of Vacuole Formation Induced by Plant Polysaccharides in Ascites Tumour Cells

S. E. LURIA, Relation of Viral Functions to Cancer Chemotherapy

R. J. C. HARRIS, Tolerance and Interference

ALICE E. MOORE, Carcinolytic Viruses

B. MCLOUGHLIN, The Connective Tissue Substrate

RIOJUN KINOSITA, Anticarcinogenesis

J. MAISIN *and* G. LAMBERT, Prophylaxis

AUTHOR INDEX.

CARCINOGENESIS

By I. HIEGER

Chester Beatty Research Institute, Royal Cancer Hospital, London

February 1961, 138 pp., illus., $5.00

The basic concept of carcinogenesis is discussed in a concise critical survey of the theoretical approaches to this complex problem which involves cytology, genetics, biochemistry, chemistry and physics. Experimental studies on chemical carcinogens are incorporated in the well documented analysis of the theories.

Although the theories are still inadequate, their re-statement serves to outline the guiding principles for achieving progress in solving the cancer problem.

CONTENTS:

Theories of Carcinogenesis. Immunological Theory of Carcinogenesis. Carcinogens. Carcinogenesis and Chemical Structure. Carcinogenesis as a Multi-Stage Process. Work of Earle, of Warburg, and of Cowdry.

REFERENCES — AUTHOR INDEX — SUBJECT INDEX.

ACADEMIC PRESS *London and New York*

HORMONES IN BLOOD

Edited by C. H. GRAY, *King's College Hospital Medical School, London*
and A. L. BACHARACH, *London*

August 1961, about 650 pp., approx. $19.00

Methods of hormone assay in blood and plasma are presented in a series of informative and fully documented chapters by scientists expert in the specific endocrine substances discussed. The book gives a critical survey of the different hormones, the methods of their determination in blood, and supplementary information on their presence in other body fluids. This material has been integrated with a summarization of the chemical contributions and physical properties of the hormones, their sources and biosynthesis, and their secretion and fate in the organism.

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By JACOB ROBBINS, J. E. RALL, *and* P. G. CONDLIFFE, *National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland*

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By V. J. O'DONNELL, *University of British Columbia and* J. R. K. PREEDY, *Emory University, Atlanta, Georgia*

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By A. M. BONGIOVANNI, *The Children's Hospital of Philadelphia and* J. DARREL SMITH, *The University of Mississippi*

Progesterone
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The Corticosteroids
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Retrospect and Prospect
By RAYMOND GREEN, *London*

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ANALYTICAL BIOCHEMISTRY 2, 287–291 (1961)

Determination of Micro Quantities of Rubber by a Modified Bromination Method

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Received January 30, 1961

INTRODUCTION

Rubber and gutta have been found to occur in many genera throughout the plant kingdom (1). In 1955 a report was made of the occurrence of rubber in microorganisms (8). With few exceptions the content of this hydrocarbon polymer in plants and microorganisms is exceedingly small, and molecular weights, where recorded, are found to be much lower than the average molecular weight of *Hevea braziliensis* rubber (5, 8, 10). Although several methods are available for determining macro amounts of rubber (4, 7, 9), no rapid, convenient method has been reported for micro or semimicro quantities. An investigation of isoprenoid compounds in the mushroom *Lactarius* has led to the development of such a method. Accurate analysis of as little as 200 μg of the polymer has been made possible by a modification of the gravimetric bromide precipitation methods of Willits *et al.* (9) and Gowans and Clark (4). Most of the substances which interfere in the described bromination procedure can be removed by successive extraction of samples with ethanol and acetone before benzene extraction. The method has not been standardized with materials other than crepe rubber. However, *cis*-polyisoprene with an average molecular weight as low as 41,000 was accurately determined by this procedure.

MATERIALS AND METHODS

Rubber Standards

Crepe rubber was milled several times through a two-roll Stewart-Bolling rubber press.[2] This treatment served to decrease the average

[1] A laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

[2] The mention of products does not imply endorsement or recommendation by the Department of Agriculture over other products of a similar nature not mentioned.

molecular weight (3). Several fractions were obtained by successive precipitation of the rubber from benzene solution with increasing amounts of methanol (6). The average molecular weight of each fraction was determined from intrinsic viscosity measurements according to Carter *et al.* (2). Each fraction was dried under nitrogen and redissolved in benzene. Three fractions representing average molecular weights of 41,000, 111,000, and 320,000 were redissolved and used as standard rubber solutions.

Brominating Solution

Two grams of C.P. iodine was dissolved in 100 ml carbon tetrachloride. The solution was filtered, and 5 ml of C.P. bromine was added.

Apparatus

Circles were cut from commercial, heavy-duty aluminum foil and stamped into light-weight weighing cups on a template shaped from softwood. Capacity, 7–10 ml; weight, 0.20–0.22 gm. Conically shaped, thick-walled centrifuge tubes with a capacity of 40 ml were used. Dissolve the sample to be analyzed in benzene and dilute to a known volume. Transfer a 5-ml aliquot containing no less than 200 μg rubber to a conically-shaped centrifuge tube. Add 1 ml chloroform and 0.2 ml of brominating solution and stir the contents of the tube thoroughly. Allow 10 min for the bromination reaction. Add 30 ml ethanol, stir, and place in a water bath at 2–5°C for 10 min. Centrifuge at 2000 rpm for 10 min in a swinging cup centrifuge and pour off supernatant. Allow the residual ethanol to evaporate until the rubber bromide is nearly dry. Quantitatively transfer the bromide to previously weighed foil cups, using benzene as a solvent. Dry the contents of the cup under a stream of air and reweigh. Calculate the rubber content of the sample aliquot by multiplying the weight of the rubber bromide by the factor 0.299. This value is the theoretical factor for the conversion of the weight of rubber bromide to weight of rubber.

RESULTS AND DISCUSSION

Dilutions of each of the three standard rubber fractions were made so that each solution contained approximately 200 μg/ml rubber. Aliquots of these solutions were used to run standard curves following the procedure outlined. Figure 1 shows standard curves obtained from the rubber fraction representing an average molecular weight of 41,000. Each point is based on at least six independent observations. The length of each bar represents deviation among individual observations for that point on the graph. Similar results were obtained when standard curves

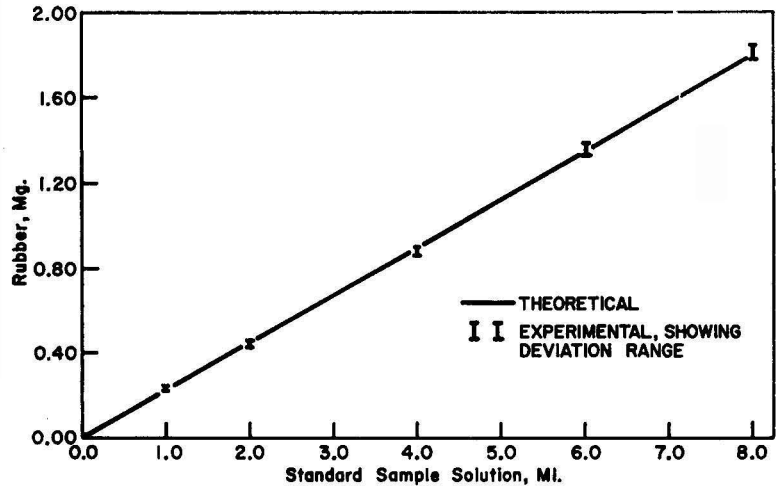

FIG. 1. Recovery of micro amounts of rubber from standard samples by use of a bromination procedure.

were obtained from fractions equivalent to rubber with average molecular weights of 111,000 and 320,000. The results of bromination reaction-time studies appear in Fig. 2.

Samples containing 3.8 mg rubber required 5 min for complete

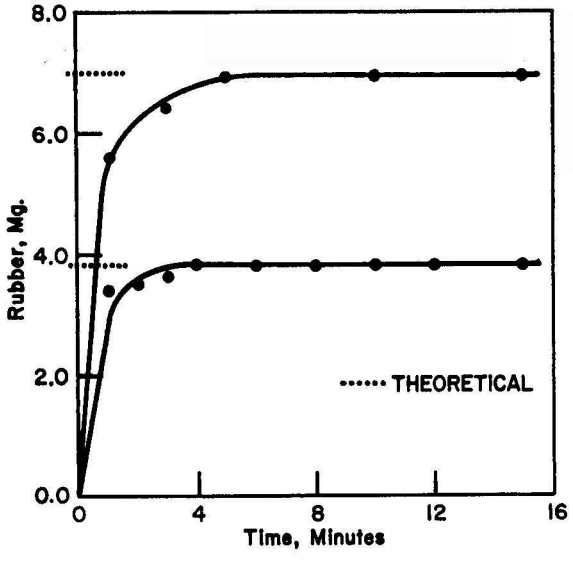

FIG. 2. Time required for complete bromination of rubber.

bromination. Further experimentation showed that 8–10 min of bromination was sufficient for any convenient sample size up to 10 mg. The time necessary for complete precipitation of rubber bromide by ethanol was investigated. Two different sample concentrations were brominated for 10 min. The bromination reaction was stopped by the addition of ethanol, and the sample was immediately placed in a 2°C water bath to facilitate bromide precipitation. Samples were centrifuged after 5, 15, and 30 min precipitation time. Complete precipitation of the rubber occurs within 5 min after addition of ethanol to the rubber bromide as shown in Table 1.

TABLE 1
PRECIPITATION TIME

Time (min)	Rubber		Maximum deviation[a]
	Added (mg)	Recovered (mg)[a]	
5	0.23	0.23	±0.02
15	0.23	0.24	±0.02
30	0.23	0.24	±0.005
5	0.45	0.45	±0.02
15	0.45	0.45	±0.0005
30	0.45	0.44	±0.005

[a] Based on four independent observations.

Gowans and Clark (4) reported the necessity of adding chloroform during the bromination of unsaturated hydrocarbon to eliminate substitution reaction. Our work confirms this observation. In several trials, using two different rubber fractions and various bromination times, recovery values obtained in the absence of chloroform ranged from 103 to 126% compared to recoveries of 97–103% when chloroform was present. It should be noted that the accuracy of this procedure, especially at its lower limits, depends primarily on the accuracy of the analytical balance employed. Maximum sensitivity can be obtained by using aluminum foil cups, as described in the procedure, or some other lightweight vessel for weighing. Also, difficulties may arise in transfer of the bromide from centrifuge tube to weighing cups when sample weights are greater than 10 mg. On centrifugation the rubber bromide normally forms a clean pellet in the bottom of the conically shaped centrifuge tubes. However, the larger samples form a precipitate which clings to the sides of the tube even after centrifugation, making quantitative transfer less convenient. Results comparing round-bottom and conically

shaped centrifuge tubes demonstrate that the round-bottom tubes give inconsistent values when used for the centrifugation of rubber bromide. Therefore, only conically shaped tubes should be used for this procedure.

The advantages of the bromination method for the determination of rubber have been discussed thoroughly by Willits *et al.* (9). Recent studies in our laboratory on the occurrence of polyisoprenoids in the microorganisms emphasized the need for a convenient, rapid, and accurate procedure to determine very small quantities of rubber. The modified bromination method presented here has been successfully employed for the quantitative determination of rubber in microorganisms. Detection of as little as 0.05–0.10% rubber in lactiferous fungi was possible. Infrared spectra of extracts confirmed that the material brominated was *cis*-polyisoprene (10). The procedure has been found to be more convenient and more rapid than the macromethod and at the same time is capable of determining as little as 200 μg rubber with an accuracy of from 96 to 97%.

SUMMARY

A method is described for determination of as little as 200 μg rubber with an accuracy of 96–97%. *cis*-Polyisoprene with average molecular weight as low as 41,000 was accurately determined employing a modification of a gravimetric bromide precipitation method.

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The Determination of Di- and Trihydroxycholanic Acids in Bile

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INTRODUCTION

In 1952, Kier (1, 2) introduced a method for simultaneous quantitative determination of the di- and trihydroxycholanic acids by differential spectrophotometry. Mosbach *et al.* (3) subsequently proposed a modification which, however, had the disadvantages that hydrolysis of the conjugated bile acids was required and applicability was limited.

In order to develop a more satisfactory procedure for routine differential bile acid analysis, a restudy was made of previously reported methods of extraction, reaction, and measurement (4–7). By modification of the extraction conditions, lengthening of reaction time, and use of differential spectrophotometry, a procedure applicable to human, canine, and rat bile has been developed and is reported below.

Analyses of human and rat bile were carried out to test the method and to determine normal ranges for both the absolute and relative concentrations of the two classes of bile acid.

MODIFIED METHOD

A. Reagents

1. *Absolute Ethanol.*

2. *Barium Reagent:* 5 gm barium hydroxide and 0.4 gm barium acetate are added to 100 ml of distilled water; the suspension is shaken, boiled, cooled, and filtered.

3. *"Alkaline" Ethyl Acetate:* 2 gm NaOH pellets is added to 100 ml ethyl acetate, previously dried over anhydrous sodium sulfate, and the mixture is agitated by magnetic stirring. This reagent is used freshly prepared.

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4. *Calcium Oxide Reagent:* 100 mg of finely pulverized calcium oxide is suspended by homogenization in 100 ml of freshly prepared "alkaline" ethyl acetate, after decantation from the NaOH pellets. The suspension is magnetically stirred during pipetting in order to assure transfer of equal amounts of calcium oxide.

5. *"Minibeck Reagent":* 1 part by volume of glacial acetic acid is added to 9 parts by volume of concentrated sulfuric acid. *Note:* The acids used for the reagent should be from freshly opened bottles and protected from external sources of contamination; all glassware in which this reagent is used must have been thoroughly cleaned with concentrated acid.

6. *Standard Solutions:* (*a*) cholic acid (2 mg/ml) and (*b*) deoxycholic acid (4 mg/ml) or chenodeoxycholic acid (4 mg/ml) or an equimolar mixture of the two (2 mg/ml of each) are dissolved in 95% ethanol.

B. Procedure

1. *Extraction, Purification, Reaction, and Measurement.* (*a*) *Canine or Rat Bile.* A 0.10-ml aliquot of bile is pipetted into a 5-ml volumetric flask, followed by 0.25 ml of barium reagent and absolute ethanol added to the mark. A blank solution containing no bile is prepared in the same manner and carried through the subsequent steps. The flask is kept at the boiling point of the mixture for 5 min by partial immersion in a water bath. After being cooled and ethanol added to the mark, the contents of the flask are mixed and filtered through fluted Whatman No. 1 filter paper. A suitable aliquot (usually 0.1 ml for canine bile, 1.0 ml for rat bile) is evaporated to dryness on a boiling water bath in an acid-cleaned Pyrex round-bottomed tube (15 × 125 mm with standard-taper fitting for glass stopper). To the residue is added 4 ml of the calcium oxide reagent; the tube is placed for 2 min in a water bath previously heated to 90°C, and then centrifuged at about 2500 for 15 min. The supernatant is decanted carefully so as not to disturb the precipitate, and the tube is allowed to drain in an inverted position. (For samples believed to contain large amounts of cholesterol or other lipids, extraction with two separate 4-ml portions of the ethyl acetate reagent, with heating and centrifuging as above, is recommended.) To the residue, 10.0 ml of the Minibeck reagent is added, and all tubes are placed simultaneously in a constant-temperature water bath at 40°C for 90 min. The stoppered tubes should be agitated until all of the residue has dissolved.

One-tenth milliliter of each of the standard solutions of cholic and deoxycholic (or chenodeoxycholic) acids and of a blank solution con-

sisting of ethanol are evaporated to dryness on a boiling water bath, 10.0 ml of the Minibeck reagent is added, and the tubes are incubated in the 40°C water bath, each of these steps being carried out simultaneously with the corresponding steps for the unknown samples.

The optical densities of the unknown and standard solutions are read against their respective blanks in 1-cm silica cuvettes at 310 and 390 mμ in an ultraviolet spectrophotometer (e.g., Beckman DU) the optical densities being constant from about 15 min to 2 hr after the time of removal from the 40°C water bath.

(*b*) *Human Bile.* A 0.05-ml aliquot of bile and 1.0 ml of the barium reagent are pipetted into a 10-ml volumetric flask and made up to the mark with absolute ethanol. After heating and filtration as described above, the filtrate is left standing overnight at room temperature and is refiltered, giving a clear, colorless solution. The rest of the procedure is as given for canine and rat bile, 1.0 ml of the filtrate being evaporated to dryness.

2. *Evaluation of Readings.* The concentrations of the di- and trihydroxycholanic acids (for which deoxycholic and cholic acids, respectively, are used as models) are calculated from the following equations (the constants [A, B, $\emptyset$, and D] being evaluated from the standards that are run simultaneously):

$$C_d = A \times \text{O.D.}^{310} - B \times \text{O.D.}^{390}, \tag{1}$$

concentration (in μg/ml) of dihydroxycholanic acids in final reaction volume (usually 10.0 ml);

$$C_t = \emptyset \times \text{O.D.}^{310} - D \times C_d, \tag{2}$$

concentration (in μg/ml) of trihydroxycholanic acid in final reaction volume;

where:

$$A = \frac{E_t^{390}}{E_t^{390} \times E_d^{310} - E_t^{310} \times E_d^{390}}; \qquad B = \frac{E_t^{310}}{E_t^{390} \times E_d^{310} - E_t^{310} \times E_d^{390}};$$

$$\emptyset = \frac{1}{E_t^{310}}; \qquad \text{and} \qquad D = \frac{E_d^{310}}{E_t^{310}}$$

with E_t the extinction coefficient, in μg/ml, of the trihydroxycholanic acid (cholic) and E_d the extinction coefficient, in μg/ml, of the dihydroxycholanic acid (deoxycholic) at the respective wavelengths. (Typical values found for the constants are: A, 40.2; B, 5.2; $\emptyset$, 110; D, 3.0.)

The di- and trihydroxy acid concentrations (in mg/ml) of the original bile samples may be calculated from the C_d and C_t values (in μg/ml),

when the specified volumes are used (cf. *Procedure*), by multiplying the C_d and C_t values by 0.5 (rat bile), 2.0 (human bile), or 5.0 (canine bile).

EXPERIMENTAL

A. Materials

Cholic (m.p. 199°C), glycocholic (m.p. 154–156°C), deoxycholic (m.p. 171–172°C), glycodeoxycholic (m.p. 178–181°C), lithocholic (m.p. 184–186°C), dehydrodeoxycholic (m.p. 187–188°C), dehydrocholic (m.p. 237°C), and chenodeoxycholic (m.p. 142–145°C) acids were employed as standards.[3] Melting points were measured on the Fisher-Johns melting point apparatus. Other chemicals employed were "Reagent Grade."

B. Reaction with the Minibeck Reagent

Wysocki *et al.* (7) observed that after reaction with the Minibeck reagent, cholic acid exhibited maximal absorption at 390 mμ, with another absorption peak at 310 mμ, while the dihydroxycholanic acids (deoxycholic and chenodeoxycholic) showed maximal absorption at 310 mμ, with slight peaks at 390 mμ. Because the extinction coefficient of cholic acid, determined at 310 mμ after heating with the Minibeck reagent for 30 min at 40°C (5) was found to vary with concentration, a study was made after different heating times (Fig. 1). At 310 mμ, although there was linearity, the line passed through the origin only after 90 min of heating. Substantially the same result was obtained at 50°C, although the heating time could be slightly reduced at this higher temperature.

The concentration–absorption relationships of deoxycholic and chenodeoxycholic acids were studied under the conditions found optimal for cholic acid, and good agreement with the Beer-Lambert law was found in each case (Fig. 2).

Molar extinction coefficients of various bile acids are shown in Table 1. Deoxycholic and chenodeoxycholic acids have almost identical extinction coefficients at 310 mμ, somewhat different extinctions at 390 mμ. In the calculation of constants A and B, however, this difference

[3] The cholic, deoxycholic, dehydrocholic, and dehydrodeoxycholic acids (Riedel-de Haen), glycocholic acid (Hoffman-LaRoche), lithocholic acid (Schering Research Division), and glycodeoxycholic acid (synthesized from deoxycholic acid) were gifts of Dr. Harry Sobotka, Chemistry Department, The Mount Sinai Hospital, N. Y. C., and the chenodeoxycholic acid was a gift of Drs. E. Mosbach and F. E. Kendall, Department of Biochemistry, Columbia University College of Physicians and Surgeons, N. Y. C.

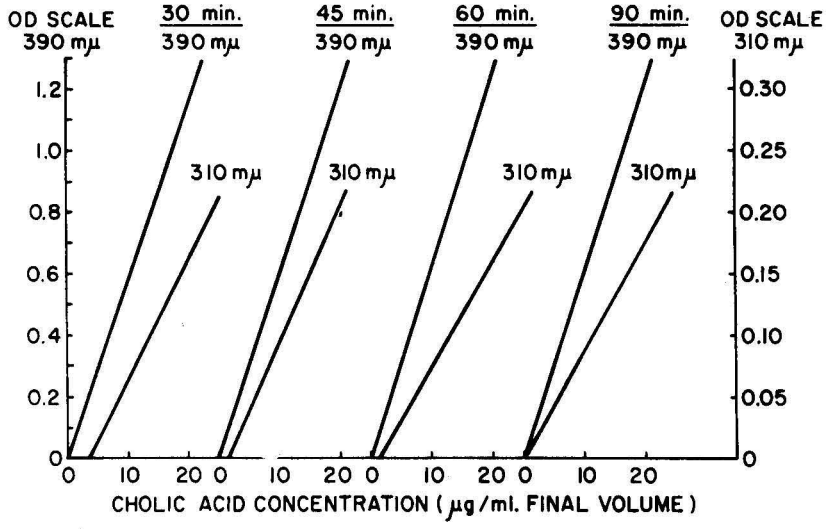

FIG. 1. Study of agreement with the Beer-Lambert law after heating the reaction mixture of cholic acid plus Minibeck reagent for various periods of time at 40°C.

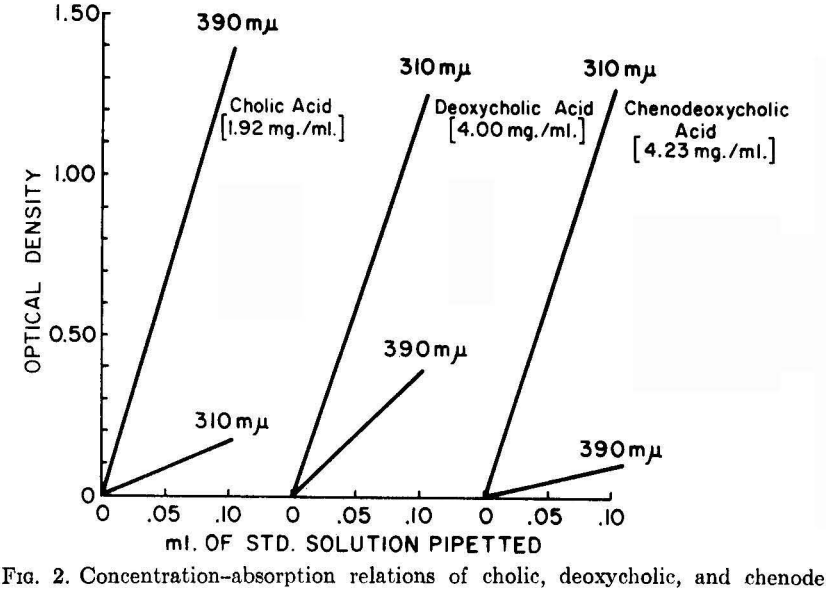

FIG. 2. Concentration–absorption relations of cholic, deoxycholic, and chenodeoxycholic acids at two wavelengths under conditions found optimal for cholic acid. To indicated volumes of the standard solutions (concentrations given in brackets) were added 10.0 ml of Minibeck reagent, and the solutions were heated for 90 min at 40°C, read after 15 min.

TABLE 1
MOLAR EXTINCTION COEFFICIENTS OF VARIOUS BILE ACIDS IN MINIBECK REAGENT

	$E_{molar}^{310} \times 10^3$		$E_{molar}^{390} \times 10^3$	
	Found	Reported[a]	Found	Reported[a]
Cholic acid	3.6	3.3	28.0	24.0
Glycocholic acid	3.6	3.3	28.0	24.0
Deoxycholic acid	11.5	9.4	3.9	2.1
Glycodeoxycholic acid	9.7	7.4[b]	4.0	2.4[b]
Chenodeoxycholic acid	12.3	8.7	1.03	2.1
Lithocholic acid	5.2	5.3	0.73	1.2
Dehydrocholic acid	0	—	0	—
Dehydrodeoxycholic acid	0	—	0	—

[a] Wysocki *et al.* (7).
[b] Taurodeoxycholic, not glycodeoxycholic acid was reported.

at 390 mμ becomes insignificant, since the first product in the denominator is so much larger than the second. In the work reported, deoxycholic acid has been routinely employed as the prototype of the dihydroxycholanic acids. In view of the lower extinction of the conjugated acid at 310 mμ (Table 1), and the fact that the bile acids are found conjugated in the bile, others may prefer to employ the glyco- or tauro-conjugate of this acid as the standard.

The equation "constants" (A, B, $\emptyset$, and D) do vary to some extent. The evaluation of these "constants" from the extinctions of pure compounds run simultaneously is necessary for each set of determinations.

C. Recovery Experiments

In order to establish the applicability of the method of differential measurement for bile acids in the presence of one another, a synthetic mixture of cholic, deoxycholic, and chenodeoxycholic acids was analyzed, the results being evaluated from the extinctions of the individual acids run separately. The "recoveries" were essentially quantitative (Table 2).

TABLE 2
RECOVERY OF BILE ACIDS DETERMINED DIRECTLY IN SYNTHETIC MIXTURE

Material analyzed	Trihydroxy acid			Dihydroxy acids		
	Added (μg)	Found (μg)	Recovery (%)	Added (μg)	Found (μg)	Recovery (%)
Synthetic mixture A[a]	9.58	9.60	100.2	10.29	10.04	97.6
	9.58	9.50	99.2	10.29	10.12	98.3

[a] Consisted of cholic, deoxycholic, and chenodeoxycholic acids, 9.58, 5.00, and 5.29 μg of each, respectively, per ml of final reaction volume (10.0 ml).

TABLE 3

RECOVERIES OF BILE ACIDS FROM SYNTHETIC AND NATURAL (RAT BILE) MIXTURES AFTER EXTRACTION[a]

Material analyzed		Trihydroxy acid					Dihydroxy acids				
		Added in bile (μg)	Added as acid (μg)	Total calcd. (μg)	Total found (μg)	Recovery (%)	Added in bile (μg)	Added as acid (μg)	Total calcd. (μg)	Total found (μg)	Recovery (%)
Synthetic mixture B[b]	(*a*)	—	9.58	9.58	9.40	98.1	—	20.00	20.00	20.52	102.6
	(*b*)	—	9.58	9.58	9.34	97.5	—	20.00	20.00	20.39	102.0
	(*c*)	—	9.58	9.58	9.29	97.0	—	20.00	20.00	20.37	101.9
	(*d*)	—	9.58	9.58	9.11	95.1	—	20.00	20.00	20.58	102.9
Rat bile[c]	(*a*)		—		9.84	—		—		7.19	—
	(*b*)		—		9.61	—		—		6.97	—
	(*c*)		—		9.54	—		—		6.81	—
	(*d*)		—		9.53	—		—		7.37	—
Rat bile + synthetic mixture B	(*a*)	4.92	9.58	14.50	14.42	99.4	3.59	20.00	23.59	23.41	99.2
	(*b*)	4.81	9.58	14.39	13.92	96.7	3.49	20.00	23.49	23.95	102.0
	(*c*)	4.77	9.58	14.35	14.29	99.6	3.41	20.00	23.41	23.34	99.7
	(*d*)	4.77	9.58	14.35	13.94	97.1	3.69	20.00	23.69	23.57	99.5

[a] (*a*) Barium hydroxide–barium acetate treatment with ethanol extraction of bile acids.
(*b*) *a* plus calcium oxide–ethyl acetate absorption–extraction.
(*c*) *b* plus one washing with ethyl acetate.
(*d*) *b* plus three washings with ethyl acetate.

[b] Synthetic mixture B contained 9.58 μg cholic, 20.00 μg deoxycholic acid per ml in the final reaction volume employed.

[c] Obtained by intubation of common bile duct.

TABLE 4

RECOVERIES OF BILE ACIDS ADDED TO HUMAN AND CANINE BILE[a]

Material analyzed	Trihydroxy acid					Dihydroxy acids				
	Added in bile (μg)	Added as acid (μg)	Total calcd. (μg)	Total found (μg)	Recovery (%)	Added in bile (μg)	Added as acid (μg)	Total calcd. (μg)	Total found (μg)	Recovery (%)
Human bile[b]	4.9	5.8	10.7	10.4	97.9	5.6	0	—	5.7	—
	5.0	8.6	13.7	12.5	91.8	5.7	0	—	5.3	—
	4.9	11.5	16.4	15.7	95.6	5.6	0	—	5.8	—
	4.9	0	—	5.6	—	4.4	12.0	16.4	14.9	90.4
	4.9	0	—	5.9	—	4.4	18.0	22.4	20.5	91.5
	4.9	0	—	6.0	—	4.4	24.0	28.4	27.2	95.7
	Average recovery				95.1					92.6
Canine bile[c]	10.3	3.8	14.1	14.6	103.6	5.4	0	—	6.4	—
	10.3	5.8	16.0	15.1	94.2	5.4	0	—	5.8	—
	10.3	7.7	17.9	16.9	94.3	5.4	0	—	5.4	—
	10.2	0	—	12.5	—	4.9	6.0	10.9	11.1	101.8
	10.2	0	—	13.2	—	4.9	9.0	13.9	15.1	108.5
	10.2	0	—	12.8	—	4.9	12.0	16.9	18.1	107.0
	Average recovery				97.4					105.4

[a] The procedure (cf. *Modified Method*) including two extractions with ethyl acetate for removal of lipid was employed.
[b] Obtained from gall bladder at autopsy.
[c] Obtained from gall bladder of living animal.

When the modified Minibeck extraction procedure was used, quantitative recovery of the components of a synthetic mixture of cholic and deoxycholic acids was realized at each of the steps of the method described (Table 3). Upon analysis of rat bile, and rat bile to which a synthetic mixture of pure bile acids was added (Table 3), there were no significant differences in the values obtained after varying treatments, indicating that all interfering substances were removed by the initial barium hydroxide–barium acetate precipitation. At the calcium oxide adsorption step, the bile acids were bound *in toto* and not removed by subsequent washings with the alkaline ethyl acetate. In similar experiments, however, interference due to added cholesterol (50 mg/ml bile) or stearic acid (60 mg/ml) was totally removed by this same procedure.

Recoveries of added bile acids from human and canine biles were satisfactory (Table 4).

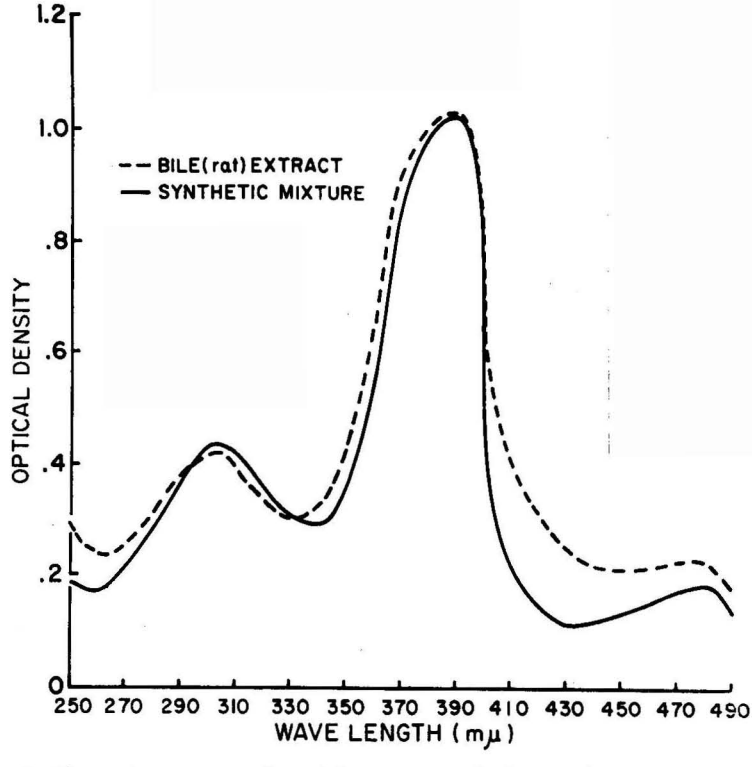

FIG. 3. Absorption spectra of rat bile extract and of a synthetic mixture of pure cholic and deoxycholic acids, in amounts corresponding to those found in the rat bile (cholic acid, 16.4 μg/ml; deoxycholic acid, 11.1 μg/ml).

A final check on the precision and specificity of the determination was made by the following procedure: Rat bile was analyzed for its bile acid content, the evaluation being based on the extinction coefficients of cholic and deoxycholic acids. A synthetic mixture composed of cholic and deoxycholic acids in amounts corresponding to those found in the analyzed bile was then analyzed simultaneously with the bile. The spectra of the respective reaction mixtures were determined in the range 250–490 mμ (Fig. 3), the results of the spectral analysis indicating good precision and absence of nonspecifically absorbing material in the region of the critical wavelengths (310, 390 mμ).

RESULTS

In Table 5 are shown the results of analyses of human biles obtained from the gall bladder at autopsy of cases having no hepatic involvement and of normal rat biles obtained by intubation of the common bile duct.

TABLE 5
BILE ACID CONTENT OF HUMAN AND RAT BILE[a]
(Expressed as free acids)

Source	Trihydroxy acid (mg/ml)	Dihydroxy acids (mg/ml)	Total acids (mg/ml)	Ratio of trihydroxy / dihydroxy
Human (10)[b]	12.5 ± 7.1[c]	19.6 ± 10.5	32.1 ± 16.9	0.69 ± 0.26
Rat (12)	7.9 ± 2.2	5.0 ± 1.3	12.8 ± 3.3	1.60 ± 0.37

[a] Human gall bladder bile was obtained at autopsy from cases with no hepatic involvement. Rat bile was obtained during 1 hr intubation of the common bile duct of 150–200 gm Sprague-Dawley female rats.
[b] Number of individuals analyzed.
[c] Means ± std. dev.

The bile acid content of human gall bladder bile is generally about twice that of rat common duct bile, and the relative amount of dihydroxy acid(s) considerably higher in human than in rat bile. No assessment was made of bacterial influence on the bile acid ratios obtained in the human samples.

DISCUSSION

In the described procedure, two of the innovations, prolongation of the time of heating at 40°C and the use of a dilute, homogeneously suspended calcium oxide solution, were necessary to give stability and consistent agreement with the Beer-Lambert law at 310 mμ and to assure constant blank values. These changes had little influence on the extinction at 390 mμ. Drying and alkalization of the ethyl acetate

extractant solutions prevented loss of the bile acids during the hot extraction procedures. The hydrolytic step considered responsible for the relatively low recoveries of cholic acid obtained by Mosbach *et al.* (3) has been eliminated.

In the present method no distinction is made between the dihydroxycholanic acids, nor is consideration given to monohydroxycholanic (lithocholic) acid, reported to be present in relatively small amounts (6). A chromatographic procedure such as that of Sjövall (8) would be the method of choice when a fine differentiation of the various bile acids is desired.

SUMMARY

A differential spectrophotometric method of bile acid determination is described, whereby unhydrolyzed di- and trihydroxycholanic acids may be quantitatively measured simultaneously. Conditions have been established under which the products of reaction with the reagent of Minibeck (9:1 conc. H_2SO_4, glacial acetic acid) are stable and show good agreement with the Beer-Lambert law at the two wavelengths concerned (310, 390 mμ).

Recoveries of pure bile acids in synthetic mixture, as well as of pure bile acids added to human, canine, and rat bile are satisfactory, and the spectra of the reaction products indicate the absence of interfering substances.

Human gall bladder bile was found to have a higher bile acid content and relatively more dihydroxy acid than rat common duct bile.

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Self-Absorption Correction in the Use of Tritium-Labeled Substrates for Enzyme Assays[1]

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INTRODUCTION

The development of methods for the direct labeling of organic compounds with tritium has resulted in the production of new radioactive tracers (1–9), but the best use of these tracers for quantitative work in many laboratories has been hindered by limitations associated with solid counting of the soft β emission of tritium (10–13). Although liquid scintillation counting is being employed with increasing frequency (14–19), the convenience of the less elaborate and expensive method of direct counting in windowless flow counters continues to promote the use of the latter, even though precise measurements are required in many of the experiments. Thus, from solid-counting data enzyme activities have been calculated as a function of the radiochemical yields of tritium in the reaction products of tritiated substrates (20–23), and deoxyribonucleic acid (DNA) specific activities have been determined to compare the extent of incorporation of tritium-tagged precursors into DNA under various experimental conditions (24–30), or to measure the replacement time of DNA in different tissues (31). To avoid self-absorption corrections, the samples have been plated at infinite thinness (20–31) or in layers approaching infinite thinness (22, 28), but where this has not been possible, or practical, attempts have been made to reproduce accurately a standard sample thickness to obtain meaningful relative counting rates (21, 23–25, 29, 30). In a few instances where known weights of a pure compound, e. g., DNA, have been plated, corrections for self-absorption have been obtained from a self-absorption curve prepared with the particular compound (26, 27, 29).

It is evident that major problems associated with experiments of this

[1] This investigation was supported by a Senior Research Fellowship (SF 191) from the U. S. Public Health Service and by a research grant (C-5066) from the National Cancer Institute, U. S. Public Health Service.

type are (*a*) excessive lowering of counting rates if attempts are made to prepare samples of infinite thinness by serial dilution of solutions of labeled substances of moderate or low specific activity and (*b*) inability to correct for self-absorption losses in samples of indeterminate weight plated in the region between infinite thinness and infinite thickness. Complementing the former difficulty is the requirement for relatively large amounts of the samples where assays are attempted at infinite thickness. In this paper it is shown that the total activity of tritium in solid samples of finite thickness and variable composition can be accurately determined by a method based on the use of internal standards[2] of tritium prepared from thymidine-H^3 of high specific activity. Measurements of sample weights are not required.

MATERIALS

Thymidine and TMP[3] were purchased from the California Corporation for Biochemical Research, Los Angeles, Calif., and thymidine-H^3 and dCMP-H^3 were obtained from Schwarz BioResearch, Inc., Mount Vernon, N. Y. ATP (disodium salt) and phosphoglyceric acid (barium salt) were purchased from Nutritional Biochemicals Inc., Cleveland, Ohio. Ba 3-phosphoglycerate was converted to K 3-phosphoglycerate with K_2SO_4, and the pH of the solution was adjusted to 7.9 with NaOH before use. Crystalline bovine plasma albumin was purchased from The Armour Laboratories, Kankakee, Ill. Tris buffer was "Sigma 121" (Sigma Chemical Company, St. Louis, Mo.).

METHODS

Determination of Radioactivity

Radioactive solutions were plated on flat aluminum disks[4] (0.1-ml aliquots in each case) within a circle drawn with a pencil 1.85 cm in diameter. The solutions were spread over the circle area with a fine glass pick and allowed to dry at room temperature. To insure clean, uniform surfaces, the disks, prior to use, were polished with steel wool, heated in

[2] While preparing this manuscript the author became aware that Paulus and Kennedy (32) have used tritium standards to correct for self-absorption losses in lipid samples containing inositol-H^3.

[3] The abbreviations used are: TMP, thymidine 5'-monophosphate; dCMP, deoxycytidine 5'-monophosphate; ATP, adenosine triphosphate; Tris, tris(hydroxymethyl)aminomethane; ABA, aqueous solution of crystalline bovine plasma albumin, 25 μg/ml.

[4] Model AF-12 flat aluminum pans obtained from Nuclear-Chicago Corp., 333 E. Howard St., Des Plaines, Ill.

boiling acetone–formic acid (50:50), rinsed with water, and wiped with tissue paper. The circles were drawn; then the disks were immersed about 2 min in boiling acetone, removed with forceps, dried in air, and used immediately. For accurate results, drying of each plated aliquot in a uniform layer within the encircled area was essential. With dilute samples plating "at" or near infinite thinness, no difficulty was encountered in duplicating the counting results in assays on replicate plates. With thicker samples, where nonuniform distribution of material on the plates can lead to large errors (28), it was found that the presence of protein in the solutions facilitated drying of the samples in uniform layers, making it possible to obtain satisfactory duplication of counts on replicate plates having surface densities up to 360 μg/sq cm. The minimal effective concentration of protein (crystalline bovine plasma albumin) was approximately 25 μg/ml. Radioactivity measurements were made in a windowless flow counter[5] operated in the Geiger region. To insure unhindered passage of "Q-gas" from the counting chamber to the exhaust holes at the bottom of the solid sample pan holders, sample pans were placed in the holders on top of aluminum disks[4] cut in the shape of three-quarter circles. Solid samples were prepared in duplicate or in triplicate, and sufficient counts were accumulated to give a 0.95 error within $\pm 5\%$ or $\pm 3\%$ (33).

Self-Absorption Correction

The availability of tritiated compounds of very high specific activity, such as thymidine-H^3, enables one to prepare by serial dilution solutions from which infinitely thin solid samples of variable activity can be obtained. If a measured volume of one of these solutions (A-type) were lyophilized, then redissolved in the same volume of a second solution (B-type) containing *unlabeled* nonvolatile solute at an appreciable concentration, and if identical aliquots of each solution were plated and counted, the fractional reduction of the counting rate of the B sample (compared with the counting rate of the infinitely thin sample from A) would be a quantitative expression of the self-absorption of tritium β's in the material on the B plate. To measure this counting-rate depression in the usual case where the nonvolatile solute of the B solution is tritium labeled, it would be necessary to assay identical samples from the B solution before, and after mixing with the lyophilized residue from A (the internal standard) to obtain by difference the net counting rate due to the internal standard. The observed activity of the B sample

[5] Nuclear-Chicago model D47 gas-flow counter with model C110B automatic sample changer, C111B printing timer, and 181A decade scaler.

obtained prior to mixing the B solution with the internal standard could then be corrected for self-absorption by dividing by the ratio (B/A) of the counting rates of the internal standard in B and A. The assumption on which such a correction is based, namely, that the percentage self-absorption of β particles emanating from the tritium of the internal standard is identical to the percentage self-absorption of β's emanating from tritium originally present in B, would appear to hold true in the strictest sense only where the internal standard corresponded exactly to the tritiated compound being assayed (12).

Preparation of Enzyme Source

Three male albino rats[6] weighing 180–220 gm were partially hepatectomized (34) and fasted until killed by decapitation 24–25 hr postoperatively. Livers were excised rapidly, dropped into ice-cold 0.15 *M* KCl solution, and transferred immediately to the cold room at 1–3° where they were washed several times by decantation with chilled isotonic KCl solution to remove blood and traces of fur. The combined livers were blotted with paper towels, weighed, and broken up initially with a motor-driven loose-fitting pestle in an all-glass homogenizer containing a volume in milliliters of 0.15 *M* KCl equal to the weight in grams of tissue. Homogenization was completed in a smooth-bore glass homogenizing tube with eight passes with a Teflon pestle at 1000 rpm. The homogenizing tubes were immersed in ice water in a round-bottom flask during homogenization. The supernatant fraction, which was the source of the enzymes assayed in these experiments, was prepared by centrifuging the whole homogenate for 30 min at 60,000 $\times$ *g* in a Spinco preparative ultracentrifuge.

Assay of Thymidine Kinase

The method was adapted from the thymidine kinase assay of Bollum and Potter (21). The reaction mixture contained 0.1 μmole thymidine-H^3 with a specific activity of 12 μcuries (μc)/μmole; 3.0 μmoles 3-phosphoglycerate; 2.5 μmoles ATP; 2.5 μmoles $MgSO_4 \cdot 7H_2O$; 100 μmoles Tris, pH 8.0; 0.05–0.3 ml. of supernatant fraction diluted 1.5:1 with isotonic KCl; and water to a final volume of 0.5 ml. The components were added together at 0–4° in 15-ml conical centrifuge tubes, then incubated at 37° for 30 min. The reaction was stopped by immersing the tubes 1 min in a boiling water bath. A zero-time control tube was heated immediately after addition of the reaction components. Carrier TMP (500 μg in 0.5 ml water) was mixed with each residue, and after

[6] Holtzman Company, Madison, Wis.

centrifugation 0.4-ml aliquots of the supernatant fluids were streaked acrss 2-in. lines on a sheet of Whatman #1 filter paper (18 × 22½ in.). The chromatogram was developed with the solvent *n*-butanol–water (86:14 v/v). Thymidine phosphates were located at the origin under ultraviolet light in bands which were cut into small pieces and eluted by shaking 2 hr in 8.0 ml ABA. The eluates were centrifuged, and 0.1-ml aliquots were plated, counted to a 0.95 error of ±5%, and corrected for self-absorption with internal standards (see text below).

Assay of Deoxycytidylate Deaminase

The conditions for the spectrophotometric assay of deoxycytidylic acid deaminase (Method II) reported by Maley and Maley (35) were employed in a more sensitive type of assay utilizing tritiated deoxycytidylic acid. The reaction mixture contained 5.5 μmoles dCMP-H^3 with a specific activity of 0.11 μc/μmole; 100 μmoles Tris, pH 8.0; 0.05–0.3 ml of supernatant fraction; and water to a final volume of 0.5 ml. The components were added together at 0–4° in 15-ml conical centrifuge tubes, then incubated at 37° for 30 min. The reaction was stopped by immersing the tubes in a boiling water bath for 1 min. A zero-time control tube was placed in the boiling water bath immediately after addition of the reaction components. The tubes were centrifuged after addition of 0.6 ml of 0.2 *N* acetic acid. Supernatant fluids were transferred to columns of Dowex 50-H^+ (2.5 ml wet resin, X-4, 100–200 mesh, in 10-ml serological pipets) prewashed with 0.1 *N* acetic acid. Pellets remaining in the tubes were stirred with 1.4 ml of 0.1 *N* acetic acid and centrifuged; supernatant fluids were transferred as before to corresponding columns and allowed to drain to the resin surfaces. The columns were rinsed with four 2.5-ml portions of 0.1 *N* acetic acid. Under these conditions dCMP and deoxycytidine remain on the columns, whereas dUMP and its derivatives pass out of the columns in the acetic acid rinse (R. E. Beltz, unpublished data).[7] Higher phosphates of dCMP, which would pass out of the columns under these conditions, are not formed in the absence of added ATP (36). The effluents were collected in large culture tubes and evaporated to dryness at reduced pressure on a rotating evaporator. The residue in each tube was dissolved in 2.0 ml ABA, and 0.1-ml aliquots were plated, counted to a 0.95 error of ±5%, and corrected for self-absorption with internal standards (see text in next section).

[7] Maley and Maley (36) reported a similar procedure for separating dCMP-C^{14} from its deamination products while this manuscript was in preparation.

RESULTS

Preparation of Thymidine-H³ Internal Standards; Estimation of Counter Efficiency

An aqueous solution of thymidine-H³ (500 μc/ml; 1.9 μc/mμmole) was diluted with water to a concentration of 0.357 μc/ml (0.046 μg thymidine-H³/ml). The extent to which this solution (I) required further dilution to yield thymidine-H³ layers of infinite thinness when 0.1-ml aliquots were plated was found to be about 30-fold (Fig. 1). If 0.1-ml

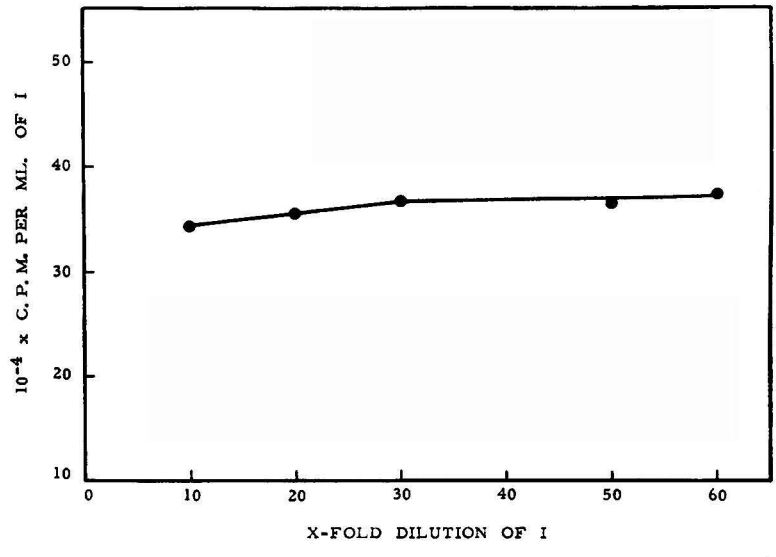

FIG. 1. Serial dilution of a solution of thymidine-H³ (I) to obtain samples plating at infinite thinness. Solid samples prepared by plating in triplicate 0.1 ml of each diluted solution were counted to a 0.95 error of ±3%. Activity/ml of I was calculated in each case from the mean counting rate of replicate plates.

aliquots of undiluted I were used as an internal standard in the manner previously described (see text under *Self-Absorption Correction,* above), at least 3 ml water (A), and an equal volume of solution B, would be required to obtain the necessary 30-fold dilution.

From the mean counting rate of infinitely thin samples prepared after 30-, 50-, and 60-fold dilutions of I, the absolute counting efficiency of the system was estimated to be 46%.[8] The calculation was based on the

[8] One hundred times counts/min in 1.0 ml I divided by disintegrations/min in 1.0 ml I.

stated specific activity of Schwarz thymidine-H^3, which is maintained within 10% of the true disintegration rate.[9]

Absorption of Radiation in Solid Samples and Correction of Counting Rates to Infinite Thinness Values

To determine the relationship of sample thickness to observed counting rate of tritium samples of constant total activity, 0.4-ml aliquots of I were mixed with 9.2 ml of solutions of nonisotopic thymidine in

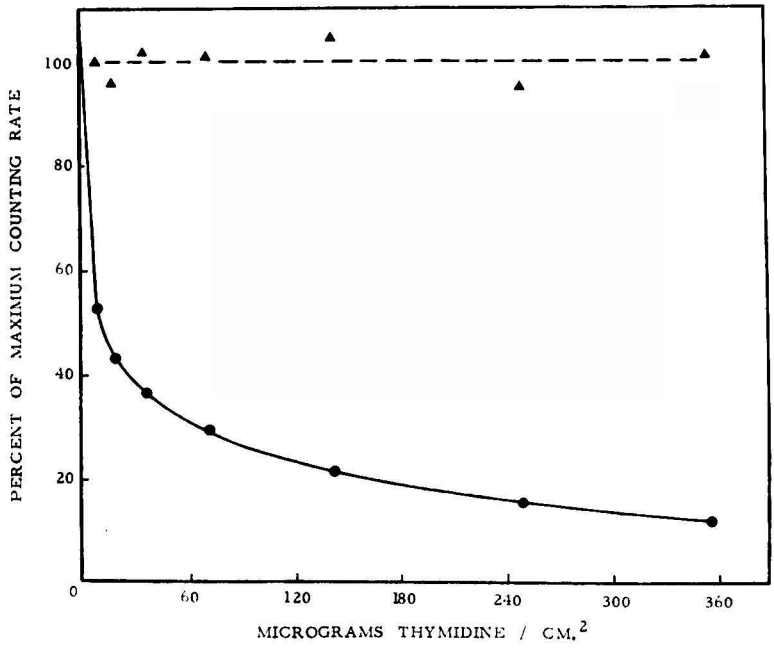

FIG. 2. Counting rate as a function of the surface density of nonisotopic thymidine in samples of thymidine-H^3 of constant total activity, and corrections for self-absorption. (●—●) Self-absorption of β-radiation with increasing surface density of thymidine. (▲- - -▲) Zero-thickness (maximal) counting rates calculated from self-absorption losses measured with internal standards of tritium.

ABA ranging in concentration from 0.25 to 10 mg thymidine/ml (B solutions). Solid samples were prepared from 0.1-ml aliquots of each solution and assayed for radioactivity as previously described.

To correct for self-absorption losses, the residues of lyophilized 0.1-ml aliquots of I (internal standards) were dissolved in 3.1 ml of each B

[9] D. R. Schwarz, Schwarz BioResearch, Inc., Mount Vernon, N. Y., personal communication.

TABLE 1

SELF-ABSORPTION OF TRITIUM β-RADIATION IN SOLID SAMPLES OF CONSTANT TOTAL ACTIVITY, AND CORRECTION OF OBSERVED ACTIVITIES TO THE ZERO-THICKNESS COUNTING RATE WITH INTERNAL STANDARDS

Nonisotopic thymidine (μg/plate)[a]	B (counts/min/plate)[b]	B + internal std. (counts/min/plate)[c]	Activity due to internal std. (counts/min/plate)[d]	Internal std. $\left(\frac{\text{counts/min in B}}{\text{counts/min in A}}\right)$[e]	B zero-thickness counts/min/plate, calcd[f]
25	783	1385	602	0.5212	1502
50	643	1159	516	0.4468	1439
100	548	962	414	0.3584	1529
200	434	766	332	0.2874	1510
400	321*	558*	237	0.2052	1564
700	231	419	188	0.1628	1419
1000	181	320	139	0.1203	1505

[a] Solid samples of B, and B + internal std. were prepared by plating 0.1-ml aliquots of B solutions before, and after mixing with the internal standard, respectively (see text).

[b] Counting rates of B samples obtained prior to mixing B solutions with the internal standard. Each number is the average of triplicate or *duplicate assays.

[c] Counting rates of B samples obtained after dissolving the residues of lyophilized 0.1-ml aliquots of I (internal standard) in 3.1-ml aliquots of B solutions. Each result is the average of triplicate or *duplicate assays.

[d] Net counting rate due to the internal standard, calculated by difference ($c - b$).

[e] A = 1155 counts/min/plate = the mean counting rate of the internal standard plated at infinite thinness. Solid samples were prepared in triplicate by plating 0.1-ml aliquots of the A solution (3.1 ml water + residue of lyophilized 0.1-ml aliquot of I).

[f] Counting rates of B samples corrected for self-absorption losses by dividing the observed activities by the ratio (B/A) of the counting rates of the internal standard in B and A. The true total activity on each B plate (zero-thickness counting rate) was 1492 counts/min $\left(\frac{1155 \times 10 \times 3.1 \times 4}{9.6 \times 10}\right)$; cf. footnote *e* and text.

solution, and in 3.1 ml water (A), and 0.1-ml aliquots were plated and counted. The data and calculations are shown in Table 1, and the self-absorption curve with self-absorption corrections for each point is represented in Fig. 2. From the data of Table 1 (cf. footnotes *e* and *f*), it can be calculated that the total activity in 0.1 ml of I was 35,805 counts/min, and in 3.1 ml of each B solution was 46,252 counts/min. The total activity of the internal standard aliquot employed in these self-absorption corrections was thus slightly less than the total activity contained in the volume of the B solutions with which it was mixed. For the other self-absorption corrections reported in this paper, the total activity of the aliquot of internal standard used was in no case allowed to exceed more than slightly the total activity in the volume of the B-type solution with which it was mixed. The advisability of this limitation was suggested by preliminary experiments in which the accuracy of self-absorption corrections was reduced if the total activity of the internal standard greatly exceeded the total activity of the solution mixed with it.

The weakness of the tritium β radiation (5.7×10^{-3} mean mev) is evident from the self-absorption curve of Fig. 2 which shows that the counting rate of the B samples was reduced to 50% of the maximal rate by the presence of as little as 31 μg of inactive material per plate (12 μg/sq cm). The observed activity of samples having the largest surface density (357 μg/sq cm; 961 μg/plate) was reduced to 12% of the maximal counting rate. Each corrected counting rate (Fig. 2) fell within $\pm 5\%$ of the observed counting rate of an equivalent amount of tritium plated at infinite thinness (cf. Table 1, footnote *f*). In the 13 triplicate and 2 duplicate assays of radioactivity from which the data of Table 1 were taken, the deviations of individual replicate samples from their respective mean values varied between 0 and 4.97% of the means, with an average deviation of 1.84%. The magnitude of the deviations was unrelated to sample thickness. Furthermore, the accuracy of the self-absorption corrections exhibited no correlation with sample thickness. These results support the conclusion based on visual evidence that uniform, or at least reproducible distribution of material on the plates was achieved throughout the range of sample thicknesses tested in these experiments.

Enzyme Assays

Self-absorption corrections were made by the method previously described (text, above, and Table 1). The results, expressed as total counts/min in reaction products, were plotted against milliliters of enzyme source in each incubation mixture (Figs. 3 and 4). A linear

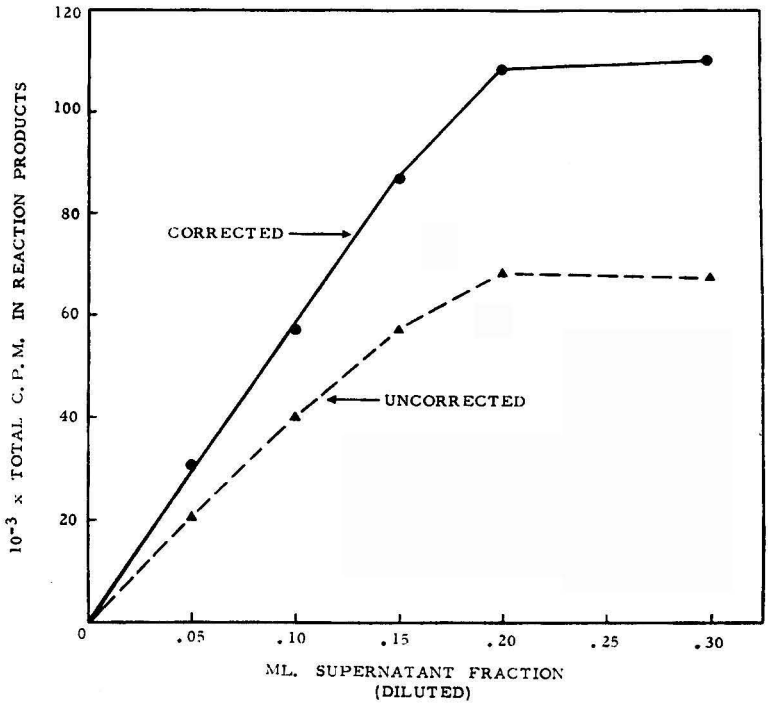

FIG. 3. Phosphorylation of thymidine by supernatant fraction of regenerating rat liver homogenate. (▲---▲) counts/min converted to mono-, di-, and triphosphates of thymidine, calculated from the observed activities of aliquots plated from solutions of the isolated reaction products. (●—●) Total counts/min in reaction products calculated from observed activities corrected for self-absorption losses with internal standards of tritium.

relationship was obtained ("corrected" curves) between enzyme activity and the volume of enzyme source added up to 0.15, and 0.2 ml, respectively, in the thymidine kinase and deoxycytidylate deaminase assays. The nonlinearity of the "uncorrected" curves in the corresponding regions was due to small self-absorption differences in the samples. Self-absorption in kinase samples (Fig. 3) was 31–40% of the total activity, and somewhat higher levels of self-absorption were found in samples of the deaminase assay (40–50%, Fig. 4). These ranges of self-absorption would be expected in samples containing less than 30 μg of material (cf. Fig. 2). Sample-counting efficiencies, calculated from the absolute efficiency of the counting arrangement (46%) and self-absorption losses in the samples (31–50%), varied from 23 to 32% of the disintegration rate. At this level of efficiency it would appear that any advantage to

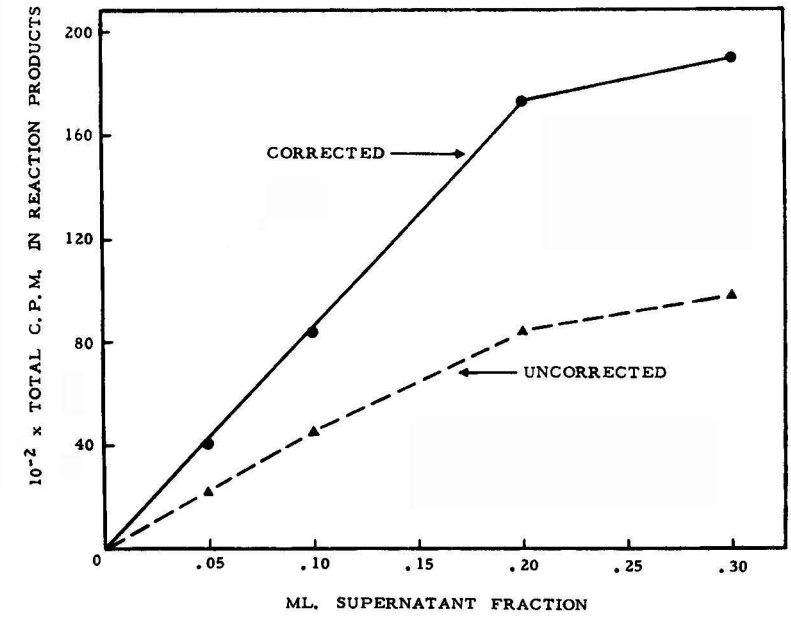

FIG. 4. Deamination of deoxycytidylic acid by supernatant fraction of regenerating rat liver homogenate. (▲---▲) Counts/min converted to deamination products, calculated from the observed activities of aliquots plated from solutions of the deamination products. (●—●) Total counts/min in deamination products, calculated from observed activities corrected for self-absorption losses with internal standards of tritium.

be gained with respect to sample counting rates by the use of C^{14}-labeled substrates in place of tritiated substrates would be insignificant.

DISCUSSION

Rydberg (12) has dealt in detail with the problem of determining absolute tritium activities in samples of finite thickness. Since Rydberg's self-absorption corrections were obtained by the standard method (37) of constructing a curve relating apparent specific activity to sample weight, correction of the observed activity of an individual sample for self-absorption by this method requires an accurate knowledge of sample weight[10] and a self-absorption correction curve based on radioactivity assays of varying thicknesses of the particular sample material. The necessity for the latter has been underscored by Rydberg's finding that

[10] Since the maximum range of tritium β radiation is 0.6 ± 0.1 mg/sq cm (12), sample weights are too small to determine by differential weighing of the plates. Aliquots of solutions of known concentration were plated in this case.

different tritiated substances can exhibit markedly discrepant self-absorption characteristics (12). Muramatsu and Sasaki (38) have proposed eliminating the need for self-absorption correction curves by converting the tritium of all tritiated organic compounds to octadecane-1-H^3, the counting rate of which has been shown to be proportional to sample thickness up to a surface density of about 100 μg/sq cm. The advantage gained would seem to be largely nullified by the chemical manipulations involved. Obviously, none of these methods is suitable for routine use with large numbers of samples of the type characteristically obtained in the enzyme assays presented in this paper, i.e., samples of finite thickness, unknown weight, and variable composition. On the other hand, the technique of using internal standards to correct samples of unknown weight and composition for self-absorption is ideally suited to total activity determinations in enzyme assays that relate enzyme activity to total radioactivity in the reaction products. This method enables one to correct the observed counting rates of tritium-tagged samples of finite thickness to the counting rates one would observe if the total radioactivity of each sample were assayed in an infinitely thin layer. The accuracy of the self-absorption corrections is not affected by the presence of impurities plated with the tritiated reaction products, provided that suitably uniform or reproducible distribution of sample material is attainable on replicate plates.

The ideal condition of exact correspondence between the internal standard and the tritiated compound being assayed cannot be met in enzyme assays of the type described, due to the presence of multiple reaction products. However, this condition can at least be approached by employing as the internal standard the same compound used as the substrate in order to minimize structural differences between the internal standard and tritiated reaction products that might lead to self-absorption differences due to differences in "pattern" of crystallization on the plate (12). Furthermore, it seems likely that the unlabeled material (protein, etc.), which is present together with the reaction products and internal standard in relatively large and constant amount on plates from assays of a given enzyme, may determine a "pattern" of crystallization of its own that would tend to eliminate self-absorption differences between individual tritiated compounds present in trace amounts.

It is evident that in the design of isolation procedures applicable to enzyme assays of the type described in this paper, the essential requirements are (*a*) complete separation of reaction products from the radioactive substrate and (*b*) quantitative recovery of the labeled products present in a measured volume of the reaction mixture. In cases where specific activity values are required, as in isotope dilution analysis,

specific activities can be calculated from total activites provided the sample weights are known (cf. footnote 10). The use of internal standards for making self-absorption corrections thus eliminates the necessity of preparing individual self-absorption correction curves in order to determine the specific activities of pure tritium-tagged compounds in solid samples.

SUMMARY

Solid tritium-tagged samples of finite thickness have been assayed in the Geiger region in a windowless flow counter and corrected for self-absorption by comparison with the counting rates of duplicate samples to which internal standards of tritium have been added. For the determination of absolute activities, measurements of sample weights are not required. Uniform or reproducible distribution of material on replicate plates has been attained through an improved plating technique. The use of this method is demonstrated in assays of thymidine kinase and deoxycytidylic acid deaminase employing thymidine-H^3 and deoxycytidylic acid-H^3 as substrates.

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ANALYTICAL BIOCHEMISTRY 2, 317–323 (1961)

Identification of Minor Flavonoid Impurities Found in Commercial Rutin[1]

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Rutin, the 3-rhamnoglucoside of quercetin (3,3′,4′,5,7-pentahydroxyflavone) is widely distributed in the plant kingdom (1) and is prepared commercially from buckwheat leaves, flower buds of *Sophora japonica*, and Eucalyptus leaves. Commercial rutin which conforms to standards for purity prescribed by the *National Formulary* (2) has been used in numerous investigations involving the possible biological effects of flavonoids, with conflicting and confusing results often having been reported, as summarized in detailed reviews (3, 4). Although *National Formulary* specifications insure a relatively high purity of N.F. quality commercial rutin, requiring that it contain no more than 5% of the aglycone quercetin and 0.004% each of chlorophyll and red pigment, it has been generally recognized that such rutin also contains small quantities of other closely related but unidentified flavonoid compounds. This paper reports the isolation and identification of small quantities of the impurities kaempferol (3,4′,5,7-tetrahydroxyflavone), isoquercitrin (quercetin-3-glucoside), kaempferol-3-rhamnoglucoside, and the 3-rhamnoglucoside of isorhamnetin (3,4′,5,7-tetrahydroxy-3′-methoxyflavone), as well as quercetin, from such rutin. Details of the preparation of chromatographically pure rutin and results of the examination of impurities in *National Formulary* Reference Rutin, which is used as the standard for spectrophotometric analysis of rutin, are also described.

METHODS AND RESULTS

Chromatographic Fractionation of Commercial Rutin, N.F.

Separation of several components present in small quantity in commercial rutin was attained by column chromatography, using Magnesol (Food Machinery and Chemical Corp., N. Y.) as adsorbent. Four grams

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of rutin N.F. IX (S. B. Penick and Co., N. Y.) was dissolved in 200 ml methyl alcohol. This was adsorbed on a 6 × 68 cm column prepared by packing with a slurry of Magnesol (dry-cleaning grade) in methyl alcohol under 5 lb air pressure. The column was eluted with ethyl acetate saturated with water at 20°C.

After the material had moved a short distance down the column, a zone, designated Z-1, yellow in visible and yellow-green in ultraviolet light, moved rapidly off the column. A narrow zone, Z-2, yellow in visible and yellow-brown in ultraviolet light, next moved off the column. As the leading edge of the major portion of the material moved slowly down the column, two more narrow zones, both yellow in visible light, moved out of it. The first, Z-3, was red-brown in ultraviolet light, and the second, Z-4, was yellow-brown. Several days of constant elution under 5 lb air pressure were required for elution of these zones from the column.

The major portion of the original material, Z-5, yellow in visible light and yellow-brown in ultraviolet light, was spread out over the entire column, and appeared to become so tightly adsorbed that further elution with water-saturated ethyl acetate became impossible after only a small portion of this zone had moved off. Most of this zone, plus a narrow zone at the top of the column, Z-6, which appeared brown in ultraviolet light, was eluted, therefore, with 80% ethyl alcohol–20% water. Each of the above fractions was next studied by paper chromatography.

Separation and Identification of Components of Z-1 (Aglycones)

On paper chromatograms of Z-1, using Whatman No. 1 paper, descending chromatography, and *n*-butyl alcohol–acetic acid–water (6:1:2,v/v/v, hereafter referred to as BAW), three spots (R_f's 0.77, 0.87, and 0.94) were visible. These appeared to be flavonol aglycones. Examination of several solvent combinations on chromatostrips (5) coated with silicic acid indicated that some separation of these components could be attained using 16% acetone in benzene. Z-1 was therefore reduced to dryness *in vacuo* and dissolved in acetone, and enough benzene was added to give a solution of 16% acetone–84% benzene. This was placed on a 4 × 56 cm silicic acid column packed from a slurry of 100-mesh silicic acid (Mallinckrodt analytical reagent) in 16% acetone-84% benzene, and the column was eluted with this same solvent.

Two major zones, both yellow in visible light and yellow-brown in ultraviolet light, moved off the column. These were further purified by rechromatographing on silicic acid columns. The first zone which moved off the column gave an ultraviolet absorption spectrum identical with

that of authentic kaempferol (max. 267 and 366 mμ). It was confirmed as kaempferol by direct chromatographic comparison with authentic kaempferol, both unknown and standard giving R_f values of 0.88 in BAW, 0.56 in 60% acetic acid, and 0.50 in nitromethane–benzene–water (2:3:5, v/v/v, hereafter called NBW).

The second zone off the column, which was in much greater quantity, was identified as quercetin by direct chromatographic comparison with authentic quercetin. R_f values obtained were 0.76 in BAW, 0.42 in 60% acetic acid, and 0.09 in NBW. The substance with an R_f of 0.94 in BAW in the original aglycone fraction was present in such small quantity that its isolation in sufficient quantity for conclusive identification has not yet been achieved.

Purification and Identification of Z-2 (Isoquercitrin)

The Z-2 eluate was reduced to dryness *in vacuo,* dissolved in methyl alcohol, and rechromatographed on a Magnesol column as above, although using a smaller column. When this procedure was repeated once more, paper chromatograms indicated a high degree of purity of this Z-2 fraction. On direct comparison with authentic isoquercitrin, purified Z-2 gave R_f values (0.55 in BAW, 0.62 in 15% acetic acid, and 0.79 in 60% acetic acid) and an ultraviolet absorption spectrum (max. 258 and 359 mμ) which were identical with those of the authentic compound.

A small quantity of purified Z-2 was hydrolyzed by refluxing with a few milliliters of 3% hydrochloric acid for 2 hr, and the aglycone was extracted from the hydrolysis mixture with ethyl acetate. By paper chromatography against reference standards, the hydrolyzate was found to contain glucose and quercetin. Hence fraction Z-2 was isoquercitrin (quercetin-3-glucoside).

Purification and Identification of Z-3 (Kaempferol-3-rhamnoglucoside)

The eluted fraction Z-3 was further purified by rechromatographing it on a Magnesol column. The ultraviolet absorption spectrum of this compound had maxima at 267 and 351 mμ, and minima at 239 and 281 mμ. By direct comparison, the R_f values in BAW (0.62), 15% acetic acid (0.66), and 60% acetic acid (0.78) were identical with those for authentic kaempferol-3-rhamnoglucoside isolated from tobacco (6). Upon hydrolysis and paper chromatographic study of the hydrolyzate by procedures such as were described for Z-2 above, Z-3 yielded rhamnose and glucose, and the aglycone kaempferol, confirming its identity as kaempferol-3-rhamnoglucoside.

Purification and Identification of Z-4 (Isorhamnetin-3-rhamnoglucoside)

The eluted fraction Z-4 was purified by rechromatographing it successively through two Magnesol columns. Purified Z-4 gave an ultraviolet absorption spectrum typical for flavonol glycosides, with maxima at 256 and 358 mμ, and minima at 237 and 289 mμ. R_f values for this compound were 0.55 in BAW, 0.62 in 15% acetic acid, and 0.79 in 60% acetic acid. On paper chromatograms Z-4 was brown in ultraviolet light. It appeared yellow–green in ultraviolet when exposed to ammonia fumes and when sprayed with 1% alcoholic aluminum chloride. Though not identical, it was very similar to both rutin and kaempferol-3-rhamnoglucoside.

Upon hydrolysis, by refluxing with 3% hydrochloric acid for 2 hr, and paper chromatographic study of the hydrolysis products, Z-4 was found to yield rhamnose, glucose, and an aglycone which cochromatographed with rhamnetin (3,3′,4′,5-tetrahydroxy-7-methoxyflavone) and isorhamnetin (3,4′,5,7-tetrahydroxy-3′-methoxyflavone) in BAW (0.82), 60% acetic acid (0.50), and NBW (0.78).

Spectral studies, according to the method of Jurd and Horowitz (7), were employed to learn more about the structure of Z-4. When anhydrous sodium acetate was added to an absolute ethyl alcohol solution of Z-4, the short wavelength band of the ultraviolet spectrum was shifted from 256 to 274 mμ, indicating a free hydroxyl group in the 7 position, and eliminating the possibility that Z-4 was a glycoside of rhamnetin. In 0.002 *M* sodium ethylate, the long wavelength band of the Z-4 spectrum was shifted from 358 to 416 mμ, indicating that free hydroxyl groups were not present in both the 3 and 4′ positions. The aglycone produced by hydrolysis of Z-4 had an ultraviolet spectrum with maxima at 254 and 370 mμ, and the long wavelength peak disappeared within 6 min in 0.002 *M* sodium ethylate, indicating that the aglycone possessed free hydroxyl groups in both the 3 and 4′ positions. The brown fluorescence of Z-4 on paper chromatograms, which indicated that the glycoside did not contain a free 3-hydroxyl group on the flavone, and the close similarity of this compound to rutin and kaempferol-3-rhamnoglucoside, indicated that it was probably a 3-rhamnoglucoside.

The aglycone of Z-4 was confirmed as isorhamnetin by alkaline degradation and identification of the degradation products by paper chromatography. About 0.5 mg of the aglycone was mixed with 20 ml of 2 *N* sodium hydroxide in 50% ethyl alcohol–50% water in a 100-ml beaker. The solution was placed in a Bell micro jar wrapped with heating tape to maintain the interior at 135°C. The solution was then evaporated to dryness, with a stream of warm nitrogen being bubbled through to

protect against oxidation. The degradation residue was acidified and extracted with ether, and the ether-soluble degradation products were compared on paper chromatograms with standard substituted benzoic acids and phloroglucinol in several solvent systems (8). The degradation products gave identical R_f values and color reactions in short wavelength ultraviolet light (2537 A), in ultraviolet light when exposed to ammonia, and when sprayed with diazotized benzidine and sulfanilic acid, as those given by standard vanillic acid (3-methoxy-4-hydroxybenzoic acid) and phloroglucinol, indicating that the aglycone of Z-4 was isorhamnetin.

Isorhamnetin-3-rhamnoglucoside, called "narcissin," has been previously isolated from narcissus petals (9) and lily pollen (10). It was reported to consist of yellow needles, m.p. 173–174°. Crystalline Z-4, when examined under the microscope, was found to consist of minute pale yellow needles, m.p. 173–175°. Thus, Z-4 was identified as isorhamnetin-3-rhamnoglucoside.

Preparation of Chromatographically Pure Rutin

The 80% ethyl alcohol washings from the original Magnesol column appeared to contain considerable rutin, plus some dark-brown, water-soluble material which had washed down the column, and water-soluble salts washed from the Magnesol. These washings were reduced to dryness *in vacuo* and extracted with hot methyl alcohol. Paper chromatograms indicated that this extract contained mostly rutin, plus some material which moved only a short distance from the origin when chromatographed in BAW. The extract was adsorbed on silicic acid, and this was slurried in 10% methanol–90% benzene and placed onto a column packed from a slurry of silicic acid in the same solvent. The column was then washed thoroughly with this solvent system. Finally, the methyl alcohol content of the solvent was increased to 20%. Thereupon, a yellow zone, red–brown in ultraviolet light, moved down the column. The yellow crystalline material produced on evaporation of the solvent from this fraction was recrystallized from boiling water. R_f values (0.46 in BAW, 0.61 in 15% acetic acid, and 0.74 in 60% acetic acid), and ultraviolet absorption spectrum (max. 258 and 361 mμ, log $\epsilon = 4.36$ and 4.29; min. 238 and 284 mμ) of the pale-yellow needle crystals produced indicated that they were rutin. The crystalline rutin melted at 190–191° (block), and elemental analysis indicated that it contained three moles of water per mole of rutin, which was removed upon drying with P_2O_5 under 0.1 mm Hg pressure at 126° for 12 hr.

Anal. Calcd. for $C_{27}H_{30}O_{16}$: C, 53.11; H, 4.95. Found: C, 53.18; H, 5.02. Calcd. for $C_{27}H_{30}O_{16} \cdot 3H_2O$: C, 48.80; H, 5.42. Found: C, 48.81;

H, 5.37. $[\alpha]_D^{26°} = +37.2$ in glacial acetic acid, +17.4 in *n*-butyl alcohol.

Although spots indicating compounds other than rutin can be seen on heavily spotted chromatograms of both Penick Rutin N.F. IX and *National Formulary* Reference Rutin when these chromatograms are sprayed with 1% alcoholic aluminum chloride and viewed under very intense ultraviolet light (3660 A, Blak Ray, Ultra-Violet Prod. Inc., San Gabriel, Calif.), similar chromatograms of the rutin produced above showed no such impurities.

The yield of rutin (127 mg) purified in this manner was poor. Much of the rutin remained on the silicic acid column, and higher methyl alcohol content in the solvent was required to remove it. With higher concentrations of methyl alcohol, a contaminating substance of short R_f value in BAW was also removed. This material may be the same as Z-6 from the original column, but its nature has not been determined.

Impurities in National Formulary Reference Rutin

A sample of *National Formulary* N.F. Reference Rutin, Lot No ERRL-226-39, produced from buckwheat and intended for use in adjustment of the spectrophotometer used in the official N.F. assay for rutin, was examined chromatographically in the same manner as described above for Penick rutin. Although the quantities of impurities were smaller than in the case of Penick rutin, the general pattern was very similar, with two exceptions. Isorhamnetin-3-rhamnoglucoside could not be identified in the reference rutin, and isoquercitrin made up a larger portion of the non-rutin glycosides than it did in Penick rutin.

A rough estimate of the amount of glycosides which moved off the Magnesol column prior to rutin was obtained. Eight hundred milligrams of reference rutin was chromatographed on a Magnesol column, and all eluate moving off the column after the aglycones had been removed and prior to elution of rutin was collected as quantitatively as possible. The absorbance of this material was obtained at 267 mμ, and the quantity of impurities, as kaempferol-3-rhamnoglucoside, was read from a standard curve for this compound. Results thus obtained indicated the presence of about 1.8% impurities, which probably represents a minimum value due to losses on the column. This value does not include the aglycones present, nor would it include any glycoside which would move off the column after rutin.

SUMMARY

The following flavonoid impurities have been isolated and identified from N.F. quality commercial rutin: kaempferol, isoquercitrin, kaempferol-3-rhamnoglucoside, quercetin, and isorhamnetin-3-rhamnogluco-

side. Details of the preparation of chromatographically pure rutin and results of the examination of impurities present in N.F. Reference Rutin have also been described.

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Application of Zone Centrifugation to the Study of Normal and Pathological Human Sera

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INTRODUCTION

Since the initial work of Pickels (7) on sedimentation in the angle centrifuge, there has been relatively little application of density-gradient centrifugation to the analysis of serum and other protein mixtures. De Lalla and Gofman (2) have applied flotation procedures to the study of lipoproteins, and Hogeboom and Kuff (6) have used the preparative ultracentrifuge in the determinations of sedimentation coefficients of proteins over a 4 to 2000 S range. The main use of density-gradient centrifugation has been, however, in the fractionation of cellular particles.

Following the success of its application in this field, it seemed worthwhile attempting to develop the technique for the quantitative analysis of protein mixtures on a molecular level. The results obtained by zone electrophoretic methods, such as those developed by Flodin and Porath (4), have already indicated the value of preparative fractionation procedures which afford a measure of the composition of the system under investigation as well as providing fractions for further analysis. Moreover, zone centrifugation of human serum should yield data complementary to that obtained from zone electrophoresis, fractions being separated according to their molecular size rather than charge. The significance of such an approach is well illustrated by the results reported by Wallenius *et al.* (10). These workers studied the distribution of ultracentrifugal components in the various zones obtained by starch-block electrophoresis of human serum and found that nearly all the major electrophoretic fractions were heterogeneous in terms of composition of sedimenting constituents.

An additional advantage of the zone-centrifugation procedure is that it provides a valuable alternative to the use of partition ultracentrifuga-

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tion cells in the characterization of biologically active substances, as was shown recently by Stanworth (9) in a study of the physicochemical properties of horse-dandruff reagin. This report will be confined, however, to a description of the technique employed and its application to the comparative analysis of normal and pathological human sera.

EXPERIMENTAL AND RESULTS

Zone-Centrifugation Technique

In all experiments a *gradient differential* technique has been employed, the tube contents being stabilized against convection and other disturbances by means of buffered sucrose gradients. This is the development of a method used previously by Edelman *et al.* (3) in the purification of rheumatoid factor. Those workers fractionated 1-ml samples of serum (in 5-ml tubes in a swing-out rotor) above layers of 10 and 30% buffered sucrose solutions. A modification of this method was used by Stanworth (9) in the characterization of skin sensitizing antibodies. More recently, however, the technique has been refined with a view to achieving greater resolution of the complex mixture of proteins comprising human serum.

Ideally, of course, maximal resolution would be expected by starting with an infinitely narrow band of an infinitely dilute protein solution. Obviously, for practical reasons, it was necessary to compromise, and so by starting with 0.5 ml serum (in a 5-mm deep band), fractions of sufficiently high concentration for subsequent analysis and biological testing were obtainable. Moreover, by narrowing down the band as compared with the width (10 mm) of the starting sample used in the initial studies, it was possible to extend the effective length of separation.

The arrangement found to give optimum resolution of human serum is that shown in Fig. 1. A combination of 10 and 30% buffered sucrose solutions proved to be the most effective of several tested. Such solutions were dense enough to uphold the starting band of serum and to stabilize the separated protein bands, both during centrifugation (at a temperature of 12°C) and afterward at room temperature. In this medium only the high density lipoproteins (of density greater than 1.063) would sediment. This means that the zone-centrifugation procedure frees the serum of the low density lipoprotein constituents, which remain in the starting layer well above any non-lipid-containing protein zone. In this respect, it is interesting to note that one serum showed three discrete lipoprotein bands near the top of the tube after zone centrifugation.

A small volume (0.3 ml) of carbon tetrachloride at the bottom of the tube is useful in avoiding pellet formation by the high molecular weight

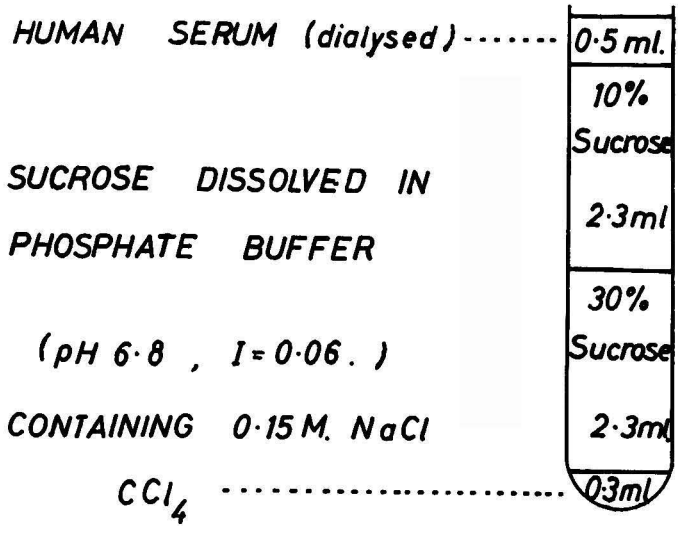

FIG. 1. Zone-centrifugation procedure, showing initial arrangement and positions of slicing of tube.

serum protein constituents—a process which can lead to insolubility and loss of biological activity. With such an arrangement it is most important to preclarify the serum by centrifugation for 30 min at 10,000 rpm and 5°C. Otherwise particulate matter will carry down adsorbed serum proteins nonspecifically, and these become layered above the carbon tetrachloride.

Lusteroid tubes, carefully selected for uniformity in shape and bore, are set up in this way, using solvents precooled to 4°C. They are then centrifuged in precooled buckets in a Spinco SW 39 swing-out rotor (refrigerator setting of —1°C). This cools the tube contents to 12.5°C during the run. Afterward the tubes are immediately sliced in a mechanical slicer of similar design to that described by Randolph and Ryan (8). By slicing systematically with a highly sharpened blade it is possible to obtain as many as 13 sections (each 3 mm deep) from a single tube.

If the tube and blade are well lubricated with silicone grease, 80% recoveries of liquid are achieved indicating an average loss of about 0.08 ml at each slicing. The final fraction is usually sucked off from above the carbon tetrachloride with a pipet. No other form of sampling the contents of the tube after centrifugation has been tried. The successive slicing of tubes containing colored protein solutions indicated that this form of sampling causes only slight disturbance of separated protein zones.

Use of Dye-Labeled Reference Proteins

Selection of suitable positions of slicing of the tubes after zone centrifugation of human sera has been guided by the use of reference proteins labeled with various colored dyes. A purified protein from each of the main sedimenting classes of serum proteins, namely 4.5, 7, and 19 *S*, has been labeled with a different colored dye and used in this way. Thus albumin has been labeled with Evans Blue (and more recently bromophenol blue). γ-Globulin has been labeled with Brilliant Procion Yellow (and Red) and 19 *S* α_2-glycoprotein[3] with Brilliant Procion Red.

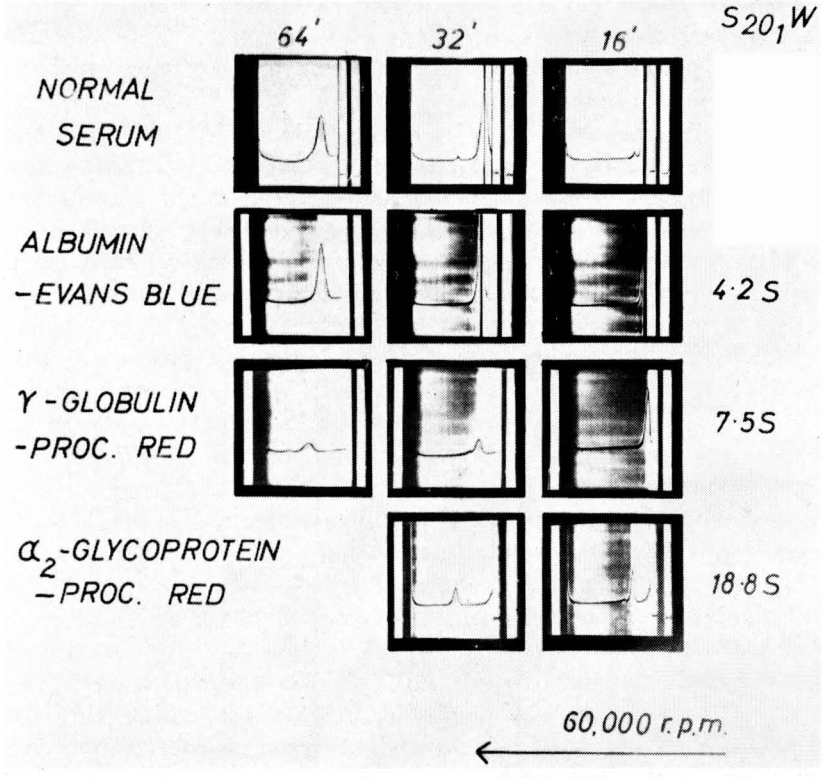

FIG. 2. Analytical ultracentrifugal patterns of dye-labeled albumin, γ-globulin, and α_2-glycoprotein. The sedimentation coefficients ($S_{20,w}$) of the labeled proteins are included.

[3] A purified preparation of this high molecular weight glycoprotein constituent of normal human serum was obtained by a method (D. R. Stanworth, 1960; unpublished work) involving zone centrifugation of a fraction (comprising only 4.5 and 19 *S* components) eluted from a diethylaminoethylcellulose (DEAE-cellulose) chromatography column with 0.05 *M* NaH_2PO_4 solution.

The procion dyes are cellulose-reactive dyes possessing a triazine chloride radical via which they become linked to proteins. They have been used by Hess and Pearse (5) for labeling antigens. It is easy to irreversibly precipitate the protein during the labeling procedure, which is accomplished merely by mixing with dye in buffered (pH 7.4) saline at room temperature for 30–60 min and then dialyzing away the unbound dye. The coupling of γ-globulin with Procion Yellow proved particularly difficult in this respect, whereas it was much easier to couple Procion Red on to this and other proteins.

As is shown in Fig. 2, ultracentrifugal analysis of the labeled proteins in a Spinco model E machine indicated that the dye-binding had not affected the sedimentation coefficients of the proteins. Here the ultracentrifugal patterns of the labeled proteins are compared with the pattern of normal human serum.

Comparative zone centrifugation of the three labeled proteins in separate tubes was carried out at varying times in order to establish optimum conditions for achieving complete resolution of a mixture in the same tube. Such a separation was ultimately accomplished by centrifuging for 16 hr at 39,000 rpm, the carbon tetrachloride layer preventing the pelleting of the labeled 19 *S* α_2-glycoprotein (Fig. 3). Primary colors

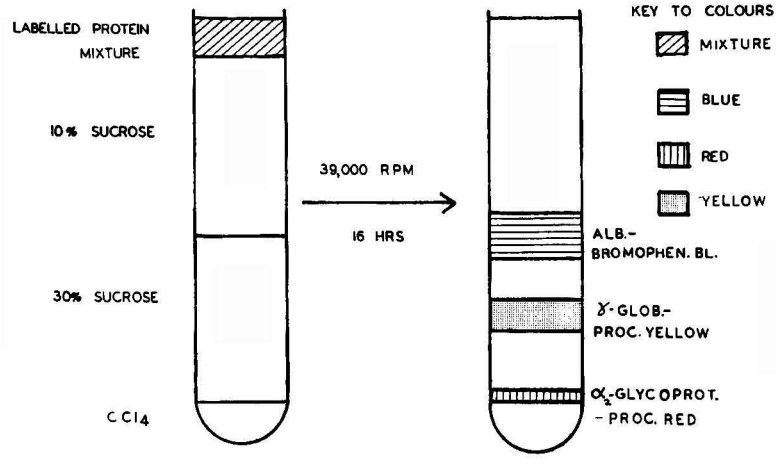

FIG. 3. Result obtained by zone centrifugation of a mixture of dye-labeled albumin (4.5 *S*), γ-globulin (7 *S*), and α_2-glycoprotein (19 *S*).

were chosen deliberately as dye labels so that any overlapping would be revealed as secondary color formation. There was no evidence of any such effect in the separation shown in Fig. 3.

The tubes containing the labeled proteins were afterward sliced according to the separation of the colored zones. Gel-diffusion precipitin analysis of the fractions thus obtained (employing rabbit antihuman serum) revealed only slight overlapping of the labeled proteins. Moreover, ultracentrifugal analysis indicated that the labeled proteins were still intact after zone centrifugation.

It has been the custom to include a tube containing a mixture of the different labeled proteins in each zone centrifugation analysis of human serum. By placing such a reference tube, after the run, directly alongside the serum tubes in a plastic holder, it is possible to select the best positions of slicing in order to obtain maximal separation of 4.5 from 7 *S* serum component and of 7 from 19 *S* component.

Application of the Zone-Centrifugation Technique to the Analysis of Pathological Human Sera

A problem which sometimes confronts the clinical biochemist is illustrated in Fig. 4, which shows the paper electrophoretic patterns of a normal and two different pathological sera (each containing about 12 gm protein/100 ml). It will be noted that both pathological sera contain an abnormally intense protein band in the γ-globulin region. Serum WA is from a patient showing typical myeloma symptoms, whereas serum WI is from a patient showing no such symptoms. The important question left unanswered by the paper electrophoresis is whether serum WI contains abnormal macroglobulins.

Zone-centrifugation patterns of the two pathological sera and the normal serum obtained by the technique just described are shown in Fig. 5. These patterns were obtained by plotting the total protein in each fraction (as obtained by optical density measurements at 280 mμ on diluted aliquots) against the mean distance from the meniscus. The demarcation positions between labeled reference proteins centrifuged simultaneously are illustrated by the vertical dotted lines. The values shown are the percentages of the total serum protein falling in each of the three main zones. It will be seen at once that serum WI can be differentiated from serum WA by means of its marked content of 19 *S* component. On the other hand, serum WA contains considerably more 7 *S* component than is found in serum WI.

These findings are confirmed by examination of the analytical ultracentrifugal patterns of these sera (shown in Fig. 6). Macroglobulin components comprise 53% of the total protein in serum WI, while 7 *S* component comprises 52% of the total protein in serum WA.

Hence, without having carried out an analytical ultracentrifugal

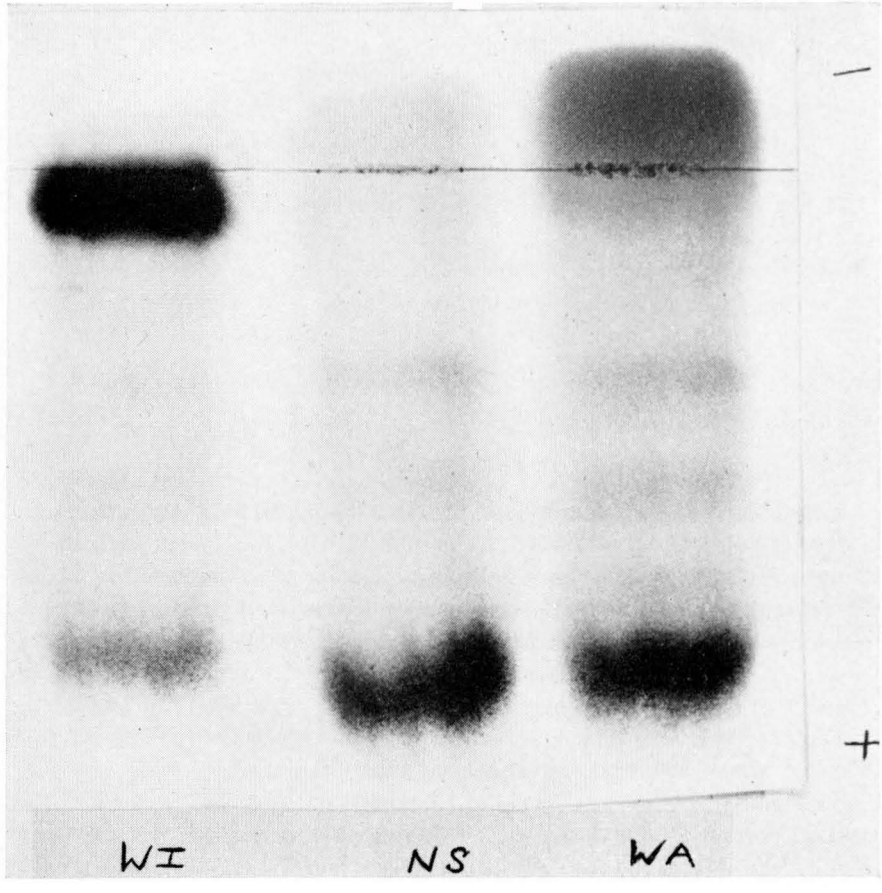

FIG. 4. Patterns obtained by paper electrophoretic analysis (barbitone buffer, pH 8.6, $I = 0.05$) of normal human serum (NS), myeloma serum (WA), and macroglobulinemic serum (WI). Each serum was diluted 1:4 before analysis.

examination of the two pathological sera, it has been possible, by means of a *single* zone-centrifugation analysis, to demonstrate that the serum from patient WI (who failed to show myeloma symptoms) contained an abnormal amount of macroglobulin responsible for the "M" band revealed by paper electrophoresis. Moreover, the pathological serum fractions obtained from the zone-centrifugation procedure were available for further study.

DISCUSSION

Normally, it would be necessary to establish the presence of abnormal macroglobulin in a serum by use of an analytical ultracentrifuge (from

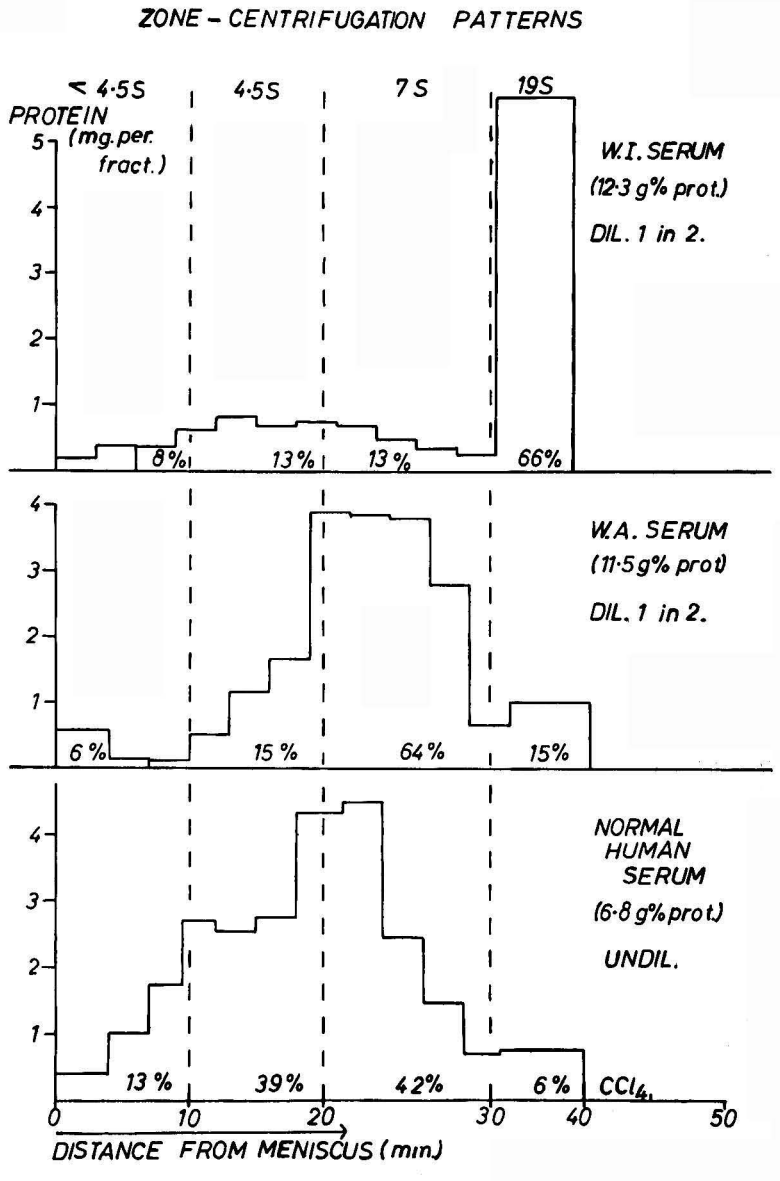

FIG. 5. Zone-centrifugation patterns of normal human serum, myeloma serum (WA), and macroglobulinemic serum (WI). The vertical dotted lines indicate the demarcation levels of the various ultracentrifugal classes of serum proteins as revealed by comparative sedimentation of dye-labeled components. Percentages of total protein falling in the different zones are given.

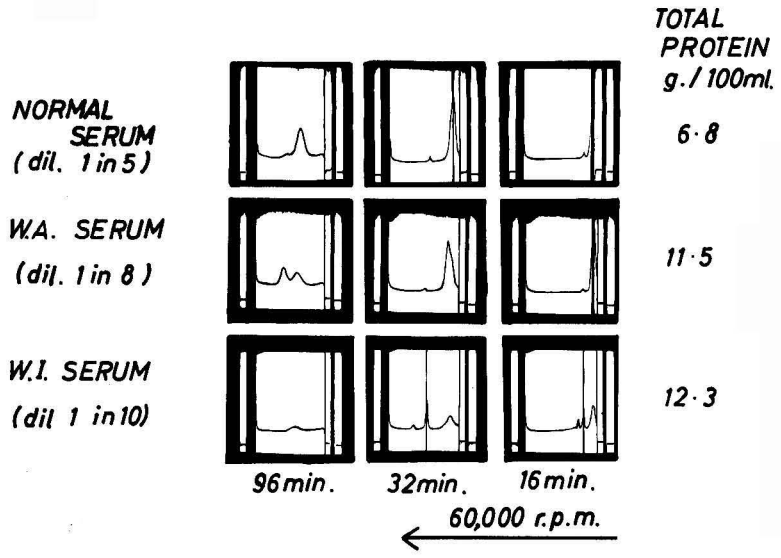

FIG. 6. Analytical ultracentrifugation patterns of normal human serum, myeloma serum (WA), and macroglobulinemic serum (WI). Analyses were carried out at 20°C on diluted sera which had been dialyzed against barbitone buffer (pH 8.6, $I = 0.05$) containing 0.2 M NaCl.

which the separated protein is not recoverable). Providing the concentration of macroglobulin is sufficiently high, however (as in typical cases of Waldenström macroglobulinemia), it can be detected and isolated simultaneously by the zone-centrifugation procedure described here. The inclusion in each run of a tube containing reference proteins labeled with different colored dyes facilitates selection of the positions of slicing of the tubes. Such a practice leads to the isolation of more clear-cut fractions besides serving as an independent index of sedimentation rate.

As might be expected, complete resolution of the relatively low molecular components (3, 4, 5, and 7 S) of whole serum was not achieved. This was particularly noticeable in the zone centrifugation of normal human serum, where comparative paper electrophoresis and gel-diffusion precipitin analyses on the fractions revealed considerable overlapping of the 4.5 S albumin component with a globulin component (probably 5 S siderophilin). In addition, there was some overlapping of the albumin with 7 S γ-globulin.

The results of preliminary zone-centrifugal analyses, involving the use of narrower (2-mm) bands of starting serum, suggest that some of this overlapping could be reduced (at the expense of a reduction in the

yields of the fractions recovered). In this connection the zone-centrifugation pattern of an analbuminemic serum is of interest. A protein deficiency was observed in the 4.5 *S* region, but there was no marked trough as might have been expected in the pattern of a serum containing only 400 mg albumin/100 ml (according to electrophoretic analysis).

There is good reason to assume that the resolution achieved in the zone-centrifugal analysis of serum could be improved even further by adoption of technical refinements such as the use of longer Lusteroid tubes and better methods of sampling. Mixing of the tube contents could be minimized by stricter stabilization of the swing-out rotor during acceleration and decceleration (as employed, for example, by DeDuve *et al.* (1) in the zone centrifugation of cellular particles).

At the moment, it seems necessary to recycle fractions in order to purify serum components other than the 19 *S* macroglobulin. Alternatively, zone centrifugation has been applied to the purification of crude serum protein preparations isolated initially by other complementary fractionation procedures (such as DEAE cellulose chromatography).

SUMMARY

The development of a zone-centrifugation technique suitable for the fractionation of soluble protein mixtures such as human sera is described. This involves gradient differential sedimentation in buffered sucrose at 39,000 rpm in a swing-out rotor.

Selection of optimum positions of slicing of the tubes (Lusteroid) after centrifugation is indicated by the use of reference proteins of various molecular sizes (4.5, 7, and 19 *S*) labeled with different colored dyes. Pelleting of the high molecular weight (19 *S*) components of serum is avoided by placing a layer of carbon tetrachloride on the bottom of the tube.

Characteristic histograms, representative of serum ultracentrifugal composition, are obtained by plotting the optical densities (at 280 mμ) of fractions (zones) against their distance from the meniscus. It is, for example, possible to distinguish between myeloma and macroglobulinemic sera.

ACKNOWLEDGMENTS

The authors are grateful to Miss P. Ratcliff for carrying out ultracentrifugal analyses in a Spinco model E machine generously donated by The Rockefeller Foundation, New York; to Dr. K. W. Walton for assistance with the labeling of protein samples with dyes. The Procion dyes were kindly supplied by Imperial Chemical Industries, Ltd., Dyestuffs Division, Blackley, Manchester.

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ANALYTICAL BIOCHEMISTRY 2, 335–343 (1961)

A Procedure for Isolating Stable Mitochondria from Rat Liver and Kidney

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In previous communications (1, 2) we have presented evidence that rat liver mitochondria, when isolated under rigorously controlled conditions, retain their capacity for oxidative phosphorylation after treatments generally considered to be highly detrimental. In particular, a study was described of the unusual stability of oxidative phosphorylation in liver mitochondria that were stored at 4° for prolonged periods. As this stability could not be related to any particular factor, we now believe that it is the meticulous attention given to the minutiae of the isolation procedure that yields stable mitochondria. Although most of the procedures for isolating mitochondria currently in use are essentially those first described by Hogeboom, Schneider, and Pallade (3), there are wide variations in the interpretation and implementation of the original scheme. The present paper describes in detail a procedure that has consistently yielded liver and kidney preparations of high stability in our laboratory.

EXPERIMENTAL

Liver Mitochondria[2]

A nonfasted, adult Sprague-Dawley rat (200–250 gm) was killed by a sharp blow in the cervical region. Adequate liver tissue was rapidly excised and transferred to approximately 200 ml of cold 0.25 *M* sucrose at 1° (total time for this operation was 60 sec or less depending on the amount of tissue required). The tissue was allowed to chill for a few minutes, during which time the temperature increased no more than 2°,

[1] With the technical assistance of C. Elwood Claggett.

[2] The entire procedure, which was carried out rapidly without any delay between the individual steps, required approximately 1.5 hr. It should be emphasized also that only scrupulously cleaned glassware was used throughout.

and was placed in the chilled cylinder of a Delepine Press.[3] A small amount of the initially extruded pulp and blood was discarded, and a desired amount of the remainder was extruded into a tared beaker containing a measured portion of cold (1°) 0.25 *M* sucrose. The pulped tissue plus sucrose was added to the tissue grinder[4] containing sufficient additional chilled sucrose solution to maintain a nine-volume ratio in respect to the wet weight of the pulped tissue. The grinding was slow until the first complete pass of the pestle; the speed was then increased to 1000 rpm, and homogenization proceeded at this speed for exactly 1 min. During the procedure the tissue grinder was kept immersed almost completely in a suitable vessel containing cracked ice and water. The temperature of the homogenate did not rise above 4° during disruption of the tissue.[5]

The homogenate (pH 7.0–7.1) was transferred to prechilled plastic centrifuge tubes which were balanced in beakers containing ample cracked ice; thus the temperature was rigorously maintained at 0–1° at all times. The homogenate was centrifuged at 600 × *g* for 10 min in an International refrigerated centrifuge, model PR-1, equipped with the multispeed attachment. The chamber temperature[6] was 1–2°. After the first centrifugation, the pellet (unbroken cells, nuclei, erythrocytes, cell debris) was discarded and the supernatant fluid was removed by chilled Pasteur pipet to other prechilled plastic tubes held in cracked ice, and then recentrifuged at 8500 × *g* for 10 min. After this first sedimentation of mitochondria, the layer of fat which rose to the top was removed carefully with cotton-tipped applicators. The supernatant fluid, along with

[3] A. H. Thomas Co., Philadelphia 5, Pa., Cat. No. 8485. Only perforated disk C was used. The press was chilled in a refrigerator about 30 min prior to its use. Overchilling (as in a deep freezer) should be avoided because of subsequent freezing of the tissue. Although other presses have not been used in our laboratory, any similar device should be satisfactory.

[4] Tissue grinders equipped with Teflon pestles (A. H. Thomas, Cat. No. 4288-B) have proven very satisfactory. The pestle was mounted in a motor of the "Mix-Master" type (Fisher Scientific Co., Cat. No. 14-499) whose original sleeve-type chuck was replaced by the more convenient Jacobs chuck. It was also advantageous to control the speed of the motor by a foot-operated rheostat.

[5] The preparation of the homogenate is possibly the most critical step of the entire procedure. Experiments with ethylenediamine tetraacetate (2) suggest that the mitochondria are particularly vulnerable during their release from the broken cells.

[6] The thermometer mounted on the instrument may not be adjusted accurately; the chamber temperature should be checked independently with an accurate thermometer.

any free-flowing material, was discarded by decantation, and sufficient cold sucrose was added so that for each gram of initial liver tissue, 2 ml of 0.25 *M* sucrose was used. The tubes, held in cracked ice, were taken into the cold (4°) room in order to avoid undue exposure of the suspension to room temperature. The mitochondrial pellet was broken by rubbing with a stirring rod, after which a uniform suspension was obtained by gentle aspiration with a Pasteur pipet equipped with a rubber bulb. Frothing was avoided as much as possible. The mitochondria were resedimented at 8500 × *g* for 10 min, and the washing procedure was repeated.[7] Usually, after the second washing, much free-flowing material was discarded. The mitochondria were suspended finally in 0.25 *M* sucrose so that each milliliter represented the mitochondria from 1.0 gm wet weight of original tissue. The average N content (micro-Kjeldahl) was 3.0 mg/ml. The pH of the mitochondrial suspension was 7.0–7.1, and it remained unchanged during storage.

Kidney Mitochondria

The organs of two or more animals were quickly excised and chilled in cold 0.25 *M* sucrose. The chilled kidneys were transferred to a glass block set on cracked ice where the perirenal fat, fascia, and capsule were rapidly removed. The organs were pulped by passing them through the chilled tissue press, and a 10% homogenate in 0.25 *M* sucrose was prepared as described for liver. The isolation of mitochondria was similar to that employed for liver. Each milliliter of the final suspension was adjusted to represent the mitochondria obtained from 1.0 gm wet weight of pulped tissue. The average N content was 2.0 mg/ml.

Analytical Procedures

Oxidative phosphorylation was determined as previously described (2). Oxygen uptake in the respiratory control experiments was measured in a rotating cuvette with a stationary platinum electrode following the procedure described by Chance and Williams (4) [cf. Hagihara (5)]. Other pertinent analytical details are given in the legends to the table and figures.

[7] The number of washings does not appear to be an important factor for obtaining stable mitochondria. For example, liver mitochondria that were washed once, twice, or four times exhibited similar stability for oxidative phosphorylation after prolonged storage at 4°. It is of interest also that repeated washings do not remove endogenous substrates from mitochondria; these substrates may be related to their stability [cf. (11)].

RESULTS AND DISCUSSION

Oxidative Phosphorylation

Table 1 summarizes the results of determinations of the stability of oxidative phosphorylation in mitochondria isolated from liver and kidney. The general plan of these experiments was the same as previously described (2). Mitochondria were assayed immediately after isolation, and again after prolonged storage at 4°. It may be seen in Table 1 that,

TABLE 1
STABILITY OF OXIDATIVE PHOSPHORYLATION[a]

Substrate	Hours after isolation	O	P	P/O	O	P	P/O
Succinate		Liver (12)[b]			Kidney (11)[b]		
	0	8.8	16.7	1.9	20.4	38.0	1.9
	24	9.1	16.0	1.8	20.9	30.8	1.5
	48	10.0	13.0	1.3	18.2	23.5	1.3
	72	12.1	9.2	0.8	15.3	9.4	0.6
β-Hydroxybutyrate		Liver (32)			Kidney (5)		
	0	5.8	17.0	2.9	13.7	25.0	1.8
	24	5.1	14.2	2.8	12.0	16.0	1.3
	48	5.1	10.8	2.1	12.6	13.5	1.1

[a] Each flask contained, in a final volume of 2 ml, 80 μmoles glycylglycine buffer, pH 7.4; 20 μmoles succinate or 50 μmoles DL-β-hydroxybutyrate; 20 or 40 μmoles orthophosphate, pH 7.4; 5 μmoles ADP; 2 μmoles DPN; 0.03 μmole cytochome c, 50 μmoles glucose; 0.5 mg hexokinase (Sigma Chemical Company, Type III), 10 μmoles $MgCl_2$, and 0.5 ml mitochondria. Incubated in air for 20 min at 30°. The values are expressed as μatoms (oxygen) or μmoles (phosphorus) per mg mitochondrial N.

[b] The figures in parentheses refer to the number of individual experiments.

with succinate as substrate, kidney mitochondria were as stable as those of liver. Even after 48 hr of storage these preparations still retained a high capacity for coupled phosphorylation. When β-hydroxybutyrate was used as the substrate, liver mitochondria displayed stability comparable to that found with succinate, although the kidney mitochondria were somewhat less stable.

Respiratory Control[8]

An important biochemical characteristic of intact ("tightly coupled") mitochondria is their negligible oxidation of added substrate in the absence of phosphate and a terminal phosphate acceptor (6); that is,

[8] These experiments were conducted while the author was a guest of the Wenner-Gren Institute in Stockholm, Sweden.

they exhibit "respiratory control." As seen in Fig. 1, the oxidation of succinate by the freshly isolated liver and kidney preparations was greatly accelerated by the addition of ADP.[9] Respiratory control ratios, obtained by dividing the rate of succinate oxidation prior to the addition of ADP into that obtained after added ADP were 8.4 for liver and 6.0 (average) for kidney. These ratios are especially favorable in view of the fact that succinate, and not a DPN-linked substrate, was used in these experiments [cf. (7)]. Where all of the added ADP was phosphorylated, the respiration decreased to a very low rate which was again markedly stimulated by the addition of more ADP.

The respiratory control was considerably diminished in mitochondria which were stored for 24 hr, although even with these preparations the addition of ADP stimulated the respiration more than twofold. However, unlike the case with the fresh preparations, the respiration did not cease after the short period of time required for all of the added ADP to be phosphorylated, but continued until the oxygen was exhausted. Indeed, as may be seen in Fig. 1, the acceleration of the rate of respiration following the addition of ADP to the stored preparations always increased after an initial lag period, rather than decreased.

A plausible explanation for the failure of the stored preparations to exhibit the same pattern of respiratory control as shown by the fresh ones may reside in their increased ATPase activity. The liver and kidney mitochondria when freshly isolated displayed negligible ATPase activity, but after 24 hr of storage usually there was a marked increase in this activity [cf. (2)]. Thus, in the stored mitochondria the increased ATPase activity would make available a continuously regenerated supply of ADP. It has been suggested by Chance and Baltscheffsky (8) that a more critical test for respiratory control is to divide the respiratory rate with added ADP by the rate observed after the ADP has been phosphorylated. By their criterion the stored preparations would lack respiratory control since the respiration did not diminish, following the initial acceleration upon addition of ADP. However, as pointed out by Gregg, Heisler, and Remmert (9), this criterion is not always suitable for a test of respiratory control, especially with preparations exhibiting high ATPase activity [cf. Azzone *et al.* (10)]. It is not known whether the loss of respiratory control and the increased ATPase activity exhibited by the stored mitochondria is owing to the method of their preparation or to the conditions under which they were stored.

It is of interest that the stored mitochondria, although greatly diminished in their capacity for respiratory control, still catalyzed

[9] Abbreviations: DPN, diphosphopyridine nucleotide; ATP and ADP, adenosine tri- and diphosphates, respectively.

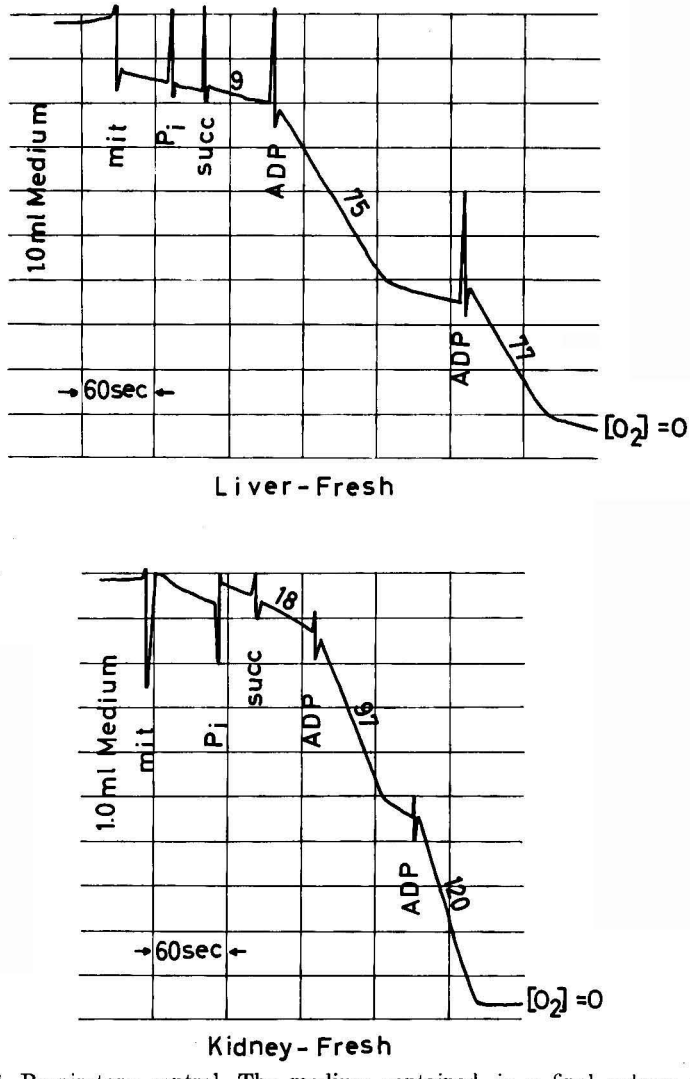
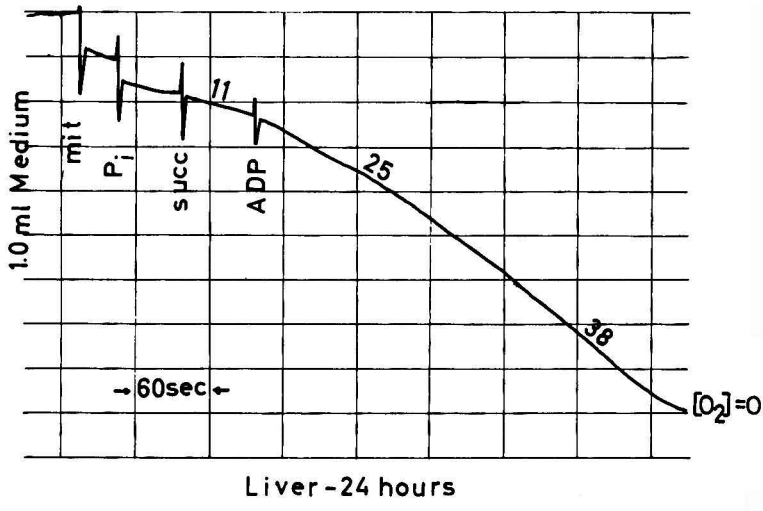


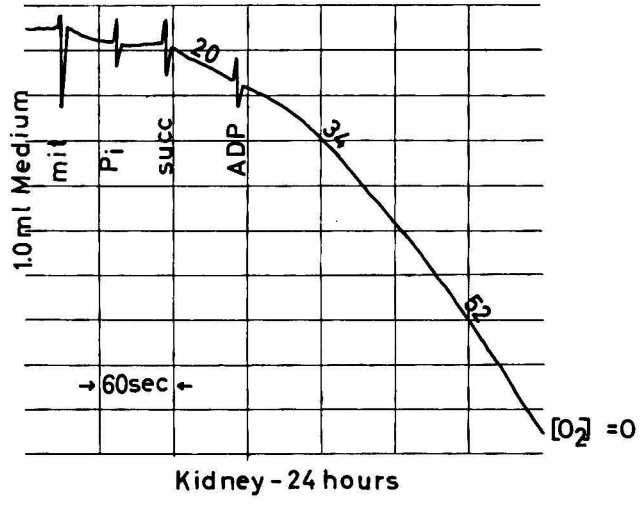
FIG. 1. Respiratory control. The medium contained, in a final volume of 1 ml, 120 μmoles KCl, 20 μmoles glycylglycine buffer, pH 7.4, and 8 μmoles $MgCl_2$. Mitochondria (mit) 0.1 ml, 5 μmoles orthophosphate (P_i), 10 μmoles succinate (succ) and

net oxidative phosphorylation with high P/O ratios (Table 1). Other examples of this interesting phenomenon are discussed in Ref. (7).

Endogenous Phosphorylation

In a separate communication (11) evidence has been presented that liver and kidney mitochondria, isolated under the conditions described





0.5 μmole ADP were added at the points indicated. The numerals refer to the oxidation rate of succinate which is given in mμmoles oxygen/min.

in this report, catalyzed a substantial uptake of phosphate coupled to the oxidation of endogenous substrates, and that this endogenous activity may be related to their stability. For example, when assayed under the conditions described in Table 1, but without added substrate, mitochondria from liver catalyzed an average P uptake of 4.3 μmoles/mg N/20 min, and those from kidney, 3.4 μmoles/mg N/20 min. It appears

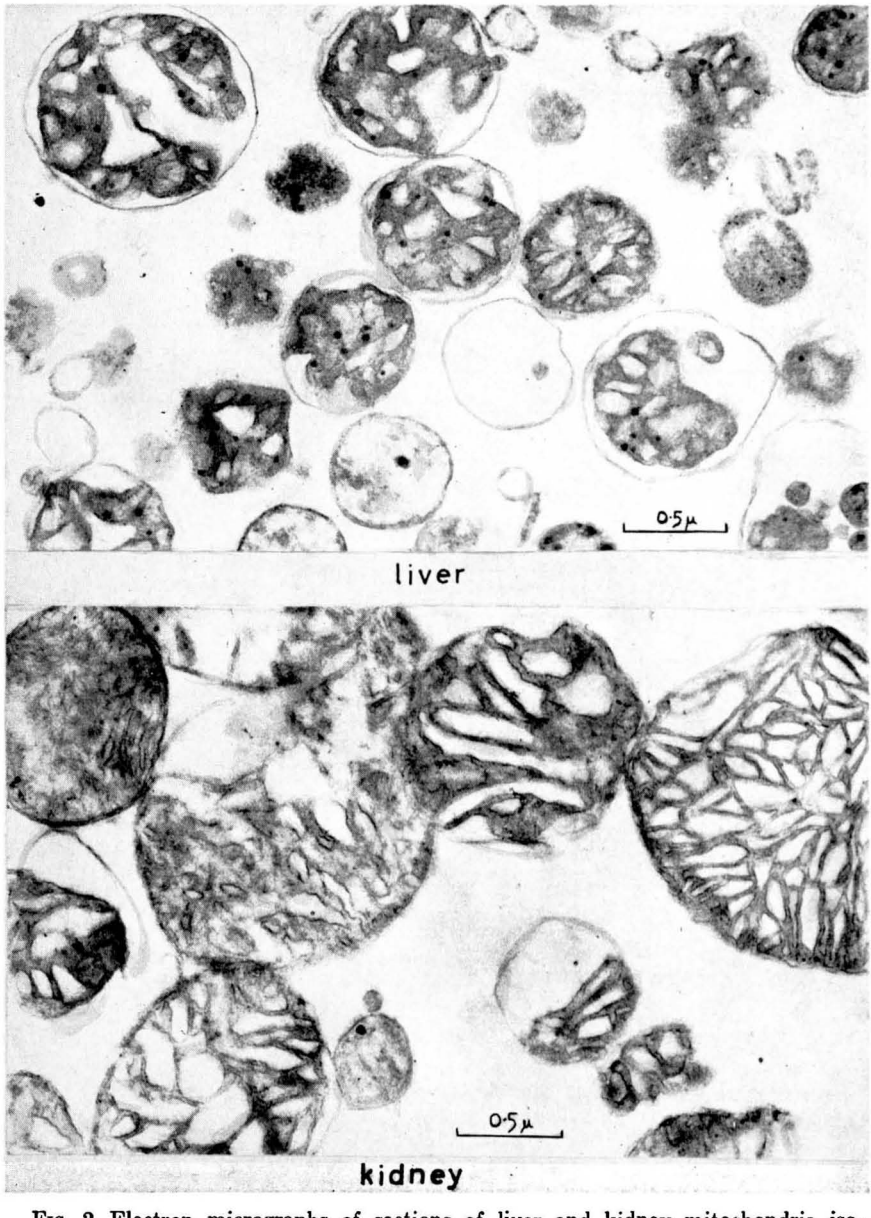


FIG. 2. Electron micrographs of sections of liver and kidney mitochondria isolated in 0.25 *M* sucrose. Samples were fixed in 1% osmium tetroxide at pH 7.2–7.4, dehydrated in graded alcohols, and examined with a Siemens microscope. Note that, as also seen *in situ*, the kidney mitochondria are larger than those of liver and have many more cristae mitochondriales.

that the capacity for endogenous phosphorylation may be an important biochemical characteristic of stable mitochondria.

Morphology[8]

In view of the unusual stability exhibited by these preparations, it was of interest to examine their fine structure with the electron microscope. Although, as illustrated in Fig. 2, no distinctive or unusual morphological features could be detected in the freshly isolated mitochondria, they did appear to be somewhat less swollen than those usually obtained in 0.25 *M* sucrose [cf. (12)]. However, no apparent morphological basis could be found for the stability of these mitochondrial preparations.

SUMMARY

A procedure based on standard methods of differential centrifugation is described in detail for the isolation of rat liver and kidney mitochondria. Mitochondria isolated under the conditions described retain their capacity for oxidative phosphorylation after prolonged storage at 4° and other procedures generally considered to be detrimental [cf. (2)]. The freshly isolated preparations are characterized by high respiratory control ratios with succinate as substrate, and a high rate of endogenous phosphorylation.

ACKNOWLEDGMENT

It is a pleasure for the author to thank Miss Jane Baxandall for her generous assistance in performing the electron microscopy.

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ANALYTICAL BIOCHEMISTRY 2, 344–352 (1961)

Two-Component Phases in Gas Chromatographic Separations of Steroids

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INTRODUCTION

One of the most valuable features of gas chromatographic techniques lies in the ability to alter separation patterns by changing liquid phases. This effect may be used in two ways. When the objective is to achieve a separation for analytical or preparative purposes, selective phases are almost always used. A second use of selective phases is to determine the nature and number of functional groups in compounds of unknown structure.

In both instances it has been customary to rely on the availability of a variety of liquid phases to obtain the desired degree of selective retention of individual components. In the present study the possibility of using two different liquid phases in a single column was explored. Experiments were carried out with columns containing a two-component liquid phase and with columns containing segments with different phases. The use of selective phases, both singly and in mixtures, was explored from the point of view of using these methods for identifying functional groups in steroids.

MATERIALS AND METHODS

The gas chromatography system (Barber-Colman) was used with glass *U*-tubes 6 ft $\times$ 3.4 mm I.D. for mixed-phase experiments, and with 6 ft $\times$ 4 or 5 mm I.D. tubes for other experiments. A Lovelock argon ionization detector containing a radium foil source was used. The support was Gas-Chrom P, 100–140 mesh, and a solution technique was used to coat the silane-treated support (1). The desired amounts of the two polymers were used in an appropriate solvent, usually toluene; coating of the support with this solution gave the desired packing. The liquid phases were silicone polymer SE-30 (General Electric Co.), ethylene glycol isophthalate (EGIP) (Applied Science Laboratories, Inc.), fluoroalkyl silicone polymer QF-1-0065 (Dow Corning Corp.),

[1] Visiting Scientist from the University of Turku, Finland.

and neopentyl glycol succinate (NGS) (a gift from Dr. J. W. Corse). The amount of liquid phase on the support was verified in each instance by exhaustive solvent extraction of a 1-gm sample of the packing. A 1% level for the liquid phase (0.75% for NGS) was chosen since this is useful for steroid separations. Weighed amounts of column packing were used in each experiment, and in each case column conditioning was carried out for 12 hr at 240°.

The techniques of column packing and instrument operation were those developed and used in recent work on the separation of steroids by gas chromatography (2). The columns were filled under argon pressure. The column temperature was 210° or as otherwise indicated in the tables. The argon pressure was adjusted in the range 10–25 psi to give a flow rate of 50–52 ml/min for each column in the two-component phase experiments. The "flash heater" was maintained at 260–270°.

Steroids were obtained from commercial sources or were made by established procedures. Cholestane was used as a reference compound in calculating retention times. Samples were introduced in acetone or tetrahydrofuran solution. The usual sample size was 1–2 μl of 5% solution for multicomponent mixtures, and 0.1–1 μl of 1–5% solution for individual compounds.

RESULTS

Table 1 contains relative (to cholestane) retention times observed with different liquid phases for a variety of steroids. These times depend

TABLE 1
RELATIVE RETENTION TIME RELATIONSHIPS FOR STEROIDS WITH DIFFERENT LIQUID PHASES

Compound	SE-30	EGIP	QF-1[a]	NGS[b]
Cholestane	1.00[c]	1.00[d]	1.00[e]	1.00[f]
Cholestanyl trifluoroacetate	1.64	1.87	3.23	2.22
Cholestanyl methyl ether	1.86	3.15	2.31	2.56
Cholestane-3β-ol	2.06	8.12	3.30	6.34
Cholestane-3α-ol	2.02	6.70	2.96	5.78
Cholestane-3-one	2.21	9.62	6.77	6.78
Cholestanyl acetate	3.06	7.05	5.76	6.03
Androstane-17-one	0.20	1.02	0.60	0.63
Androstane-3,17-dione	0.42	11.4	4.82	5.15
Pregnane-3,20-dione	0.62	13.1	5.43	6.45
5α-Pregnane-3β,20β-diol	0.65	12.0	1.94	6.47
5α-Pregnane-3,20-dione	0.69	15.4	5.93	7.20

[a] 195°, 12 psi.
[b] 24 psi.
[c] Time, 9.2 min.
[d] Time, 4.8 min.
[e] Time, 4.15 min.
[f] Time, 3.65 min.

upon the degree of solute–solvent interaction. In general, the presence of an oxygen-containing functional group leads to an increase in retention time when a polar phase is used, but the results show a high degree of specificity. For example, the relative retention time for cholestane-3β-ol varies from about 2 to 8, depending upon the phase. The order of increasing solute–solvent interaction for these phases with respect to the hydroxy group is SE-30 < QF-1 < NGS < EGIP. The retention time relationships observed for cholestane-3-one, in terms of increasing solute–solvent interaction, is SE-30 < NGS = QF-1 < EGIP. These effects extend to relatively nonpolar functional groups, and they may be emphasized for polyfunctional compounds. For example, for cholestanyl methyl ether and cholestanyl acetate the order of increasing solute–solvent interaction is the same as that observed for cholestanol. The effect of a trifluoracetoxy group did not parallel that of the acetoxy group; the order observed for cholestanyl trifluoracetate was SE-30 < EGIP < NGS < QF-1. A very striking effect was noted for diols and

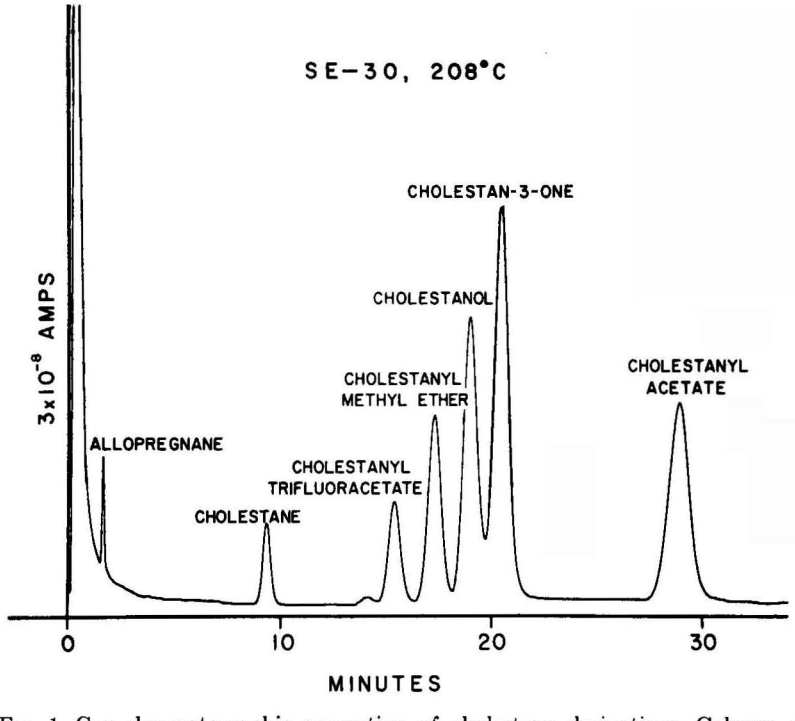

FIG. 1. Gas chromatographic separation of cholestane derivatives. Column conditions: 6 ft × 4 mm I.D. glass column; 0.75% SE-30 silicone polymer on 100–140 mesh Gas Chrom P; 208°; 16 psi.

diones. The order of phases showing increasing retention times was SE-30 < QF-1 < NGS < EGIP, with a very large increase occurring for the EGIP phase. The selective effect shown by EGIP for diols and diones is reflected in the data for monofunctional alcohols and ketones when compared with those for diols and diones.

Two-component phase effects were studied by making a comparison of retention times observed with five columns. An SE-30 phase was taken as the most nearly nonpolar of all phases now known (in other experiments it has been shown that SE-30 is less polar than Apiezon L or M), and Fig. 1 shows a separation of cholestane derivatives observed with an SE-30 column. A polar extreme was an EGIP column, and three columns with 3:1, 1:1, and 1:3 mixtures of SE-30/EGIP were employed to give the data in Table 2. In order to compare functional

TABLE 2
VALUES OF T INDICATING SELECTIVE RETENTION OF STEROIDS WITH TWO-COMPONENT COLUMNS OF SE-30/EGIP

Compound	$T_{25\%}$	$T_{50\%}$	$T_{75\%}$	$T_{100\%}$
Cholestane	0	0	0	0
Cholestanyl trifluoroacetate	0	−0.03	0.01	0.15
Cholestanyl methyl ether	0.04	0.06	0.28	0.70
Cholestane-3β-ol	0.24	0.53	1.53	2.94
Cholestane-3α-ol	0.19	0.41	1.08	2.32
Cholestane-3-one	0.28	0.60	1.56	3.36
Cholestanyl acetate	0.10	0.21	0.62	1.30
Androstane-17-one	0.36	1.21	2.02	4.10
Androstane-3,17-dione	2.30	5.75	11.5	26.0
Pregnane-3,20-dione	1.76	4.38	9.30	20.5
5α-Pregnane-3β,20β-diol	1.47	3.48	7.72	17.6
5α-Pregnane-3,20-dione	1.87	4.65	9.65	21.9

group effects, the value T was taken as:

$$T = \frac{t_s' - t_n'}{t_n'}$$

where t_s' is the relative (to cholestane) retention time observed with a selective phase and t_n' is the corresponding relative time observed with a column containing the same amount of nonselective (SE-30) phase. Table 2 contains values of T for the per cent selective phase indicated by the subscript. These values reflect the extent of solute–solvent interaction for each compound and phase. When they are presented in graphical form, as in Fig. 2, three families of curves are evident. Tri-

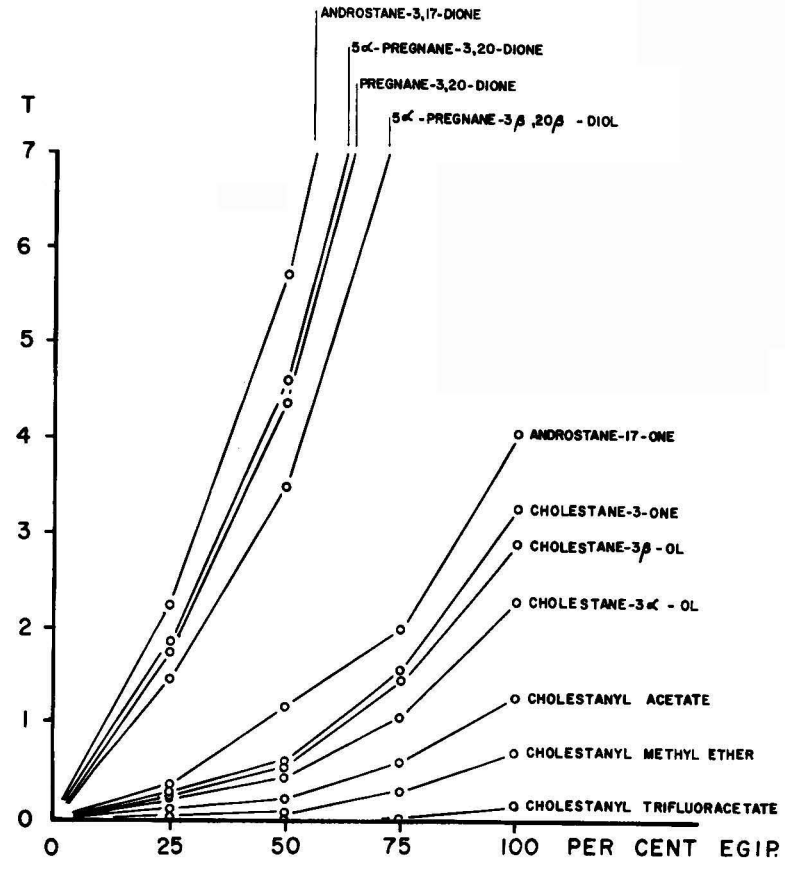

FIG. 2. Effect of polarity of the stationary phase on the retention times of several steroids given as relative to cholestane and SE-30 values (for definition of T, see text). Note the selective retention observed for various functional groups.

fluoroacetoxy, methoxy, and acetoxy functional groups show very little evidence of selective retention. Compounds containing a single hydroxy or keto group show a selective retention behavior. The effect is slightly greater for ketones than for alcohols, but the effects are of the same magnitude. For diols and diones, a marked selective retention behavior leads to another family of curves in which the selective retention effect is again slightly greater for ketones than for alcohols.

Values of T may also be calculated for selective phases by using data obtained with appropriate columns. Table 3 contains T values for QF-1 and NGS phases, and EGIP values are included for comparison. Each

TABLE 3
VALUES OF T' FOR SELECTIVE PHASES FOR STEROIDS WITH DIFFERENT FUNCTIONAL GROUPS

Compound	EGIP	QF-1	NGS
Cholestane	0	0	0
Cholestanyl trifluoroacetate	0.15	0.97	0.35
Cholestanyl methyl ether	0.70	0.24	0.38
Cholestane-3β-ol	2.94	0.60	2.08
Cholestane-3α-ol	2.32	0.47	1.86
Cholestane-3-one	3.36	2.01	2.02
Cholestanyl acetate	1.30	0.88	0.97
Androstane-17-one	4.10	2.00	2.17
Androstane-3,17-dione	26.0	10.5	11.3
Pregnane-3,20-dione	20.5	7.78	9.43
5α-Pregnane-3β,20β-diol	17.6	1.98	8.96
5α-Pregnane-3,20-dione	21.9	7.59	9.43

functional group or combination of functional groups gives characteristic values.

The preparation of two-component phase columns requires the preparation of individual column packings for each experiment. A more convenient method for preparing columns containing two different phases lies in using weighed proportions of individual column packings which are placed in the column in segments. If there is no interaction between solvents when two-component phases are used, it would be expected that relative retention times would be the same for these two circumstances. A 3:1 SE-30/EGIP segmented column was prepared and tested to determine column behavior. There was no change in the general appearance of the gas chromatographic separations with a column of this kind; Fig. 3 shows a typical separation. The observed relative (to cholestane) retention times are in Table 4, along with data from a column with a 3:1 SE-30/EGIP two-component phase. For monofunctional compounds, the column behavior was identical under both circumstances. For difunctional compounds, the behavior was slightly different. Lower retention times were observed for the segmented column than for the two-component phase.

No evidence was found during the course of several weeks for a change in column behavior due to unequal bleed rates (both phases are quite stable at 190–220°), but if phases of widely differing stability are used together it may be expected that the behavior of a column with a two-component phase or segmented packing would change with use.

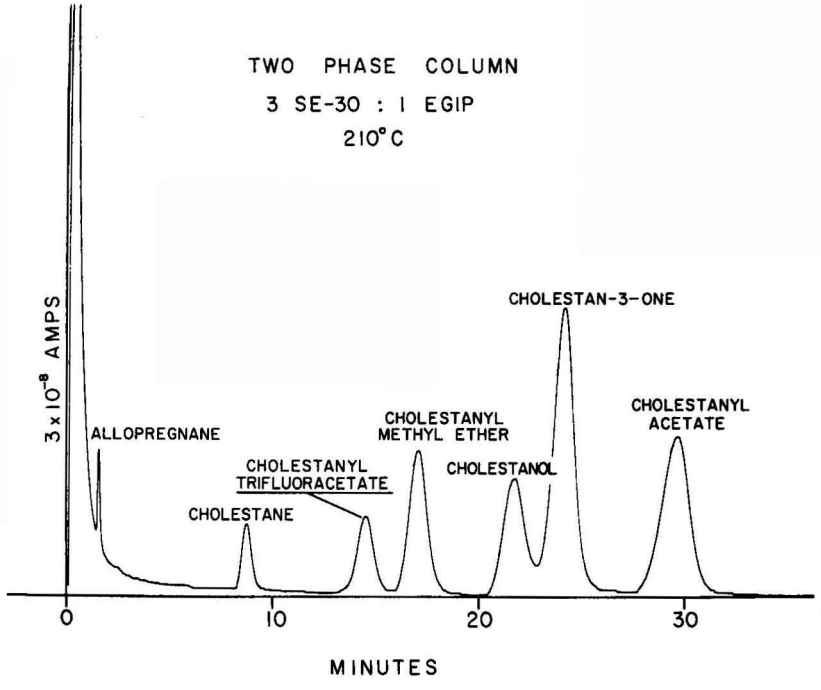

FIG. 3. Gas chromatographic separation of cholestane derivatives on a "segmented packing" column. Column conditions: 6 ft × 3.4 mm I.D. glass column; three parts 1% SE-30 silicone polymer on 100–140 mesh Gas Chrom P and one part 1% ethylene glycol isophthalate on 100–140 mesh Gas Chrom P; 210°; 24 psi.

TABLE 4

RELATIVE RETENTION TIMES FOR STEROIDS WITH 3:1 SE-30/EGIP COLUMNS

Compound	Two-component phase	Segmented packing
Cholestane	1.00[a]	1.00[b]
Cholestanyl trifluoroacetate	1.64	1.66
Cholestanyl methyl ether	1.93	1.95
Cholestane-3β-ol	2.56	2.50
Cholestane-3α-ol	2.42	2.36
Cholestane-3-one	2.82	2.79
Cholestanyl acetate	3.36	3.39
Androstane-17-one	0.27	0.26
Androstane-3,17-dione	1.37	1.20
Pregnane-3,20-dione	1.72	1.51
5α-Pregnane-3β,20β-diol	1.60	1.42
5α-Pregnane-3,20-dione	1.97	1.74

[a] Time, 7.0 min.

[b] Time, 7.85 min.

DISCUSSION

Selective phases are widely used in gas chromatography to separate compounds of approximately the same molecular size but with different kinds of functional groups, or with different stereochemical arrangements of functional groups. Since selective retention is due to solute–solvent interaction, it is possible to use retention time data obtained with selective phases as a means of characterizing functional groups. Two very broad approaches to this problem were developed by Wehrli and Kováts (3) and by Brown (4). Both approaches were designed for use with compounds of unknown size containing functional groups of unknown kind and number. Three sorts of phases—nonselective, selective electron-attracting, and selective electron-contributing—have been described by Brown (4), and it is clear that retention data obtained with two or three phases would be extremely useful in characterizing a compound whose structure is unknown in all respects. However, in many biological problems the class of compound is known even though the structure may be uncertain, and in these instances gas chromatographic data may have still greater usefulness in determining rather precisely the number and kind of functional groups present in the compounds under study. The T values calculated for a variety of steroids suggest that phases SE-30, EGIP, and QF-1 are particularly useful for steroid work, and that hydroxy, keto, methoxy, acetoxy, and trifluoroacetoxy compounds may be differentiated from each other.

Columns containing two-component phases and segmented packings are just as satisfactory from the point of view of column efficiency and column behavior as those prepared with single phases, although the column properties may change with time if there is a great difference in bleed rate for the phases. No particular advantage is gained by using two-component phases with monofunctional steroids, but for polyfunctional compounds this might be desirable in order to introduce relatively small selective effects. For example, diols and diones could be differentiated from monofunctional steroids with a 1:1 SE-30/EGIP column as well as with a column containing EGIP alone. For tri- and tetrafunctional alcohols or ketones it might be helpful to use mixed columns of low polarity in order to obtain reasonably short retention times.

SUMMARY

The possible use of two-component phases and segmented packings of varying degree of selectivity in the gas chromatographic separation of steroids has been investigated. This highly potent technique permits

the preparation of columns which exhibit specificity toward various functional groups, making it possible to distinguish between steroids differing in type and degree of substitution.

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ANALYTICAL BIOCHEMISTRY 2, 353–359 (1961)

Interference by Amino Acids and Peptides with the Photometric Estimation of Proline

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INTRODUCTION

When proline is heated with ninhydrin under acid conditions, a red reaction product is formed (1). This observation was used by Chinard (2) to develop a method for estimating proline.

According to Chinard, the only amino acids which interfere with the method are ornithine, citrulline, lysine, hydroxylysine, and cysteine; other amino acids had no significant effect when present at molar concentrations 10 or 100 times as great as that of proline. Finch (3) and Finch and Hird (4), however, have noted that amino acids other than the above-named interfere with the method.

The present results show that Chinard's method is subject to interference by almost all the common amino acids and by some di- and tripeptides, when they are present at concentrations 10 or 20 times that of proline. On the basis of these results, a simple modification of Chinard's method is proposed by which the interference by many amino acids and peptides can be eliminated or considerably reduced.

METHODS

Except where otherwise indicated, the estimation of proline was carried out as described by Chinard (2). Briefly, the method consists of adding 1.0 ml of glacial acetic acid and 1.0 ml of a solution of ninhydrin (25 mg/ml) in a mixture of glacial acetic acid (3 vol) and 6 *M* phosphoric acid (2 vol) to 1.0 ml of the solution to be analyzed, whereupon the mixture is heated in stoppered tubes for 1 hr at 100°C and then made to 5.0 ml with glacial acetic acid, the optical density of the red solution being estimated at a wavelength of 515 mμ.

All the amino acids used were obtained from British Drug Houses Ltd., U. K., except for glutamine, hydroxyproline and histidine, which were products of Hoffman-LaRoche and Co., Ltd., Switzerland. All the peptides used were obtained from Mann Research Laboratories, United

States. The ninhydrin used was a British Drug Houses product of "Analar" grade.

RESULTS

Amino Acids

Twenty amino acids were tested for their effects on the color yield from proline, using Chinard's method. The concentration of proline was 0.1 μmole/ml of solution analyzed, and that of the amino acids either 1.0 or 2.0 μmoles/ml, the molar ratio of amino acid to proline thus being 10 and 20, respectively.

It was found that the amino acids could be divided into four groups according to their effects on the color yield from proline (Table 1, first column).

The first group, consisting of aspartic acid, β-alanine, and γ-aminobutyric acid, had no effect whatever on the color obtained from proline.

The second group consisted of 10 amino acids of which none produced any color in the absence of proline, but all markedly increased the color yield from proline. Moreover, at equal concentrations, all 10 amino acids produced the same increase. The amino acids belonging to this group were glycine, alanine, valine, leucine, isoleucine, serine, threonine, arginine, asparagine, and glutamic acid.

The remaining seven amino acids all produced a certain amount of color in the absence of proline. Methionine, phenylalanine, and tyrosine each produced a faint yellow; glutamine, hydroxyproline, and histidine gave varying shades of orange; while tryptophan, as previously noted by Chinard (2), produced a bright yellow color.

When these amino acids were tested in the presence of proline and the color produced by them was subtracted from the total color yield, two different effects could be seen (Table 1). Methionine, glutamine, tryptophan, and hydroxyproline (group 3) increased the color yield to varying extents, thus behaving qualitatively like the amino acids of group 2, whereas phenylalanine, tyrosine, and histidine (group 4) produced a decrease in color yield which tended to mask the color produced by them in the absence of proline.

Further experiments were carried out in an attempt to elucidate the mechanism by which the amino acids of group 2 stimulated the color yield from proline.

It was found that glycine greatly accelerated the rate of color formation and lessened the time taken for optimal color development from about 50 to 30 min. Glycine also increased the rate at which the color disappeared when the solutions were heated for a longer time. Identical

TABLE 1

EFFECTS OF AMINO ACIDS AND PEPTIDES ON COLOR YIELD FROM PROLINE[a]

	Color yield: % of control	
	Chinard's method	Modified method
Amino acid		
Group 1 (mean)[b]	101	99
Group 2 (mean)[b]	143	98
Methionine	144 (137)	98 (93)
Glutamine	171 (140)	125 (97)
Tryptophan	181 (146)	121 (103)
Hydroxyproline	172 (104)	135 (97)
Phenylalanine	93 (88)	88 (86)
Tyrosine	81 (75)	78 (75)
Histidine	112 (58)	91 (64)
Peptide		
Glycylglycine	115	100
Glycylalanine	113	98
Glycylvaline	113	101
Glycylleucine	115	99
Glycylglutamic acid	115	99
Glycylphenylalanine	116	101
Glycyltyrosine	114 (112)	101
Glycylglycylglycine	112	101
Glycylproline	311 (118)	137 (101)
Alanylglycine	100	100
Valylglycine	100	102
Leucylglycine	100	100
Prolylglycine	116 (103)	104 (100)
Alanylalanine	99	100
Leucyltyrosine	100	101
Glutamylasparagine	101	101
Alanylglycylglycine	103	100

[a] Proline, 0.1 μmole/ml; amino acid or peptide, 2.0 μmoles/ml. The figures in parentheses are the values obtained after subtracting the color yield obtained in the absence of proline.

[b] See text for groups.

results were obtained when leucine, arginine, or glutamic acid was used in place of glycine. When the absorption spectra of the red solutions obtained in the absence and presence of glycine were compared, they were found to be identical.

The greater the ninhydrin concentration, the less the increase in color produced by glycine; this, however, was mainly due to an increase in the control values. In the presence of glycine a ninhydrin concentration of 20 mg/ml was optimal.

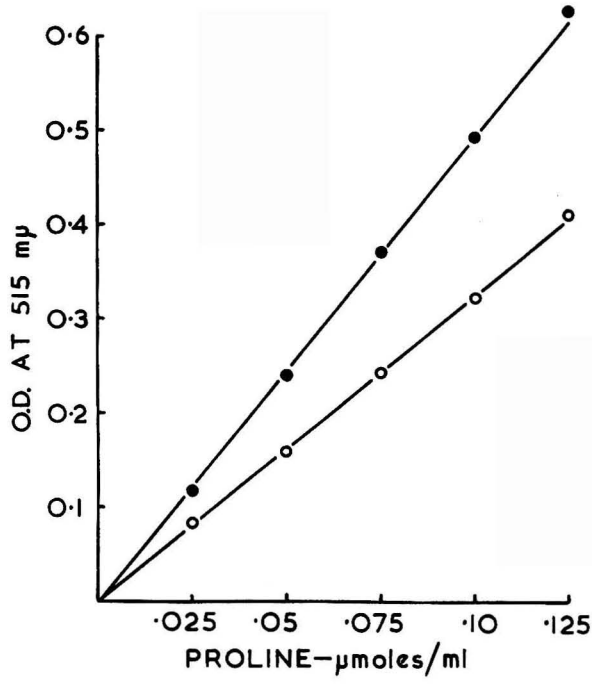

FIG. 1. Relationship between proline concentration and optical density at 515 mμ in the absence and presence of glycine. Lower curve: proline alone. Upper curve: proline plus glycine (2.0 μmoles/ml), heating time, 30 min; ninhydrin concentration, 20 mg/ml.

Figure 1 illustrates the effect of varying the proline concentration in the absence and presence of glycine. It is seen that the increase in color produced by glycine was independent of the proline concentration.

When the effects of increasing concentrations of glycine on the color yield from proline were investigated using a 30-min heating period and a ninhydrin concentration of 20 mg/ml, it was found that there was a linear increase in the amount of color as the concentration of glycine was raised from 0 to 1.8 μmoles/ml; thereafter a plateau extended to a concentration of about 3 μmoles/ml, followed by a gradual decrease in color yield as the glycine concentration was raised still further. When leucine was used instead of glycine under otherwise identical conditions, an identical curve was obtained. Moreover, the effects of glycine and leucine were additive, 1.0 μmole of each having the same effect as 2.0 μmoles of either.

It seemed likely that if 1.8 μmoles of one of the amino acids of group

2 was added per milliliter of the solution of proline to be analyzed, the further addition of up to 2.0 μmoles/ml of any one of the amino acids of this group should have little or no effect on the color yield. This was found to be the case; when Chinard's method was modified by (*a*) adding glycine to a concentration of 1.8 μmoles/ml, (*b*) reducing the time of heating to 30 min and (*c*) reducing the ninhydrin concentration to 20 mg/ml, none of the amino acids of group 2 produced any effect at a concentration of 1.0 μmole/ml, and 'an almost negligible effect when added to a concentration of 2.0 μmoles/ml (Table 1, second column).

It is apparent from Table 1 that the above modifications did not abolish the interference produced by the remaining seven amino acids. However, they appeared to eliminate the stimulating effect on the color yield of the amino acids of group 3, thus considerably lessening their interference.

Peptides

Fifteen dipeptides and two tripeptides were tested for their effects on the color yield from proline, using Chinard's method. The results (Table 1, first column) show that all the *N*-glycyl peptides produced an increase in color whereas, except for prolylglycine, none of the other peptides, including some with C-terminal glycine, had any effect.

The interference by the two proline-containing peptides greatly increased when the time of heating was extended beyond 60 min; this indicated that these peptides interfered as a result of being hydrolyzed to proline and glycine during the heating process. Estimation of the proline produced from the two peptides when they were heated in the absence of added proline under the conditions of Chinard's method showed that glycylproline was hydrolyzed to the extent of about 10%, and prolylglycine to about 0.6%.

Since glycylproline was shown to be very much more unstable than prolylglycine, it seemed likely that the interference produced by the other *N*-glycyl peptides was not caused by the peptides per se but as a result of their hydrolysis to amino acids; in that case their interference should be abolished by the modifications of Chinard's method described in the previous section. It was in fact found (Table 1, second column) that, with the exception of glycylproline, none of the *N*-glycyl peptides interfered when the modified method was used. Due, mainly, to the shorter heating period, the interference by the proline-containing peptides was much smaller, prolylglycine now having an almost negligible effect at 2.0 μmoles/ml.

When the proline-containing peptides were heated in the absence of added proline using the modified conditions, glycylproline was found to be hydrolyzed to the extent of about 2% and prolylglycine to about 0.2%.

Other Substances

The following substances had no effect on the color yield from proline, using either Chinard's method or the modified method: leucinamide, hippuric acid, and ammonium sulfate.

DISCUSSION

The results show that of 18 α-amino acids tested, all except aspartic acid interfered with Chinard's (2) method of estimating proline, in addition to the five amino acids whose interference was previously observed by Chinard and whose effects were not investigated in the present work.

For most of the amino acids, the interference consisted of an increase in color yield; three amino acids, however, produced a decrease. With seven of the amino acids the situation was complicated somewhat by the fact that they produced a certain amount of color in the absence of proline.

It appears that the interference is produced only by unsubstituted α-amino acids; leucinamide, hippuric acid, β-alanine, and γ-aminobutyric acid had no effect, nor did peptides, except insofar as they were hydrolyzed to amino acids during the estimation. This hydrolysis was sufficient to affect the color yield only in the cases of prolylglycine and *N*-glycyl peptides.

The results indicate that by adding glycine to the solution to be analyzed, the interference by 10 amino acids and by all the peptides except glycylproline can be eliminated. At the same time the sensitivity of the method is increased by about 50% (cf. Fig. 1) and the time required for heating is halved. It is apparent, however, that even with the present modification it is not possible to estimate proline in the presence of glutamine, tryptophan, hydroxyproline, tyrosine, and histidine, except at low concentrations (0.1–0.5 μmole/ml) of these amino acids. Nor is the method applicable in the presence of more than 2.0 μmoles/ml of any of the other 10 amino acids. It would seem that when large amounts of other amino acids are present, they should first be removed, e.g., by the nitrous acid treatment recommended by Schweet (5). The modification of Chinard's method proposed by Troll and Lindsley (6), in which lysine, hydroxylysine, and ornithine are first removed by shaking with Permutit, would appear, from the present results, to be

subject to the same interference by the remaining amino acids as is Chinard's original method.

The present work does not provide sufficient data to enable one to propose a mechanism by which the amino acids produce their interference.

SUMMARY

The effects of amino acids and peptides on Chinard's (2) method of estimating proline were investigated.

Of the 18 α-amino acids tested, all except aspartic acid interfered with the method. Of the 17 di- and tripeptides tested, only prolylglycine and *N*-glycyl peptides interfered. The interference by peptides was found to be secondary to their hydrolysis to amino acids during the estimation.

By adding glycine to the solution to be analyzed, the interference by 10 amino acids and by all the peptides except glycylproline could be eliminated and the sensitivity of the method increased by 50%.

ACKNOWLEDGMENT

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ANALYTICAL BIOCHEMISTRY 2, 360–369 (1961)

The Direct Microdetermination of Tissue Calcium by Flame Photometry[1]

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A number of methods have been reported for the determination of calcium in biological materials by means of flame photometry (1–5), and by titration employing chelating agents such as (ethylenedinitrilo)-tetraacetic acid (6–12). These various methods are less time consuming than the Clark and Collip (13) modification of the Kramer and Tisdall procedure (14). Kingsley and Schaffert (5) determined the calcium in small serum samples by flame photometry using organic solvents to enhance the luminosity. Humoller and Walsh (15) modified the method of Kingsley and Schaffert (5) and employed trichloroacetic acid (TCA) to precipitate the proteins and isopropyl alcohol to enhance the luminosity. Their experimental conditions prevented flame blowouts and capillary clogging. Interference from sodium was overcome by having approximately 3 meq sodium in all solutions and standards.

Mori (10), using a microtitration procedure, found the TCA extraction of tissue was adequate to obtain release of all of the calcium. Since TCA does not affect the luminosity of calcium (2, 15), the present micromethod was developed to utilize both the convenience of TCA extraction and that of flame photometry. Under the conditions employed, tissue sodium, potassium, and phosphate caused no significant interference, and calcium in concentrations of 0–2 μg/ml was readily determined.

MATERIALS AND APPARATUS

Deionized distilled water having a specific conductance of $<0.66 \times 10^{-6}$ mho was used throughout. The organic diluting fluid contained 500 ml isopropyl alcohol, 300 ml water, 0.2 ml Non-Ion-Ox (Aloe

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Scientific Co., St. Louis, Mo.), 74.6 mg KCl, and 292 mg NaCl. The trichloroacetic acid solution for tissue extraction was 6.2% (w/v) and that for preparing standards was 8.3% (w/v) and contained 19.5 mg NaCl/100 ml. A primary calcium solution was prepared by dissolving 0.2500 gm dry $CaCO_3$ in 25 ml of 2 *N* HCl and diluting to 1 l to give 100 μg Ca/ml. All glassware was given exhaustive rinsing with deionized distilled water, and at no time was calcium contamination detected.

Flame photometric determinations were done with the Beckman spectrophotometer, model DU, equipped with a flame photometry attachment, model 9200, a hydrogen–oxygen burner, and photomultiplier tube attachment, model 4300. The homogenizer employed was model 4288-B, Arthur H. Thomas Co., Chicago, Ill., which has a Teflon rotor on a stainless steel shaft.

METHODS

Standards

Standards and reagent blanks containing 0–3 μg Ca/ml were prepared by mixing 80 ml of organic diluting fluid, 15 ml of 8.3% TCA, 0–3 ml of primary calcium solution, and water to 100 ml. As suggested by Humoller and Walsh (15), TCA was included, even though it had no effect on the luminosity.

Instrument Operation

Organic solvents are prone to cause flame blowouts (3, 15). In the present study it was found that if adjustment of the burner was made while aspirating the organic solvent to give a fairly generous flame, full enhancement of the luminosity was obtained without danger of such blowouts. A wavelength setting of 422.7 mμ was used, and the instrument was set at 50% *T* using the 1 μg Ca/ml standard; the blanks, other standards, and unknowns were then analyzed. Although the stability of the instrument was excellent, standards were frequently checked.

Extraction of Tissues

Male rats of the Charles River C. D. Sprague-Dawley Strain (Charles River Breeding Laboratories, North Wilmington, Mass.) weighing 150–250 gm and maintained on Purina Laboratory Chow (Ralston Co., St. Louis, Mo.) were used as the source of tissues. After having sacrificed the animals by a blow on the head, the tissues were quickly excised and chilled. Using a Stadie blade, slices of tissue approximately 0.5 mm

thick were prepared freehand. As each slice was obtained, it was momentarily dipped in calcium-free incubation solution (16), carefully blotted on filter paper, and accurately weighed on a rapid-weigh balance to yield samples in the range of 100 mg wet weight. To the sample contained in a small calibrated centrifuge tube, enough 6.2% TCA solution was added to bring the volume to 2 ml. The tubes were covered with several layers of Saran (Dow Chemical Co., Midland, Mich.), held in place by means of small rubber bands. Tubes provided with screw caps having thin Teflon liners are more convenient, but the Saran is considerably less expensive and is disposable. With a thumb pressing down firmly on the Saran, the tubes were vigorously shaken three times during a 30-min period. Eight milliliters of organic diluting fluid was added, the tubes were centrifuged, and an aliquot of the supernatant liquid was analyzed directly. Tissue-free blanks were always obtained. In some instances the intact liver was first perfused with isotonic sucrose solution as described by Thiers and Vallee (17).

The adequacy of the extraction procedure as applied to thin tissue slices was tested by employing the TCA extraction procedure of Mori (10) in which the tissue is homogenized. Results of these analyses are given in Table 1. Samples of tissue were also analyzed by the method of

TABLE 1

CALCIUM CONTENT OF RAT TISSUES DETERMINED BY FLAME PHOTOMETRY AND THE CLARK AND COLLIP METHOD

Tissue	Flame photometric method[a]		Clark and Collip method[b]
	Slices (μg Ca/100 mg)	Homogenates (μg Ca/100 mg)	Slices (μg Ca/100 mg)
Liver	3.96 ± 0.11 (27)	3.82 ± 0.10 (13)	3.92 ± 0.08 (8)
Heart	3.95 ± 0.06 (24)	4.06 ± 0.41 (6)	3.87 ± 0.09 (7)
Kidney	5.52 ± 0.18 (12)	5.36 ± 0.16 (6)	5.35 ± 0.20 (7)
Diaphragm muscle	4.83 ± 0.23 (18)	4.76 ± 0.31 (5)	4.98 ± 0.74 (6)

[a] Mean ± standard error; wet basis. Figures in parentheses refer to number of different samples analyzed.

[b] Approximately 1-gm samples of liver, heart, and kidney and 0.3 gm of diaphragm muscle were used in the Clark and Collip procedure.

Clark and Collip (14). For this purpose, approximately 1-gm samples were ashed at 510°C in platinum crucibles. The use of 2% NH_4OH in equal volumes of ethyl alcohol, ethyl ether, and water in the washing of the calcium oxalate (18) helped prevent loss due to solubility, which

is appreciable when only small amounts of precipitate are available. Data pertaining to these studies are included in Table 1.

Successful use of flame photometry for the microdetermination of calcium depends in large measure on controlling interference from sodium (Fig. 1), potassium (Fig. 2), and phosphate. Humoller and Walsh (15) employed 1–3 meq Na/l to circumvent enhanced luminosity from this source when analyzing serum. In the present study, 5–7.5 meq Na/l (Fig. 1) was the range in which analyses for calcium could be performed satisfactorily. The sodium content of the TCA extracts, assayed by means of flame photometry, varied from approximately 100 to 150 μg Na/100 mg fresh tissue. Thus, about 0.5 meq Na/l was derived from the tissue in the final solution assayed for calcium. Therefore, 5.5 meq Na/l was added to the standards and 5.0 meq Na/l to the unknowns.

In the absence of sodium, potassium had a pronounced effect on the luminosity from calcium (Fig. 2). This was less in the presence of 3 meq Na/l and was essentially abolished at 5 meq Na/l (Fig. 2). However, 1 meq K/l was added routinely to all solutions. This is the approximate final concentration of K derived from fresh tissue samples weighing about 100 mg.

The TCA extracts of the tissues were analyzed for phosphate by the method of Lowry and Lopez (19) and ranged from approximately 40 to 90 μg P/100 mg fresh tissue or 4–9 μg P/ml in the final solution (Table 2). Calcium analyses were therefore done with final phosphate concentration of 0, 5.45, 11.2, 16.3, 21.7, 27.2, 32.9, and 54.5 μg P/ml. Only at the highest concentration was an effect observed, and this amounted to a 7% reduction in luminosity.

TABLE 2

SODIUM AND PHOSPHATE CONTENT OF RAT TISSUE EXTRACTS[a]

Tissue	TCA extractable electrolyte from fresh tissue	
	μg Na/100 mg	μg P/100 mg
Liver	106 ± 4.93 (7)	45.86 ± 2.73 (5)
Heart	140.7 ± 3.21 (7)	64.8 ± 1.93 (6)
Kidney	142.6 ± 3.66 (5)	42.6 ± 2.35 (4)
Diaphragm muscle	106.2 ± 4.16 (6)	89.6 ± 4.94 (5)

[a] The trichloroacetic acid extracts used for these analyses were chosen at random from those used for the calcium analyses given in the other tables. The numbers in parentheses refer to the number of samples analyzed. Values given are the mean ± standard error.

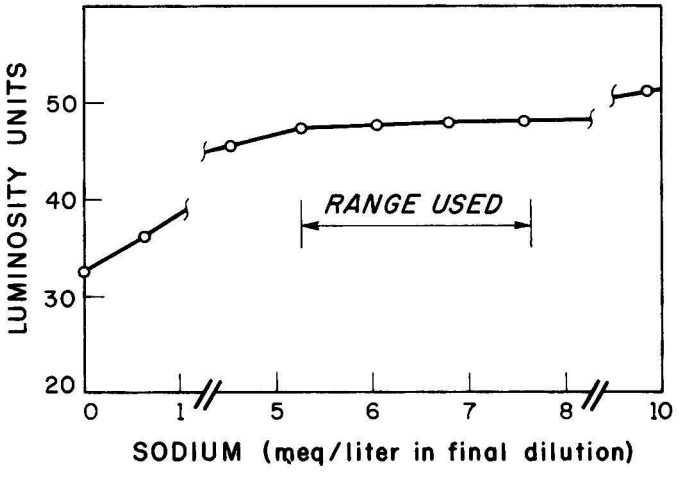

FIG. 1. Effect of sodium on calcium luminosity.

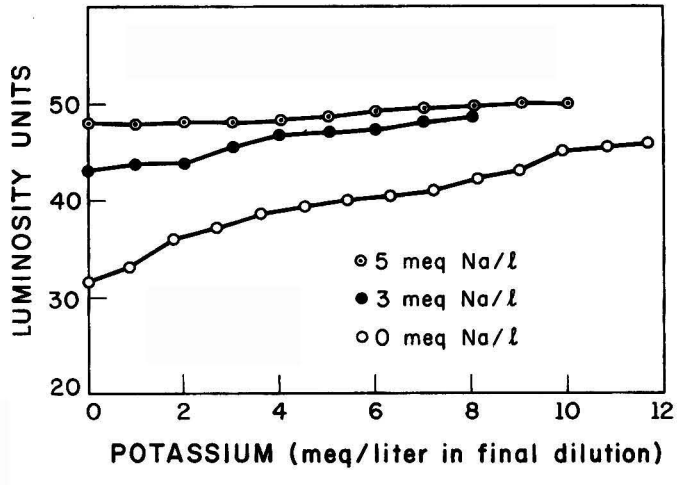

FIG. 2. Effect of potassium on calcium luminosity.

Other aspects of the study concerned the effects of tissue weight, recovery of Ca, and the nature of the samples analyzed. Pertinent data and results of these studies are given in the appropriate tables.

RESULTS AND DISCUSSION

The calcium content of fresh rat liver, heart, kidney, and diaphragm muscle was determined by flame photometry using TCA extracts of the

tissues and without prior separation of interfering ions (Table 1). Humoller and Walsh (15) had applied such a procedure to the analysis of serum with good results using 3 meq Na/l to avoid serum sodium interference. By means of 5 meq Na/l in the present method, not only was such interference essentially abolished (Fig. 1), but that of potassium as well (Fig. 2). Under these conditions a linear response between calcium concentration and luminosity was still obtained (Fig. 3). The

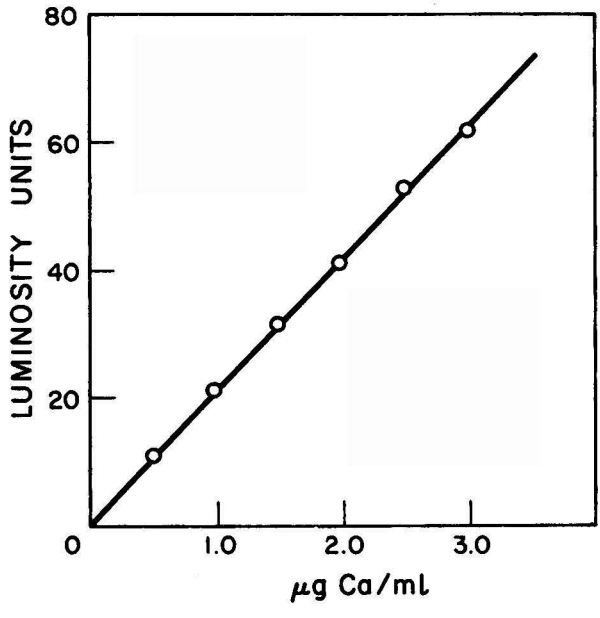

FIG. 3. Relationship between luminosity and calcium concentration.

phosphate contents of the TCA extracts (Table 2) were in the range reported by Mori (10) and were well below the 35–55 μg/P ml needed for interference. The complex nature of phosphate interference has been demonstrated by Chen and Toribara (2). This was shown in the present experiments where the omission of the isopropyl alcohol was not only accompanied by a greatly decreased luminosity from calcium, but as little as 1 μg P/ml caused further depression. On the other hand, when 30 μg P/ml was added to all samples, standards, and blanks, and the isopropyl alcohol content was raised to 70% at the expense of water in the usual organic diluting fluid, an additional 70 μg P/ml could be added with no effect upon the luminosity. The use of correction factors,

or the addition of the phosphate to the standard solutions has been suggested by others (2, 3). West and Cooke (20) found the sodium salt of (ethylenedinitrilo)tetraacetic acid effective in overcoming phosphate interference. However, they used a specially modified Beckman flame photometer employing continuous sample feeding and recording devices, and were thereby able to circumvent sodium interference. In the present studies the sodium salt of this chelating agent gave rise to excessive interference, while the ammonium salt was ineffective even at concentrations of 10 μg P/ml. West and Cooke (20) found the latter much inferior to the sodium salt.

As judged by a *T* test (21, 22), there was no significant difference between the means of the analyses done by flame photometry on thin tissue slices and homogenates (Table 1), indicating that extraction of the calcium was similar in both cases. These means were not significantly different from those based on the Clark and Collip method (Table 1). Tissue weight did not influence the results (Table 3), and the over-all recovery of added calcium was 101.7 $\pm$ 3.43% (Table 4).

TABLE 3
CALCIUM CONTENT OF LIVER SAMPLES OF DIFFERENT WEIGHTS

No. samples	Average tissue weight[a] (mg)	Calcium content[b] (μg/100 mg)
12	128.5 (78.1–160.7)	4.04 $\pm$ 0.10
15	197.3 (171.8–227.9)	3.91 $\pm$ 0.12
14	542.3 (500.4–789.8)	3.96 $\pm$ 0.09

[a] Numbers in parentheses refer to range. The samples were derived from the livers of 18 rats.

[b] Mean $\pm$ standard error; wet basis.

The standard errors given in Table 1 are close to those given by Mori (10) for rat kidney and abdominal muscle and by Gilbert and McGann (23) for frog skeletal muscle. Kratzing *et al.* (24) reported the calcium content of rat liver was 0.76 μmoles/gm fresh tissue with a standard error of $\pm$0.06. The results given in Table 1 are in close agreement with their data. Thiers and Vallee (17) found appreciable variation in the calcium content of rat liver even though perfused tissue was used. In the present study no significant reduction in variation was found by employing perfused tissue.

TABLE 4
RECOVERY OF ADDED CALCIUM

	Liver extract number[a]													
	1		2		3		4		5		6		7	
Theoretical[b]	1.59	2.08	1.62	2.11	1.39	1.88	1.31	1.80	1.02	1.51	1.21	1.70	1.25	1.74
Found	1.62	2.00	1.55	2.10	1.45	1.95	1.35	1.90	1.06	1.59	1.19	1.67	1.31	1.80
Difference	+0.03	−0.08	−0.07	−0.01	+0.06	+0.07	+0.04	+0.10	+0.04	+0.08	−0.02	−0.03	+0.06	+0.06

[a] Each extract from tissue of a different animal.
[b] The amount found in the extracts by direct analysis plus 0.50 or 0.99 μg Ca/ml added as $CaCl_2$.

The method for calcium proposed here is simple and is adaptable to large series of samples. Only under unusual circumstances would the TCA extracts contain sufficient amounts of interfering ions, since there is a four- to fivefold leeway from normal in this respect. Even should such a condition exist, it can be circumvented either by using minimum weights of tissue or, as suggested by others (2, 3, 5, 15), by determining the exact concentration of the interfering ion and adding an equal quantity to the standards and blank.

SUMMARY

A simple direct micro flame photometric determination for tissue calcium was developed. Approximately 100 mg fresh tissue containing a total of 0.5–3.0 μg Ca was extracted with trichloroacetic acid solution, and the extract was analyzed after the addition of 5 meq Na/l, 1 meq K/l, and isopropyl alcohol. Under these conditions tissue sodium, potassium, and phosphate did not cause significant interference. The values and standard errors for fresh rat liver, heart, kidney, and diaphragm muscle were 3.96 ± 0.11, 3.95 ± 0.06, 5.52 ± 0.18, and 4.83 ± 0.23 μg Ca/100 mg, respectively. Good agreement with the results of analysis by the Clark and Collip method was obtained.

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ANALYTICAL BIOCHEMISTRY 2, 370–379 (1961)

An Improved Test for the Quantitative Determination of Trypsin, Trypsin-Like Enzymes, and Enzyme Inhibitors[1]

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INTRODUCTION

As part of a study of specific proteolytic enzymes in malignant tissues (1) and unaffected tissues of tumor-bearing animals, we investigated substrates which lend themselves to quantitative biochemical tests as well as histochemical localization of the enzymes. One of the activities of interest is the trypsin-like cathepsin B (2) activity present in most tissues. Benzoylarginine-β-naphthylamide (BANA) synthesized in 1959 by Riedel and Wuensch (3) lends itself to both biochemical and histochemical (4) testing. For quantitative tests it is preferable to unspecific substrates, such as casein (or nitrocasein) (5) and hemoglobin (6), because, in conjunction with other specific substrates, it can be used to obtain multienzyme patterns and ratios of different tissue enzymes. In this respect it is comparable to the more commonly used (7, 8) benzoylargininamide (BAA) substrate. However, digestion of BAA releases ammonia, and many tissues and fluids give high blanks in this test. Using the same substrate for both biochemical and histochemical analysis has the further advantage that the same enzymes are sure to be measured in both tests.

In this paper we shall describe a quantitative biochemical procedure and some examples illustrating the usefulness of the method.

METHOD

Riedel and Wuensch in their original paper (3) describe a method of analysis involving a diazonium salt. This method calls for extraction of the color with an organic solvent. If this step can be eliminated, the test becomes more convenient and can be used with all tissues. The Goldbarg and Rutenburg (9) assay for amino peptidase applies the Bratton-

[1] This work was supported by a grant from the American Cancer Society.

Marshall (10) test for naphthylamine, i.e., formation of an azodye by coupling the naphthylamine released with naphthylethylenediamine, and thus avoids the necessity for color extraction. We have adapted this procedure (9) to assay trypsin and tissue enzymes with trypsin-like specificity using benzoylarginine-β-naphthylamide as the substrate. Enzyme concentration is proportional to the amount of naphthylamine released, and this can be measured colorimetrically by the amount of azodye produced. When the reaction is allowed to go to completion, the theoretically calculated amount of naphthylamine is released and can be recovered from the system. Comparison with calibration curves shows that none of the naphthylamine remains bound to the tissue. Klett readings or any other optical density measurements can be converted to μg naphthylamine from a straight-line calibration curve as given by Goldbarg and Rutenburg (9). Elimination of Fast Blue or equivalent dye salts also eliminates the necessity for fresh calibration curves with fresh batches of reagent (3). The absolute colorimetric values are reproducible, and percentage digestion is easily calculated from them.

Because complete racemization occurs during synthesis (3) of benzoyl-arginine-β-naphthylamide, only the DL form is available and maximum hydrolysis cannot exceed 50%. The amount of substrate which gives equivalent color release is therefore almost four times as much benzoyl-DL-arginine-β-naphthylamide (mol. wt. 441) as L-leucine-β-naphthylamide (mol. wt. 258). In the interest of conserving the relatively expensive substrate, lower amounts can be used. We found the following procedure satisfactory and sensitive to 0.2 μg trypsin.

Tissue Homogenate

Fresh tissues from normal 150–170-gm Wistar rats were homogenized in cold distilled water (100 mg wet tissue/ml), the homogenates were centrifuged 5 min at 2000 rpm, and 1 ml of the supernatant was withdrawn for each test. The N content of the solutions was established by micro-Kjeldahl determination, and enzyme activities are expressed per mg N.

Substrate Solution

Eighty milligrams of *N*-benzoyl-DL-arginine-β-naphthylamide[2] is dissolved in 100 ml of distilled water. Equal volumes of this solution and solutions of 0.2 *M* citrate–phosphate buffer (11) of desired pH are mixed, and 1 ml of the buffered substrate solution is withdrawn for

[2] Commercially available from Mann Laboratories, N. Y.

each test. The solutions are stable in the refrigerator at +4°C for at least 2 weeks.

Determination of Tissue Enzymes with Trypsin-Like Specificity

A mixture of 1 ml of buffered substrate solution and 1 ml of tissue homogenate is incubated at 37°C for 2 hr. One milliliter of 40% trichloroacetic acid is then added to stop the reaction, precipitate proteins, and establish the acid pH needed for subsequent steps. Any precipitate formed is centrifuged off, and 1 ml of the centrifugate is withdrawn. Free naphthylamine in the solution is assayed essentially by the Goldbarg and Rutenburg adaptation (9) of the Bratton-Marshall test (10). To 1 ml centrifugate the following reagents are added, mixing thoroughly after each new addition: (*a*) 1 ml of 0.1% aq. $NaNO_2$, (*b*) after exactly 3 min 1 ml of 0.5% aq. ammonium sulfamate to decompose excess $NaNO_2$, (*c*) after exactly 2 min 2 ml of 0.05% solution of *N*(1-naphthyl)ethylenediamine in 95% ethyl alcohol to develop the color.

The $NaNO_2$ solution must be prepared fresh daily, the ammonium sulfamate and the ethylenediamine (in a dark bottle) are stable for at least 1 month at +4°C. The blue color formed is stable for several hours, but usually read after 15 min in a Klett photoelectric colorimeter with a green (540) filter or in any other similar instrument between 540 and 560 mμ. Colorimeter readings can be converted to μg naphthylamine released from a calibration curve as described by Goldbarg and Rutenburg (9).

Determination of Trypsin Activity and Preparation of a Standard Curve

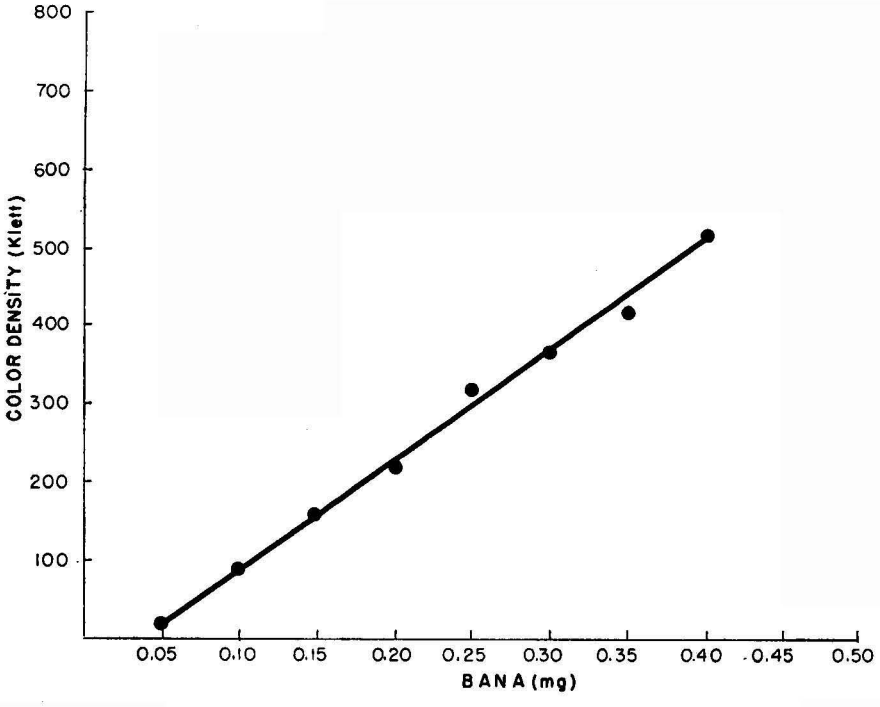
The same assay procedure is performed with solutions containing different concentrations of crystalline trypsin (Armour tryptar) in place of the homogenate. A standard curve is drawn (see Fig. 2), and tissue enzyme activity is expressed as mg trypsin equivalents.

Determination of Trypsin Inhibitor Activity

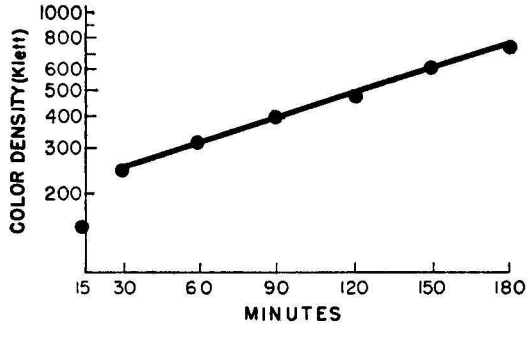
A mixture of 1 ml of a 0.1% trypsin solution and 1 ml of tissue homogenate is allowed to react for 2 min at room temperature; 1 ml of buffered substrate, pH 7.2, is then added, and the assay is performed exactly as above. In control experiments the buffered substrate solution is added to 1 ml trypsin + 1 ml water, respectively, 1 ml homogenate + 1 ml water.

Modifications of the method described may, at times, be desirable:

(*a*) *Buffer.* The citrate–phosphate buffer (11) was chosen because it enabled us to use the same system over a wide range of pH values.



A



B

FIG. 1. *A*. Effect of substrate concentration on BANA digestion by 0.1 mg Tryptar in 2 ml of 0.05 *M* phosphate–citrate buffer, pH 7.2. Incubation time: 2 hr. *B*. Rate of digestion of 0.4 mg BANA by 0.1 mg Tryptar in 2 ml of 0.05 *M* phosphate–citrate buffer pH 7.2.

However, the method works equally well with Tris, phosphate, or acetate buffers. Acetate or Tris may be preferable when the effect of metal ions is to be investigated.

(*b*) *Kinetics, Incubation Time and Substrate Concentration.* The action of trypsin on BANA has already been studied by Riedel and Wuensch (3) and also by Glenner and Cohen (4). Kinetics are essentially the same as those observed with other synthetic substrates. Figure 1 shows that first-order kinetics are followed over a wide range of tryptic BANA digestion. The same amount of naphthylamine is therefore released at shorter incubation times with higher substrate concentration. Shorter times or lower substrate concentrations may be used for the assay of trypsin itself or other enzymes of high specific

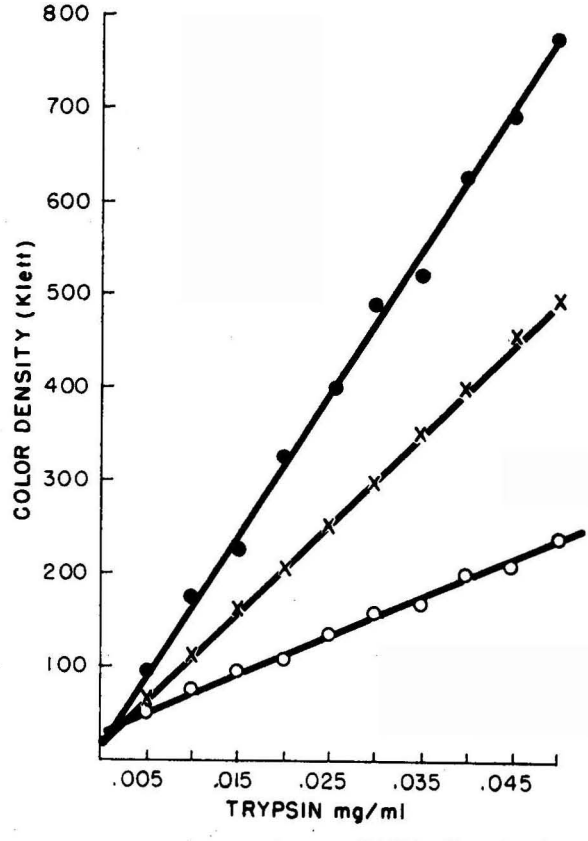


FIG. 2. Effect of enzyme concentration on BANA digestion by Tryptar. Substrate concn.: 0.2 mg/ml; incubation time: 2 hr. Buffers: 0.05 *M* phosphate–citrate, pH 7.7 ●——●, 7.2 ×——×, and 6.2 ○——○.

activity; higher substrate concentrations may be needed in the assay of enzyme-poor tissues such as muscle. During the initial phases of the reaction, however, zero-order kinetics are followed. Increasing the substrate concentration in this region will therefore be without effect, and results obtained after short incubation (10 min) cannot be converted or compared to results at incubation times of 30 min or more.

RESULTS

Figure 2 shows that proportionality between enzyme concentration and color release is good, not only at the optimum pH but also at pH values of lesser activity. Calibration curves at different pH were constructed because the tissue enzymes (cathepsin B) have pH optima very different from those of trypsin (2, 8). Figure 2 also shows the great sensitivity of the method.

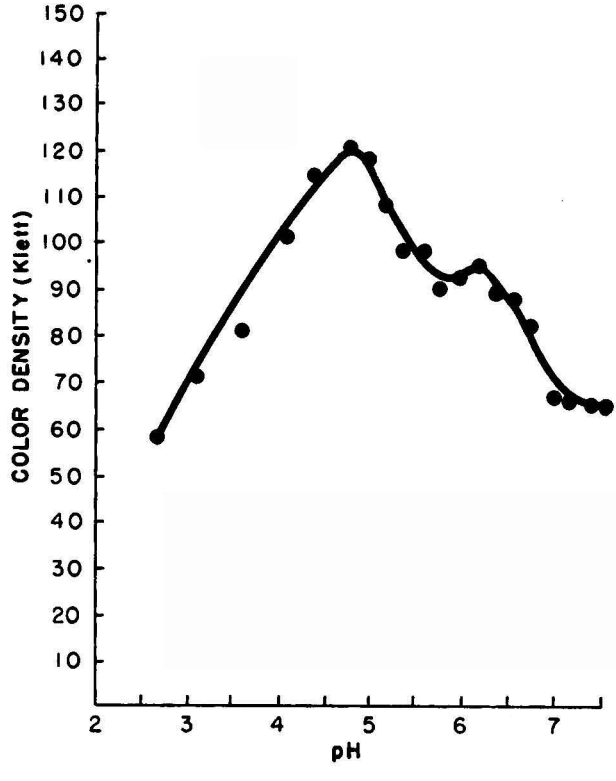


FIG. 3. Effect of pH on BANA digestion by a liver homogenate (0.5 mg N/ml) Substrate concn.: 0.2 mg/ml. Incubation time: 2 hr. Buffers: 0.05 *M* phosphate-citrate.

Figure 3 gives an optimum pH curve for the trypsin-like liver enzyme(s), showing two maxima, the principal one at pH 4.8 and a secondary one at pH 6.2. Above pH 7, where trypsin activity is optimum, tissue enzymes show minimal activity. Trypsin, on the other hand, is inactive at pH 4.8. This is important when the same method is applied to the study of trypsin inhibitors present in tissue homogenates. At pH 7.2 catheptic activity is sufficiently low not to obscure trypsin inhibition.

Table 1 lists range and average values of the trypsin-like enzymes in homogenates of different tissues and relates them to trypsin equivalents (at pH 7.2). No activator had to be added, possibly because the necessary constituents were present in the crude tissue homogenates. Values are expressed per mg Kjeldahl nitrogen and are highest for kidney, lowest for muscle. The results refer only to a particular strain of rats; they are meant to illustrate the general applicability of the method and were chosen from normal controls to experiments still in progress (1). No study has been made of the effects of age, sex, strain, or species difference on these enzymes. On the other hand, similar relative values for kidney, liver, spleen, and muscle have been found by others (5).

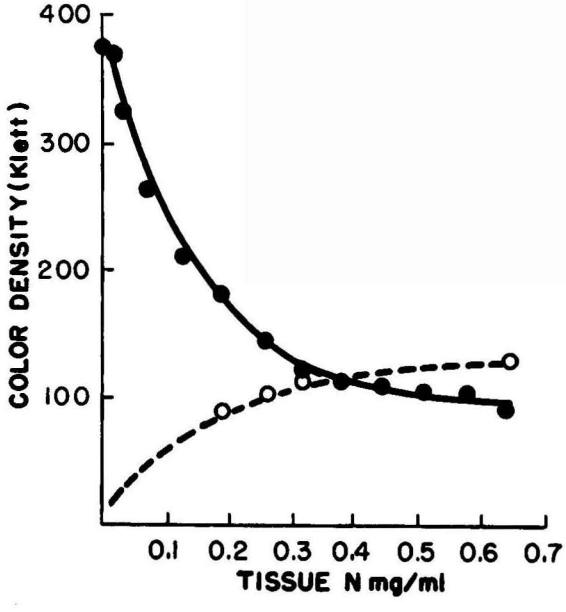


FIG. 4. Inhibition of tryptic BANA digestion by a liver homogenate. Trypsin concn.: 0.33 mg/ml; substrate concn.: 0.2 mg/ml. Incubation time: 2 hr. Buffer: 0.033 *M* phosphate-citrate, pH 7.2. Homogenate + substrate control — — — — —.

TABLE 1
BANA DIGESTION BY NORMAL RAT TISSUE HOMOGENATES

Tissue	No. of rats tested	pH 7.2			pH 6.2			pH 4.8		
		Klett reading		Mg trypsin equivalents	Klett reading		Mg trypsin equivalents	Klett reading		Mg trypsin equivalents
		Range	Average		Range	Average		Range	Average	
Liver	10	30–72	58	0.010	118–151	131	0.024	148–174	162	0.03
Kidney	10	113–290	188	0.035	110–360	202	0.040	168–420	316	0.063
Spleen	8	30–88	64	0.010	134–174	152	0.028	161–210	186	0.036
Muscle	6	20–28	24	0.002	26–35	30	0.003	44–68	54	0.008

Powerful trypsin inhibitors were found to be present in all the tissues investigated. With liver, complete inhibition of 0.2 mg trypsin has been possible with 1 ml homogenate containing 1.93 mg N. The effect of different amounts of inhibitor on 0.1 mg trypsin is illustrated in Fig. 4.

Partial tryptic digestion of the tissue enzyme can account for the proteolytic activities below the control values at high tissue concentrations.

The tissue inhibitors have greater resistance to heat and iodoacetate inactivation than the tissue enzymes. Homogenates heated to 56°C for 30 min show minimal proteolytic activity but still inhibit trypsin. They also inhibit BANA hydrolysis by fresh homogenates at pH's 4.8 and 6.2. In this they differ from soy trypsin inhibitor which has no effect on these tissue enzymes.

DISCUSSION

The test as described is applicable to all enzymes with trypsin-like specificity and all those attacking the similar substrate benzoylarginin-amide. It is as specific as the hydrolysis of BAA and tests are more sensitive and more reproducible than the ammonia determination. Papain, ficin, bromelin, etc. attack BANA as does the amidase–esterase (Clostripain) (12–14) present in *Clostridium histolyticum* filtrates. In addition BANA can frequently be used with crude extracts or bacterial filtrates where other constituents, especially NH_3, interfere, e.g., the determination of the amidase in *C. histolyticum* filtrates. The test is also applicable to the determination of trypsin inhibitors in serum and other fluids as well as other inhibitors or activators of trypsin and trypsin-like enzymes.

SUMMARY

A modification of the Bratton-Marshall test (10) developed by Goldbarg and Rutenburg (9) for the assay of amino peptidase has been adapted to the determination of naphthylamine released from the specific trypsin substrate benzoylarginine-β-naphthylamide (3). Advantages of this method over others used in similar procedures include specificity, sensitivity, good reproducibility, easy colorimetric assay, lack of interference by contaminants present in crude enzyme preparations, tissues, and fluids, and the possibility of using the same substrate for parallel histochemical tests. The procedure has been applied to the determination of trypsin itself, other enzymes with similar specificity for arginine side chains, and the trypsin-like cathepsin B enzymes of liver, kidney, spleen, muscle, and other tissues. It has also been used to assay trypsin inhibitors, in particular those found in tissues.

ACKNOWLEDGMENT

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A Glucose Oxidase Reagent for Maltase Assay

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INTRODUCTION

Assay of chromatographic column fractions for maltase (α-glucosidase) activity requires a sensitive method adaptable to routine use and not unduly affected by varying salt concentrations in eluting solutions. The reducing nature of the substrate also limits the procedures available for this purpose. Dahlqvist (1) has used Tauber and Kleiner's acid copper reagent for reducing disaccharide substrates. The shortcomings of the various methods are noted by Dahlqvist; he used Caputto's modified acid copper reagent when the salt content of substrates interfered with the Tauber-Kleiner reagent. Later (2) he used glucose oxidase for determination of turanase, lactase, cellobiase, and gentiobiase activity, but since the glucose oxidase preparations hydrolyzed maltose and isomaltose, he could not use it with these substrates.

Whistler, Hough, and Hylin (3) removed amylase and maltase activities from commercial glucose oxidase with a 20% recovery of dehydrogenase activity. Dahlqvist (1) was not successful in preparing glucose oxidase free from maltase and isomaltase activity. Friedman (4) removed trehalase from glucose oxidase by diethylaminoethylcellulose (DEAE-cellulose) column chromatography, but was unable to remove maltase.

The inhibition of maltase and 1,6-oligoglucosidase by several amines, including tris(hydroxymethyl)aminomethane (Tris) has been noted by Larner and Gillespie (5) who found such inhibition much more pronounced at alkaline than at acid pH. Friedman (4) noted that Tris was inhibitory to trehalase and maltase contaminants in commercial glucose oxidase, but made no analytical use of this. Salomon and Johnson (6) have described a glucose oxidase–peroxidase–tolidine reagent for blood glucose. We have modified this reagent by substituting Tris buffer for the acetate, under which conditions it has negligible maltase activity

[1] Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

but retains high glucose dehydrogenase action. Color developed by the reagent is a yellow–brown rather than blue; it must be stabilized after the reaction by adding acid, which produces a violet color. Acid stabilization of the glucose oxidase–peroxidase–dianisidine color has recently been reported (7).

EXPERIMENTAL

All developed colors were measured in 18 × 150 mm unmatched test tubes. Displacement spectrophotometry (8) eliminates the need for matching tubes, since each tube acts as its own blank. Although round tubes are used, results are essentially as accurate as though the solution were transferred to parallel-side cuvettes for measurement, and the procedure is much more suited to routine use. Tubes were read in a Lumetron[2] photometer with a 500-mμ interference filter.

The Salomon and Johnson reagent (6), which we had used for invertase assay, was tried for maltase assay, but considerable maltase activity was shown by all glucose oxidase preparations available. After unfruitful attempts to separate the two activities in practical yield by

TABLE 1
INHIBITION OF MALTASE CONTAMINANT IN GLUCOSE OXIDASE[a]

			Glucose		Maltose	
			100 μg	700 μg	1000 μg	7000 μg
Reagent comp.	pH	Min.	Absorbance		Absorbance	
Salomon-Johnson alone	4.1	30	0.054[b]	0.431	0.019	0.147
		60	0.100	0.635	0.106	0.432
With 0.02 *M* Tris	4.1	30	0.074	0.445	0.032	0.198
		60	0.116	0.650	0.102	0.445
With 0.2 *M* Tris	4.3	30	0.048	0.535	0.002	0.074
		60	0.076	0.830	0.044	0.276
With 0.2 *M* Tris buffer[c]	7.6	60	0.073	0.499	0.000	0.004
With 0.2 *M* phosphate buffer[c]	7.2	30	0.090	0.425	0.000	0.006
		60	0.136	0.463	0.000	0.044
With 0.2 *M* borate buffer[c]	7.4	30	0.023	0.314	0.000	0.000
		60	0.060	0.725	0.000	0.000

[a] Each tube contained indicated sugar in 2 ml with 5 ml reagent added, incubated 30 and 60 min at 26°C.

[b] Absorbance at 600 mμ of 7.3 mm thickness, by displacement photometry.

[c] Original acetate buffer omitted, replaced by indicated buffer, final concentration 0.2 *M*. Two drops HCl was added to each tube before reading Tris-buffered solutions.

[2] Mention of trade names does not constitute endorsement by the Department over others not named.

the procedure of Whistler *et al.* (3), selective inhibition of maltase by Tris was investigated.

Table 1 shows the results of the addition of Tris to the reagent, making it 0.02 and 0.2 *M* in Tris. No inhibition was shown at 0.02 *M*, but some was evident at 0.2 *M*. Elimination of the pH 4.1 acetate buffer from the original reagent and addition of a 0.2 *M* Tris buffer at pH 7.6 produced the results in the fourth entry of the table. In this case, as the reaction proceeded, the glucose solutions became yellow–brown, rather than blue. This yellow color appears proportional to glucose content at very low concentrations, but not above 50 μg/2 ml original solution. By adding acid subsequent to incubation, a violet color results which follows Beer's law between about 30 and 500 μg glucose/2 ml. Its visible spectrum has an absorption maximum at 530 mμ with minima at 442 and 740 mμ.

When the pH 4.1 acetate buffer in the original reagent was replaced by borate or phosphate at slight alkalinity, most of the tolidine was precipitated. Sufficient remained in solution, however, to give the results in the last two entries in the table. Absorbance values in the glucose tubes show that low values for maltose are not due to insufficient tolidine. Reduction of maltase activity was complete in borate and substantial with phosphate at the indicated pH values, both in the absence of Tris. The tolidine in the reagent appears to be inhibitory toward maltase at these pH values, though not under the original acid conditions of Salomon and Johnson, as noted for other amines by Larner and Gillespie. This was confirmed by adding *o*-tolidine to Huggett and Nixon's (8) glucose oxidase–dianisidine reagent, which is phosphate-buffered at pH 7. Their reagent shows maltase activity, but addition of *o*-tolidine dihydrochloride, filtration, and readjustment of the pH to 7 reduces maltase activity to one-third. A more stable reagent, in which no precipitation takes place, can be made with Tris and tolidine; the color obtained after acidification is much more suitable for measurement. The color becomes quite stable 1 hr after acid addition, remaining unchanged for 90 min thereafter. However, if a timed routine is used, reproducible results can be obtained by reading the tubes 1 min after acid addition. This eliminates an additional hour's wait in the procedure, so that 60 tubes can be analyzed for glucose in 2 hr.

The sensitivity of the reagent varies with the particular lot of glucose oxidase. A calibration curve must be established with each lot to determine the upper limit of reliability of the reagent. For example, one lot of enzyme gave a reagent with a range of 30–500 μg glucose in 2 ml; that prepared with another lot was reliable only between 30 and 350 μg

in 2 ml. Where the upper limit is exceeded, color after acidification is a brownish violet and shows completely unreliable absorbance values, unrelated to the glucose concentration. Thus, a maximum absorbance value should be determined for the procedure as it is to be used. We have found a value of 0.400–0.425 to be the limit under our conditions (7.2 mm depth), corresponding to the upper limits given above with different lots of glucose oxidase.

Interference by salt from gradient elution is negligible from 0 to 0.5 *M* NaCl in the substrate. Color is increased about 5% at 0.5 *M* NaCl, 7% at 1 *M* NaCl, and at 2 *M* NaCl about 15% more color is produced. However, if tubes are not measured until an hour after acidification, salt interference is negligible (<5%) to at least 2 *M* NaCl.

Reagent and Procedure

Tris Buffer. A volume of 384 ml of 0.8 *M* HCl is added to 500 ml of 0.8 *M* Tris ("Sigma 7–9", Sigma Chemical Company, St. Louis, Mo.) solution, pH adjusted if necessary to 7.6, and the solution is made to 1 l with water.

A solution containing 800 mg glucose oxidase (crude, Sigma Chemical Company) and 16 mg peroxidase (horse-radish, type I, Sigma Chemical Company) in 200 ml of Tris buffer (above) and 200 ml water is filtered into an amber glass bottle. A solution of 180 mg *o*-tolidine dihydrochloride in 260 ml water is also filtered into the bottle. The contents are mixed and stored under refrigeration. The reagent should stand overnight before use and is stable in cold storage for at least 6 weeks.

For routine measurement of maltase or invertase activity of column filtrates, aliquots are incubated with the appropriate substrate in 2 ml volume. The buffer used must not be strong enough to affect materially the pH of the reagent added later. After incubation, 5 ml of the glucose oxidase–Tris reagent (at room temperature for accurate work) is added to tubes at exactly 1-min intervals, to allow time to acidify and read them. After 60 min at room temperature (25°C) 0.1 ml of 4 *N* HCl is added to each of the first two tubes, each being immediately mixed (a rod with a flat tip or touching the tube to a rubber stopper revolving on a stirring motor mixes rapidly). The first tube is read, using a displacement bar,[3] following Hamilton's (8) procedure. Then acid is added to the third tube; it is mixed, replaced in the rack, and the second tube is read, and so on. About 1 min is required for acidification and mixing

[3] The bar is a 220 × 12 mm piece of plate glass, 7.3 mm thick, polished on all six surfaces.

of a tube and reading the preceding tube. The color should be a clear violet, with no brown cast. Two tubes containing 300 μg glucose in 2 ml water are included in each lot for calibration.

If a blank with zero absorbance is desired, it is necessary to use substrates containing less than 0.005% glucose when 0.139 *M* saccharide substrates are employed. The glucose content of 0.4% found in a commercial maltose sample was reduced by one charcoal column treatment to less than 0.005%. Commercial sucrose gives no color with this reagent at this concentration.

SUMMARY

Inhibition of maltase contamination of glucose oxidase used for glucose assay is described, which provides a glucose oxidase reagent suitable for routine maltase assay of chromatographic fractions.

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ANALYTICAL BIOCHEMISTRY 2, 385–400 (1961)

SHORT COMMUNICATIONS

Prestaining Serum Lipoproteins with Protoporphyrin IX[1]

Protoporphyrin IX in aqueous solution has been found capable of staining lipids (1) and lipoproteins (2) on filter paper. Lipid materials treated with protoporphyrin IX show brilliant red fluorescence when viewed in ultraviolet light. Searcy *et al.* (3) have demonstrated that α- and β-lipoprotein cholesterol may be isolated and quantitated following direct elution of protoporphyrin-stained electrophoretic strips. More recently it has been observed that serum treated with small quantities of crystalline protoporphyrin becomes intensely fluorescent in ultraviolet light. Furthermore, electrophoresis or chromatography may be used to separate protoporphyrin-stained serum into two well-defined fluorescent areas.

Lipoproteins were stained by adding approximately 1 mg of commercially available protoporphyrin IX (California Corporation for Biochemical Research, Los Angeles) to 1 ml serum. The mixture was incubated at 37° for 1 hr, and excess dye was removed by centrifugation. Electrophoretic separations were performed in a Shandon Universal cell (Consolidated Laboratories, Chicago Heights, Illinois) on cellulose acetate paper (Oxoid No. 11-53 G). A 50-μl aliquot of stained-serum supernatant was placed on strips saturated with 0.07 M barbitone acetate buffer of pH 8.6 (Oxoid No. Br 11 G). A constant current of 0.4 ma/cm width of strip applied for 3 hr resulted in migrations of approximately 6 cm. When viewed under ultraviolet light two distinct bands were visible (Fig. 1). These zones were marked and subsequently stained for protein with Ponceau S. Thus, it was demonstrated that the two fluorescent areas were located in the β-globulin and α_1-globulin–albumin regions.

Protoporphyrin-stained serum was also applied to Whatman No. 3

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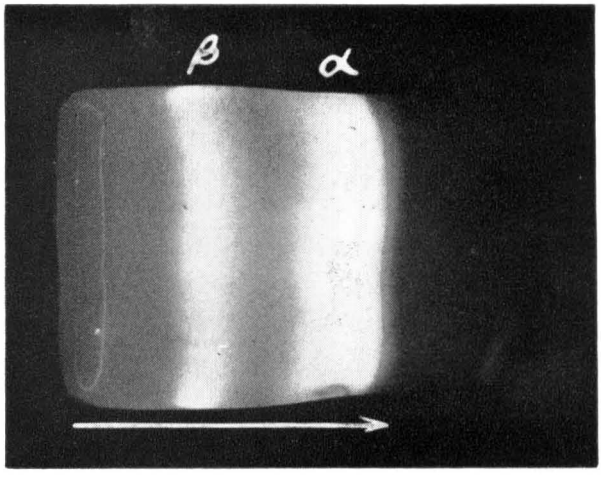

FIG. 1. A fluorescent electrophoretic strip obtained from 50 μl serum prestained with protoporphyrin showing α- and β-lipoprotein zones.

MM filter paper and developed by descending chromatography for 1 hr with a mixture of Veronal–oxalate–citrate buffer and isopropyl alcohol (3:2 v/v) (4, 5). Under ultraviolet light, separation into two fluorescent zones could be observed in the chromatographic chamber (Fig. 2). Fluorescent patterns from prestained serum separated chromatographically were similar to those obtained by poststaining with protoporphyrin.

Treatment of serum with crystalline protoporphyrin provides a simple

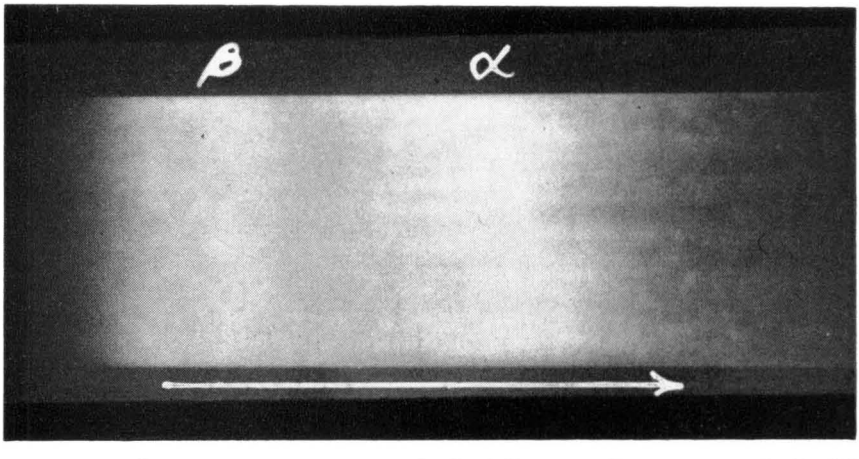

FIG. 2. A fluorescent chromatogram obtained from 50 μl serum prestained with protoporphyrin showing α- and β-lipoprotein zones.

technique for identification of lipoproteins separated chromatographically or electrophoretically. Attempts are being made to apply this method for measurement of lipoprotein fractions by eluting protoporphyrin bound to prestained serum. In addition, fluorescent lipoprotein patterns obtained from protoporphyrin-treated serum are currently being studied by densitometry.

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A Method for the Isolation and Separation of Catechol Amines and Their Transformation Products from Biological Media[1]

The methods in general use for the determination of adrenaline, noradrenaline, and other catechol amines and their derivatives from biological sources first involve the extraction of these substances in the free state (1). During preliminary *in vitro* experiments, with an aim towards isolating metabolites of 3-hydroxytyramine, we have found that

[1] Part of this work will be presented in a thesis by Miasnig Hagopian in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Department of Chemistry, Clark University. This investigation was supported in part with funds from contract AT(30-1)-918, U. S. Atomic Energy Commission.

certain advantages were gained by acetylating the catechol amines directly in the aqueous phase before extraction. The acetylation procedure employed is that described by Welsh (2) who found that dilute aqueous pharmaceutical preparations of adrenaline and noradrenaline could be quantitatively converted to the O^3,O^4,N-triacetyl derivatives. Some of the advantages of working with acetylated derivatives are as follows:

(*a*) The acetates are readily soluble in organic solvents such as ethyl acetate and methylene chloride; whereas the free catechol amines are very sparingly soluble.

(*b*) The esters are relatively stable substances in marked contrast to the free catechols which are easily oxidized.

(*c*) Since phenolic hydroxyls and primary and secondary amino groups are acetylated by this technique (3), the recovery of substances into the organic phase will include a wide range of possible transformation products of catechol amines. Other methods (4, 5) which utilize alumina for adsorption of catechol amines from biological fluids are specific for the catechol group; hence such possible non-catecholic metabolites as monohydroxyphenyl derivatives may pass undetected.

(*d*) A mixture of acetylated catechol amines may be chromatographically separated using mild nonaqueous systems.

A modified toluene–propylene glycol chromatographic system (6) was found to be most satisfactory for separating mixtures of triacetylated derivatives of known catechol amines. The material to be separated was placed on Whatman No. 1 paper strips which had been impregnated with a solution of propylene glycol in acetone (1:4 by volume). When chromatographed at 28° with propylene glycol-saturated toluene, the following mobilities (in cm/hr) were obtained for the O^3,O^4,N-triacetyl derivatives of the catechol amines listed:

Noradrenaline	0.5
Adrenaline	2.25
3-Hydroxytyramine	3.25
Epinine	7.0

The very sensitive Turnbull's blue reagent for phenolic substances (7) was used to locate the positions of the above substances on the paper strips after pretreatment with 5% alcoholic KOH. Alkaline blue tetrazolium reagent (8) was found to be equally useful for detecting catechol derivatives on paper.

The following adaptation of Welsh's method (2) exemplifies an application of this procedure for the isolation of metabolites which arise

during the incubation of 3-hydroxytyramine-1-C^{14} with bovine hypothalamic tissue in pH 7.35 phosphate buffer. The substrate to fresh tissue ratio was approximately 1:5000. The incubations were run for 3 hr at 37.5° in an atmosphere of 95% O_2 and 5% CO_2. At the end of the incubation period, the incubation mixture was acidified with 10% HCl to pH 1–2. The proteinaceous material was precipitated by the addition of 4 vol of acetone–methanol (1:1). The supernatant was concentrated *in vacuo* to an aqueous residue and extracted four times with equal volumes of ethyl acetate. The amine derivatives which are retained in the acid aqueous phase were acetylated by adding a large excess of sodium bicarbonate followed immediately by the addition of acetic anhydride in four equal portions with thorough mixing (a weight to volume ratio of 11:5 $NaHCO_3$–acetic anhydride was used). The mixture was allowed to stand 5 min after the evolution of CO_2 had ceased, and then was extracted with six equal-volume portions of methylene chloride. The residue obtained after removal of the solvent from the combined extracts was subjected directly to paper chromatographic analysis as described for the known compounds above. This resulted in the separation of this fraction which contained 6% of the starting radioactivity into six zones of radioactivity. The mobilities of two of these zones correspond to those of noradrenaline triacetate and 3-hydroxytyramine triacetate. The identities of the remaining metabolites in the amine fraction have not yet been determined.

The nonbasic fraction (ethyl acetate extract) contained 66% of the radioactivity. Paper chromatography of this material in an ascending system of benzene–acetic acid–50% ethanol (4:2:1) resulted in the appearance of one major radioactive zone which had the same mobility as 3,4-dihydroxyphenylacetic acid. The infrared spectrum of the substance eluted from this zone was identical with that of an authentic sample of 3,4-dihydroxyphenylacetic acid. This finding is consistent with the reported high level of monoamine oxidase activity in the hypothalamus (9) and the relatively fast rate of oxidative deamination of 3-hydroxytyramine by this enzyme (10).

This method has been successfully applied to other studies, and in some instances modifications have been made to adapt the method to the isolation and estimation of catechol amine metabolites in urine (11, 12).

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A Rapid Enzymic Determination of L(+)-Lactic Acid[1]

The standard procedure for the measurement of lactic acid is that of Barker and Summerson (1). Although very sensitive, it is laborious, time consuming, and somewhat nonspecific since it determines any compound that can be converted to acetaldehyde. A need arose in this laboratory for a procedure that would overcome these objections and which would be specific for the naturally occurring L(+) isomer found in tumor tissue and blood (2). An enzymic procedure is thus indicated.

The equilibrium between lactate and pyruvate lies so enormously in the direction of the former that it is not feasible, on the basis of enzyme action alone, to force it to completion in the direction of pyruvate. Gercken (3) and Warburg *et al.* (4) have attempted this by trapping

[1] Supported by a grant (A-3049) and a Senior Research Fellowship (SF-186) from the U. S. Public Health Service.

the pyruvate as the hydrazone or destroying it with H_2O_2 and reading the increase of optical density at 340 mμ. Neither of these methods were, however, considered adequate since they depend on the relatively low molar extinction coefficient of reduced diphosphopyridine nucleotide (DPNH), and their precision left much to be desired.

The method presented here utilizes a hydrazone trap (3) and the dye 3-*p*-nitrophenyl-2-*p*-iodophenyl tetrazolium chloride (INT) (5) as a hydrogen acceptor. A commercial preparation of diaphorase[2] from *Clostridium kluyveri* is used as the intermediate electron carrier. This preparation gave none of the difficulties cited by Nachlas *et al.* (6) who used instead a preparation from pig heart for the assay of lactic acid dehydrogenase (LDH).

A standard solution of 34 μg/ml of L(+)-lactic acid in 5% trichloroacetic acid (TCA) is prepared. Aliquots of 0.05–0.5 ml, representing a range between 1.7 and 17 μg are added to test tubes. Additional TCA is added to all tubes to bring the volume to 0.5 ml. Either 0.5 ml of standard, or TCA is added to the blank, as there is no demonstrable difference between the two in the absence of LDH. Then 1.3 and 1.5 ml of 0.1 *M* As_2O_3 buffer (adjusted to pH 9.6 with NaOH) are added to the standard and blank tubes, respectively. This preliminary addition of buffer is required to prevent denaturation of the enzyme and turbidity. Two milliliters of a freshly prepared reaction mixture is added to all tubes. This mixture is constituted so that each assay tube contains the following: 0.01 ml diaphorase;[2] 1.0 ml aqueous INT,[3] 1 mg/ml; 0.1 ml hydrazine sulfate, 0.1 *M* adjusted to pH 7.0; 0.1 ml gelatin, 0.5%; 4 mg DPN^+;[4] and 0.79 ml of the As_2O_3 buffer. Two-tenths milliliter of crystalline LDH[5] (diluted 1:10 with 0.1 *M* arsenate, pH 9.6 buffer) is added immediately to all tubes except the blanks. The tubes are incubated at room temperature for 30 min, and the reaction is stopped by the addition of 0.5 ml of 1 *N* HCl. The formazan produced is read at 500 mμ against the blank, using a Beckman DU spectrophotometer.

Unknowns from tissue, blood, etc., are deproteinized with TCA and the supernatant diluted to appropriate levels, maintaining the TCA concentration at 5%. The consistent use of unknown blanks containing no LDH is advised.

It is important that the final concentration of TCA be the same in all tubes, and that the reaction mixture be prepared immediately before

[2] Worthington Biochemical Company, Freehold, N. J.

[3] Dajac Laboratories, 5000 Langdon Street, Philadelphia, Pa.

[4] Pabst Laboratories, Milwaukee, Wis.

[5] Sigma Chemical Company, St. Louis, Mo., Type I, crystalline LDH, 57 mg/ml. 50,000 units/mg.

using. The addition of LDH should follow immediately if a low blank is to be obtained. It should be pointed out that other L(+)-α-hydroxy acids can serve to a limited extent as substrates for LDH (7). In most cases concerning biological systems, this problem is of little consequence.

In Table 1, the data indicate that a straight line for the range of concentrations between 2.8 and 17.4 μg is obtained. The method as presented is specific for the naturally occurring L(+) isomer of lactic acid and is several fold more sensitive than methods which depend on DPNH formation. It offers the additional advantage of simplicity and permits the assay of small quantities of tissue.

TABLE 1
FORMAZAN PRODUCTION WITH VARIOUS LEVELS OF LACTIC ACID

Standard[a] (ml)	μg/aliquot	ΔE_{500}	$\Delta E_{500}/\mu g$ lactate
0.08	2.78	0.068	0.024
0.10	3.47	0.087	0.025
0.20	6.94	0.169	0.024
0.30	10.4	0.264	0.025
0.40	13.9	0.335	0.024
0.50	17.4	0.416	0.024

[a] Standard solution contains 34.7 μg/ml of L(+)-lactic acid in 5% TCA.

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Protective Action of Bovine Serum Albumin in Measurements of Activity of Low Concentrations of Cellulase

Low apparent recovery of enzymic activity following starch block zone electrophoresis of small quantities of cellulase can be counteracted by extracting sections of the blocks with bovine serum albumin solution instead of with water (1). The present communication shows that this effect is attributable to a protective action of the albumin on the enzyme under the conditions of the assay for activity, and is increasingly apparent as the concentration of enzyme is lowered.

Samples of cellulase from *Myrothecium verrucaria* QM 460 (2) were incubated in different solutions for different periods of time at 50°C, and activities of the enzyme (3) were determined at the end of the treatment. The different solutions used were (*a*) 0.05 *M* pH 5 citrate, (*b*) 0.05 *M* pH 5 citrate–0.5% carboxymethylcellulose, (*c*) 0.05 *M* pH 5 citrate–0.005% bovine serum albumin, and (*d*) 0.05 *M* pH 5 citrate–0.5% carboxymethylcellulose–0.005% bovine serum albumin. Solutions having these particular compositions were selected because of their relationships to those used in the determination of activity of the enzyme: The citrate is the buffer which is employed in the method, while the citrate–carboxymethylcellulose solution is the mixture of buffer and substrate which is used. The retention of enzymic activity of different-sized samples of cellulase in the different solutions is given in Table 1, which

TABLE 1

RETENTION OF ENZYMIC ACTIVITY OF CELLULASE AFTER INCUBATION AT 50°C IN DIFFERENT SOLUTIONS FOR DIFFERENT TIMES

Concentration of cellulase (units/ml)	Per cent retention of enzymic activity					
	Citrate		Citrate–CMC[a]			Citrate–BSA[b] or Citrate–CMC–BSA
	1 hr	24 hr	½ hr	3½ hr	40 hr	½ hr–40 hr
1.0	87	81	102			100
0.2	58	16	90			100
0.1			81	86		100
0.02	33	0		71		100
0.01				67	71	100

[a] CMC = carboxymethylcellulose.

[b] BSA = bovine serum albumin.

shows that in the absence of bovine serum albumin the retention decreased as the concentration of the enzyme decreased. The table shows further that in tests carried out in citrate solution the retention also decreased with increasing periods of incubation; however, in tests carried out in the citrate–carboxymethylcellulose solution, the activity appeared to decrease only to equilibrium levels beyond which further inactivation did not occur on prolongation of incubation. In additional

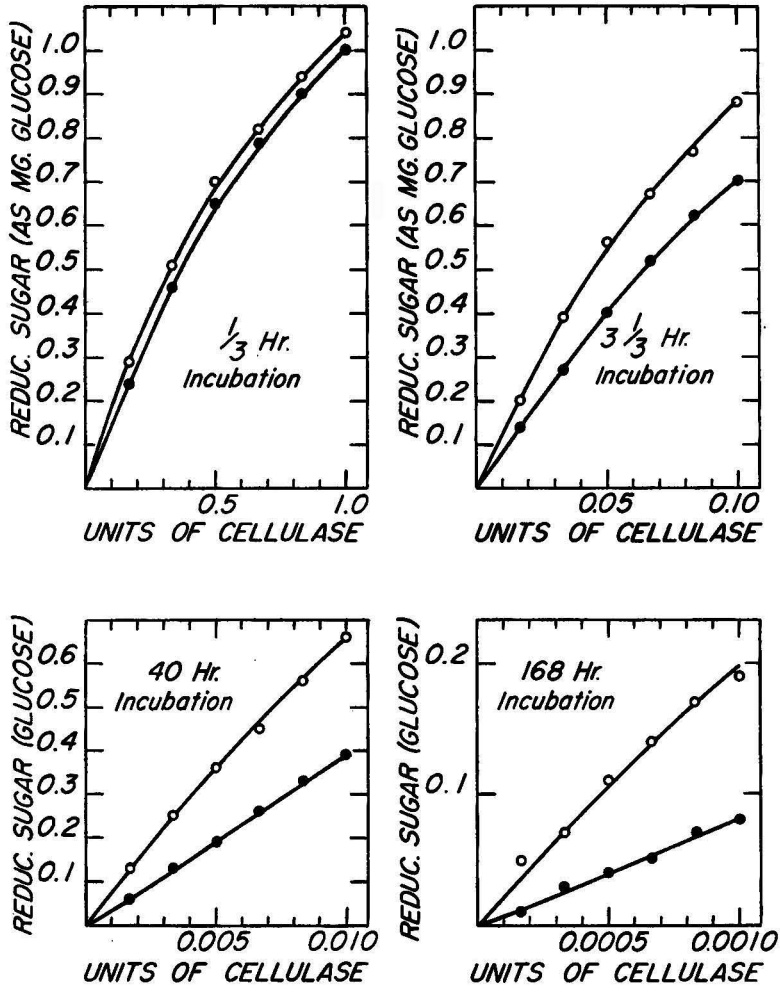

FIG. 1. The relation between amount of cellulase and amount of reducing sugar produced over different ranges of activity in the presence (○—○) and absence (●—●) of bovine serum albumin.

tests, now shown, it was found that the same protective effect was afforded by 0.02% albumin, that 0.002% albumin was only slightly less effective, and that 1% albumin caused marked inhibition.

The effects of bovine serum albumin upon the relationship between the amount of cellulase and amount of reducing sugar produced, as determined under conditions used for measurement of enzymic activity (3), are shown in Fig. 1. The results confirm and extend those of Table 1. They also demonstrate that the deviations from normal enzymic behavior which was previously noted for cellulase (3) are partially explained by the destruction of the enzyme which occurs at low concentrations in the absence of a protective agent. Thus the slight *S*-shape of the dilution curve is eliminated by the presence of the bovine serum albumin. Furthermore, constant products of enzyme concentration and time of incubation yield more nearly constant amounts of reducing sugar when tests are carried out in the presence of the albumin than in its absence.

A stimulatory effect of protein on cellulase has been reported previously (4). However, this effect was applicable only to tests using cellulose as the substrate, not to tests using carboxymethylcellulose. The lack of effect in tests using carboxymethylcellulose as the substrate might have been due to the use of too high concentrations of enzyme to show the effect.

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An Internal Standard for the Colorimetric Determination of Monoamine Oxidase Activity

The colorimetric procedure of Green and Haughton (1) for determining monoamine oxidase (MAO) activity is convenient, but extraction of the aldehyde hydrazone formed is not quantitative. To determine the actual amount of product formed, the authors corrected the results by a factor based on the proportion of added aldehyde hydrazone recovered in their extraction procedure. Such corrections are not reliable when applied to different tissue preparations, such as crude homogenates (see below), where the aldehyde produced via MAO is not quantitatively recovered.

In a colorimetric procedure similar to that of Green and Haughton, but developed independently in this laboratory, we have used as an internal standard the semicarbazone of *p*-hydroxyphenylacetaldehyde (p-HPA). After vain attempts to prepare this compound via the procedure outlined by Langheld (2), we applied the "hydramine splitting" reaction of Auterhoff and Roth (3) to sympatol (1-(4-hydroxyphenyl)-2-methylaminoethanol, K & K Laboratories, Inc., Jamaica, N. Y.) with success. Three grams of sympatol were mixed with 30 gm of 95% H_3PO_4 (prepared by boiling the 85% reagent until the temperature reached approximately 200°C). After heating the sympatol–phosphoric acid mixture for 3 hr on the steam bath, the brown solution was mixed with 450 ml of cold water, and subsequently extracted three times with equal volumes of ether. The ether was then removed under vacuum in a rotary evaporator (Rinco Instrument Company, Greenville, Ill.) without heating, the residue being kept from freezing by rotating the flask in cool water. The crude aldehyde was stored at —18°C. Upon reacting the cold crude p-HPA with an equal volume of saturated semicarbazide hydrochloride, aqueous 4-nitrophenylhydrazine (free base), or solid 2,4-dinitrophenylhydrazine hydrochloride, we obtained products with the expected melting points, ultraviolet and infrared characteristics, and elemental composition of the respective hydrazones (Table 1). The p-HPA semicarbazone so derived exhibited the same spectral characteristics when reacted with 4-nitro- or 2,4-dinitrophenylhydrazine as did the product of MAO-catalyzed tyramine oxidation.

Recoveries of p-HPA after adding known quantities of tyramine to rat liver homogenates are shown in Table 2. In this experiment, 2.0 ml of 2.5% rat liver homogenate in 0.2 *M* pH 7.4 buffer were agitated with

TABLE 1
COMPOUNDS RELEVANT TO THE COLORIMETRIC MAO ASSAY

Compounds[a]	Absorption maxima (mμ)	ε	Melting point (°C)[b]		Elemental analysis					
					C		H		N	
			Literature	Obtained	Theo.[c]	Obt.	Theo.	Obt.	Theo.	Obt.
p-HPA via Ref. (3)	285, ether	—	—	—	—	—	—	—	—	—
p-HPA semicarbazone	248, 275, 5% DMF	3320 (248 mμ)	—	194	56.0	56.1	5.7	5.8	21.4	21.8
4-NP derivative of p-HPA (2)	460, 0.1 *N* base	19,340	158	160	62.0	61.5	4.8	5.0	15.5	15.2
	420, 0.0375 *N* base	16,090	—	—	—	—	—	—	—	—
4-NP derivative via assay[d]	460, 0.1 *N* base	—	—	—	—	—	—	—	—	—
	420, 0.0375 *N* base	—	—	—	—	—	—	—	—	—
DNP derivative via Ref. (4)	430, 0.1 *N* base	—	183	180	53.4	53.8	3.8	4.2	17.8	17.6
DNP derivative via Ref. (3)	430, 0.1 *N* base	15,870	184	186	53.2	53.7	3.8	4.2	17.8	17.6

[a] p-HPA, *p*-hydroxyphenylacetaldehyde; 4-NP, 4-nitrophenylhydrazine; DNP, 2,4-dinitrophenylhydrazine; DMF, *N*,*N*-dimethylformamide.

[b] Uncorrected data taken with a Fisher-Johns melting point block.

[c] Theo., theory; Obt., obtained.

[d] Not isolable from biological mixture.

TABLE 2
RECOVERIES OF *p*-HYDROXYPHENYLACETALDEHYDE FORMED ENZYMICALLY FROM TYRAMINE

Tyramine added (μmoles)	Aldehyde formed[a] (μmoles)	Per cent recovery
2.0	1.9	95
4.0	4.1	102
6.0	5.7	95

[a] Averages of triplicate determinations. See text for details.

0.5 ml of buffer and 0.5 ml of neutralized 0.06 *M* semicarbazide–0.006 *M* KCN solution containing 2, 4, or 6 μmoles tyramine at 37°C in oxygen for 60 min. Standard mixtures containing 2 μmoles of p-HPA semicarbazone (stock solution containing 2 μmoles per 0.5 ml made by dissolving 19.3 mg in 3 ml of *N,N*-dimethylformamide and diluting to 25 ml with buffer) added in 0.5 ml buffer, 0.5 ml of KCN–semicarbazide solution without tyramine, and 2.0 ml homogenate as above were incubated simultaneously with the mixtures containing tyramine. Unincubated mixtures were used as zero-time blanks. Proteins were removed by

TABLE 3
MONOAMINE OXIDASE ACTIVITY OF WHOLE HOMOGENATE AND MITOCHONDRIA OF RAT LIVER

Preparation[a]	Tyramine oxidized (method)	
	Colorimetric	Manometric
Liver homogenate Trial 1	129.0 μmoles/hr/gm liver	—
2	119.2	—
3	129.6	—
Over-all average ± S.D.[b] and (range)	126.0 ± 9.6 (106.0–139.0)	
Mitochondria average ± S.D. and (range)	13.0 ± 1.8 (11.0–15.2) μmoles/hr/mg N	14.3 ± 1.6 (12.7–15.5) μmoles/hr/mg N

[a] Each homogenate trial represents the average of values for 5 rats; 1.0 ml of 2.5% homogenate was incubated for 30 min as described in the text with 30 μmoles of tyramine substrate. The data presented with mitochondria [isolated by the method of Schneider (6)] are for five rats by the colorimetric method; results with the manometric method (5) represent determinations on pooled liver samples from 30 groups of three rats each. The data from both methods are expressed as "tyramine oxidized" for convenience of expression. The difference between colorimetric and manometric values for mitochondria is not significant ($p \sim 0.3$).

[b] Standard deviation.

adding 0.5 ml of 50% trichloroacetic acid (TCA) to each mixture and centrifuging. Colorimetric analyses for p-HPA were performed by reacting 0.5-ml aliquots to the supernatants made to 3.0 ml in ice water with 1.0 ml of ice cold 1.5% 4-nitrophenylhydrazine hydrochloride in 1 *N* HCl at 0°C for 15 min. Upon extraction of the hydrazone into 2.0 ml chloroform, re-extraction of 1.0 ml of the chloroform with 5 ml of ice-cold 0.1 *N* NaOH, and dilution of 3.0 ml of the basic solution to 8.0 ml with cold water, absorbancies were determined in a colorimeter at 420 mμ. As is evident in Table 2 calculations of product formed based on the absorbancies given by the standard indicated quantitative recovery was obtained of p-HPA formed enzymatically from tyramine.

Monoamine oxidase activities of Wistar strain rat liver mitochondria were the same whether determined by Creasey's manometric method (5) or by the above colorimetric method; 2.5% rat liver homogenate activities determined colorimetrically were reproducible from day to day (Table 3).

When 10% rather than 2.5% liver homogenate was used, the recovery of p-HPA declined (Table 4). Since little added p-HPA semicarbazone was destroyed, and since prolonged incubation did not decrease the recovery of aldehyde from tyramine, it appeared that oxidation of the aldehyde was occurring before the semicarbazone was formed, and was possibly due to high concentrations of aldehyde dehydrogenase or its cofactors in the 10% homogenate. This view was partially supported by decreased recovery of p-HPA from tyramine when diphosphophyridine nucleotide was added to the incubation mixture (Table 4).

TABLE 4

EFFECT OF INCREASED HOMOGENATE CONCENTRATION OR DIPHOSPHOPYRIDINE NUCLEOTIDE (DPN) ADDITION ON RECOVERIES OF *p*-HYDROXYPHENYLACETALDEHYDE

Addition[a]	p-HPA recovery (μmoles)
2 μmoles tyramine plus 2.5% homogenate	2.0
10% homogenate	0.6
2.5% homogenate + 1 μmole DPN	1.0

[a] Ten per cent homogenate was prepared by homogenizing 4 gm of fresh rat liver in 36 ml of cold 0.2 *M* phosphate buffer at pH 7.4 and straining through two layers of gauze; 2.5% homogenate was prepared by making a 1:4 dilution of the 10% preparation in buffer. Reaction mixtures consisted of 2.0 ml homogenate, 0.5 ml of 0.2 *M* phosphate buffer alone or containing 1 μmole DPN, and 0.5 ml of 0.06 *M* semicarbazide–0.006 *M* KCN solution containing 2 μmoles tyramine. An internal standard was prepared with 2 μmoles p-HPA semicarbazone added in 0.5 ml buffer (see text,) 2.0 ml homogenate, and 0.5 ml semicarbazide–KCN solution without tyramine. Incubation was at 37°C for 1 hr in oxygen; assays were performed on 0.5-ml aliquots of TCA-treated supernatant solutions as described in the text.

Because of possible aldehyde losses as demonstrated above, it is important to determine that a stoichiometric relation exists between tyramine utilized and product formed in a given system before using it routinely. Such determinations are not difficult with purified preparations on which oxygen consumption or NH_3 formation may be estimated; however, with crude preparations neither of the above procedures may be practical. Under these conditions, the use of a colorimetric method and an internal standard is essential for accurate estimations of MAO activity.

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Quantitative Determination of the Minor Hemoglobin Component Hb-A_2 by DEAE-Cellulose Chromatography[1]

Quantitative analyses of the minor hemoglobin fraction Hb-A_2 in different hemoglobin samples have been successfully carried out using starch electrophoresis (1, 2) and chromatography with the cation exchanger carboxymethylcellulose (CMC) as adsorbent (3, 4). With both methods the amount of Hb-A_2 in the blood of normal adults was found to range from 1.8 to 2.5% of the total hemoglobin, while the percentages of Hb-A_2 were increased to 4–6% in most cases of thalassemia trait.

In recent investigations[2] the chromatographic behavior of different

[1] Supported by U. S. Public Health Service Grant No. H 5168.
[2] T. H. J. Huisman and A. M. Dozy, *J. Chromatography.* In press.

human hemoglobin types on the anion exchanger diethylaminoethyl-cellulose (DEAE-cellulose) was studied. It was found that the elution rates of many different hemoglobin types (with the exception of fetal hemoglobin) were in accordance with differences in their isoelectric points. With the use of this anion exchanger it was possible to separate the hemoglobin of a normal adult into three components: the minor Hb-A_2 fraction being eluted first followed by the main hemoglobin component (A_0) and then the electrophoretically fast moving Hb-A_1 fraction. Since the separation between the Hb-A_2 and the Hb-A_0 components was complete, with both fractions, after isolation, behaving electrophoretically pure [tested by starch gel electrophoresis (5, 6)], new possibilities were presented for the determination of Hb-A_2 percentages in blood samples.

The following routine procedure was developed:

A large quantity of DEAE-cellulose (Selecta cel-DEAE,[3] type 40) is carefully equilibrated by repeated washings with 0.005 *M* sodium phosphate buffer pH 8.6 containing 100 mg KCN/l. A small quantity of absorbent is washed once again with the same buffer just prior to the experiment. The equilibrated DEAE-cellulose is poured into columns of 20×0.9 cm and packed under atmospheric pressure until a column height of 15 cm is obtained. Prior to chromatography hemoglobin solutions are dialyzed for 24 hr against the buffer used for the equilibration. From 10 to 25 mg oxyhemoglobin, dissolved in 0.5–2.0 ml, is chromatographed. Elution of the Hb-A_2 fraction is performed by applying a 0.01 *M* sodium phosphate buffer pH 8.6 (with 100 mg KCN/l) to the column from a separatory funnel. The flow rate is adjusted to 30–40 ml/hr. Fractions of 3.5–4.0 ml are collected in tubes graduated to 4 ml. After the elution of the Hb-A_2 fraction (about 40 ml of the effluent is required), the column is mounted above a volumetric flask (200 ml) and the remaining hemoglobin eluted with the use of 0.01 *M* sodium phosphate buffer pH 6.0 to which 0.3 *M* NaCl is added. The volumes of the fractions are finally adjusted to 4 and 200 ml, respectively. Examination of the fractions is carried out at 415 mμ in the Beckman DU spectrophotometer. The percentage of Hb-A_2 is calculated using the formula

$$\frac{A}{A + B \times 50} \times 100\%,$$

in which A represents the combined optical densities of the Hb-A_2-containing fractions and B the optical density of the hemoglobin solution collected in the 200-ml volumetric flask.

[3] Brown Company, 55 Main Street, Berlin, N. H.

Examples of elution diagrams for the hemoglobins of a normal adult and a patient with thalassemia trait are presented in Fig. 1. The Hb-A_2 was completely separated from the main component. In starch gel electrophoresis the isolated Hb-A_2 was found to be free of any other Hb fraction (Fig. 1). Quantitative data are presented in Table 1. The

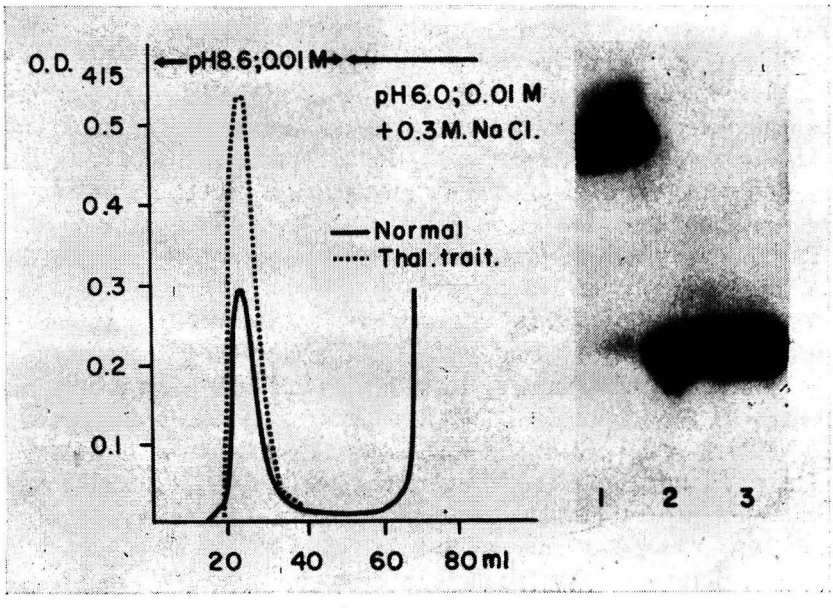

FIG. 1. Left: Elution diagrams of the hemoglobins of a patient with thalassemia trait and of a normal control. Right: Starch-gel electrophoresis of the isolated Hb-A_2 fraction (2 and 3) and of normal Hb-A. The eluted Hb-A_2 was concentrated by CMC chromatography (3).

mean Hb-A_2 level of 2.1% found in blood samples of 34 normal individuals is in close agreement with the percentages determined either by starch-block electrophoresis or by CMC chromatography.

In the blood of 15 patients with thalassemia minor the Hb-A_2 level was increased to 4.4% with a range of 3.8–5.2%. The method offers reproducible data since repeated Hb-A_2 analyses in one hemoglobin sample carried out at the same time revealed similar results (Table 1).

The new chromatographic technique can also be applied to hemoglobin samples from cases with various hemoglobinopathies. Electrophoretically slow-moving hemoglobins, however, will interfere with the elution of the Hb-A_2 component. Application is therefore restricted to hemoglobin samples of normal individuals, patients with thalassemia minor, and carriers of hemoglobin anomalies possessing higher electrophoretic mobil-

ities than Hb-A. Since fetal hemoglobin does not interfere with the elution of the Hb-A_2 component,[2] the method can be used in the study of samples from cord blood and from patients with the persistent high

TABLE 1

Hb-A_2 PERCENTAGES IN BLOOD SAMPLES OF NORMAL ADULTS AND OF PATIENTS WITH THALASSEMIA MINOR DETERMINED BY DEAE-CELLULOSE CHROMATOGRAPHY

Case	No.	Hb-A_2	Range
Normal	34	2.1 ± 0.2	1.6–2.5
Thal. minor	15	4.4 ± 0.3	3.8–5.2

Case	Col. 1	Col. 2	Col. 3	Col. 4	Mean
Normal	1.8	1.95	1.85	1.95	1.9
Normal	2.0	2.2	2.2		2.1
Thal. trait	5.25	5.25	5.05	5.25	5.2
Thal. trait	4.5	4.3	4.1	4.1	4.25
Thal. trait	4.6	4.8	4.5		4.6
Thal. trait	4.8	4.7	4.3	4.4	4.55
Thal. trait	4.3	4.3	4.1	4.0	4.2

fetal hemoglobin abnormality or thalassemia major. The use of DEAE-cellulose chromatography for the preparation of large quantities of different hemoglobin fractions will be reported elsewhere.[2]

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Microbiological Assay of Intact Proteins Using *Tetrahymena pyriformis* W I. Survey of Protein Concentrates

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Received March 13, 1961

INTRODUCTION

An investigation of best methods of processing cottonseed meals for feeding nonruminant animals requires some means of evaluating the nutritive quality of the meals and their intact protein fractions. This quality may be determined by small animal (chick or rat) feeding tests, but there is a need for a more rapid assay requiring smaller amounts of research material. Rosen and Fernell (1, 2) and Rosen, Stott, and Smith (3) determined the relative nutritive values of intact proteins with *Tetrahymena pyriformis* W. The advantages of using this organism for protein assays is discussed by these authors and by Kidder and Dewey (4), Grau and Carroll (5), and Rosen (6); and the status of microbiological assays of proteins is reviewed by Rosen (6).

A study was made of the feasibility of using the Rosen (6) method with modifications as a guide to processing research to develop oilseed meals of high nutritive quality. A preliminary study on the use of the method in assaying a variety of intact proteins and a mixture of amino acids and a statistical study of the results obtained with the reference standard, "vitamin-free," purified casein are reported. This microbiological assay is apparently satisfactory for the intended purpose. A survey of the use of the modified method in assaying some intact proteins and mixture of amino acids is reported.

EXPERIMENTAL

The procedure followed in this study was essentially that of Rosen and Fernell (1, 2).

[1] One of the Laboratories of the Southern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

Test Organism

The test organism, *Tetrahymena pyriformis* W, was obtained from G. W. Kidder, Biological Laboratories, Amherst College.

1. *Maintenance.* Axenic stock cultures were maintained at 25 ± 2°C in screw-cap test tubes (16 × 130 mm) containing 10 ml of nutrient broth of the following composition (% w/v): proteose peptone (Difco)[2]

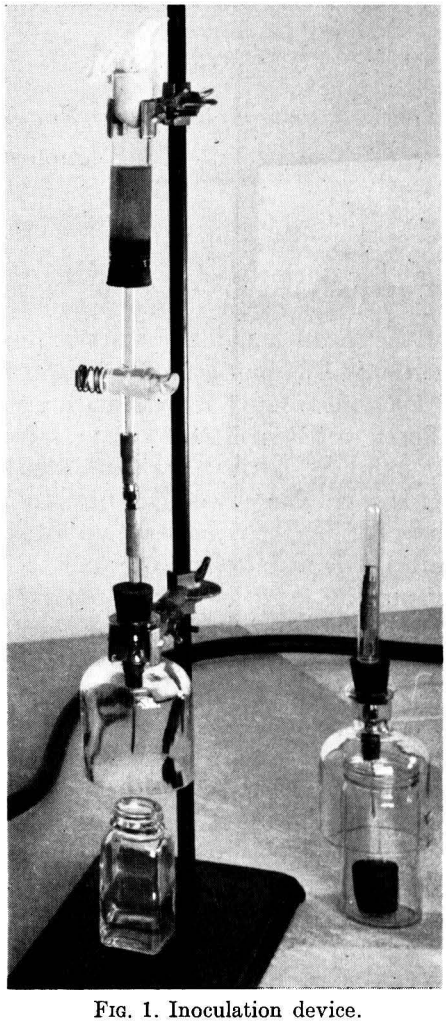

FIG. 1. Inoculation device.

[2] The mention of this or other commercial products does not constitute an endorsement of them by the U. S. Department of Agriculture.

2.0, Yeast Extract (Difco) 0.1, glucose 0.5, sodium chloride 0.1, adjusted to pH 7.1, and sterilized by autoclaving (120°C for 15 min). Stock cultures were transferred at approximately weekly intervals and checked periodically for contamination.

2. *Inoculum.* Each assay bottle received an inoculum of two drops (approximately 0.03 ml each) of a composite of two 3-day, 10-ml broth cultures, from the device illustrated in Fig. 1. It consisted of a piece of glass tubing 22 mm i.d. and 135 mm long, fitted with a rubber stopper as a bottom. The glass capillary tubing and stopcock have a 1.5-mm i.d. bore. The needle is No. 15. The capillary tube is extended through the stopper for 22–25 mm to provide a raised outlet. The "funnel" used to protect the needle from contamination is a 250-ml Pyrex bottle with the bottom removed. The inoculation setup, with a cotton stopper at the top of the container and wrapped in Dispo-wrap paper,[3] was autoclaved for 15 min at 120°C. Two bottles of the stock culture were poured aseptically into the sterilized container. The nonmotile and inactive organisms settled below the level of the tip of the capillary and the actively motile organisms were used as inoculum.

Basal Medium

Table 1 shows the composition of the basal medium. It was prepared from a series of stock solutions.

1. *Preparation of Stock Solutions. Group A.* For Group A, stock solutions of all the vitamins except riboflavine and biotin, were mixed and lyophilized, and stored in brown, wide-mouth bottles, over P_2O_5 in a desiccator. During preparation and storage the vitamin mixture was protected from light. This stock mixture retained its usefulness for more than 18 months. Riboflavine and biotin solutions were prepared as needed.

Groups B, C, and D. Stock solutions of Groups B, C, and D (100 $\times$ final concentration) were each sterilized by Millipore filtration.

Group E. The components of Group E were dissolved, mixed, and dried by lyophilization, ground to a coarse powder, and stored over P_2O_5 in a desiccator. This stock mixture was useful for more than 18 months, as indicated by good check determinations with casein.

Group F. Two per cent soluble starch autoclaved with the protein was used as the source of energy as suggested by Rosen (6).[4]

2. *Preparation of Basal Medium.* A solution of 2.5 $\times$ final concen-

[3] Manufactured by Scientific Products, Division of American Hospital Supply Corporation, Evanston, Ill.

[4] Also, G. D. Rosen, private communication.

TABLE 1
COMPOSITION OF THE BASAL MEDIUM

Group	Component	Concentration in assay medium (μg/ml)
(A)	Pantothenic acid	0.625
	Nicotinamide	0.625
	Pyridoxine hydrochloride	6.25
	Pyridoxal hydrochloride	0.625
	Pyridoxamine hydrochloride	0.625
	Riboflavine	0.625
	Folic acid	0.0625
	Thiamine hydrochloride	6.25
	Inositol	0.625
	Choline chloride	6.25
	p-aminobenzoic acid	0.625
	Biotin	0.0625
	Lipoic acid	0.02
(B)	$MgSO_4 \cdot 7H_2O$	140.0
	$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	62.5
	$MnCl_2 \cdot 4H_2O$	1.25
	$ZnCl_2$	0.125
(C)	$CaCl_2 \cdot 2H_2O$	30.0
	$CuCl_2 \cdot 2H_2O$	3.0
	$FeCl_3 \cdot 6H_2O$	0.75
(D)	K_2HPO_4	175.0
	KH_2PO_4	175.0
(E)	Guanylic acid	150.0
	Adenylic acid	100.0
	Cytidylic acid	125.0
	Uracil	50.0
(F)	Soluble starch	20,000

tration was prepared from the stock solutions of Groups B, C, and D and the lyophilized mixture of Group E. The pH was adjusted to 7.1 with NaOH. A slight precipitate formed even though the stock solutions were diluted before they were mixed, and various orders of mixing were tried. The solution was stored in approximately 120-ml lots in a deep freezer until they were needed.

A solution of Group A, 10 × final concentration, was made of the lyophilized vitamin mixture and the riboflavine and biotin solutions noted above. The pH was adjusted to 7.1 with NaOH. The solution was sterilized by filtration (Millipore) because panthothenic acid (7), pyridoxal hydrochloride (7), thiamine hydrochloride (7–9), and *p*-amino-

benzoic acid (10) are reported to be unstable to autoclaving. The sterilized solutions protected from light and stored frozen were good for at least 2 months, as indicated by the good checks for assays on casein.

Preparation of Proteins for Assay

As suggested by Rosen,[4] the proteins were ground to pass through a 40-mesh screen. The material was suspended in distilled water, the pH adjusted to about 8.2 with NaOH, also as suggested by Rosen,[4] and refrigerated overnight. After warming to room temperature the pH was readjusted to 8.2.

Assay Procedure

The medium containing Groups B, C, D, and E (4 ml, 2.5 × concentration), starch (1 ml of 20% solution, hot), and the protein or other nitrogen source (4 ml) were autoclaved in an assay bottle at 120°C for 15 min. After cooling to room temperature, 1 ml of Group A (10 × final concentration) was added aseptically.

After the cultures were inoculated, the bottles, with loose screw caps, were placed in special racks at a slope of 15° to the horizontal to insure a high surface to volume relationship of the culture medium. The cultures were incubated at 24.5 ± 1°C, relative humidity 80–90%, for 4 days. A high relative humidity was used to lessen evaporation since the results were reported on a volume basis. Triplicate cultures shaken for *ca.* 3 min were composited. Each composite was then shaken for *ca.* 3 min before taking aliquots for analyses.

Reference Standard

The reference standard was "vitamin-free," purified casein (Control No. 408315) prepared by Difco Laboratories, Detroit, Michigan.

Nitrogen Determinations

Nitrogen determinations were made by a modified Kjeldahl method (11) on controls containing 4 ml of material to be assayed plus 6 ml water (instead of the basal medium, starch, and vitamins) autoclaved as for an assay.

Growth Response

The growth response was measured by determining microscopically the numbers of organisms per milliliter present in the inoculated test materials (termed "cultures") after 4 days' incubation.

A composite of triplicate cultures of each test material at each level of nitrogen concentration assayed was used in estimating the population of *T. pyriformis* W. One milliliter (one aliquot) was removed with a bacteriological blow-out pipet and transferred to a screw-cap vial (2-dram capacity, 17 mm o.d., 60 mm over-all length) containing 1 ml of preserving fluid. This fluid was 6% formaldehyde in phosphate buffer of five times the concentration of the D component of the assay medium (Table 1). In preparing the material for counting the organisms, it was found necessary to agitate the vial containing the preservative while adding the culture to avoid clumping of the organisms. At least two aliquots from each composite at each nitrogen level assayed were analyzed.

The numbers of organisms were determined microscopically in a Spencer, improved Neubauer, double-cell hemacytometer. The cultures were transferred to the cells of the counting chamber with capillary pipets made of 6 mm o.d. (*ca.* 4 mm i.d.) tubing with an orifice of *ca.* 1 mm i.d. The organisms on five of the 9 sq mm areas of the network of each cell were counted. From each of at least two aliquots, the organisms on 20 or more sq mm areas were counted. The mean of the numbers of organisms in a minimum of 40 sq mm areas multiplied by the dilution $\times 10^4$ gave the population per milliliter of the original culture. Before counting, all of the network of each cell was examined to determine that even filling had been obtained. One operator made all the determinations on all of the samples.

Statistical Validation of Counting Technique

Before the study of experimental proteins was begun, a statistical study was made of the sources of variance in estimation of *Tetrahymena* populations in the assay of casein. In this study two composites were prepared for each nitrogen level by shaking and pouring together the contents of three culture bottles for each composite. Two aliquots were withdrawn from each composite, and two pipettings were made from each aliquot filling both cells of each of three chambers at each pipetting. The numbers of organisms on each of five areas of 1 sq mm from each cell were counted. These data were transformed to logarithms and subjected to analysis of variance procedures which enabled the isolation of mean squares and estimation of variance components as shown in Table 2.

From Table 2 it is evident that the additional variability due to different aliquots from the same composite and the additional variability due to different pipettings from the same aliquot are negligibly small.

TABLE 2

MEAN SQUARES AND ESTIMATED COMPONENTS OF VARIANCE FOR SOURCES OF VARIABILITY AFFECTING MEANS OF LOGARITMHS OF COUNTS OF *Tetrahymena pyriformis* W

Source of variability	Degrees of freedom	Mean square	Estimate of component	
Nitrogen Levels (L)	4	7.8373		
Composites (S) in L	7	.1012	.0003	.0014
Aliquots (A) in S	12	.0780	.0001	
Pipettings (P) in A	23	.0730	.0010	
Chambers (CH) in P	45	.0545	.0028	
Cells (C) in Ch	89	.0266	.0022	
Squares (Sq) in C	724	.0156	.0156	

composites as indicated by the bracketing of these three sources of variability in Table 2 and as would happen in practice if only one pipetting of one aliquot is used.

Taking into account the estimated costs of counting more cells vs. more squares, more chambers vs. more cells, etc. as compared to the increase in precision from increased cells, chambers, etc., it is indicated that the optimum sampling schemes for a number of cost situations do not differ widely from the scheme described in the main body of this paper. Estimating the losses due to such things as cells overflowing, poor distributions of organisms in the cell, etc., at one-third, this procedure is estimated to yield an average of four chambers (40 squares) counted per composite.

The standard deviation of a single count (square) from a single cell by the foregoing system is about 40%, whereas the standard deviation of the average count of four chambers is estimated to be about 13%.

Test Materials

The commercial egg albumin (Control No. 6682), crystalline egg albumin (Control No. 9060, 1 gm, and Control No. 2351, 1.5 gm), lactalbumin (Control No. 2659), and α-protein from soy (Control No. 8667) were obtained from the Nutritional Biochemicals Corporation, Cleveland, Ohio, the bovine hemoglobin (Lot No. 206-9) from the Armour Laboratories, Chicago, Ill. The compositions of the other test materials are mentioned later.

Notes on the Assay Method

In the first run made, the growth of *T. pyriformis* W was tested in the basal medium without added nitrogen. A count made on the composite of two cultures used for the inoculum showed there were 0.029 million

organisms/0.06 ml, the amount used per assay bottle. Therefore each inoculated culture for the assay contained approximately 0.0029 million organisms/ml at the zero hour. After inoculation and 4 days of incubation, the basal medium with 2% soluble starch, but without added nitrogen, contained less than 400 organisms/ml. This medium without added nitrogen did not support the growth of the test organisms.

The use of starch instead of glucose as the source of energy simplified the assays.

RESULTS AND DISCUSSION

According to Rosen (6),[4] the amount of ammonia nitrogen excreted by the test organisms is not required in evaluating the nutritional quality of proteins by this assay, and therefore ammonia was not determined.

Evaluation of Growth Response

The growth response was evaluated by computing the linear regression coefficient for the regression of numbers of organisms per unit volume of medium on the nitrogen content of the test substance in the medium.

TABLE 3

GROWTH RESPONSE OF *Tetrahymena pyriformis* W TO SEVERAL SOURCES OF NITROGEN (AUTOCLAVED)

Nitrogen source	Range of nitrogen concentration (mg/ml)			
	0–1		0–1.7	
	Regression coefficient[a]	Nutritive index	Regression coefficient[a,b]	Nutritive index
Casein (vitamin-free)	1.05	1.00	0.92	1.00
Egg albumin (commercial)	0.92	0.88	0.74	0.80
Lactalbumin	0.91	0.87	0.85	0.92
Soybean meal (#100, toasted)	0.63	0.60	0.48	0.52
Bovine albumin (crystalline)	0.62	0.59	[d]	[d]
Amino acids mixture[c]	0.57	0.54	0.39	0.42
α-Protein from soybean	0.52	0.49	0.38	0.41
Incaparina 9B	0.31	0.29	0.31	0.36
Soybean ration, No. 50	0.29	0.27	0.33	0.36
Egg albumin (crystalline)	0.18	0.18	[d]	[d]
Bovine hemoglobin	0.04	0.04	0.04	0.04

[a] The regression coefficient is the increase in number of organisms/ml for each added mg nitrogen/ml.

[b] Most of the regression coefficients in this nitrogen range are smaller than those in the lower range, indicating that the response to nitrogen is probably curvilinear rather than linear.

[c] Amino acids in concentrations used in Medium D of Kidder *et al.* (4, 16).

[d] Insufficient data.

The regression coefficient b was calculated from the data of nitrogen levels ranging from zero through 1 mg/ml, and the regression coefficient b' was computed from observations in the nitrogen levels ranging from zero to 1.7 mg/ml. The coefficients were computed with the restriction that the line of response must pass through the origin, "no growth at 0 mg nitrogen/ml" (12). Table 3 shows the growth response expressed as the linear regression coefficients and the nutritive indices for the nitrogen sources studied.

Casein Reference Standard

Casein, "vitamin-free," purified, was assayed at several nitrogen levels in 16 runs to determine its growth curve (Fig. 2). The response was the

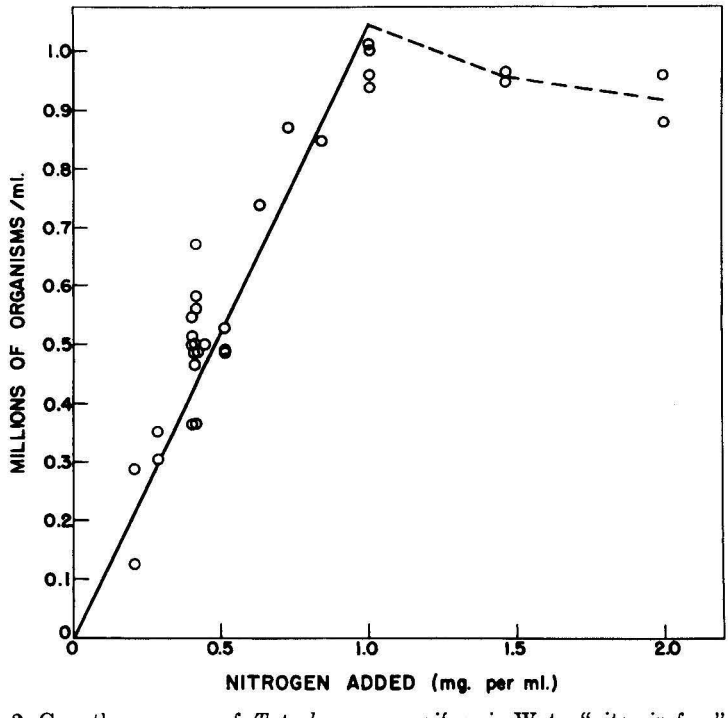

FIG. 2. Growth response of *Tetrahymena pyriformis* W to "vitamin-free" casein at various nitrogen concentrations, assayed in 16 runs. Solid line: slope of regression b; broken line: trend of growth at nitrogen concentrations above 1 mg/ml medium.

same as that reported by Fernell and Rosen (1), maximum growth of 1 million organisms/ml occurring with 1 mg nitrogen/ml of final medium and lesser growth at higher concentrations. The response was linear with

the nitrogen concentration up to that observed for maximum growth. Analysis of variance for the regression is shown in Table 4. A standard

TABLE 4

ANALYSIS OF VARIANCE OF GROWTH RESPONSE OF *Tetrahymena pyriformis* W TO CASEIN ("VITAMIN-FREE") IN 27 ASSAYS IN 16 RUNS AT VARIOUS CONCENTRATIONS OF NITROGEN IN THE RANGE OF 0–1 MG/ML OF MEDIUM

Source of variation	Degrees of freedom	Sum of squares	Mean square	Probability[a]
Total	27	10.4299		
Reduction in organism sum of squares due to the regression	1	10.1568	10.1568	0.0005
Residual or error	26	0.2731	0.0105	

[a] Probability of a reduction sum of squares this large due to random arrangement of data.

deviation of 0.1025 was found. As noted by Rosen,[4] it was not necessary to use a casein standard with every run. It was always included when new solutions of basal medium were used.

Casein was adopted as the reference protein for comparative purposes because it is used by so many investigations as a control in feeding tests of higher animals and in other microbiological assays. However, it may not be the best reference for comparing proteins whose growth response continues to increase at nitrogen concentrations of 2, 3, or even 4 mg nitrogen/ml. Some of the proteins which were inferior to casein at a range of 0–1 mg nitrogen/ml were superior to it at higher nitrogen levels.

"Commercial" egg albumin (as distinguished from crystalline egg albumin) or lactalbumin, which yield increasingly higher growth at higher nitrogen levels (1–3 mg/ml), may be better references for comparing proteins at a range of 1–3 mg of nitrogen/ml.

Protein Concentrates and a Mixture of Amino Acids

The growth response of the protein concentrates and a mixture of amino acids at all the nitrogen levels assayed is shown in Figs. 3 and 4. The curves were calculated from the regression coefficient for each set of data. The curve for casein, without the experimental points, is shown in each figure for comparison.

Two regression coefficients were calculated for each test substance. One of these was over the range of nitrogen 0–1 mg/ml to compare results in the range in which casein is most effective, and the other was over the range 0–1.7 mg/ml to show the increasing effectiveness of other

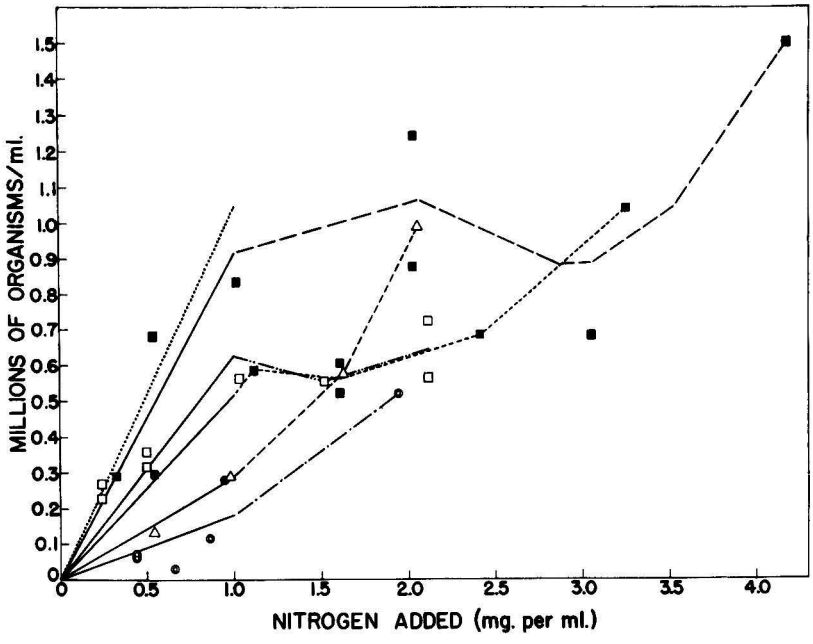

FIG. 3. Growth response of *Tetrahymena pyriformis* W to five protein concentrates compared with casein. For casein dotted line is slope of regression coefficient *b*. For the other concentrates: solid line = slope of regression *b*; broken line = trend of growth at nitrogen concentrations above 1 mg/ml medium.

Casein, "vitamin-free" .....
Egg albumin, commercial ■——■
α-Protein soy ■--------■
Soy ration #50 △——△
Soy meal #100, toasted □—-—-—□
Egg albumin, crystalline ⊙—•—•—⊙

proteins relative to casein. Nutritive indices from these results were calculated and are shown in Table 3. In most cases the regression coefficient *b* (for the nitrogen range 0–1 mg/ml) is noticeably larger than *b'* (for the nitrogen range 0–1.7 mg/ml). However, the nutritive indices calculated from these values are fairly close for both ranges.

Additional information on the nutritive value of these proteins is provided by the data shown in Figs. 3 and 4 at nitrogen levels higher than those used in computing the above coefficients. While most of the nitrogen sources had higher nutritive values at the low ranges of nitrogen concentrations, some have much higher nutritive indices when compared with casein in the 0–2+ mg nitrogen/ml range. These nitrogen sources

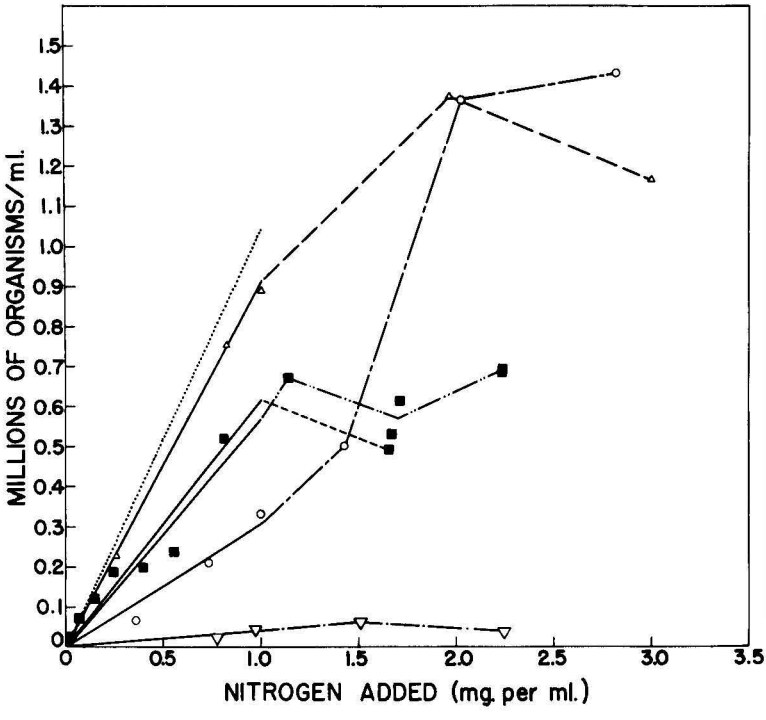

FIG. 4. Growth response of *Tetrahymena pyriformis* W to four protein concentrates compared with casein. For casein: dotted line is slope of regression coefficient *b*. For the other concentrates: solid line = slope of regression *b*; broken line = trend of growth at nitrogen concentrations above 1 mg/ml medium.

Casein, "vitamin-free"	.....
Incaparina 9B	○—-—-—○
Lactalbumin	△———△
Amino acids mixture	■—••—••■
Bovine albumin, crystalline	■———---■
Bovine hemoglobin	▽—•—•—▽

are egg albumin (both commercial and crystalline), lactalbumin, and the mixed diets, Soy Ration No. 50 and Incaparina. Rosen and Fernell (2) also found that the relative nutritive values depended markedly upon the range selected for comparison, peanut germ being superior to casein in the range of 0–3 mg nitrogen/ml but inferior in the range of 0–1 mg nitrogen/ml. This difference in behavior is not accounted for in these data.

Figures 3 and 4 also show that different proteins supported the maximum growtm attained for each at different nitrogen concentrations.

Incaparina, Mixture No. 9B. This is a vegetable mixture which N. S. Scrimshaw (private communication) and Scrimshaw *et al.* (13) report to be of high protein value (26% protein) prepared for feeding to infants and small children in areas of Central America where animal proteins are in short supply. According to Scrimshaw (private communication), the mixture has the following composition:

Whole corn	29%
Whole ground sorghum	29%
Cottonseed flour	38%
Torula yeast	3%
Vitamin A	100,000 international units/kg
Free gossypol	0.045%
Total gossypol	0.95%

Although this mixture supported only 0.066 million *T. pyriformis* W at 0.358 mg nitrogen/ml, whereas casein supported *ca.* 0.350 million; at 2.091 gm the mixture supported 1.366 million and casein *ca.* 0.920 million. At this nitrogen level, the mixture's nutritive index would be about 1.5 compared with casein.

Soy Ration No. 50. Soy Ration No. 50 was used as a control diet in a large collaborative study by V. L. Frampton (private communication) on the nutritional quality of cottonseed meals in chick feeding tests. The ration contained:

Basal ration	62.90%
Ground yellow corn	50.00%
Fish solubles	2.50
Dried whey (50% lactose)	2.50
Dehy. alfalfa meal (17% protein)	2.00
DL-Methionine	0.05
Limestone, ground	1.60
Dicalcium phosphate (20% P, 24% Ca)	1.75
Sodium chloride	0.50
Tallow (stabilized)	2.00
Soybean meal	27.14%
Trace minerals	0.10
Fat	0.72
Glucose	8.14

The nutritional indices for this ration obtained with *Tetrahymena pyriformis* W are very low (Table 3). Approximately 30 mg of this ration/ml was required to obtain 1 mg nitrogen/ml in the final medium. However, the protein from it supported the 0.988 million organisms/ml at 2.058 mg nitrogen/ml. Compared with casein at this same nitrogen concentration only, its nutritive index was about 1.0.

Crystalline Egg Albumin. Crystalline egg albumin supported only

0.062 million organisms/ml at 0.436 mg nitrogen/ml whereas casein supported 0.500 million at this level. The test protein supported 0.520 million at 1.944 mg nitrogen/ml at which the standard supported *ca.* 0.920 milion. At this level, the nutritive index of the crystalline egg albumin would be *ca.* 0.5 compared with casein. In a similar sample of crystalline egg albumin, Martinez (14) reported 6.5 gm lysine, 2.4 gm histidine, and 6.0 gm arginine, as determined by the Moore, Spackman, and Stein method (15), and 6.6 gm lysine as determined with 2,4-dinitrofluorobenzene, per 16 gm nitrogen.

Amino Acids Mixture. The concentrations of the amino acids used in the mixture are those in Medium D of Kidder *et al.* (4, 16) from which Rosen and Fernell's medium (1) is derived. Figure 4 shows the growth response at the original concentration (2.24 mg nitrogen/ml) and dilutions thereof. The growth response at one-half the original dilution (0.670 million/ml) was almost equal to that of the original strength (0.689 million/ml). These results cannot be compared with those reported by Kidder *et al* (4, 16) since they report growth in terms of optical densities in a study of the optimum concentrations of various amino acids required for the best growth. Fernell and Rosen (1) report populations of one million ciliates/ml obtained with an amino acid medium (formula and nitrogen content not stated).

This microbiological method should be useful in evaluating the nutritional quality of intact proteins of cottonseed meals and other oilseed meals; especially as compared with that obtained in higher animal feeding tests; in determining the effect of various processing methods on these meals, and in studying the effect of supplementary substances, such as various amino acids, gossypol, etc., on the nutritional quality of the protein.

SUMMARY

Results are reported of a survey of the relative nutritional quality of 10 protein concentrates and of a mixture of amino acids determined by assay with *Tetrahymena pyriformis* W. The method is essentially that of Rosen and Fernell, with the simplifying modification proposed by Rosen in 1958, except that utilization of the nitrogen source was evaluated by growth-response curves and lyophilized mixtures of some of the basal medium components were employed.

The soundness of the counting procedure used in estimating this growth response was investigated. The reproducibility of the results at several nitrogen levels of the reference standard, "vitamin-free" purified casein, was established by statistical studies.

Nutritional quality is estimated as an index: the ratio of the regression coefficient of growth on the nitrogen content of the test material to the regression coefficient of growth on the nitrogen content of casein. These were estimated for the ranges 0–1.0 and 0–1.7 mg nitrogen/ml. These ranges were adopted because there was better growth at these concentrations for most of the nitrogen sources studied. Assays at higher nitrogen concentrations yield further information on the nutritional quality, particularly for diets containing mixed proteins.

The same general response occurred with casein and most of the materials tested: Growth increased with increasing nitrogen concentration up to a maximum and then decreased with higher nitrogen levels. Growth response curves are characteristic of each nitrogen source.

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The Determination of α-Ketoglutaric Acid by 2,4-Dinitrophenylhydrazine; Salting-Out Extraction Method

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INTRODUCTION

Many methods have been proposed for the determination of α-keto acids (1). The most sensitive are those which are based upon the reaction of this acid with DNP.[3] Several years ago, it was demonstrated by the authors (2) and others (3, 4) that there existed *cis* and *trans* isomers in the DNPH of most α-keto acids derived from α-amino acids.

In most cases, the determination of KGA is disturbed by the presence of PA, because these two keto acids are distributed most widely in biological materials. In order to separate and determine KGA–DNPH from PA–DNPH, the following methods have been adopted by many workers: (*a*) differential extraction by solvents (5), (*b*) calculation based on their different spectra (6), and (*c*) separation by chromatography (7). In the first method, no satisfactory solvent has been discovered. Further, the existence of *cis* and *trans* isomers in PA–DNPH gives rise to too many difficulties. The second one is also incomplete owing to the presence of isomers of PA–DNPH as in the first case. The third chromatographic method is troublesome to practice although separation of KGA–DNPH from others is possible.

During the course of the authors' studies (2, 8, 9) on the DNPH of α-keto acids, it was found that some of them were extracted with organic solvents from sodium carbonate solution on the addition of neutral salts, and that the degree of salting-out depended on the kind of DNPH of keto acids.

[1] Kyoto Women's College.

[2] Mukogawa Women's College.

[3] Abbreviations used are DNP, 2,4-dinitrophenylhydrazine; DNPH, 2,4-dinitrophenylhydrazone(s); KGA, α-ketoglutaric acid; PA, pyruvic acid.

This paper deals with a new method for the determination of KGA by differential salting-out extraction of DNPH of various keto acids.

REAGENTS, MATERIALS, AND APPARATUS

Isoamyl Alcohol. This alcohol is washed with dilute hydrochloric acid, dried with potassium carbonate, and distilled.

Carbonate Solution. Anhydrous sodium carbonate, 5.3 gm and sodium bicarbonate, 0.84 gm, are dissolved in distilled water and diluted to 1 l.

Carbonate–Sulfate Solution. Anhydrous sodium sulfate, 200 gm, is dissolved in carbonate solution and made up to 1 l.

Sulfate Solution. Ten per cent solution of anhydrous sodium sulfate.

Ethyl Acetate. This is washed with 5% sodium carbonate solution and water successively and then dried with calcium chloride for a few days. Then it is filtered and dried again with potassium carbonate and distilled. A fraction from b.p. 74 to 77° is used. It may be stored for several months over anhydrous sodium sulfate.

Saturated Ethyl Acetate. Ethyl acetate and the carbonate–sulfate solution are mixed in a volume ratio of 10:2 and shaken well at 30°. After standing for a while, the lower layer is discarded and the upper layer is kept in a bath of 30°. Freshly prepared solution should be used at every determination.

Saturated Ethyl Acetate–Alcohol. Ethyl acetate and isoamyl alcohol are mixed in a volume ratio of 10:1. To 10 parts by volume of this mixed solvent, 2 parts by volume of the carbonate–sulfate solution is added and shaken at 30°. After there is a separation into two layers, the lower layer is poured down and the upper layer is kept in a bath at 30°. The lower layer, "saturated carbonate–sulfate solution" is also kept at 30° for use. Both solutions should also be freshly prepared for each experiment.

DNP Reagent. DNP, 0.1 gm, is dissolved in 100 ml of 2 *N* hydrochloric acid by gentle heating.

Sodium Hydroxide. A 2.0 *N* solution is used.

KGA was purchased from Wako Chemicals Ind. Co., Japan. Lithium pyruvate was prepared according to the directions of Wendel (10). KGA–DNPH was prepared as usual and recrystallized from acetone–benzene mixture. *cis*- and *trans*-PA–DNPH were prepared as reported previously (2, 9) and recrystallized from acetone–benzene mixture, respectively.

Extraction Tube. Colored test tubes, 150 × 20 mm, with a sharp-pointed bottom are used. They have two marks at 2.5 and 12.5 ml.

Wright's Capillary Pipet. The extraction is performed by blowing air

through this pipet (the upper portion, which is a 10-mm o.d. glass tubing is 50 mm long; the lower portion, drawn to fine capillary, is 100 mm long; the bulge lies between those portions; total length 170 mm, and the bulge volume about 7 ml). After extraction, the upper layer or the lower layer can be removed by means of this pipet which is fitted with a rubber bulb.

This extraction process might be performed also by using a specially designed separatory funnel.

EXPERIMENTAL AND RESULTS

Salting-Out Effect of Neutral Salts on the Extraction of trans-PA–DNPH

Part of DNPH of keto acid dissolved in carbonate solution is extracted with organic solvent in the form of its sodium salt. Addition of neutral salt to the carbonate solution increases the degree of extraction of DNPH. In this salting-out extraction, KGA–DNPH, (in general, DNPH of keto dicarboxylic acid) is hardly extracted with organic solvent compared with DNPH of keto monocarboxylic acid. By repeating these extractions, DNPH of keto monocarboxylic acid can be completely separated from KGA–DNPH, most of which remains in the lower layer.

In the following experiments, *trans*-PA–DNPH, which forms predominatingly compared with the *cis* form under the usual selected conditions, was chosen as a representative of DNPH of keto monocarboxylic acid. At first, the salting-out effects of several inorganic salts on the extraction of *trans*-PA–DNPH were determined. The *trans*-PA–DNPH was dissolved in 2 ml of carbonate solution containing each of the following salts. To this solution was added 10 ml of ethyl acetate–isoamyl alcohol mixture (volume ratio = 10:0.5), and the solution was aerated for 2 min at 30°. After the mixture was completely separated into two layers, the upper layer was removed by using a capillary pipet with a rubber bulb. To remove the DNPH in the residual upper layer completely, about 3 ml ethyl acetate was added from the pipet, washing the inner wall of the tube, and the upper layer was removed again. This washing treatment was repeated twice again. Then the solution was aerated until the odor of ethyl acetate had disappeared. The DNPH solution thus obtained was transferred into a measuring flask and diluted with carbonate solution to a constant volume, and the absorbancy was measured at 370 mμ. The data in Table 1 show the percentage of residual DNPH in the carbonate solution.

TABLE 1
SALTING-OUT EFFECTS OF INORGANIC SALTS ON THE EXTRACTION OF *trans*-PA–DNPH

Inorganic salts added	Concentration of salt (gm/100 ml)	Residual DNPH in carbonate solution after extraction (%)[a]
Control (no salt)		82
Sodium chloride	30	43
Sodium sulfate, anhydrous	20	23
Sodium chlorate	70	74
Sodium bromide	44	69
Tertiary potassium phosphate[b]	70	22

[a] Initial amount of *trans*-PA–DNPH: 0.182 μmoles.
[b] The pH of the solution was adjusted to 7.0 with phosphoric acid.

Tertiary potassium phosphate was found to be as effective as sodium sulfate, but it was abandoned because it did not give consistent results. Ammonium sulfate was also excluded because some troubles were expected to occur during the following alkali addition process. Besides the inorganic salts described above, sodium salts of organic acids, such as salicylic, benzoic, benzenesulfonic, phthalic, and acetic acids, were tested but were found to give unsatisfactory results.

Effect of the Composition of Organic Solvents on the Extraction

In order to find the best solvent which makes the extraction of *trans*-PA–DNPH maximal and of KGA–DNPH minimal, mixtures of ethyl acetate and isoamyl alcohol in different proportions were tested. The experimental conditions were the same as in Table 1, and the results obtained are shown in Fig. 1.

For the purpose of separating each DNPH, it was found that a mixed solvent which consisted of ethyl acetate and isoamyl alcohol in a volume ratio of 10:1 was most effective.

Range of DNPH Concentration in the Extraction

When the extraction by the above solvent is repeated several times, the *trans*-PA–DNPH should be transferred into the solvent completely, while most of the KGA–DNPH should be expected to remain in the carbonate–sulfate solution. In order to examine the concentration range of DNPH in which this expectation is valid, the following experiment was performed. In this experiment, the "saturated solvent system" was used to prevent the volume decrease of the lower layer by repeated extraction. Various amounts of DNPH were dissolved in 2 ml of

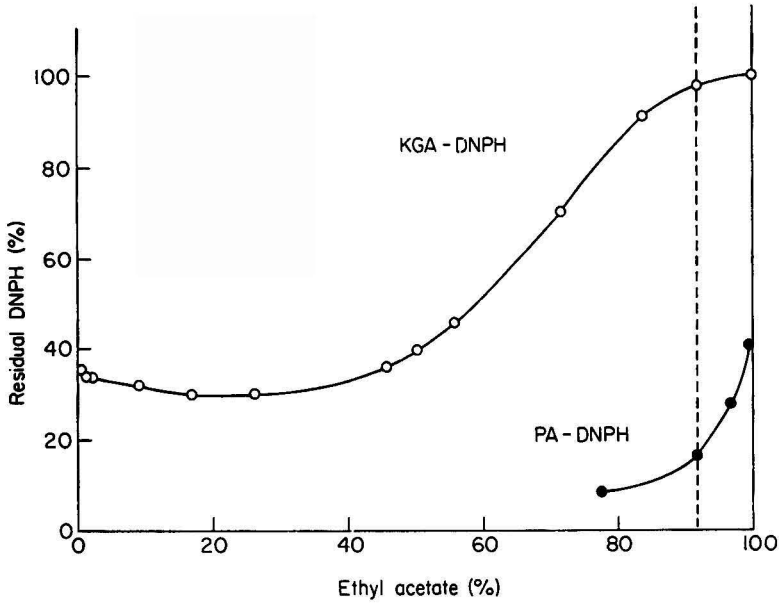

FIG. 1. The extracting effects of the mixtures of different proportions of ethyl acetate and isoamyl alcohol for *trans*-PA–DNPH and KGA–DNPH. The following amounts of DNPH were added to 2 ml of the carbonate–sulfate solution, respectively: KGA–DNPH, 0.268 μmole; *trans*-DNPH, 0.255 μmole.

saturated carbonate–sulfate solution and were extracted with 10 ml of saturated ethyl acetate–alcohol. The percentage of the residual DNPH in the carbonate–sulfate solution is shown in Table 2.

TABLE 2
PERCENTAGE OF RESIDUAL KGA–DNPH AND *trans*-PA–DNPH AFTER EXTRACTION

	DNPH added (μmoles)	Residual DNPH (%)	
		Extracted once	Extracted thrice
KGA–DNPH	0.128	95.5	92.7
	0.256	95.5	92.7
	0.512	95.5	92.7
	1.02	94.8	92.7
	2.04	94.8	91.4
trans-PA–DNPH	0.520	16.0	0
	1.04	15.0	0
	2.08	14.4	0
	4.15	14.4	0
	8.30	16.0	1.7

From these results, the percentage of the residual DNPH was found to remain essentially constant over a considerably wide range of concentration when single DNPH was present; and by carrying out this extraction three times, it was proved that the *trans*-PA–DNPH was completely removed, while more than 90% of KGA–DNPH remained unextracted.

Extraction of KGA–DNPH in the Presence of PA–DNPH

When *trans*-PA–DNPH and KGA–DNPH coexisted in various proportions, the percentage of residual KGA–DNPH after three extractions was as shown in Table 3.

TABLE 3
EFFECTS OF COEXISTING *trans*-PA–DNPH ON THE EXTRACTION OF KGA–DNPH

KGA–DNPH added (μmoles)	*trans*-PA–DNPH added (μmoles)	Molar ratio	Residual KGA–DNPH (%)[a]
0.521	0.50	1:1	93.2
0.521	1.00	1:2	93.2
0.521	2.00	1:4	95.9
0.521	4.00	1:8	98.6

[a] Extractions were carried out three times.

As shown in Table 3, the residual percentage of KGA–DNPH was constant only when the amount of coexisting *trans*-PA–DNPH was less than twice that of KGA–DNPH. But the applicable range was successfully extended by carrying out the extraction with ethyl acetate ("preliminary extraction") three or four times before the ethyl acetate–alcohol extraction ("main extraction"). From these experimental results, the following method was devised for the KGA determination.

Procedure for KGA Determination

Before carrying out the determination, a sample solution is treated with metaphosphoric acid to remove protein. To 4 or 5 ml of the deproteinized solution, containing less than 1.5 μmoles of keto acids, 1 ml of DNP reagent is added and the mixture is shaken. After reacting for 30 min at 25–30°, 8 ml ethyl acetate is added and aerated vigorously through a Wright's capillary pipet for 2 min. After standing, the mixture is separated into two layers. The lower layer is removed with the aid of the Wright's pipet. To remove the residual lower layer completely, 1 ml of sulfate solution is added, washing the inner band of the tube, and the water layer is discarded. This washing is repeated twice again. Then the reaction tube is kept in a bath of $30 \pm 2°$, and 2 ml of

carbonate–sulfate solution is added and the lower layer is made to 2.5 ml with sulfate solution. After filling to the upper mark with ethyl acetate, the mixture is aerated for 2 min. On standing, the mixture is separated into two layers, and then the upper layer is discarded.

This preliminary extraction is repeated again twice or thrice using saturated ethyl acetate. After the upper layer of the last preliminary extraction is discarded, 10 ml of saturated ethyl acetate–alcohol is added and aerated for 2 min, and the upper layer is removed. This main extraction is repeated thrice in all. After the upper layer of the last extraction is removed, the lower layer is aerated through Wright's pipet until the odor of ethyl acetate is gone. The solution is transferred into a measuring flask and filled to 10 ml with the carbonate–sulfate solution. This solution can be measured at 382 mμ. But in most cases the next alkali addition procedure gives better results. To 5 ml of this solution, 5 ml of 2.0 *N* NaOH is added. After mixing and standing for 10 min, the solution is measured at 417 mμ within 10 min.

If the sample contains any acidic colored substances, the control absorbancy, which is obtained by replacing the DNP reagent by 2 *N*

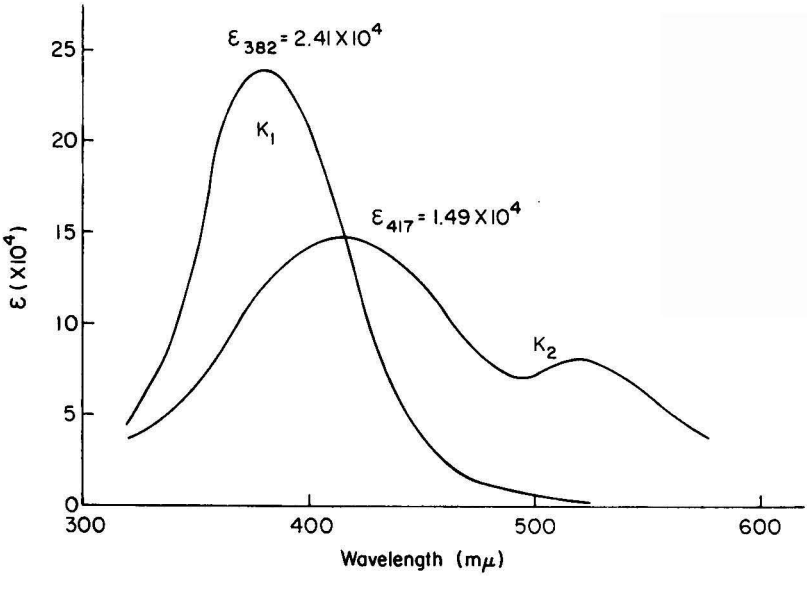

FIG. 2. Molecular absorption spectra of KGA–DNPH in carbonate–sulfate solution and sodium hydroxide solution. k_1: KGA–DNPH in 0.1 *N* Na_2CO_3, containing 0.01 *N* $NaHCO_3$ and 4% sodium sulfate. k_2: KGA–DNPH in 1 *N* NaOH, containing 0.05 *N* Na_2CO_3, 0.005 *N* $NaHCO_3$, and 2% Na_2SO_4.

HCl throughout the procedure, has to be subtracted from the absorbance obtained.

The calculation procedure will be described later, because the calculation factor is determined according to the number of extraction times.

The absorbancy of KGA–DNPH in 0.1 *N* Na_2CO_3, containing 0.01 *N* $NaHCO_3$ and 4% sodium sulfate, at 382 mμ is 0.165/μg KGA/ml. The absorbancy of this DNPH in 1 *N* NaOH, containing 0.05 *N* Na_2CO_3, 0.005 *N* $NaHCO_3$, and 2% Na_2SO_4, at 417 mμ is 0.102/μg KGA/ml. These spectra of KGA–DNPH are shown in Fig. 2.

Analysis of Authentic KGA

When authentic KGA was analyzed according to the procedure described above (the preliminary extraction was carried out three times), the percentage of recovered KGA was found to be constant over a wide range of its concentration. These results are shown in Table 4.

TABLE 4
DETERMINATION OF KGA STANDARD

KGA added (μmoles)	Absorbance observed[a]	Residual KGA after extraction (%)[b]	Recovery of determination (%)[c]
0.202	0.141	94.3	103
0.300	0.210	94.2	103
0.429	0.303	95.0	104
0.957	0.675	94.7	104
1.49	0.526 × 2	94.8	104
2.64	0.920 × 2	94.0	103

[a] Measured at 417 mμ.

[b] The preliminary and the following main extractions were carried out three times respectively.

[c] Calculated from the formula (see *Calculations*).

But when the determination of KGA was performed in the presence of PA, a lower recovery of KGA was obtained as shown in Table 5.

When the amount of coexisting PA was less than 10 times that of KGA, it was found to be sufficient to carry out the preliminary extraction three times.

But when more PA, less than 15 times, coexisted, it was necessary to carry out preliminary extraction four times. The recovery of KGA in the former cases was found to be from 90.5 to 92.5%, in the latter cases, from 87.4 to 88.0%. If more than 15 times of PA coexisted, this method was shown to be inapplicable.

TABLE 5
DETERMINATION OF KGA IN THE PRESENCE OF PA

Keto acid added (μmoles)		Molar ratio	Absorbance observed[a]	Residual KGA (%)	Recovery of determination (%)[c]
KGA	PA				
0.957	0	1:0	0.675	94.7[b]	103
0.957	0.10	1:0.1	0.659	92.5[b]	102
0.957	0.20	1:0.2	0.659	92.5[b]	102
0.957	0.50	1:0.5	0.657	92.2[b]	101
0.957	0.80	1:0.8	0.646	90.6[b]	99.5
0.957	1.00	1:1	0.644	90.3[b]	99.3
0.301	0.30	1:1	0.202	90.5[b]	99.5
0.301	0.60	1:2	0.202	90.5[b]	99.5
0.301	1.50	1:5	0.202	90.5[b]	99.5
0.301	3.00	1:10	0.202	90.5[b]	99.5
0.301	4.50	1:15	0.176	78.6[b]	86.4
0.301	6.00	1:20	0.097	43.5[b]	47.8
0.150	0	1:0	0.101	91.0[d]	103
0.150	1.50	1:10	0.098	88.1[d]	100
0.150	2.20	1:15	0.097	87.4[d]	99.4
0.150	3.00	1:20	0.093	83.3[d]	94.4

[a] Measured at 417 mμ.
[b] Preliminary extraction: three times.
[c] See *Calculations*.
[d] Preliminary extraction: four times.

Calculations

From the data described above, the amount of KGA can be determined by the following formula calculations.

When preliminary extraction was carried out three times, for those cases without addition of alkali

$$\text{KGA}(\mu\text{g}) = \frac{\text{absorbance at 382 m}\mu}{0.165} \times 10 \times \frac{100}{91}$$

For those cases with addition of alkali

$$\text{KGA}(\mu\text{g}) = \frac{\text{absorbance at 417 m}\mu}{0.102} \times 20 \times \frac{100}{91}$$

When preliminary extraction was carried out four times, for those cases without addition of alkali

$$\text{KGA}(\mu\text{g}) = \frac{\text{absorbance at 382 m}\mu}{0.165} \times 10 \times \frac{100}{88}$$

For those cases with addition of alkali

$$\text{KGA}(\mu\text{g}) = \frac{\text{absorbance at 417 m}\mu}{0.102} \times 20 \times \frac{100}{88}$$

For all cases, errors are within ±3%.

When the amount of coexisting PA is smaller than that of KGA, the calculation factor should be rather 100/92.

Application to Biological Materials

This method was applied to the determination of KGA in parsley and in the fluid of a biotin-deficient culture of *Bacillus macerans*, and good recovery was obtained as shown in Table 6.

TABLE 6
DETERMINATION OF KGA IN BIOLOGICAL MATERIALS

Sample (ml)		Preliminary extraction	KGA added (μg)	Absorbancy observed[b]	KGA (μg)	Recovery of added KGA (%)
Parsley extract[a]	3	3		0.087	18.8	
	3	3		0.087	18.8	
	5	3		0.152	32.9	
	5	3		0.152	32.9	
	3	3	22.0	0.192	41.4	103
	3	3	22.0	0.192	41.4	103
Culture fluid of	3	4		0.070	15.7	
Bacillus macerans	3	4		0.070	15.7	
	3	4	52.0	0.308	68.5	101
	3	4	52.0	0.296	66.0	97

[a] Parsley, 1.73 gm (wet weight), was extracted with 10 ml of 5% metaphosphoric acid.

[b] Measured at 417 mμ.

Interference by Other Keto Acids

In the extraction from carbonate–sulfate solution with ethyl acetate and in that from carbonate–sulfate solution with ethyl acetate–alcohol mixture, 44 and 17% of *trans*-PA–DNPH was found to remain in the lower layer, respectively, as mentioned above. It may be expected, therefore, that coexistence of large amounts of other keto acids–DNPH, which are less extractable than *trans*-PA–DNPH, will interfere with the KGA determination. The percentages of several keto acids–DNPH remaining in the carbonate–sulfate solution by single extraction in both extraction systems are shown in Table 7.

TABLE 7
PERCENTAGE OF RESIDUAL DNPH OF SEVERAL KETO ACIDS AFTER EXTRACTION

DNPH of keto acids	Residual DNPH (%) after extraction with	
	Ethyl acetate	Ethyl acetate–alcohol
KGA	99	98
Oxalacetic	98	96
Glyoxylic		
(*trans*)	81	57
(*cis*)	23	26
PA		
(*trans*)	44	17
(*cis*)	37	9.8
Acetoacetic	36	19
α-ketoisovaleric	9.1	6.7
α-keto-β-methylvaleric	2.4	4.3
α-ketoisocaproic		
(*trans*)	6.7	0.93
(*cis*)	12	11

From these results, the DNPH of acetoacetic, α-ketoisovaleric, α-ketoisocaproic, and α-keto-β-methylvaleric acids, and perhaps other higher keto acids, were found to be more extractable than *trans*-PA–DNPH. Therefore the coexistence of less than 15 times of these keto acids, except glyoxylic acid, should not interfere with the KGA determination. Oxalacetic acid, on the other hand, was found to be scarcely extracted as in the case of KGA. But this keto acid could be removed by complete conversion into PA with cobaltic chloride treatment before DNPH formation.

DISCUSSION

The KGA–DNPH used as the base of KGA determination throughout these experiments was prepared in the ordinary way and should have the *trans* structure according to our test. In 1955, Isherwood *et al.* reported the isolation of two isomers of KGA–DNPH, although they did not give any elementary analysis for their "*cis*" form. Thus far the present authors have never succeeded in obtaining the *cis* isomer. At least, the absorbancy of the DNPH which was obtained by treating the authentic KGA by DNP reagent, extracting with ethyl acetate, and then transferring into the carbonate solution accorded completely with that of the "*trans*-KGA–DNPH" calculated from an equivalent amount of KGA. So it is probable that Isherwood's "*cis*" form is a semi-ester or a

degradation product. The authors believe there would be no problem even if it were assumed for the present that KGA produce only *trans*-DNPH under ordinary conditions.

SUMMARY

A new method was reported for the determination of α-ketoglutaric acid (KGA). This acid was separated from the other keto acids by the salting-out extraction of 2,4-dinitrophenylhydrazones (DNPH). It was shown by repeated extractions with an ethyl acetate–amyl alcohol mixture that DNPH of keto monocarboxylic acids were completely extracted from the sodium carbonate solution containing sodium sulfate, while most of KGA–DNPH remained unextracted. Coexistence of pyruvic acid or higher keto monocarboxylic acids in an amount less than 15 times that of KGA did not interfere with the present determination.

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The Determination of Pyruvic Acid by 2,4-Dinitrophenylhydrazine Method

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INTRODUCTION

In the previous paper (1) the authors reported a method for the determination of KGA[3] by salting-out extraction of other keto acids in the form of DNPH. Among the keto acids which are often found with KGA in natural biological materials, PA is the one distributed most widely, and so the separative determination of these two keto acids has been studied by many investigators. Numerous methods (2) using DNP have been devised for this purpose. The chromatographic determination of PA (3), however, gives only incomplete results because of the presence of *cis* and *trans* isomers in PA–DNPH. Isomerization, which occurs between them in the chromatographic procedure, causes further disturbance in the results unlike the case of KGA. Of the extraction methods with solvents, the most frequently used is the one by Friedemann and Haugen (4). In 1955, Friedemann, improving it, proposed a new method (5). However, even with his new method, satisfactory results were not necessarily obtained.

The present paper is concerned with a new method for PA determination based on the chemistry of the isomers of PA–DNPH[4] (6). Further, the determination of PA in the presence of KGA was also studied by combining the present method with the salting-out extraction method for KGA determination reported previously (1).

MATERIALS AND APPARATUS

Previously reported (1) materials and apparatus were used except for petroleum ether which was purified as follows. After washing with

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[3] Abbreviations used are KGA, α-ketoglutaric acid; PA, pyruvic acid; DNP, 2,4-dinitrophenylhydrazine; DNPH, 2,4-dinitrophenylhydrazone(s).

[4] Katsuki, H., Kawano, C., Kanayuki, H., Tokushige, M., and Tanaka, S., unpublished.

concentrated sulfuric acid, 5% sodium hydroxide, and water, successively, it was dried with calcium chloride and then distilled. A fraction from b.p. 50 to 80° was used.

EXPERIMENTAL AND RESULTS

Reaction with DNP Reagent

The sample solution must be treated with a deproteinizing agent before reaction with DNP reagent. The effect of deproteinizing agent on the formation and extraction of keto acid–DNPH was studied, and metaphosphoric acid was found to give the best results. Trichloroacetic acid gave unsatisfactory results because a considerable amount of it transferred into ethyl acetate and then into carbonate solution in the extraction procedure. This interfered with the extraction of PA–DNPH with carbonate solution, as dilute carbonate solution was used in the present method.

The proportion of both isomers of PA–DNPH, which formed instantaneously when DNP reagent was added to the sample solution, varied with the conditions of reaction. At first, a considerable amount of the *cis* isomer was found to form, but its isomerization into the *trans* form occurred slowly when the solution was allowed to stand. With previous methods, considerable error in the results would be unavoidable, unless the reaction conditions are strictly maintained as indicated. In the present method, on the other hand, fluctuation in the proportion of both isomers was proved not to influence the results.

Extraction of PA–DNPH with Ethyl Acetate

Keto acid–DNPH is almost completely transferred into ethyl acetate from its hydrochloric acid solution by a single extraction. The isomerization between both isomers occurs slowly in ethyl acetate solution catalyzed by the hydrogen ion in it, and when this solution is kept for a long time, the equilibrium state is attained where the ratio of both isomers is nearly 1:1 (7).

Extraction of the PA–DNPH from Ethyl Acetate with Sodium Carbonate Solution

To separate PA–DNPH from the remaining DNP and DNPH of the neutral carbonyl compounds in ethyl acetate, extraction with sodium carbonate solution has been usually adopted. In Friedemann's method, 1 *N* sodium carbonate was used for this purpose. However, as shown in Table 1, extraction with 1 *N* sodium carbonate proved to be unsatis-

factory. This is attributed to the fact that both isomers are not completely extracted under the influence of the salting-out effect of sodium carbonate in high concentration. Application of a dilute sodium carbonate solution gave a satisfactory result for the extraction of the *trans* isomer, but was unsatisfactory for the *cis* isomer. However, by the addition of an equal volume of petroleum ether to the ethyl acetate solution, the complete extraction of both forms of PA–DNPH with 0.1 *N* sodium carbonate was made possible. On the other hand, DNPH of neutral carbonyl compounds and DNP reagent remained in the organic solvent layer.

TABLE 1

EXTRACTION OF DNPH FROM ORGANIC SOLVENT WITH CARBONATE SOLUTION

DNPH	Upper layer[a]: / Lower layer:	Ethyl acetate (%) / 1.0 *N* Na_2CO_3 + 0.1 *N* $NaHCO_3$	Ethyl acetate (%) / 0.1 *N* Na_2CO_3 + 0.01 *N* $NaHCO_3$	Ethyl acetate + Petroleum ether (%) / 0.1 *N* Na_2CO_3 + 0.01 *N* $NaHCO_3$	Ethyl acetate (%) / 0.1 *N* NH_4OH
KGA		100[b]	100	100	100
PA (*trans*)		79	98	100	96
PA (*cis*)		11	59	100	56
Acetone				0	
Acetaldehyde				0	
(DNP)				0	

[a] Extraction conditions: upper layer, 10 ml; lower layer, 10 ml.

[b] The figures show the percentage of DNPH transferred into the lower layer.

Determination of PA–DNPH

PA–DNPH in carbonate solution, extracted from ethyl acetate, consist of *cis* and *trans* isomers. The proportion of them varies according to the conditions of treatment. Usually, the *trans* form predominates. To determine the total amount of PA–DNPH photometrically, it is necessary to measure the absorbancy at a wavelength at which the molecular absorption spectra of both isomers cross each other. The intersecting point in carbonate solution is 355 mμ. In those cases where there is no interfering substance, it would be sufficient to measure that of the carbonate solution at this wavelength. In general, however, it is preferable to measure that of the sodium hydroxide solution in the longer wavelength region to avoid interference by colored substances in the sample.

On the addition of sodium hydroxide, the *trans* isomer produced a deep red color while the *cis* isomer produced a faint red one. The sta-

bility of the color produced from each isomer of PA–DNPH by adding alkali and the change of the coloration of each isomer with the increase of alkali were studied. Sodium hydroxide in a final concentration of 1.0 *N* was found to give the most satisfactory result, and measurements were therefore carried out within 10–20 min after the addition of alkali. The molecular absorption spectra of both isomers of PA–DNPH obtained under these conditions are shown in Fig. 1. Both spectra intersect each other at 417 mμ.

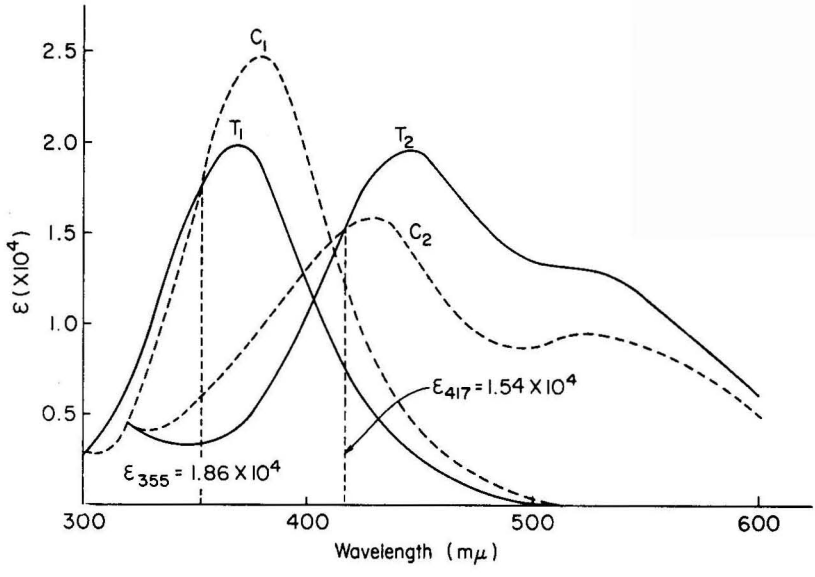

FIG. 1. Molecular absorption spectra of *cis*- and *trans*-PA–DNPH in carbonate solution and sodium hydroxide solution. c_1 and t_1: *cis*- and *trans*-PA–DNPH in 0.1 *N* sodium carbonate containing 0.01 *N* sodium bicarbonate, respectively; c_2 and t_2: *cis*- and *trans*-PA–DNPH in 1.0 *N* sodium hydroxide containing 0.05 *N* sodium carbonate and 0.005 *N* sodium bicarbonate, respectively.

PROCEDURE FOR THE DETERMINATION OF PA

The technique used in this procedure is nearly the same as that reported in the previous paper (1). To 4 or 5 ml of the deproteinized sample solution, containing less than 1.5 μmoles of keto acids, 1 ml of DNP reagent is added and the mixture is shaken. The mixture is left to stand at 25–30° for 10 min, and then 5 ml ethyl acetate is added and aerated vigorously. After the mixture separates into two layers on standing, the lower layer is removed with a pipet and discarded. The remaining ethyl acetate solution is washed twice with a small amount

of water in the same way as reported in the previous paper. Then 5 ml of petroleum ether and 2 ml of carbonate solution are added to the solution, and the mixture is aerated vigorously. After the mixture is completely separated into two layers, the upper layer is removed with a pipet and discarded. Then the carbonate solution is washed thrice with a mixture of ethyl acetate and petroleum ether (1:1) and aerated until the odor of ethyl acetate vanishes. Finally this is brought to 10 ml with carbonate solution. The absorbancy of this solution is measured at 355 mμ. In another procedure, 5 ml of 2.0 *N* sodium hydroxide is added to 5 ml of this solution, and the absorbancy of the mixed solution is measured at 417 mμ after 10 min. The color is stable for about 10 min. If any colored substance in the carbonate solution, derived from the sample, interferes with the determination, the latter method is preferable. When necessary, the absorbancy of the control, which is obtained by using 2 *N* HCl instead of DNP reagent throughout the procedure, is to be subtracted from the absorbancy obtained with the sample.

The value for PA is derived from the following equations. For the case without addition of sodium hydroxide:

$$\text{PA}(\mu\text{g}) = \frac{\text{absorbancy at 355 m}\mu}{0.211} \times 10$$

For the case with addition of sodium hydroxide:

$$\text{PA}(\mu\text{g}) = \frac{\text{absorbancy at 417 m}\mu}{0.175} \times 20$$

Analytical results obtained by this method with known amounts of authentic lithium pyruvate are shown in Fig. 2.

With less than 1.5 μmoles PA, the absorbancy was found to be strictly proportional to the amount of PA added. Further, it is important to note that these values corresponded completely to the theoretical ones which were obtained by adding sodium hydroxide to the corresponding equimolar amount of *trans*- or *cis*-PA–DNPH.

Determination of PA in the Presence of KGA

In the previous paper (1) it was shown that KGA can be determined by using the method of salting-out extraction even when as much as 15 times PA coexist with KGA. Fortunately, only one isomer of KGA–DNPH is known to occur, the absorption maximum of which is at 417 mμ, the same wavelength as used in the PA determination. When both keto acids coexist in a sample, their total amount can be determined by applying this method described above. To determine both keto acids

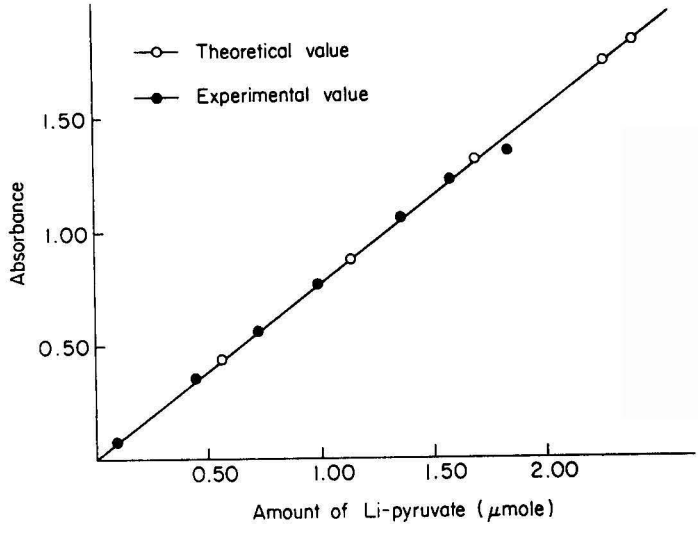

FIG. 2. Determination of authentic lithium pyruvate.

separately, another aliquot of sample solution is analyzed by the salting-out extraction method. Table 2 shows the analytical data and recovery of PA and KGA in the presence of both keto acids.

TABLE 2
SEPARATE DETERMINATION OF PA AND KGA

Added amount (μmoles)		Absorbancy obtained for		Value obtained (μmoles) for		Recovery (%) of	
PA	KGA	PA	KGA[a]	PA	KGA	PA	KGA
0.100	0.099	0.151	0.074	0.099	0.100	99.0	101
0.200	0.099	0.227	0.074	0.196	0.100	98.0	101
0.500	0.099	0.456	0.075	0.495	0.101	98.0	102
1.000	0.099	0.417 × 2	0.075	0.975	0.101	97.5	102
1.500	0.099	0.608 × 2	0.076	1.462	0.102	97.5	103
0.900	0.099	0.382 × 2	0.075	0.880	0.102	98.0	103
0.800	0.200	0.381 × 2	0.152	0.780	0.206	97.5	103
0.500	0.500	0.378 × 2	0.375	0.490	0.510	98.0	102
0.200	0.800	0.382 × 2	0.620[b]	0.186	0.832	93.0	104
0.100	0.900	0.370 × 2	0.668[b]	0.095	0.972	94.8	108

[a] Calculating factor 100/88 was used because preliminary extraction was carried out four times (1).

[b] Calculating factor 100/92 was used (1).

In this case the error was a little greater than that for the sole occurrence of PA. This was presumably due to the lower accuracy of the salting-out extraction method compared with the present method for PA determination.

DISCUSSION

For the purpose of the comparison with the authors' method, Friedemann's new method (5), the most representative of all known methods, was reinvestigated. As the result of the experimental work, data were obtained which confirmed that the present method shows higher stability than his method especially when PA and KGA are in mixtures. The lower stability of his method seems to be due to incomplete extraction of DNPH and neglect of the existence of isomers in PA–DNPH and also of the isomerization between them. The present method would not require so strict a control as his method.

The principles of this method could be applicable to other higher α-keto monocarboxylic acids, and the details will be described in another paper.

In the present method, a bubbling method was used for the extraction procedure. However, a specially designed separatory funnel can also be used for this purpose.

SUMMARY

A modified method for pyruvic acid (PA) determination was devised based on the following two facts: (*a*) *cis* and *trans* isomers of 2,4-dinitrophenylhydrazones (DNPH) of PA, by adding an equal volume of petroleum ether to an ethyl acetate layer, could be completely extracted from the organic solvent layer with 0.1 *N* sodium carbonate. (*b*) PA–DNPH could be determined by measuring either the absorbance of their sodium carbonate solution at 355 mμ or that of their sodium hydroxide solution at 417 mμ, irrespective of the proportion of both isomers. PA, in the presence of α-ketoglutaric acid (KGA), was found to be determined by combining the present method with the salting-out extraction method for KGA determination.

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ANALYTICAL BIOCHEMISTRY, 2, 441–446 (1961)

Estimation of Diethylstilbestrol and Identification of Related Synthetic Estrogens by Gas Chromatography[1]

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The synthetic estrogen, diethylstilbestrol (DES), has attracted much attention in agriculture, medicine, and public health. While reliable chemical methods are available for the determination of pure DES, none appear satisfactory when applied to estimation of DES obtained from biological materials. Because of this, many studies requiring estimations of this estrogen in small amounts rely on bioassay procedures (1). In the course of our studies on the estrogen-induced renal tumor of the male golden hamster (2), it was necessary to study the excretion levels of this compound quantitatively.

This report illustrates the use of gas chromatography for specific identification and quantitative determination of DES in the presence of closely related compounds (hexestrol and dienestrol) including application to samples of biological origin.

The chemical methods of analysis of DES isolated from biological sources require elaborate, time-consuming purification procedures for eliminating interfering substances that readily react with the commonly used colorimetric reagents. [See Warren *et al.* (3), employing $SbCl_5$ and Munsey (4), using an irradiation procedure.] The classic isolation procedures developed for estimation of the natural estrogens isolated from urine testify to the difficulty in eliminating interfering substances when the final stage of quantitation depends upon a delicate, sensitive colorimetric reaction (5). In the procedure to be described, complete isolation of the estrogen is unnecessary as the methyl ethers of the

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[2] Submitted in partial fulfillment of the Ph.D degree in Biochemistry, Baylor University College of Medicine, Houston, Texas.

phenolic substances that interfere in a colorimetric reaction (using $SbCl_5$) were widely separated by gas chromatography from the area of DES dimethyl ether. The procedure developed employs chloroform extraction, talc chromatography, methylation to form the DES dimethyl ether, and quantitation by gas chromatography on ethylene glycol adipate polyester columns.

METHODS

Urine or aqueous suspensions of feces (1 gm/10 ml) from hamsters treated with DES (2) were adjusted to pH 7.0 and extracted with three equal volumes of chloroform, centrifuging the mixtures in order to obtain separation of the phases. The combined chloroform extracts were filtered through 8.0-gm talc columns in 2.2 × 10.2 cm Allihin tubes. Porphyrins and other chromophores were retained while DES appeared in the filtrate. The chloroform extract was evaporated under a stream of nitrogen, and the residue was dissolved in 5.0 ml of absolute methanol. The methanol solution was transferred to a 50-ml Erlenmeyer flask, and 1.0 ml of 50% (w/v) NaOH was added followed by 1.2 ml dimethyl sulfate from a safety pipet. After the reaction ceased, another 5.0 ml methanol, 1.0 ml NaOH, and 1.2 ml dimethyl sulfate were added. Three more additions of NaOH and dimethyl sulfate were made in the same way without adding more methanol. The five additions of dimethyl sulfate are essential to assure over 99% conversion of the DES to its dimethyl ether. A 10–15-min interval was permitted before the addition of more reagents, and the reaction flask was not agitated until the initial vigorous reaction had ceased. The reaction flask was allowed to stand 4 hr or overnight before adding 10 ml of 20% NaOH and extracting the DES derivative (plus any other methylated phenols) with two 50-ml portions of ethyl ether. The combined ethyl ether extracts were washed with three 5.0-ml portions of water, and the ether was evaporated to a volume of a few milliliters. The sample then was transferred to 1 × 7.5 cm test tubes. Remaining droplets of water were removed by capillary pipet, and evaporation of ether was completed under a stream of nitrogen. The dried samples were dissolved in cold acetone and kept at ice-box temperature until aliquots were taken for gas chromatographic analysis. In practice the final volumes ranged from 100 to 1000 μl, and the aliquot taken for injection onto the column was 5 μl or less. The instrument was calibrated by injecting known concentrations of pure DES dimethyl ether and measuring the area under the curve of the resulting peaks (obtained by multiplying the peak height by the width at one-half the peak height).

The gas chromatographic instrument used was the Barber-Colman,

model 10, equipped with an argon ionization detector. A 6-ft glass column of 4 mm bore packed with 80–100 mesh Chromosorb W coated with 13.5% ethylene glycol adipate polyester (obtained from Applied Science Laboratories, Inc., State College, Pa.) was used. The column was maintained at 200°C with a column inlet pressure of 39.5 lb/sq in. The temperature of the detector cell was maintained at 225°C and the flash heater was maintained at 275–280°C. These conditions result in symmetrical peaks with no indication of decomposition of the methylated derivative of DES in the column. For a stable base line the voltage was maintained at 650 v with a recorder current response of 3×10^{-8} amp. For identification purposes, a reference or "marker" compound (in this case *o*-phenylphenol methyl ether) was run with pure DES dimethyl ether at the beginning of each period of analysis. The retention ratio of the DES dimethyl ether relative to *o*-phenylphenol methyl ether was constant over several months of use. The actual retention time of the DES derivative decreased with prolonged use of a given column (e.g., from 45 min down to 20 min for one column over a 5-month period). The free phenols of the synthetic estrogens (DES, hexestrol, and dienestrol) have such long retention times (several hours) that diffusion within the column results in low broad peaks unsuitable for quantitation on adipate columns. The monomethyl ethers are suitable for chromatography but cannot be prepared quantitatively.

RESULTS AND DISCUSSION

Figure 1 illustrates a typical chromatogram of DES isolated from biological sources. The several early peaks have not been identified, but presumably are methylated phenols. Using the methylation procedure described by Bauld and Greenway (6), 10–25% DES monomethyl and less than 60% dimethyl ether is formed from pure DES or from DES isolated from biological sources. The retention time of the monomethyl derivative is listed in Table 1 along with some other methylated phenols investigated with this procedure. Table 2 lists the recovery of DES from pure standards and from feces by the method described in the text.

Figure 2 is the chromatogram of a mixture of three closely related compounds used as synthetic estrogens. From data obtained on the simple phenols, lowering the inlet pressure to 20 lb/sq in. increases the retention time permitting better separation. Such a modification should afford better resolution of the materials shown in Fig. 2, although none of our studies of biological materials has yet required the simultaneous measurement of these estrogens. Moreover, our studies have shown there

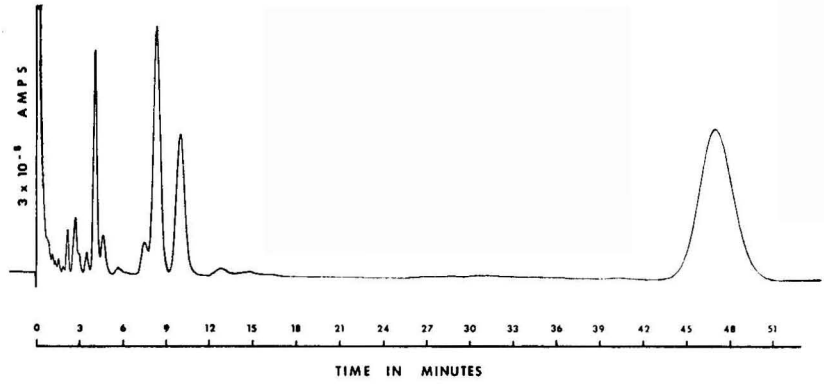

FIG. 1. Gas chromatogram of phenolic fraction extracted from 1 gm feces from a hamster implanted with DES (2); methylation as described in text. Freshly packed column; DES dimethyl ether retention time 47 min. Retention ratio relative to *o*-phenylphenol methyl ether = 7.8; 5 μl from 50 μl total sample volume. Peak area equivalent to 17.4 μg DES. The retention time of the DES monomethyl ether would have been 34 min in Fig. 1 if it had been present in the final reaction mixture.

TABLE 1

RETENTION RATIOS OF METHYLATED PHENOLS RELATIVE TO *o*-PHENYLPHENOL METHYL ETHER

Compounds tested		Retention ratio
A. Pure compounds		
Anisole		0.33
o-Dimethoxybenzene		0.22
m-Dimethoxybenzene		0.22
p-Dimethoxybenzene		0.22
Resorcinol (as the free phenol)		7.53
B. Methylated phenols reported as the major peaks obtained with the described methylation procedure[a]		
Resorcinol	peak 1	0.67
Orcinol	peak 1	0.31
	peak 2	0.40
Phloroglucinol	peak 1	0.67
	peak 2	0.77
	peak 3	1.39
	peak 4	1.72
DES-monomethyl ether		5.64
DES-dimethyl ether		7.80

[a] Methylation in borate buffer (6).

TABLE 2

RECOVERY OF DES FROM BIOLOGICAL SOURCES AND STANDARD SOLUTIONS

Sample	DES added (μg)	DES recovered (μg)[a]	Recovery (%)[b]
DES standard	1000	970	97
	1000	990	99
	1000	990	99
	1000	990	99
Hamster feces (1 gm)[c]	1000	980	98
	1000	920	92
	1000	1020	102
	1000	1000	100

[a] A 5-μl sample volume was used for gas chromatography; total sample volume, 1 ml.

[b] The monomethyl ether derivative is sometimes present in trace amounts as may be noted by a slight elevation of base line at a retention ratio of 5.64 relative *o*-phenylphenol methyl ether. This is too small to measure for quantitation relative to the DES dimethyl ether.

[c] Normal hamster feces processed as indicated in the text after addition of DES.

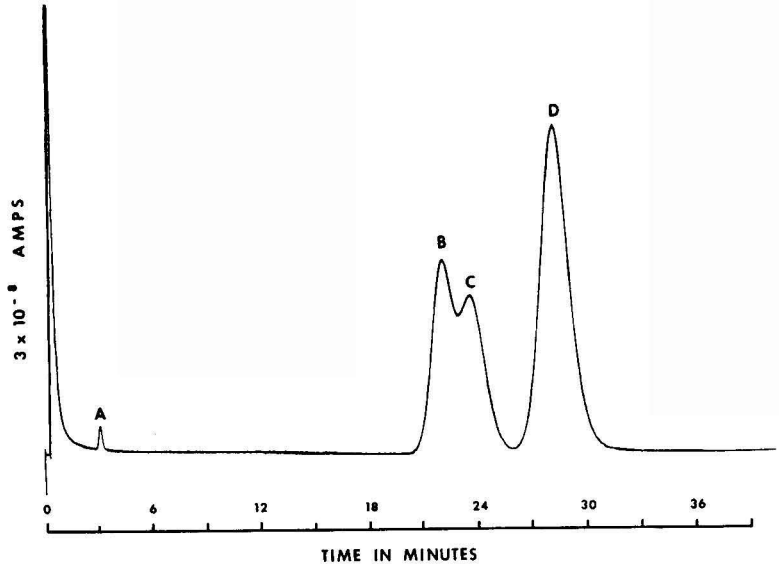

FIG. 2. Gas chromatogram of the dimethyl ethers of three synthetic estrogens. *A*, *o*-phenylphenol methyl ether (marker compound); *B*, DES dimethyl ether; *C*, hexestrol dimethyl ether; *D*, dienestrol dimethyl ether. Retention ratios relative to *A*: $B = 7.80$; $C = 8.25$; $D = 9.87$. Column has been in use 5 months.

is no detectable biological conversion of DES to either hexestrol or dienestrol.

The methods have been successfully applied to urine, feces, and tissue extracts. For tissue, the acetone extraction procedure of Jones and Deathrage (7) was found most efficient. The adipate column provides for resolution of C_{20} stilbene derivatives of similar structure differing in molecular weight by only 2 or 4 hydrogens. The minimum sensitivity with the above conditions is 1 μg DES, but more sensitive detection systems for gas chromatography are becoming available which should detect amounts in the range of 0.01–0.10 μg. The reproducibility, specificity, and rapidity of analysis suggest wide applicability to studies involving synthetic estrogens or biologically important phenols.

SUMMARY

An analytical procedure is described for the estimation of diethylstilbestrol in extracts of tissue, feces, or urine. The method involved extraction with chloroform or acetone and removal of pigments by adsorption on talc. Diethylstilbestrol in the filtrate was converted quantitatively to the dimethyl ether by treatment with dimethyl sulfate and was estimated by gas chromatography.

ACKNOWLEDGMENT

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ANALYTICAL BIOCHEMISTRY, 2, 447–462 (1961)

Liquid Scintillation Counting of C^{14} Paper Chromatograms

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Wang and Jones (1) have recently described the radioassay of C^{14} paper chromatograms by a liquid scintillation technique with custom-made vials which hold paper sections in vertical position. The method was extended by Geiger and Wright (2) using standard vials. They concluded that the angle of rotation of the radioactive paper section in the vial had only a small effect on the counting rate, thus permitting the use of an automatic sample changer where the position of vials in counting assembly could not be rigorously controlled. Bousquet and Christian (3) recently described the appropriate solvent system for counting paper chromatograms.

Direct counting of C^{14} paper chromatograms described by these workers by immersion of the paper strip in the liquid scintillation solvent has several advantages compared with the conventional Geiger-Müller (GM) counting. For example, the counting efficiency is much higher, and the wide range of radioactivity can be assayed by the scintillation technique. The method has, however, a pitfall unavoidable in liquid scintillation counting, that is, "quenching" by the sample. Although it was already established that the observed counting rate of the paper section was proportional to applied radioactivity (2, 3), this does not necessarily mean that it is proportional to the absolute radioactivity of any sample, since the quenching properties vary with the samples used. One example of such quenching effect has been reported by Loftfield and Eigner (4).

The method described here involves the extraction of radioactive materials from the paper section by hyamine, appropriate addition of the solvent system followed by counting. The radioactivity of the sample can be corrected either with or without the addition of internal standards. The methods were applied to C^{14} amino acids and C^{14} sugars with excellent reproducibility.

MATERIALS AND METHODS

Standard Source and Radioactive Compounds

Benzoic acid-C^{14} and toluene-H^3 standard source compounds were purchased from Packard Instrument Company (La Grange, Illinois). C^{14} *Chlorella* protein hydrolyzate was prepared as described earlier (5). Uniformly labeled glutamic acid-C^{14}, glucose-C^{14}, maltose-C^{14}, levulinic acid-C^{14}, and chlorophyll-C^{14} fraction were isolated from *Chlorella* cells. Fructose-C^{14} and sucrose-C^{14} were prepared from Canna leaves exposed to carbon dioxide-C^{14} (6). The specific activities of these C^{14}-labeled compounds ranged between 5 and 10 mc/mmole C. Adenosine-H^3 (199 mc/mmole) and leucine-H^3 (102 mc/mmole) were purchased from the Radiochemical Centre (Amersham, England).

Methanolic Hyamine Hydroxide Solution

Hydroxide of hyamine 10-X, *p*-(diisobutylcresoxyethyl)dimethylbenzylammonium hydroxide, 1 molar solution in methanol, was purchased from the Packard Instrument Company. This solution will be called "hyamine" for convenience in this text.

Liquid Scintillation Counting Method

A Tri-Carb model 314A liquid scintillation spectrometer equipped with an automatic sample changer (Packard Instrument Co.) was used. Standard 5-dram screw-top vials were used, and the usual technique for liquid scintillation counting (8, 9) was followed. Operating temperature of a freezer chamber was in a range of −8 to −9°. Scintillation solvent contained 4 gm 2,5-diphenyloxazole (Scintillation grade, Packard Instrument Co.) and 100 mg 1,4-bis-2-(5-phenyloxazolyl)benzene (Scintillation grade, Packard Instrument Co.) in 1000 ml of reagent-grade toluene.

Chromatography

One- and two-dimensional paper chromatography was carried out with phenol–water and butanol–propionic acid–water solvent systems according to Benson and his co-workers (7). Toyo No. 51 filter paper (Toyo Roshi Co., Tokyo) was used throughout.

Detection of Radioactive Spots and Radioassay of Paper Chromatograms with a GM Detector

C^{14}-labeled spots were located either by a radioautographic technique

or by a scanning rate meter equipped with a GM detector and a recorder (Kagaku Kenkyujo, Tokyo).

After the spots were located, they were surrounded by lead sheets (approximately 2 mm thick), and the radioactivity of each spot was assayed by a scaler (Irigaku Kenkyujo, Tokyo) with a GM detector (Type 1001T, Anton Electronic Lab., Inc., New York).

RESULTS

Extraction of Radioactive Material from Paper Section

It was shown by Geiger and Wright (2) that acetate-C^{14} was eluted considerably by a toluene scintillator solvent which contained 20% ethanol. These authors observed that 70–80% of the counts due to acetate were still retained in the counting vials after the paper sections were removed. Since immersion of nonradioactive paper section (2×5 cm) in the benzoic acid-C^{14}– or toluene-H^3–toluene scintillator system had little effect on the counting rates, counting the vials followed by the removal of paper and recounting would give a measure of the extraction efficiency by the solvent. Unfortunately, most of the biological materials are not soluble in toluene, and obviously quantitative extraction is not possible even with an ethanol-containing solvent system.

In order to solubilize various biological materials such as proteins, amino acids, purines, and pyrimidines, either hyamine (9) or alcoholic potassium hydroxide (10) has been widely applied for liquid scintillation work. This led the authors to examine the extraction of labeled compounds on the filter-paper section by hyamine.

A paper section, 2×2 cm, of which four corners were cut off in order to fit on the bottom of the counting vials, was placed in the vial. Radioactive compounds either with or without carrier were applied to the paper. After the papers were dried in an air oven, 1 ml hyamine was added. The vials were kept at 40° with occasional shaking for 4 hr or longer. To the vials were added 7–12 ml of the toluene scintillator solvent, and these were kept in the freezer chamber for 4 hr or longer. They were then counted at an appropriate high-voltage setting which gave the highest counts in the 10–50 window. After counting, papers were removed followed by recounting under the same conditions as before. The percentage of recovery in counts after removal of the paper was expressed as extraction efficiency, shown in Table 1. As can be seen, the efficiencies were in the range of 92.5–104.9% either with or without added carrier compounds, indicating that the extraction was almost quantitative.

TABLE 1
EXTRACTION OF RADIOACTIVE PAPER SECTIONS BY HYAMINE[a]

Sample	With filter paper (counts/min)	Without filter paper (counts/min)	Extraction efficiency (%)
Adenosine-H^3	8,726	8,073	92.5
Adenosine-H^3 carrier 20 μg	14,515	13,853	95.4
Adenosine-H^3 carrier 40 μg	11,511	11,705	93.0
Adenosine-H^3 carrier 100 μg	9,785	9,250	94.5
Leucine-H^3	12,044	12,630	104.9
Leucine-H^3 carrier 20 μg	11,966	12,252	102.4
Leucine-H^3 carrier 40 μg	12,931	12,994	100.5
Leucine-H^3 carrier 100 μg	12,519	12,240	97.8
Glutamic acid-C^{14}	30,927	30,309	98.0
Glutamic acid-C^{14} carrier 33 μg	29,319	28,800	98.2
Glutamic acid-C^{14} carrier 100 μg	26,001	25,768	99.1
Glutamic acid-C^{14} carrier 165 μg	30,387	30,259	99.6
C^{14} protein hydrolyzate	19,287	18,984	98.4

[a] Radioactive materials on the paper sections were extracted by hyamine. Scintillator solvent was then added, and the samples were counted with or without the papers. The applied radioactivity was almost equal for each group of samples, but no correction for quenching was made for the data presented above.

Correction for Quenching

Since the extraction of amino acids and adenosine by hyamine was almost complete and the presence of the paper sections in counting vials had little effect on the counting rates, the correction of the counting data for quenching could be done according to the usual liquid scintillation technique.

In order to recognize whether or not quenching occurs in a given sample, three ways are well established (8, 9, 11). One method involves dilution; an increased counting rate after dilution of a sample by the scintillation solvent means the existence of quenching. The degree to which the sample is quenched is a function of the concentration of the quencher; therefore a measure can be obtained by the dilution method. The second method is the use of an internal standard, by which the counting efficiency of that particular solvent system can be measured. Absolute radioactivity of the sample can be calculated with the counting efficiency observed with the internal standard. The final method involves an inspection of the ratio in counting rates in two separate windows of the spectrometer. If quenching occurs in a sample, the pulse heights are decreased, with a consequent effect on the ratio. All of these three methods may be applicable to direct counting of filter paper if the samples can be solubilized.

Dilution Method

During the study of quenching properties of phenol, which is known to be a potent quencher, on the counting rates of standard benzoic acid-C^{14}, it was found that there existed a linear relationship between phenol concentrations and the maximum counting rates in the 10–50 window obtained by applying a suitable high voltage to the photomultipliers at each phenol level.

Stepwise addition of scintillator solvent to the vials resulted in the increase in maximum counting rates in the 10–50 window. When the concentrations of phenol in the scintillator solvents were plotted versus the maximum counting rates at each phenol concentration, a straight line was obtained as shown in Fig. 1. Upon extrapolating the line to

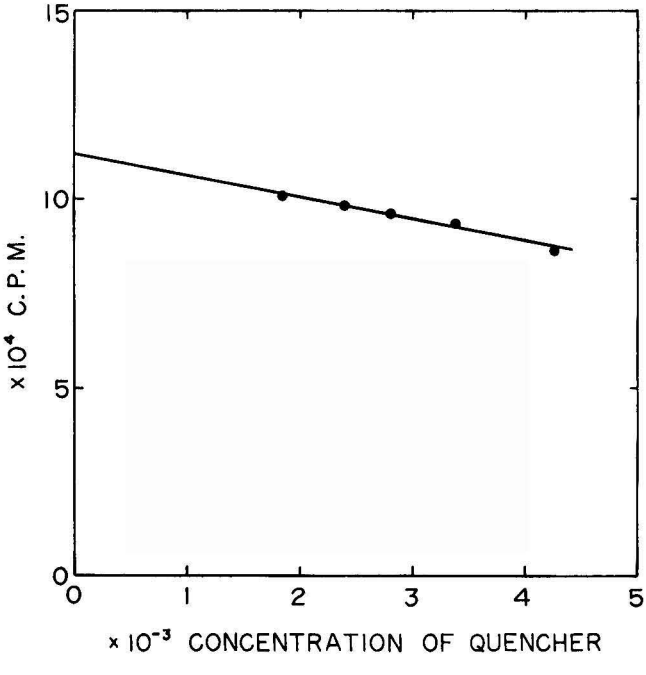

FIG. 1. The relationship between phenol concentrations and the maximum counts in the 10–50 window. Benzoic acid-C^{14}, 4.2 mg (21,336 dpm), and 0.033 ml phenol were dissolved in 7.62 ml of the toluene scintillator solvent and were diluted step by step with 2 ml each of the solvent. The maximum counting rate in the 10–50 window was recorded at each phenol level and was plotted versus phenol concentration which was expressed as follows: ml of phenol (0.033)/total volume of the liquid in the vial in ml.

zero quencher concentration, the counting rates without quenching were obtained. Since the counting efficiency of C^{14}-nonquenched sample in the 10–50 window of the spectrometer was known, the absolute radioactivity could be calculated.

A similar straight-line relationship was obtained with the chlorophyll-

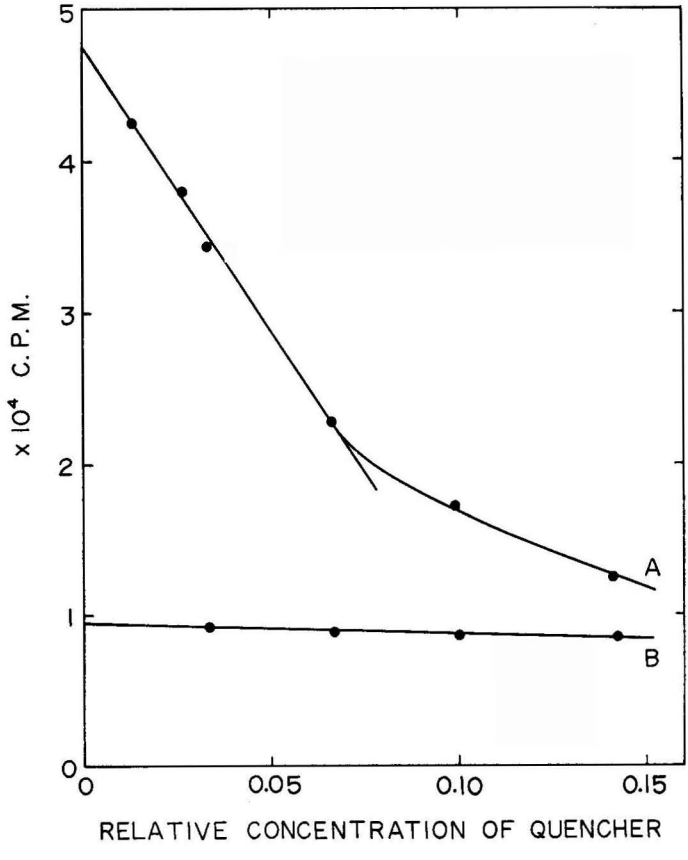

FIG. 2. The relationship between chlorophyll-C^{14} concentrations and the maximum counts in the 10–50 window. Samples (*A*: chlorophyll-C^{14} fraction diluted with cold carrier in toluene, 0.05 ml. *B*: the same fraction, 0.01 ml) were dissolved in 7.0 ml of the scintillator solvent and diluted with appropriate additions of the solvent. Aliquots were withdrawn to the new vials in order to dilute samples when needed. Countings were made at each step of dilution, and the maximum counts were plotted against the relative amounts of chlorophyll in the solvent which were expressed as 0.05/total volume of the liquid and 0.01/total volume of the liquid, respectively. The radioactivities obtained by extrapolation were 47,500 and 9500 counts/min, respectively.

C^{14} fraction, a strong color quencher; and with the benzoic acid-C^{14}–hyamine system, the latter compound is known as a moderate quencher (12). In every case, however, the linear relationship was not held at high concentrations of the quenching substance. One example of such properties with chlorophyll-C^{14} was shown in Fig. 2. Although this effect was a problem in correcting the counting data by the dilution method, it was observed only in severely quenched samples with which the maximum counting rates were obtained at high-voltage settings larger than 4.5 taps[1] (1020 v) by the instrument.

The validity of the correction by the dilution method was examined with C^{14} protein hydrolyzate on paper sections. To three counting vials was added a paper section, each 2×2 cm, and various amounts of aqueous solution of C^{14} protein hydrolyzate were added. They were then dried at 55°. One milliliter each of hyamine was added and allowed to extract the sections for 90 min at 55°. Seven milliliters of the toluene scintillator was added to each vial. After the maximum counts in the 10–50 window were obtained, the samples were then counted after 3-ml and then another 5-ml additions of the scintillator solvents. The maximum counting rates were recorded at each step, and were plotted against the relative concentrations of the quenching substance which were expressed as 1/8, 1/11, and 1/16, respectively (Fig. 3). Figure 3 shows again the straight-line relationship between the maximum counting rates and the relative concentrations of the quencher. Upon extrapolating the lines, the counting rates without quenching were obtained. By dividing the data with 0.50, which was the counting efficiency of nonquenched C^{14} samples at the 10–50 window, the absolute radioactivity of the protein hydrolyzate sample was calculated. Three calculated values ranged between 98 and 103% of the disintegration events of the samples which were obtained with the internal standard without filter paper.

Use of the Internal Standard

Table 2 summarizes the correction of counting data of C^{14} protein hydrolyzate on filter-paper sections by the use of the internal standard. The radioactive samples were the same as those used for the dilution

[1] The voltage applied to the photomultipliers is approximately 650 v on tap 0 and raises approximately 83 v per tap up to tap 9. A potentiometer type fine control which has a scale of 0 to 1000 adjusts the voltage between taps. The high voltage, therefore, raises approximately 8.3 v per 100 units of the fine control. Taps 4.5 mean that the high voltage tap on 4 and the fine adjustment control on 500 units. This type of explanation about high voltage will be made throughout the text for convenience. The voltage appeared in the text in parentheses represents the reading of a voltmeter of the instrument.

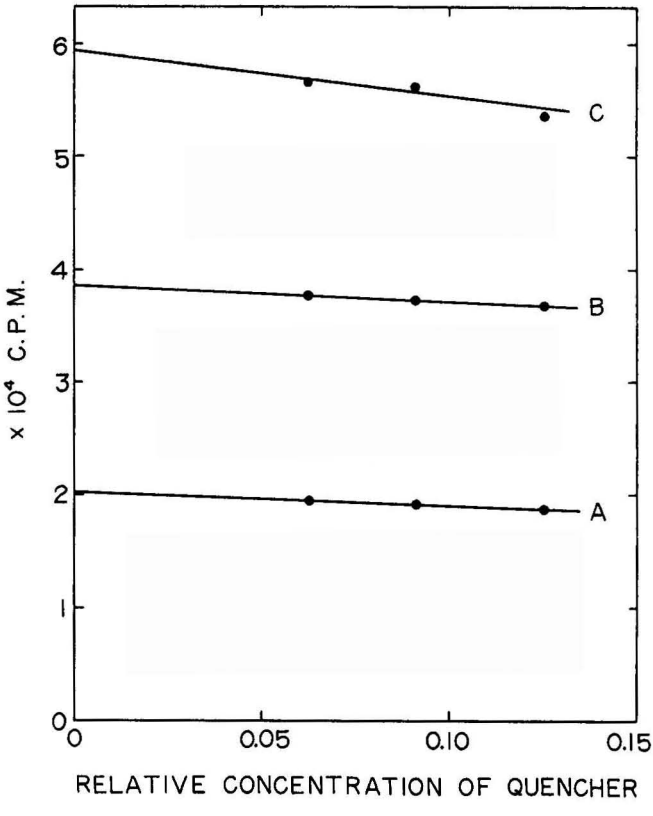

FIG. 3. The relationship between the maximum counts of C^{14} protein hydrolyzate and the relative concentrations of the quencher. The following radioactive samples were applied to the vials. *A*: C^{14} protein hydrolyzate 0.03 ml, 39,420 dpm; *B*: 0.06 ml, 78,840 dpm; *C*: 0.09 ml, 118,260 dpm. Other experimental conditions are given in the text. The calculated radioactivities obtained from the data were as follows: *A*: 40,400 dpm recovery 102.5%; *B*: 77,000 dpm, recovery 97.7%; *C*: 119,000 dpm, recovery 100.6%.

method already described. As can be seen, the corrected values were in agreement with applied radioactivity.

Correction by Ratio of Counts in Two Windows

The high-voltage setting was experimentally chosen which gave the maximum counting rates in the 10–100 window with nonquenched C^{14} sample. It was found that the taps 3.25 (920 v) gave the maximum by the spectrometer. In order to investigate the ratio of counts in two windows and to minimize the counting error, it was necessary to choose

TABLE 2
CORRECTION OF THE OBSERVED RADIOACTIVITY BY THE USE OF THE INTERNAL STANDARD[a]

C^{14} protein hydrolyzate (ml on paper sections)	Observed radioactivity (counts/min)	Corrected by internal standard (dpm)	Per cent of disintegration events
0.03	19,320	41,220	104.6
0.06	37,620	81,700	103.6

[a] The samples were the same as in Figs. 3*A* and 3*B*, respectively. One vial contained C^{14} protein hydrolyzate 39,420 dpm, and the other contained 78,840 dpm. After the samples were counted in the 10–50 window at 3.25 high-voltage taps (920 v) which gave the maximum counts at this window, 0.1 ml each of benzoic acid-C^{14} standard source (2.1 mg and 11,684 dpm) in toluene was added and the samples were recounted under the same conditions as before. The counting efficiencies were obtained from the increase of counts in the window. The corrected radioactivity of each sample was calculated from the counting efficiencies which were 46.9 and 46.0%, respectively.

the appropriate setting for the middle discriminator in the 10–100 window. According to Davidson (11), the middle discriminator was set at 40.5 which was chosen experimentally to give equal counts in both windows. With nonquenched samples, the ratio of counts in the 10–40.5 window to those in the 40.5–100 window was unity.[2] When quenching happened, the counts in the 10–40.5 window increased and those in the 40.5–100 decreased, which resulted in an increase in ratio of counts in two separate windows. Obviously, the setting of the middle discriminator was specific for this particular solvent system and the instrument.

A definite amount of standard benzoic acid-C^{14} in vials was dissolved in the scintillator solvent, and various amounts of pyridine, a strong quenching substance, were added to each vial. After cooling, they were counted as described above, and the counting efficiencies in the 10–100 window were plotted versus ratio of counts in two windows. The result is shown in Fig. 4. A similar experiment with phenol as a quencher gave the curve exactly the same as in Fig. 4. Therefore, it appears possible to determine the absolute radioactivity by inspection of the ratio.

[2] The middle discriminator setting 40.5 was chosen with the vial which contained 5.4 mg of benzoic acid-C^{14} standard source [27,432 disintegrations/min (dpm)] in 7 ml of the scintillation solvent and gave the ratio of counts 0.99 in this setting. The scintillation solvent used here was saturated by air. As a result, oxygen, a quenching substance, was included in the solvent. Therefore, the "nonquenched" sample described here which gave equal counts in two windows was slightly quenched by dissolved oxygen. A ratio of 0.95 was obtained under the assay conditions with similar sample, which was flushed with argon in order to remove oxygen from the counting vial.

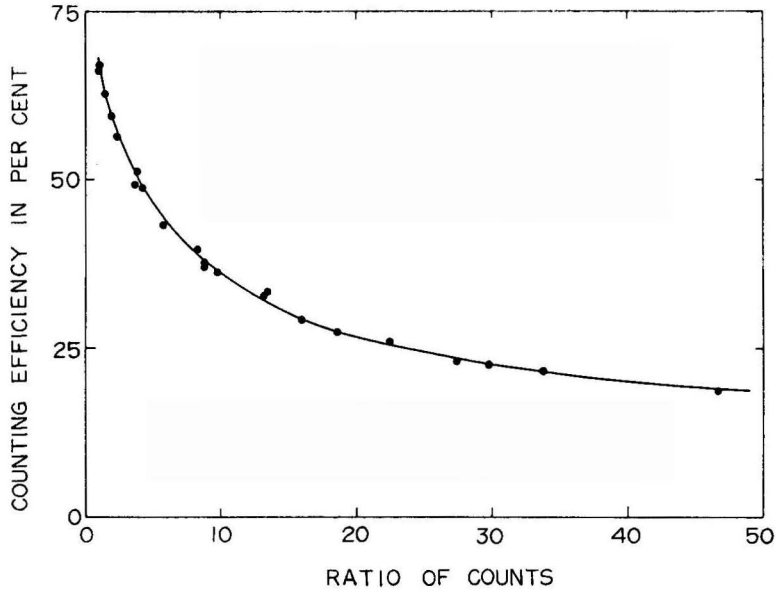

FIG. 4. The relationship between counting efficiency and the ratio of counts in two windows. Benzoic acid-C^{14} standard source was added to two vials. One contained 5.4 mg (27,432 dpm) and the other contained 8.2 mg (41,656 dpm). Seven milliliters of the scintillation solvent was added to each vial. The ratios of counts and the counting efficiencies in the 10–100 window were 0.99 and 1.07, and 65.5 and 66.8%, respectively. Various amounts of pyridine up to 0.25 ml were added step by step, and then the samples were diluted with stepwise addition of the scintillator solvent up to 8 ml. Final volume of the solvent was 15 ml. The ratio of counts and counting efficiency were determined at each pyridine concentration. The counting efficiencies were plotted against the ratios of counts as shown in the figure. The most quenched sample which contained 0.25 ml pyridine and 7 ml of the scintillator solvent gave a counting efficiency of 18.8%, and the ratio of counts was 46.8.

To test the validity of the correction, a definite amount of C^{14} protein hydrolyzate with or without carrier (salt-free casein hydrolyzate) was added to vials with or without paper section, 2×2 cm, in the bottom. After drying, 1 ml hyamine was added to each vial and kept standing overnight at 40°. Seven milliliters of the scintillator solvent was then added, and the samples were counted at 3.25 high-voltage taps (920 v) and at 10–40.5–100 discriminator settings. The ratio of counts in 10–40.5 to those in 40.5–100 windows was calculated for each sample, and the counting efficiency was obtained graphically from the standard curve

(Fig. 4). The radioactivity of each sample assayed in this manner was shown in Table 3. The correction of observed activity by the ratio method gave satisfactory recovery in absolute radioactivity.

TABLE 3
CORRECTION OF THE OBSERVED RADIOACTIVITY BY THE RATIO OF COUNTS IN TWO WINDOWS

C^{14} protein hydrolyzate	Filter paper	Corrected activity (dpm)	Per cent of theoretical[a]	Ratio	Counting efficiency (%)
0.02 ml	−	27,660	103	4.12	48
0.02 ml	+	27,420	102	4.52	46
0.02 ml carrier 109 μg	+	28,980	108	5.41	43
0.04 ml	−	53,730	100	4.23	47.5
0.04 ml	+	57,710	107	4.56	46.5
0.04 ml carrier 109 μg	+	52,720	98	6.54	41

[a] The absolute radioactivity was determined by the use of the internal standard without filter-paper section.

Counting of Paper Chromatograms

Glutamic acid-C^{14} was applied as a spot to filter paper, 2×40 cm, with various amounts of carrier glutamic acid, and one-dimensional chromatography was carried out with either phenol–water or butanol–propionic acid–water solvent systems. After the run, the radioactive spot was located by the GM scanning, and the radioactivity of each spot was determined by the GM counter. Each spot was then cut off from the paper and was put in the vials as two or three pieces in order to settle at the bottom. One milliliter of hyamine was added to each vial and was kept standing overnight at 30–40°. Seven milliliters of the scintillator solvent was added, and the maximum counting rates in the 10–50 window were recorded. Thereafter, the samples were diluted twice with the solvent, first with 3 ml and then with 5 ml more. At each step, the maximum counting rates in the 10–50 window were determined. The linear relationship was again observed and, by extrapolation, the radioactivity of each sample corrected for quenching was calculated.

Each sample was then counted at 3.25 high-voltage (920 v) and 10–40.5–100 discriminator settings, and the ratio of counts in two windows was obtained. The radioactivity of each sample was calculated

from the standard curve. Table 4 summarizes the counting data by these two different correction methods.

TABLE 4
COUNTING OF GLUTAMATE-C^{14} ON THE PAPER CHROMATOGRAM[a]

Sample No.	GM (counts/min)	Dilution method		Ratio method	
		(dpm)	LS/GM	(dpm)	LS/GM
1	417	68,520	164	69,240	166
2	557	69,310	124	71,760	129
3	306	57,810	189	60,310	197
4	439	74,030	169	76,660	175
5	384	66,000	172	67,070	175
6	433	61,840	143	64,030	148
7	341	54,600	160	58,290	171
8	379	64,150	169	67,700	179

[a] Equal amounts of glutamic acid-C^{14} with various amounts of carrier (see below) were applied at the origin of each paper, and chromatography was carried out with either butanol–propionic acid–water system (No. 1 to No. 4), or phenol–water system (No. 5 to No. 8).

No. 1, No. 5: No carrier.

No. 2, No. 6: 33 μg of carrier monosodium glutamic acid.

No. 3, No. 7: 100 μg of the carrier.

No. 4, No. 8: 165 μg of the carrier.

Note the coincidence of the corrected activities by dilution and ratio methods for each sample. Relatively large variation in radioactivity between paper sections might be due to the incomplete recovery of glutamate by paper chromatography (13).

In order to determine whether or not the extraction was satisfactory, the paper sections were removed from each vial and recounting was done. The recovery of radioactivity ranged from 98.2 to 101.0% indicating the extraction was almost complete and the counting data were dependable.

A similar type of experiment was carried out with various C^{14} compounds on two-dimensional paper chromatograms. After counting the spots by the GM detector, the spots were cut off from the paper and were put in the vials. Each vial was counted as before except the correction by the internal standard was done finally for some samples. Table 5 shows the counting data and the extraction efficiencies of these compounds.

It can be seen from Tables 4 and 5 that all of the correction methods gave similar radioactivity indicating the dependability of the methods for paper chromatogram countings. It was experienced, however, that

TABLE 5
COUNTING OF VARIOUS C^{14} COMPOUNDS ON THE PAPER CHROMATOGRAMS[a]

Sample No.	GM (counts/min)	Dilution method		Int. std. method		Ratio method	
		(dpm)	LS/GM	(dpm)	LS/GM	(dpm)	LS/GM
1	1,575	312,000	198	—	—	323,400	205
2	487	82,000	168	83,560	172	—	—
3	89	16,700	188	18,470	208	16,840	189
4	104	18,200	175	18,250	175	17,840	172
5	918	198,000	216	—	—	199,500	217
6	106	16,200	153	17,590	166	16,550	156
7	161	32,400	201	31,870	198	34,050	211
8	147	27,000	184	26,910	183	27,970	190
9	221	29,400	133	30,650	139	29,090	132
10	2,440	398,000	163	314,600	129	383,370	171
11	1,326	294,000	222	—	—	313,600	237
12	213	47,600	223	—	—	49,240	231
13	985	206,000	209	—	—	205,200	208
14	876	190,000	217	—	—	186,900	213

[a] Sample No. 1, aspartic acid (extraction, 94.6%). No. 2, glutamic acid (extraction, 96.7%). No. 3, serine (extraction, 93.3%). No. 4, glycine (extraction, 91.5%). No. 5, threonine (extraction, 96.6%). No. 6, alanine (extraction, 84.3%). No. 7, tyrosine (extraction, 92.8%). No. 8, proline (extraction, 88.0%). No. 9, glucose (extraction, 95.5%). No. 10, fructose (extraction, 95.2%). No. 11, sucrose (extraction, 95.5%). No. 12, maltose (extraction, 99.1%). No. 13, levulinic acid (extraction, 100.1%). No. 14, levulinic acid 2,4-dinitrophenylhydrazone (extraction, 98.8%).

generally the correction by the use of the internal standard gave a little higher activity compared with the other two methods.

As shown in the tables, the counting efficiency of direct counting of paper sections by the conventional GM detector under the assay conditions was approximately 0.4–0.8% of the disintegration events of the C^{14} samples. This was not surprising in view of the fact that the counting efficiencies of C^{14} samples on the filter paper by conventional GM counting was poor and varied considerably due to a minor change in geometry and to heterogeneity of the sample in the paper.

DISCUSSION

The method described here for the counting of C^{14} paper chromatograms by liquid scintillation technique involves the extraction of samples from the paper by hyamine. Although no other solubilizing agent has yet been tested, any other solvents which have an appropriate solubilizing capacity for biological materials and have a little or no quenching property can be applied as solubilizing system.

Obviously, a counting error should be expected if the extraction is not quantitative. The radioactive samples, which are not eluted from the paper section and settle in the bottom of the vials, however, also contribute to the observed counts in the spectrometer. The counting efficiency of such insoluble sample was examined with C^{14} protein hydrolyzate on the paper section (2×2 cm) in the toluene scintillator system, and was found to be approximately 47% at 3.25 high-voltage taps (920 v) and 10–100 window. If the correction by the ratio method was applied to such insoluble samples, the corrected activity was calculated as approximately 80% of disintegration events.

The dilution of the scintillator solvent by addition of fresh solvent also increases the counting rates of insoluble samples on the paper by diluting out the quenching substances if present in the solvent. The correction of the observed counts by the use of the internal standard also tends to correct the activity of the insoluble sample by revealing the quenching property of the solvent.

A model experiment was carried out with the vial which included both benzoic acid-C^{14} and the paper section which contained C^{14} protein hydrolyzate. The former substance is soluble in the toluene scintillator but the latter is not. Various amounts of phenol were added in order to quench the sample. Three correction methods: the dilution method, the use of the internal standard, and the ratio method were applied, and the corrected activities were compared with added radioactivity. It was found that the corrected activities by either of these three methods were over 94% of the disintegration events, provided the soluble fraction carried more than 80% of the total radioactivity. Therefore, it appears that the methods can be applied to C^{14} samples if the extraction is better than 80%. In keeping with this, the corrected activities of various C^{14} compounds by three different methods showed an excellent agreement as recorded in Tables 4 and 5 in spite of the fact that the extraction varied considerably.

Although the ratio method was simplest in correcting the counting data, care should be taken in a drift of the high voltage of the spectrometer. A minor variation in the high voltage caused an appreciable change in the ratio of counts in two windows which resulted in an error of the correction. Therefore, the use of a standard vial whose ratio is known is recommended. By applying appropriate high voltage to the photomultipliers, it is possible to set the spectrometer to give standard ratio in two windows.

The dilution method, although it is slow and tedious, seems to be the most dependable, since it involves series of counting at balancing posi-

tions. As shown in Fig. 2, the straight-line relationship was not held for severely quenched samples, which gave the maximum counts in the 10–50 window at high-voltage setting over 4.5 taps (1020 v). Therefore, such samples should be diluted first before the correction by the dilution method is made. Obviously, this type of correction needs quantitative extraction by hyamine.

Most of the samples so far tested gave a straight-line relationship between the maximum counting rates and the relative concentrations of the quenching substances. One exception so far experienced was a sample containing pyridine, which did not give the linear relationship. Therefore, the method should not be applied to such samples.

Thus far, spurious counts which are known to occur as a result of photoluminescence by hyamine-containing samples (10) or by C^{14} paper sections (3) have not been observed; the inclusion of an acid in counting vials may be needed if the sample shows photoluminescence. The appropriate solvent system for this problem has been described by Herberg (10) and by Bousquet and Christian (3).

Counting of tritium samples on the paper chromatograms was also examined in the manner as described here. Both dilution and ratio methods were found not to be suitable for tritium countings. The dilution method did not give the linear relationship due to the low energy nature of tritium and the ratio method resulted in erroneous corrected activity. The use of the internal standard, however, can be applied for tritium chromatogram countings, but the correction was not so reliable as C^{14} counting. The corrected activities were usually in the range of 80–90% of the disintegration events for tritium work.

SUMMARY

A method is described for the counting of C^{14} biological materials on paper chromatograms. Paper sections are placed in counting vials and are extracted by the solubilizing agent, hyamine. Scintillation solvent is then added, and the samples are counted by a liquid scintillation spectrometer. Absolute radioactivity of each spot can be obtained by any of the following three correction methods. One method involves the dilution of the sample by scintillator solvent, the other uses an internal standard, and the last method involves correction of the observed radioactivity by the counting efficiency obtained from the ratio of counts in two separate windows of the spectrometer. Any of the three methods will give quantitative data if the extraction of the sample from the paper by a solubilizing agent is over 80%. The methods have been applied to the counting of various C^{14} amino acids and C^{14} sugars on

paper sections with excellent recovery in radioactivity and reproducibility.

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Fluorometric Determination of Pyridoxamine by Conversion to Pyridoxal Cyanide Compound

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The determination of pyridoxine, pyridoxal, and pyridoxamine in foods is largely dependent on microbiological assays which are suitably sensitive to the low concentrations of these compounds in extracts prepared for assay. The three forms of vitamin B_6 can be determined separately after fractionation of the hydrolyzed food extracts on Dowex 50 ion-exchange columns (1, 2). This treatment also served as a purification step by removing most extraneous materials. In fluorometric determinations of pyridoxine, pyridoxal, and pyridoxamine in these fractions, interfering fluorescence was encountered. Considerably greater fluorescence of the pyridoxal cyanohydrin than that of pyridoxal was described by Bonavita (3) in studies to develop a proposed quantitative assay for pyridoxal 5-phosphate in certain mammalian tissues based on use of the reaction between pyridoxal 5-phosphate and cyanide. The reaction was expected by us to be suitable for the quantitative fluorometric assay for pyridoxal in the fractions from the food extracts separated on Dowex columns.

Metzler, Olivard, and Snell (4) reported that the transamination of pyridoxamine to pyridoxal with glyoxylic acid proceeded rapidly at pH 5 when catalyzed by aluminum salts. Due to side reactions, yield was 70% of theoretical. The possibility existed that this reaction could be used to determine pyridoxamine, in its proper column fraction, as pyridoxal. In order to be successful, the reactions for the transamination of pyridoxamine and the formation of pyridoxal cyanohydrin would need to be quantitatively reproducible. The studies reported here demonstarted that this sequence of reactions would permit the fluorometric determination of pyridoxamine under the conditions described.

EXPERIMENTAL

Materials and Apparatus

Pyridoxal and Pyridoxamine Solutions. Stock solutions were prepared to contain 25 μg/ml pyridoxal or pyridoxamine (Nutritional Biochemicals Corp.) by dissolving 30.45 mg pyridoxal hydrochloride or 35.84 mg pyridoxamine dihydrochloride in *M* HCl and bringing the volume to 1 l with *M* HCl.

Potassium Cyanide, 0.03 and 0.5 *M*.

Dipotassium Acid Phosphate, molar, adjusted to pH 7.4 with *N* HCl.

Quinine Reference Standard, 0.3 μg/ml of quinine sulfate in 0.1 *N* H_2SO_4.

Potassium Alum, $AlK(SO_4)_2 \cdot 12H_2O$, 0.005 *M*.

Sodium glyoxylate monohydrate was prepared from tartaric acid and periodic acid as described by Metzler, Olivard, and Snell (4). The crystals were used to prepare a 0.05 *M* solution.

The Aminco-Bowman spectrophotofluorometer described by Bowman *et al.* (5) was used for all measurements of activation and fluorescence spectra and fluorometric determinations. The spectrophotofluorometer was equipped with a Hanovia xenon light source and a 1P21 photomultiplier tube (Radio Corp. of America). The instrument was standardized at 0.03 sensitivity to read 50 for the quinine sulfate reference standard.

Procedure

The studies were carried out in a darkened laboratory since pyridoxal and pyridoxamine are sensitive to light. For the reactions, the stock solutions of pyridoxal and pyridoxamine were diluted with water to contain 0.01–10 μg/ml. The cyanide reactions with pyridoxal were carried out essentially as described by Bonavita (3). For example, to 5 ml of a solution containing 1 μg/ml pyridoxal were added 10 ml of *M* K_2HPO_4 at pH 7.4 and 2 ml of 0.03 *M* KCN; the pH of the resulting solution was adjusted to 7.2–7.4 with HCl or KOH. The final volume was 50 ml, yielding a concentration of 0.1 μg/ml pyridoxal. For reactions involving other concentrations of pyridoxal, only its concentration was varied. The 50-ml solution was held at 50°C for 2½ hr. After cooling to room temperature, 10–15 ml of the solution was adjusted to pH 9.5 with a few drops of 6 *M* NH_4OH and placed in the quartz cell for fluorescence readings.

Transamination reactions were carried out between pyridoxamine and glyoxylate essentially as described by Metzler, Olivard, and Snell (4), followed by the cyanide reactions with the pyridoxal product. These

reactions were carried out with the same amounts of reagents, varying only the concentration of pyridoxamine. For example, to 5 ml of a solution containing 1 μg/ml pyridoxamine were added 10 ml of *M* K_2HPO_4 pH 7.4, 1 ml of 0.005 *M* potassium alum, and 1 ml of 0.05 *M* sodium glyoxylate. The mixture was heated for 15 min at 100°C. When cooled to room temperature, 1 ml of 0.5 *M* KCN was added, the pH was adjusted within 7.2–7.4, and the volume was adjusted to 50 ml. This solution was held at 50°C for 2½ hr, and fluorescence readings were taken as before, after adjusting to pH 9.5.

RESULTS

Under the conditions described, the reaction between cyanide and pyridoxal required 2 hr to reach completion. As shown in Fig. 1, fluores-

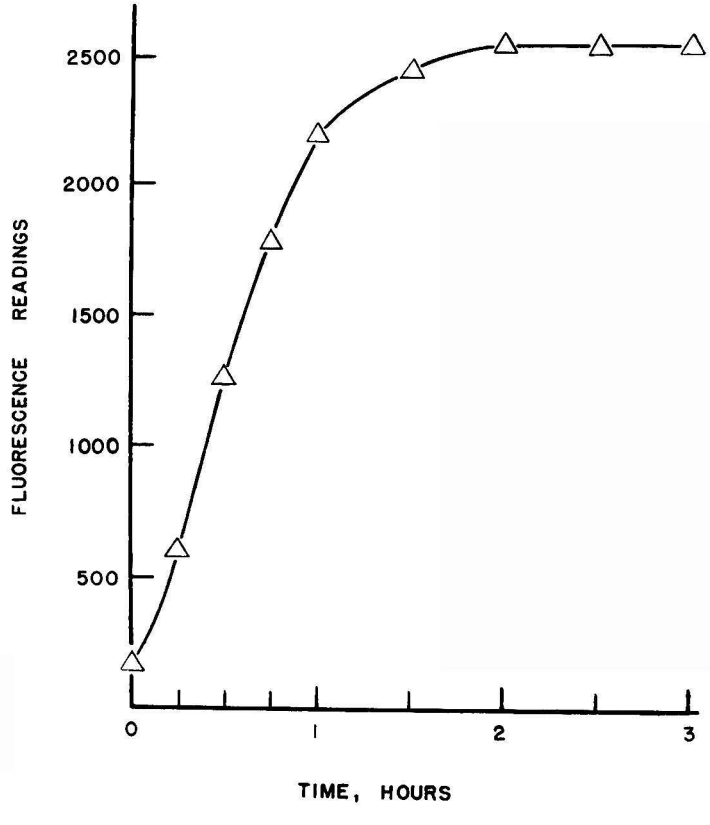

FIG. 1. Reaction between pyridoxal, 0.1 μg/ml in 0.2 *M* K_2HPO_4, and 0.0012 *M* KCN at pH 7.35, to form pyridoxal cyanide compound for which fluorescence readings were obtained at pH 9.5.

cence readings of solutions containing 0.1 μg/ml pyridoxal increased rapidly, reaching a maximum reading of 2500. Readings were made at pH 9.5 with 358 mμ activating wavelength and 435 mμ fluorescent wavelength. Fluorescence of pyridoxal or pyridoxamine was negligible under these conditions. The fluorescence spectra at activating wavelength of 358 mμ of 0.1 μg/ml pyridoxal as the cyanide compound in 0.2 *M* K_2HPO_4 at pH 9.5, and of 1.0 μg/ml each of pyridoxal or pyridoxamine are shown in Fig. 2. Fluorescence spectra of pyridoxal and pyridoxamine

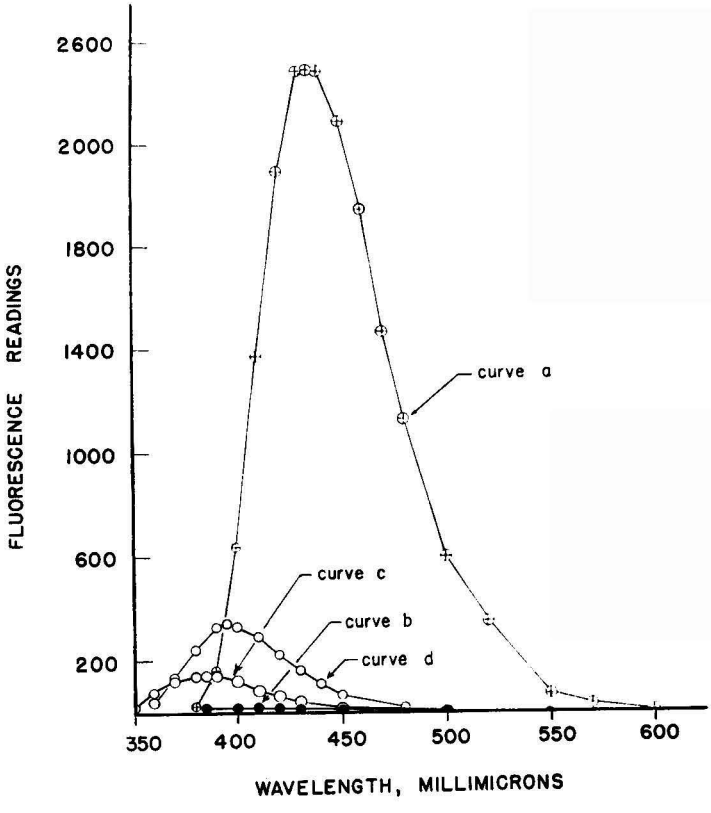

FIG. 2. Fluorescence of pyridoxal cyanide compound (*a*) 0.1 μg/ml; pyridoxal or pyridoxamine (*b*) 1.0 μg/ml in 0.2 *M* K_2HPO_4 at pH 9.5 with 358 mμ activating wavelength; pyridoxal (*c*) and pyridoxamine (*d*) 0.1 μg/ml in aqueous solution at pH 6.0 with activating wavelengths of 325 mμ for pyridoxal and 330 mμ for pyridoxamine.

at 0.1 μg/ml in aqueous solution at pH 6.0 were obtained with activating wavelengths for pyridoxal and for pyridoxamine at 325 and 330 mμ with

maximum fluorescences at 385 and 395 mμ, respectively. Interfering fluorescences for readings of the cyanide compound at pH 9.5 and 435 mμ were shown to be negligible.

Transamination of pyridoxamine with glyoxylate in 0.2 *M* K_2HPO_4 at pH 7.4 proceeded very rapidly, and the subsequent reaction between the pyridoxal product and cyanide provided a quantitative and reproducible transformation of pyridoxamine to the pyridoxal cyanide compound. No reaction occurred between pyridoxamine and cyanide as determined fluorometrically in an independent experiment. The data in Fig. 3 show the linear relationship between concentrations of pyridoxal

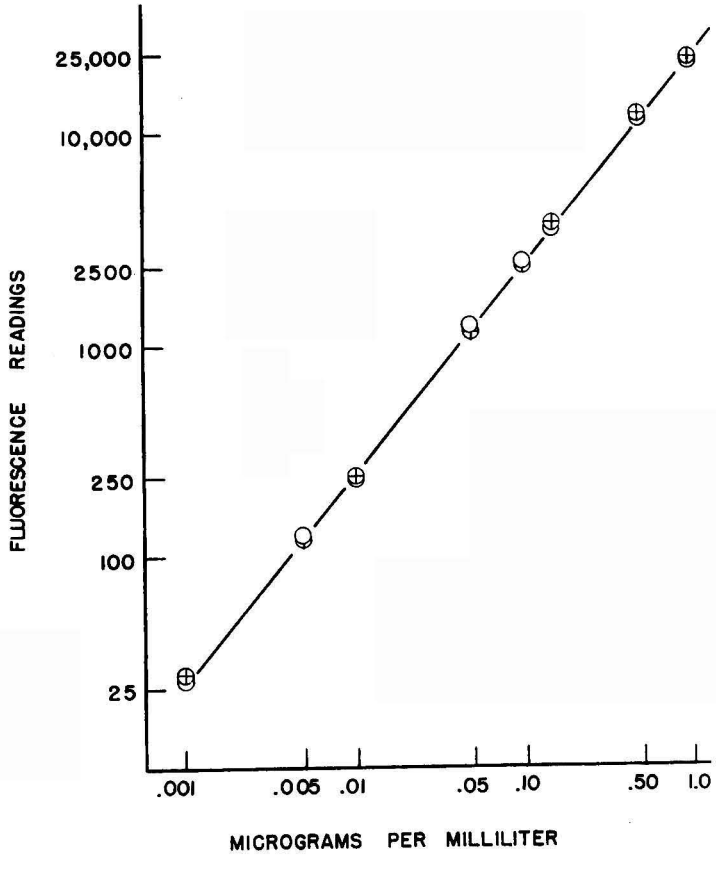

FIG. 3. Fluorescence of pyridoxal cyanide compound as the reaction product of pyridoxal —○— and pyridoxamine —⊕— in concentrations from 1.0 to 0.001 μg/ml in 0.2 *M* K_2HPO_4 at pH 9.5 with 358 and 435 mμ activating and fluorescent wavelengths, respectively.

from 0.001 to 1.0 μg/ml and fluorescence readings. Each point was determined independently by carrying out the reaction at a given concentration. Data are also shown in Fig. 3 for pyridoxamine after transamination and conversion to the pyridoxal cyanide compound. These readings agreed with those obtained for pyridoxal.

DISCUSSION

The linear relationship between fluorescence readings and concentrations of pyridoxal or pyridoxamine indicates that the reaction of cyanide with pyridoxal goes to completion under the conditions described. The readings can be reproduced independently, and the losses due to side reactions, discussed by Metzler *et al.*, were not observed in the reactions carried out in considerably lower concentrations of reactants. In determinations made on different days and times, 19 readings for pyridoxal cyanide compound from 0.05 μg/ml pyridoxal or pyridoxamine had a mean reading of 1239.6 with a standard deviation of 30.4, and 18 readings similarly obtained from 0.1 μg/ml had a mean value of 2500.5 with a standard deviation of 63.6. The average coefficient of variation was only 2.5%.

As part of these studies, an attempt was made to determine the dissociation constant of pyridoxal cyanohydrin by the method described for a number of cyanohydrins by Lapworth *et al.* (6, 7). The presence of phosphate interfered with such a determination; therefore other methods need to be devised to demonstrate the equilibrium of the reaction, if one exists. Preparation of pyridoxal cyanohydrin from 200-mg quantities of pyridoxal was undertaken in order to compare its fluorescence readings with those for the compound obtained with the reactions in dilute solutions. In this case both red and yellow crystals were obtained, both with fluorescence spectra identical to that of the compound obtained from reactions in dilute solutions. Recrystallizing red crystals from alcohol yielded yellow crystals, perhaps because of molecular rearrangement. A similarity between fluorescence spectra of pyridoxal cyanide compound and 4-pyridoxic acid lactone was noted. Both have maximum fluorescence at 435 mμ at pH 9.5 with the activating wavelength in the 355–358-mμ range. Fluorescence readings at 0.1 μg/ml were also of the same order of magnitude.

Studies of these and other reactions to form pyridoxal cyanide compounds will be continued. The present procedures promise to be of considerable value, in conjunction with column chromatography, for the determination of pyridoxal and pyridoxamine in foods and biological materials. Subsequent studies will include comparisons by microbiolog-

ical and fluorometric methods of pyridoxal and pyridoxamine in food extracts.

SUMMARY

A fluorometric assay for pyridoxamine is described based on the fluorescence of pyridoxal cyanide compound obtained by transamination of pyridoxamine to pyridoxal which was then reacted with potassium cyanide. The reactions were reproducible from 0.001 to 1.00 μg/ml pyridoxamine. Fluorescence spectra of pyridoxamine and pyridoxal show little or no interference with fluorescence readings of pyridoxal cyanide compound at 358 and 435 mμ, activating and fluorescence wavelengths.

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A Modified Cartesian Diver Method Permitting Measurement of Oxygen Uptake in the Presence of Carbon Dioxide[1]

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INTRODUCTION

Because of the important role of CO_2 in the metabolism of mammalian cells (2, 6, 9, 14, 15), it has seemed desirable to devise a method to maintain constant CO_2 tension within physiological limits while measuring respiration. An early attempt to do this was made by Warburg (14) using carbonate–bicarbonate solutions. However, he pointed out that only very low CO_2 pressures of the order of a few millimeters Hg could be maintained by this system.

The Cartesian diver technique as commonly employed (7) has the same disadvantage as the Warburg apparatus in that measurements are usually carried out in the absence of CO_2. In order to maintain constant carbon dioxide tension, Danes and Kieler (6) replaced the NaOH seal with a solution of 0.15 *M* $NaHCO_3$ equilibrated with the gas phase and obtained some buffering of CO_2 in the gas phase. Complete absorption of excess CO_2 by this method is not always obtained, however, as will be shown.

In 1949 Pardee (11) introduced the use of "CO_2 buffers" into manometric technique. These buffers are solutions of weak organic bases which form readily dissociable compounds with CO_2 and can thus be used to maintain a practically constant CO_2 pressure. He found that diethanolamine had a large enough capacity for the CO_2 tensions usually used in respiration studies. Krebs (8) subsequently confirmed these observations. However, in order to maintain pressures equivalent to

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[2] American Cancer Society Postdoctoral Fellow.

5% CO_2 in the gas phase in the Warburg apparatus, a very large reservoir for the buffer solution is required.

In consideration of this work and also in view of the demonstration by Boell *et al.* (1) that CO_2 passes readily through the oil seal in the Cartesian diver, we undertook to investigate the possibility of including diethanolamine in the flotation medium in the Cartesian diver technique. In this way it was anticipated that the flotation medium would act as a very large reservoir of CO_2 acceptor and would permit the maintenance of a constant CO_2 tension throughout the measurement period. This paper describes the development of such a method.

EXPERIMENTAL

Apparatus

The Cartesian diver technique as originally described by Holter (2, 4, 5, 7) was used as the basic method, and only the specific modifications introduced to maintain constant CO_2 tension will be described.

Flotation Medium. Diethanolamine (30% v/v) was employed as the "CO_2 buffer" (11, 8). At higher concentrations the flotation medium was too viscous. When diethanolamine was added to Holter's medium (7) at this concentration, a precipitate formed when the medium was gassed. A modified medium was therefore developed in which potassium iodide was used to raise the density to approximately 1.4.

The flotation medium finally developed was prepared as follows: A solution containing 30% diethanolamine (v/v) was cleaned by passing through activated charcoal. Fifty grams of potassium iodide was dissolved in 100 ml of this solution. One milliliter of 1% phenolphthalein per liter was added as an indicator. The flotation medium had a specific gravity of approximately 1.414 (gm/ml).

The method described by Krebs (8) was followed for equilibrating the flotation medium with the desired CO_2 gas mixture. One-third of the total volume was saturated with CO_2 at atmospheric pressure. The remaining two-thirds of the untreated solution was then added, and the gas mixture to be used in the Cartesian diver was passed through the solution at 37.5°C for 2 hr, using a sintered-glass distributor. Careful attention to this procedure was essential to obtain reproducible results.

Flotation Vessel. Using the original Cartesian diver apparatus, it was found that CO_2 leaked readily from the system, mainly through the rubber tubing. The flotation vessel was therefore modified to prevent this. A side arm, with a *U*-tube, was attached to the usual flotation vessel (Fig. 1), and a mercury seal (5–10 mm high) was placed on top

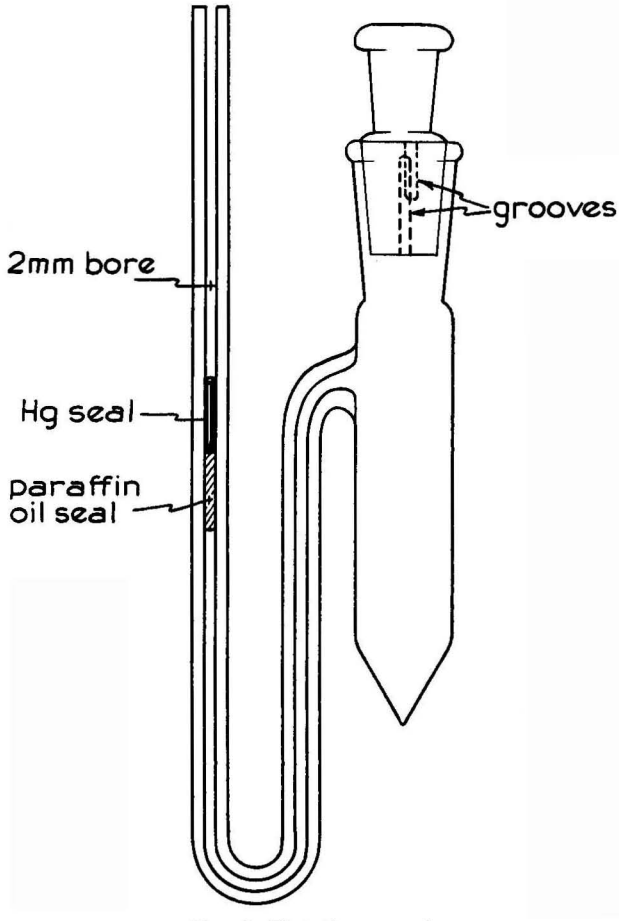

FIG. 1. Flotation vessel.

of the flotation medium in the side arm to prevent CO_2 leakage. In order to prevent the mercury from sinking, a seal of paraffin oil was placed under the mercury seal to act as a buoy.

The flotation vessel was completely filled with flotation medium in order to eliminate the necessity of obtaining equilibrium between it and an overlying gas phase. The stopper and neck of the flotation vessel were each provided with a groove which formed a continuous channel when the stopper was turned to bring them into line. By this means it was possible to allow the excess fluid and gas to pass out so that the flotation vessel was completely filled with flotation medium. This device also permitted adjustment of the mercury seal in the side arm to a constant

level by the application of a positive pressure to the flotation vessel after the Cartesian diver had been placed in it.

Double Manifold for Constant Pressure. Because of the very high solubility of CO_2 in this flotation medium, the equilibrium between gas and fluid phases was profoundly influenced by applied pressure. It therefore became necessary to include a device to permit the system to be maintained under constant pressure almost all the time.

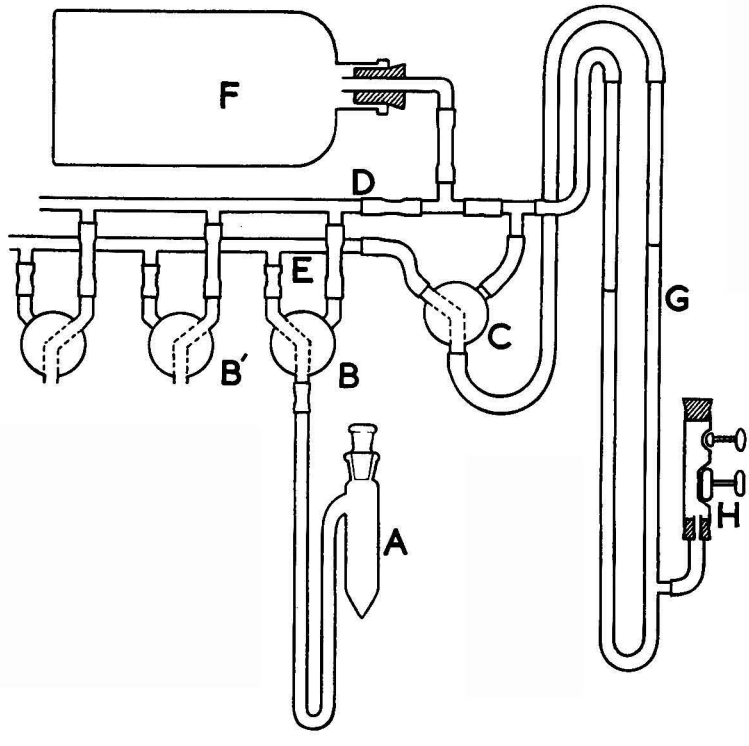

FIG. 2. Outline drawing of closed constant-pressure system for Cartesian diver manometry. *A*, flotation vessel; *B*, *B'*, stopcocks, flotation vessel to manifolds; *C*, stopcock, manifolds to manometer; *D*, manifold to air bottle; *E*, manifold to manometer; *F*, air bottle (4 l); *G*, manometer; *H*, pressure regulator.

A double manifold (Fig. 2) was used to connect each flotation vessel through a two-way stopcock to either the manometer or the air bottle. The flotation vessel was connected to the air bottle except when actual readings were being taken so that each Cartesian diver was maintained at a constant pressure for the major part of the experimental period.

Neck Seal. The seal to prevent exchange of water between the interior

of the diver and the flotation medium (7) posed a problem as it had to permit the diffusion of carbon dioxide. Carbon dioxide diffuses through paraffin oil (10) but too slowly to be ideal for this system. Diphenyl ether was tried but proved to be only slightly better than paraffin oil. As shown in Fig. 3, dibutyl phthalate proved best. Since the thickness

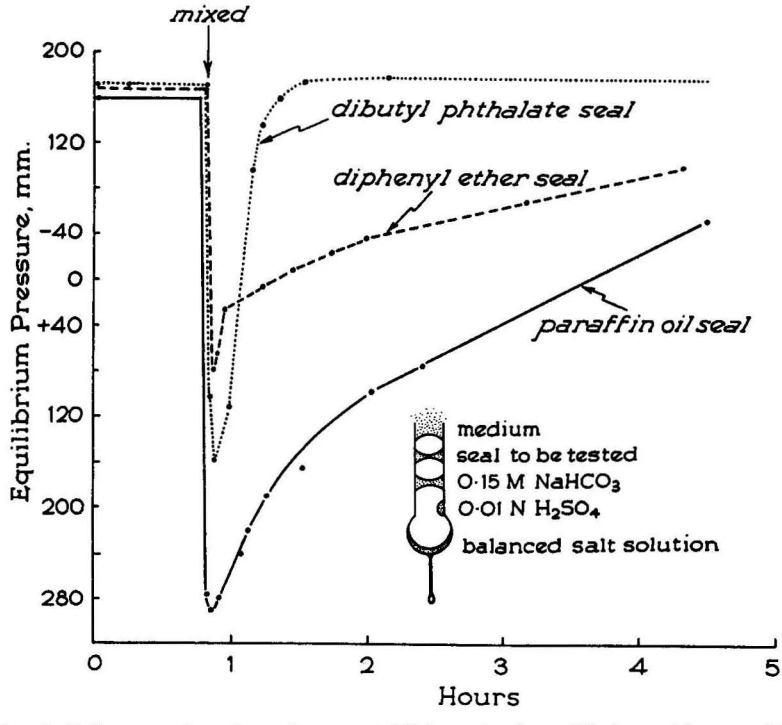

FIG. 3. Influence of neck seals on establishment of equilibrium. After establishment of initial equilibrium, bicarbonate seal and acid side drop were mixed with resulting liberation of CO_2. (V of divers and amount of CO_2 liberated same for all divers.) Equilibrium pressure of each diver was followed for the next 4 hr.

of the phthalate seal was found to be the limiting factor in the time required for establishment of equilibrium (Table 1), it was always made as thin as possible. Usually 0.25 μl dibutyl phthalate was used which resulted in a seal of less than 0.25 mm depth. It proved impossible to have a seal much thinner than this because it then disintegrated when pressure was applied (7).

Filling the Diver. The Cartesian diver used (Fig. 4) had the following dimensions: Inside diameter 1.2–1.4 mm, thickness of wall 0.12–0.14 mm, volume approximately 10 μl, weight 18–22 mg.

TABLE 1
EFFECT OF DIBUTYL PHTHALATE SEAL THICKNESS ON EQUILIBRATION TIME

Volume (μl)	Mean depth (mm)	Time for equilibration (hr)
0.25	0.22	0.8
0.50	0.44	2.5
1.0	0.89	>4
2.0	1.77	>4
4.0	3.53	>4

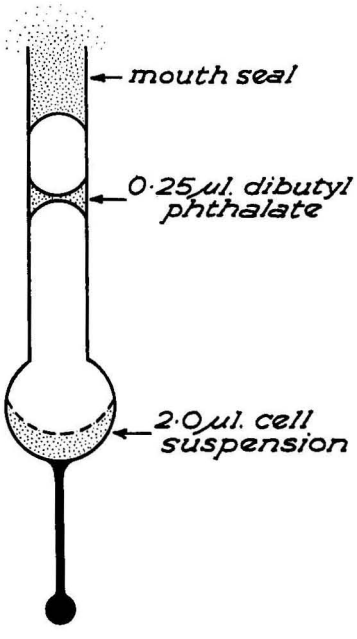

FIG. 4. Cartesian diver filled with cell suspension (2.0 μl) as a bottom drop, dibutyl phthalate seal (0.25 μl) and a mouth seal (flotation medium).

Since only one neck seal was normally introduced, it proved unnecessary to treat the inner surface of the diver with silicone (16). [Linderstrøm-Lang (10) has shown that theoretically this is desirable.]

The divers were charged under water at 37°C in a gassing chamber (Fig. 5). With this apparatus six divers could be filled at one time, and the introduction of cell suspension into all the divers at one time facilitated obtaining replicate cell samples. The bath (7-cm cube) made of Perspex (3 mm thick) was mounted on a rack and pinion. Metal spring

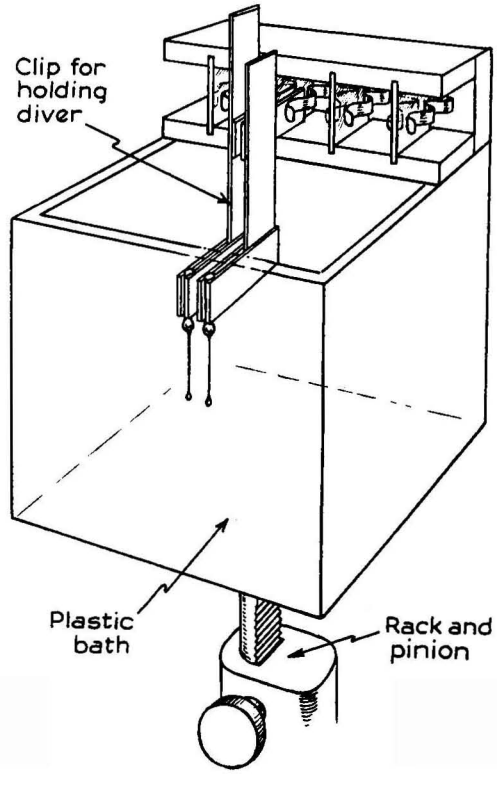

FIG. 5. Apparatus for filling and gassing six divers under water at the same time.

clips were mounted on a frame at the back of the bath to hold the plastic diver holders. These holders were made of strips of Perspex (1.5 mm thick). Each holder could be removed from the bath and used as a forceps to pick up a diver thereby eliminating excess handling of the divers.

After introduction of the cell suspension (2 μl) as a bottom drop, the diver was submerged. It was gassed for 1 min, and a dibutyl phthalate seal (0.25 μl) was then placed in position. Finally water was drawn into the neck of the diver to form the mouth seal.

Placing Diver in Apparatus. After the diver was filled, it was transferred to a flotation vessel (Fig. 2). By use of a fine pipet the mouth seal was rinsed and gas removed from the upper gas phase until the diver just sank. A small quantity of warmed, gassed flotation medium was then added to the flotation vessel so that when the stopper was placed in position, the vessel would be completely filled with medium.

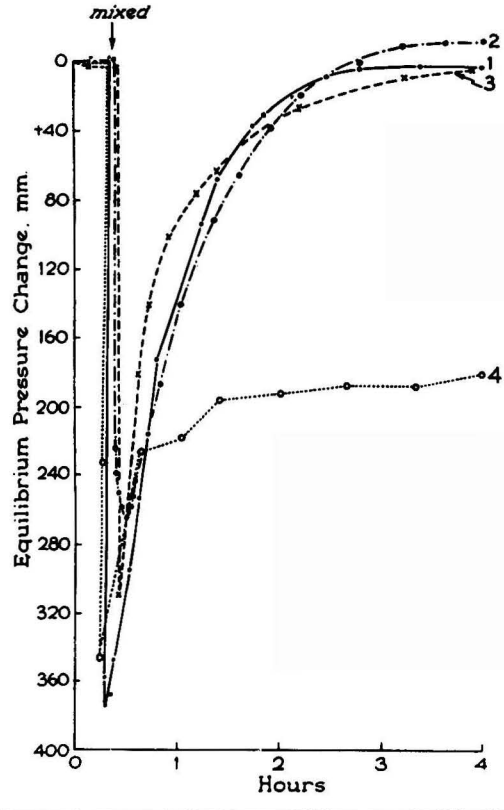

FIG. 6. A comparison of other Cartesian diver methods with the diethanolamine flotation medium as CO_2 absorber.

The level of the mercury seal in the side arm was then adjusted as previously described. Finally, the stopcock was turned to the position connecting the flotation vessel to the air bottle (Fig. 2).

Evaluation of the Method

In the following experiments the divers contained a bottom drop of nutrient fluid (2 μl), a phthalate neck seal (0.25 μl), and a mouth seal of flotation medium unless otherwise specified.

Effectiveness of Diethanolamine Flotation Medium as a CO_2 Absorber. In order to determine if diethanolamine in the flotation medium would absorb CO_2 as efficiently as the other methods (6), the following experiment was done. Four divers were prepared as shown in Fig. 6. Each contained a bottom drop and a side drop of 0.01 N H_2SO_4 and a neck seal of 0.15 M $NaHCO_3$. In addition the first diver contained a 0.1 N NaOH seal and a paraffin oil seal (7); the second contained a seal of diethanolamine and a paraffin oil seal; the third contained a dibutyl phthalate seal; the fourth diver contained a 0.15 M $NaHCO_3$ seal and a paraffin oil seal (6). The gas phase in all divers was room air. After establishing their equilibrium pressures in flotation medium equilibrated with room air, the acid side drops were mixed with the $NaHCO_3$ seals in order to liberate carbon dioxide. As shown in Fig. 6, the rate of return to equilibrium after the liberation of CO_2 was similar for the first three divers. The uptake of CO_2 in the fourth diver proved to be much slower. Warburg's (14) calculations suggest that the bicarbonate buffering system might be reasonably effective at lower CO_2 tensions. This proved to be the case in the Cartesian diver apparatus (Fig. 7), but at CO_2 tensions of the order of 5% in the gas phase the superiority of the diethanolamine buffer was indisputable.

Rate of Equilibration. It is necessary to know the time required for the establishment of CO_2 equilibrium after the diver is placed in flotation medium, and this was determined in two ways.

First, flotation medium was equilibrated with 5% CO_2. A diver filled with air, another with 5% CO_2, and a third with 10% CO_2 were placed in this medium. The equilibrium pressure of each diver was then followed to determine how long it would take for each diver to reach equilibrium. As shown in Fig. 8, the diver filled with 5% CO_2 soon became stable whereas the diver filled with room air progressively became lighter for 1 hr and then achieved equilibrium. The one filled with 10% CO_2 became heavier in a similar manner but again achieved equilibrium in 1 hr.

In a second experiment divers were filled with 10% CO_2 and placed

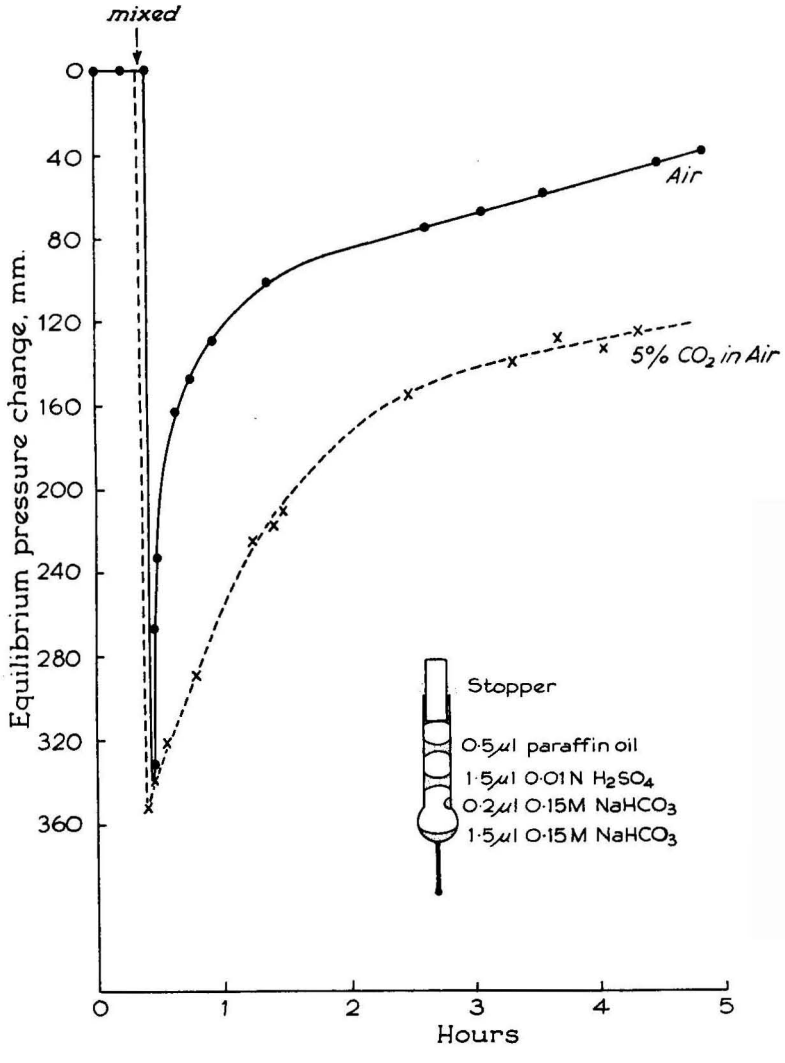

FIG. 7. A comparison of the effectiveness of bicarbonate as a CO_2 absorber when equilibrated with a gas phase of air or 5% CO_2 in the Cartesian diver. Approximately 0.2 µl CO_2 was liberated in each diver (V of diver = 7).

in flotation medium which had been gassed with different concentrations of CO_2. As shown in Fig. 9, the rate of establishment of equilibrium was directly related to the difference in CO_2 tension between the gas phase in the diver and flotation medium.

Effect of Applied Pressure on Equilibrium. It was found that the

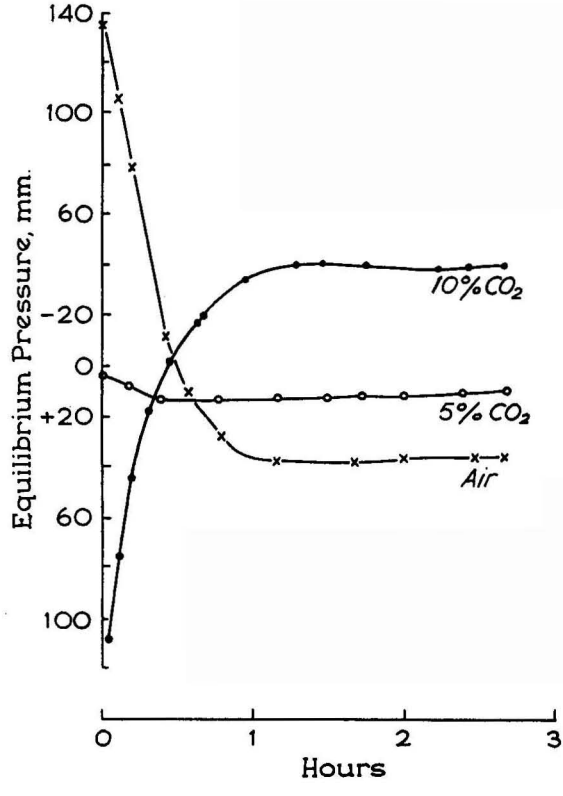

FIG. 8. Rate of establishment of equilibrium of divers gassed with 0.03% (air), 5 or 10% CO_2, and placed in flotation medium equilibrated with 5% CO_2.

divers became progressively heavier if kept under a positive pressure between readings and lighter if kept under a negative pressure, due to the establishment of a new equilibrium between the gas and fluid phases, according to Henry's law (Fig. 10). As already described, artifacts from this cause were minimized by maintaining a constant pressure in the flotation vessels between readings.

Effect of Other Seals. Any seal in the neck of the diver which did not allow rapid diffusion of CO_2 impeded or prevented the establishment of equilibrium. It was found that a seal of physiological salt solution or nutrient medium acted as such a barrier. For this reason, the cell suspension must be placed in the bottom of the diver as a drop [not greater than 0.5 mm high (10)], and the phthalate seal only should be placed in the diver neck.

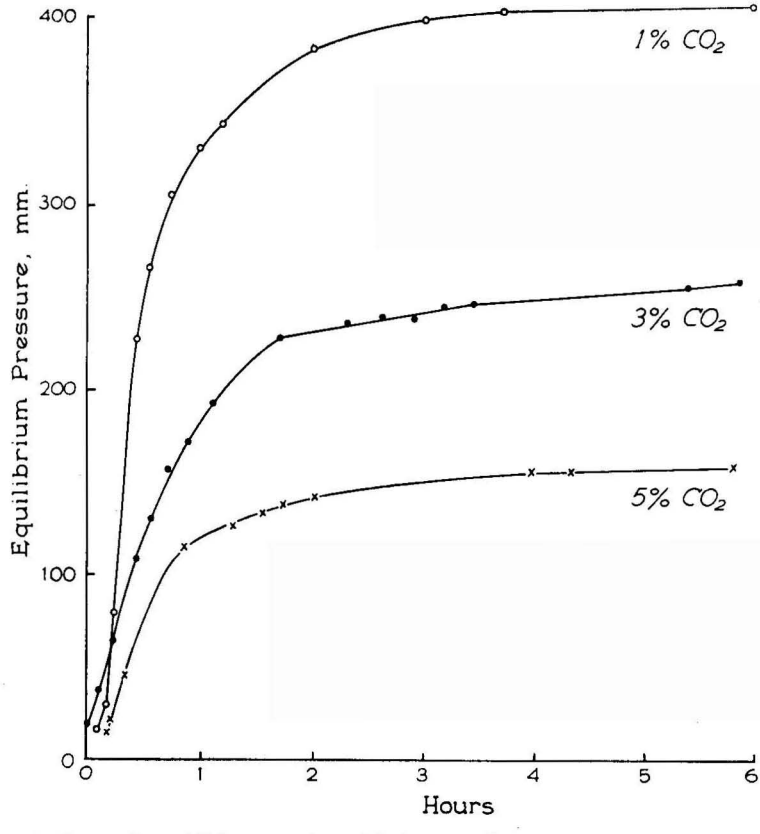

FIG. 9. Rate of establishment of equilibrium of divers gassed with 10% CO_2 and placed in flotation medium equilibrated with 1, 3, and 5% CO_2.

Absorption of Oxygen by Flotation Medium. When the above-mentioned factors were controlled, the equilibrium pressure of a control diver containing only nutrient fluid decreased by approximately 2 mm negative pressure per hour. This could be due to either (*a*) oxygen equilibration between the diver and the flotation medium and/or (*b*) oxidation of the flotation medium (8, 11).

To provide some information about this point, divers filled with 15, 20, 25, and 100% oxygen were placed in flotation medium equilibrated with 20% oxygen. As shown in Table 2, the equilibrium pressures of divers filled with 15, 20, and 25% oxygen decreased by approximately 2 mm negative pressure in all cases irrespective of oxygen concentration. It therefore appears that in this range, oxygen equilibration is not an

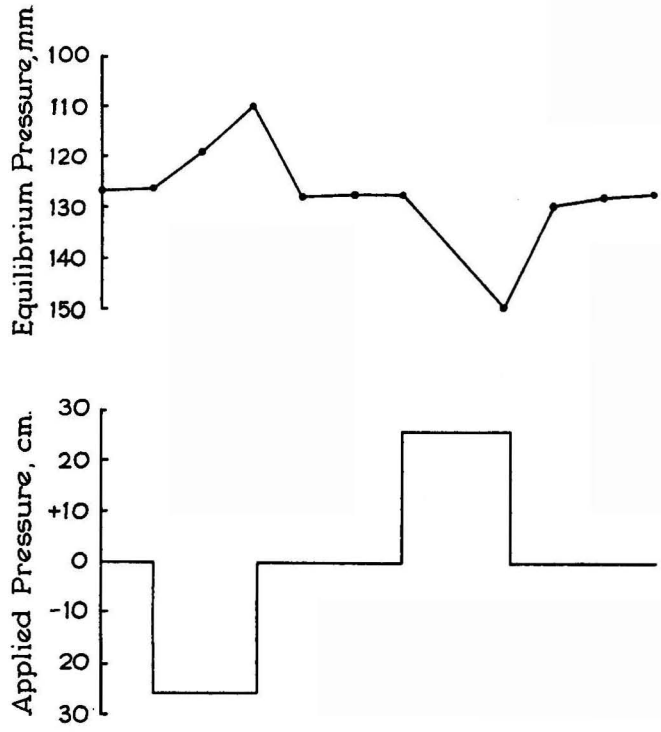

FIG. 10. Effect of changing applied pressure on equilibrium pressure.

TABLE 2
ABSORPTION OF OXYGEN BY THE FLOTATION MEDIUM[a]

Gas phase in diver (% O_2)	Change in equilibrium pressure/hr (mm negative pressure)
15	1.0
20	1.5
25	1.4
100	19.0

[a] Divers were gassed with varying concentrations of O_2 in nitrogen and placed in ungassed flotation medium.

important factor and that autoxidation occurs at a slow rate. However, as the oxygen concentration was increased to 100%, diffusion of oxygen out of the diver and/or autoxidation became significant. If high oxygen concentrations are used, this factor must be taken into consideration.

Measurement of Cell Respiration

Preparation of Cell Suspension. Mouse subcutaneous fibroblasts, strain L, clone 929, were grown in Roux flasks on glass in 60 ml of nutrient fluid for varying periods of time.

When required for respiratory measurements, cells were scraped from the flask floor by means of a "policeman" and suspended in the culture medium. A sample was examined microscopically to ensure that the cells had been dispersed. The cell concentration was then determined by means of an electronic cell counter. The cell sample was centrifuged and the cells resuspended in a volume of HERT I medium (12) giving a cell concentration of approximately 3000 cells/μl.

After the introduction of 2 μl of this suspension into the bottom of a diver, the cells soon adhered to the floor of the diver in a monolayer.

Respiration Measurements. The respiration of strain L cells grown in Roux flasks under the above conditions for 7 days was approximately 8×10^{-6} μl/hr/cell. This respiratory activity is in the range of that reported previously (6).

The influence of CO_2 tension on the respiration of the strain L mouse fibroblast was studied. As shown in Table 3, respiration appeared to be dependent on the pressure of carbon dioxide.

TABLE 3

INFLUENCE OF Co_2 ON RESPIRATION OF STRAIN L CELLS

CO_2 in gas phase (%)	O_2 consumption (μl/hr/cell $\times 10^6$)
0	1.74
0.03	4.32
1.00	7.38
5.00	5.61

[a] Cells grown in Roux flask for 4 days before respiratory measurements.

The respiration of this cell strain was found to be influenced by various other culture conditions which will be reported separately.

DISCUSSION

Warburg's prediction (14) concerning the limitations of the use of bicarbonate as a CO_2 buffer in respirometers is clearly confirmed by the results reported here, and the superiority of a substance such as diethanolamine is verified.

Both Pardee (11) and Krebs (8) reported that in Warburg flasks,

diethanolamine might not control CO_2 pressure effectively in equilibrium with 5% CO_2 in air unless a very large reservoir of diethanolamine was provided. By incorporating the diethanolamine in the flotation medium in the Cartesian diver apparatus this requirement can be met. Consequently, CO_2 produced does not exceed its capacity, and in the present system it operates almost as efficiently at 5% as at lower CO_2 tensions.

This modification of the Cartesian diver technique has another advantage. Since only two additions to each diver are required (a bottom drop containing cells and a neck seal), the manipulations are greatly simplified and a person with moderate manual dexterity can learn to use it with no great difficulty.

SUMMARY

A modification of the Cartesian diver method is described which permits the measurement of respiration at known constant CO_2 tension. Diethanolamine is employed as a CO_2 buffer and incorporated into the flotation medium. The paraffin oil seal in the diver is replaced by a dibutyl phthalate seal which permits CO_2 diffusion to occur. Consequently, the gas phase in the diver attains equilibrium with the CO_2 buffer in the flotation medium. Because of the high solubility of CO_2 in diethanolamine, it is necessary to keep the divers under constant pressure, and modifications of the apparatus to ensure this are described.

The system is compared with other CO_2 buffering systems which have been proposed, and the conditions necessary for equilibration are defined.

Data are presented which confirm that respiration of the strain L mouse fibroblast is dependent on the CO_2 tension of the medium.

ACKNOWLEDGMENTS

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ANALYTICAL BIOCHEMISTRY 2, 486–496 (1961)

An Improved p-Nitrophenol Method for Estrogen Determination Employing a Commercial Fluorimeter[1]

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INTRODUCTION

Simplified methodology for the determination of estrogens is highly desirable for our current studies on the metabolism of breast tumors. Since 1931, when Kober (1) found that the green-fluorescing orange solution, obtained by heating estrogens with concentrated sulfuric acid, turned pink on dilution with water, numerous studies have been undertaken in an attempt to utilize these fluorescent phenomena in estrogen determinations. Interference of impurities has limited their application. Ittrich in 1958 (2) described a simple method for increased purification by extraction of the fluorescing estrogen with *p*-nitrophenol in chloroform, and in 1960 (3) presented a modification of the method utilizing *p*-nitrophenol in tetrabromoethane. Both methods utilized a mercury arc lamp as the source with a mercury line at 546 mμ as the activation wavelength. He did not investigate the possibilities inherent in the fall-off of fluorescing power with time, as reported by Strickler *et al.* (4, 5). In evaluating and adapting Ittrich's method, the activation and fluorescent characteristics of the pure estrogens: estrone, estradiol-17β, estriol, estriol 3-methyl ether, 16-epiestriol, and 16-ketoestradiol were studied using the Ittrich technique for the Kober reaction. There was made available to this laboratory more sensitive and precise fluorescence equipment than that used by Ittrich, which was attached to our scanning spectrophotometer. The opportunity was therefore afforded of evaluating the Ittrich procedure with more exact equipment which gave greater

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[2] With the technical assistance of Nancy Arnold.

exciting power and sensitivity. Determinations of activation and fluorescent characteristics were studied using the constant-energy spectrum from a xenon arc lamp with a monochromator and a prism spectrophotometer. Application to estrogen assay was made with both macro- and microcuvettes in a commercial fluorimeter. Characteristics of solvents and effect of temperature and other factors were also studied in the development of this method.

APPARATUS

The light source used for exploration consisted of an 800-w Hanovia xenon arc lamp (lamp #418C1) giving an almost constant-energy level source in the ultraviolet and visible spectrum. The light beam from the xenon lamp entered a #33-86-40 Bausch and Lomb grating monochromator modified for scanning (Ho-Par, Inc.) with the entrance and exit slits opened 1.5 mm wide. The light from the monochromator served as the activation source. The specimen was placed in a 4 ml-Aminco type C quartz cell which was placed in a holder (Ho-Par, Inc.) in the path of the beam from the monochromator. Secondary radiation was collected from the specimen on a concave mirror approximately 3.5 cm from the specimen at about a 40° angle from the axis of the activation beam. The concave mirror was focused on the entrance slit of a Beckman model DK-1 spectrophotometer. The exit slit of the spectrophotometer was set at 0.5 mm [see Figs. 1(*a*) and (*b*) and Ref. (6)]. Constant amplification and sensitivity levels were maintained throughout the study.

The Farrand fluorimeter was used at aperture 5 with a primary 546-mμ interference filter (Baird type B1) and a secondary combination of a 585-mμ interference filter (Fisher Scientific Co.) and a 600-mμ glass filter (Klett). The reference solution was a dilute eosin solution made daily with 10 ml of stock solution[3] plus 90 ml water to which were added 0.4 ml of 0.1 *N* NaOH. The stock solution consisted of 50 mg eosin in a liter of 0.002 *N* NaOH, kept refrigerated.

PROCEDURES

Activation and Fluorescent Characteristics of Pure Estrogens

Samples of 5, 10, 15 and 20 μg of estrone, estradiol, estriol methyl ether, 16-epiestriol and 16-ketoestradiol each in alcohol solution were evaporated to dryness under nitrogen. To each sample 0.4 ml of a 5% hydroquinone solution in 95% alcohol was added during evaporation,

[3] Considerably more dilute eosin solutions can be used.

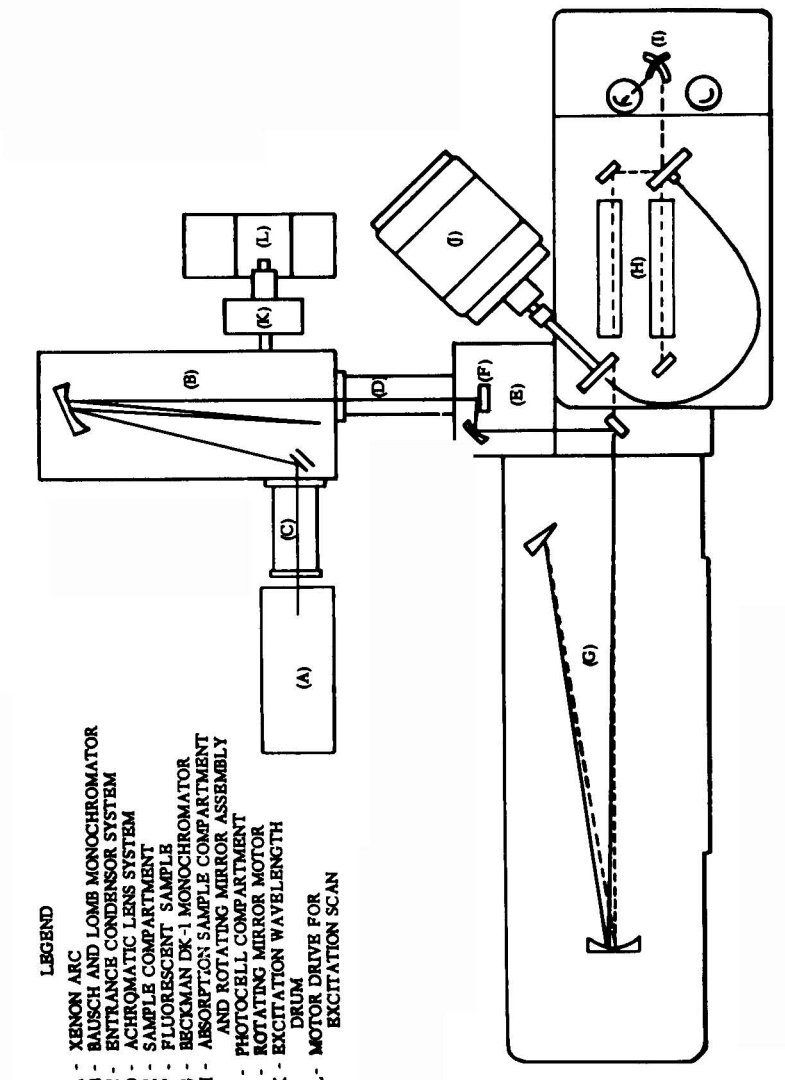

FIG. 1(*a*)

sample holder replace conventional source box of Beckman (*b*) Detail of sample holder including controls for position- ce beam. Plate at lower right replaces cuvette when solid sam- per strip). (Ho-Par, Inc.)

FIG. 1. (*a*) Arc, monochromator, and samp DK spectrophotometer (Ho-Par, Inc.). (*b*) I ing of cuvette and mirror for fluorescence bea ples are used (as smear on filter paper str

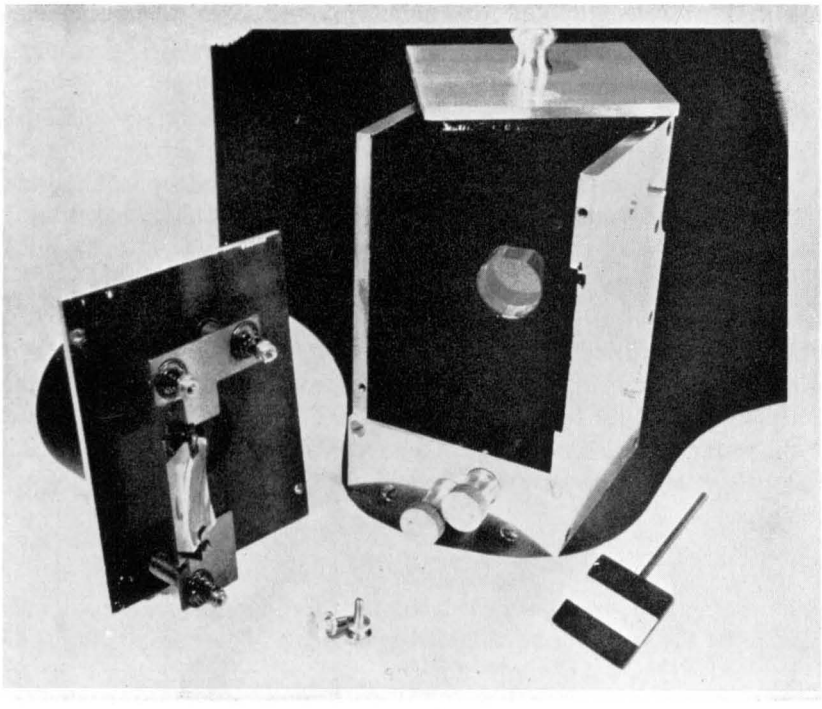

FIG. 1(*b*)

or the hydroquinone was added with the water as 0.4 ml of a 5% freshly prepared aqueous solution. Each dry sample had 0.4 ml water added. The tubes were chilled in a Dry Ice–alcohol solution, and 0.77 ml of conc. sulfuric acid was added with shaking. Samples were then heated in boiling water for 40 min allowing fluorescence to develop. Specimens were again chilled in Dry Ice–alcohol, and 1.5 ml water was added. Four milliliters of a 2% recrystallized *p*-nitrophenol solution in 1% ethanol in chloroform was then added with the sample in an ice bath. The samples were then shaken vigorously for 20 sec and then centrifuged for 10 min. The upper layer was removed by pipet, and the lower phase was transferred to the quartz cell, care being taken to avoid formation on the outside surface of the quartz cell of *p*-nitrophenol crystals which were found to reflect the incident beam. With the activation wavelength set at 580 mμ, the sample was scanned with the spectrophotometer in the range from 700 to 500 mμ to determine the presence or absence of reflected light. When no reflected light was detected, the samples were then scanned with the spectrophotometer, the activation wavelength being set at 535 mμ. The spectrophotometer was then set at the fluores-

cent peak wavelength, and the monochromator was then scanned to determine the activation peak.

Distribution Coefficient

The apparent distribution coefficient was determined by a 10-tube, 14-transfer countercurrent distribution using 2% *p*-nitrophenol solution in 1% ethanol in chloroform as the lower phase and 31% sulfuric acid as upper phase. The coefficient was found to be 0.2.

Assay of Estrogens

The samples, dried under nitrogen at $\leqslant 60°C$, were treated as described above, except for use of 6 ml *p*-nitrophenol solution for macrofluorimetry and 2 ml for microfluorimetry. The time of dilution with the 1.5 ml water was also noted. Readings were made as soon as practicable after centrifuging, because the fluorescing power fell off sharply with time.

RESULTS

The activation peak wavelength and the fluorescence peak were found to be the same for all the estrogens tested. These results are shown in Table 1. The wavelengths for the different concentrations did not

TABLE 1
ACTIVATION AND FLUORESCENT CHARACTERISTICS OF PURE ESTROGENS
(ITTRICH METHOD)

Sample	Activation peak wavelength (mμ)	Wavelength range of 75% energy (mμ)	Wavelength range of 25% energy (mμ)	Fluorescent peak wavelength (mμ)	Wavelength range of 75% energy (mμ)	Wavelength range of 25% energy (mμ)
Estrone	537	526–547	492–557	550	543–563	534–576
Estradiol-17β	538	526–545	496–556	550	542–558	531–576
Estriol	537	525–547	500–556	550	543–559	532–578
Estriol methyl ether	537	526–546	500–556	549	541–559	530–576
16-Epiestriol	537	525–547	498–556	550	540–560	532–576
16-Ketoestradiol	537	524–546	500–554	551	542–561	532–576
Eosin standard	515	503–525	463–532	535	525–548	512–568

vary qualitatively. There was a change in amount of energy emitted by different concentrations, but no attempt was made to correlate energy emitted and concentrations. This establishes the physical constants needed for the application of this method to commercial fluorimeters. The activation peak is 537 mμ and the fluorescence peak 550 mμ.

Twenty-five and 75% energy limits are given in Table 1. Ittrich (2), using a mean fluorescence peak of 548 mμ, found his primary 75% limits to be 523–548 mμ, close to our findings.

It was noted that estriol showed a marked lag compared to estrone and estradiol-17β in the first stage reaction (boiling 40 min).

The stability of the complex was studied by reading the fluorescent energy values at intervals of 1, 2, 3, 5, 7, and 24 hr after the formation of the complex by the addition of the *p*-nitrophenol. Marked instability of the complex with rapid decrease in fluorescence energy during the first 2–3 hr, with a slower degree of fading after 5–7 hr was found. When plotted on semilogarithmic paper, it was found that the ratio of fading is a logarithmic function of the concentration of the complex (see Fig. 2).

The effects of different solvents on the stability were studied by

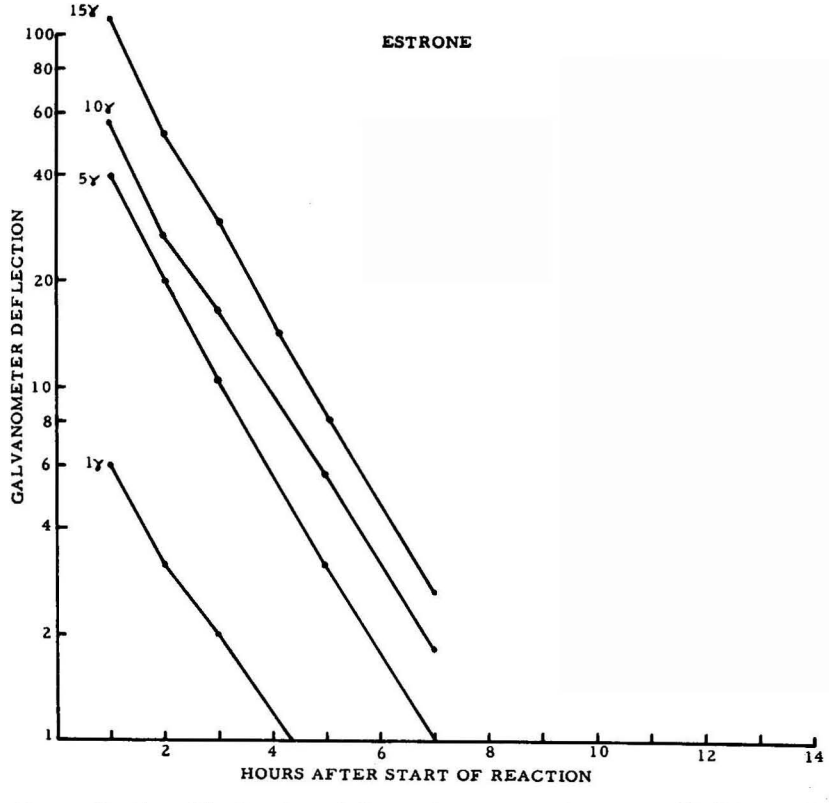

FIG. 2. Semilogarithmic plot of fluorescing power of estrone (Ittrich reaction) against time elapsed after dilution and extraction.

using 2% *p*-nitrophenol in chlorobenzene, tetrabromoethane (acetylene tetrabromide), cyclohexane, and carbon tetrachloride, as well as chloroform. All solvents tested showed a decrease in fluorescent activity with time. Tetrabromoethane showed the greatest decrease. However, when fresh it also demonstrated the highest amount of fluorescent activity. Cyclohexane showed a marked decrease in the amount of fluorescent activity. However, the decrease was not as great, due to the lower activity level. The other three solvents tested showed decreases similar to each other.

Temperature was also demonstrated by Ittrich (3) to affect the stability of the complex. We extended his work on the effect of this variable. Samples kept at 1°C demonstrated a slow decrease in fluorescent energy when compared to samples kept at 20 or 35°C, which showed a more rapid decrease in fluorescent energy with the increasing temperature. The amount of fluorescent activity within the same sample varies with the temperature of the solution. When a specimen's fluorescent activity is determined at 0°C, a higher value is obtained than when determined at a higher temperature. Samples which were alternately chilled to 0°C and warmed to 30°C would demonstrate a fluctuation of values with the amount of fluorescent activity returning to the earlier reading minus the amount of decrease due to time. The increased fluorescent activity at these low levels may be due to an increase in the density of the solvent with a concomitant increase in the concentration of the fluorescent complex or to a decreased dissociation of the complex. Due to the different factors influencing the degree of fluorescent activity, constant conditions as to time and temperature must be maintained throughout the determination after the formation of the complex by the addition of the *p*-nitrophenol [cf. also Breuer and Gertz (7)].

The instability of the complex was utilized in the determination of biological extracts. The fluorescent energy level was determined immediately after formation of the *p*-nitrophenol estrogen complex and determined again at the end of 24 hr. The difference in the energy levels was attributed to the destruction of the estrogen *p*-nitrophenol complex. Any measurable energy after 24 hr was attributed to fluorescence of other compounds. This aspect, which we have previously reported with the Slaunwhite-Engel procedure (4, 5), is currently being further explored with various biological extracts. By the use of internal and/or external standards, the degree of fluorescent energy level change for a given amount of estrogen could be determined and compounds having similar fluorescent properties could be evaluated in any given specimen.

In selecting a wavelength for the activation energy source, the strong

mercury line at 546.1 mμ was chosen. This wavelength will activate most of the potential fluorescent energy in a given sample. A 546.1-mμ interference filter (Baird type B 1) was then used as the primary filter. Numerous secondary filters were screened, utilizing the Beckman DK 1 spectrophotometer and determining energy transmission at different wavelengths. The most satisfactory combination found was a 585-mμ interference filter backed by a 600-mμ glass filter. Background radiation transmittance as determined by open-well readings were nearly zero with this combination. While this combination of secondary filters permits only a small percentage of the fluorescent energy to be transmitted, any attempt to use a filter system transmitting at a lower wavelength nearer the peak of fluorescent energy (550 mμ) resulted in a great increase in background and reflected radiation using this commercial fluorimeter.

In Table 2 are shown some fluorimeter readings obtained with this filter system, as well as the optical densities (at three of the wavelengths) noted on scanning the solutions in the Beckman DK 1 spectrophotometer. These solutions were chloroform extracts obtained on assaying the crude estriol fraction of a tissue extract by the Ittrich technique. The upper (aqueous) layer was brown; the lower (chloroform) layer was pink. This, however, is not a true Kober pink, as the data show. The Allen correction for optical density data is not very adequate here, but the combination of a well-chosen filter system for fluorimetry and the use of our time-decrease technique afford truer values.

TABLE 2

FLUORIMETRIC AND ABSORBANCE DATA FROM ITTRICH ASSAY OF CRUDE[a] ESTRIOL FRACTIONS FROM BREAST TUMORS

Cut No.[b]	G_0[c]	G_1[d]	Δ	Absorbance (mμ) 510	543	576	Allen cor.
14	3.8	2.1	1.7	0.069	0.048	0.003	+.012
14 + 0.3 E_3	16.4	2.8	13.6	0.094	0.076	0.038	+.010
15	2.2	2.3	−0.1	0.092	0.065	0.021	+.008
15 + 0.3 E_3	11.0	2.8	8.2	0.094	0.069	0.023	+.010
16	2.3	2.9	−0.6	0.135	0.098	0.034	+.013
16 + 0.3 E_3	9.5	2.8	6.7	0.111	0.079	0.015	+.016

[a] Impurities are largely very hydrophilic.

[b] Cut No. of countercurrent distribution $r = 13$, $n = 19$ with system 70 methanol–30 water–40 chloroform–60 carbon tetrachloride.

[c] G_0, fluorescence reading half hour after dilution and extraction.

[d] G_1, reading next morning.

Table 3 shows comparison of results on assay of some urine extracts by the Ittrich and Slaunwhite-Engel methods using the time decrease values for both procedures.

TABLE 3
COMPARISON OF DETERMINATIONS OF URINARY ESTROGENS BY ITTRICH AND SLAUNWHITE-ENGEL ASSAY[a,b]

	Fraction					
	Estrone		Estradiol-17β		Estriol (3 methylether)	
Urine No.	Ittrich	S-E	Ittrich	S-E	Ittrich	S-E
E-61-264	24.9	20.5	6.8	7.4	15.0	19.6
E-61-276	0.7	0.6	0.3	≤0.5	3.0	1.5
E-61-340	9.9	8.8	2.0	1.6	11.9	10.9
E-60-1440	8.4	6.8	1.3	1.8	6.2	4.1
E-60-1467	1.7	1.4	0.4	0.5	2.0	1.8
E-61-225	—	—	—	—	0.3	1.1
E-61-236	—	—	1.8	1.4	—	—

[a] Time difference method for each.

[b] Extracts were considerably purified by countercurrent distribution, and column chromatography on florosil (estrone and estradiol) or methylation (estriol).

DISCUSSION

Since the activation peak wavelength and the fluorescence peak wavelength appear to be identical for all the estrogens tested, it is suggested that a similar or identical mechanism and linkage are responsible for the fluorescent properties of all estrogens when determined by the Ittrich method of sensitization and extraction. This does not hold for the Slaunwhite-Engel method which showed very little or widely variable fluorescence values for different estrogens (8).

The slow development of fluorescing power which was noted with estriol is probably due to an initial dehydration to estrone at ring D as observed by Brown (11) and by us in a previous report (4). We substituted chloroform for tetrabromoethane because of the toxic and disagreeable nature of the latter solvent. Chloroform, although having a lower specific gravity than tetrabromoethane, did act as a satisfactory solvent for our determinations. The solution was kept refrigerated for over a month without apparent deterioration. Tetrabromoethane does have a higher viscosity than chloroform ($\eta = 10$ vs. 0.5 cp). However, these values are probably too low to extend the life of the excited estrogen molecules; hence the fall-off of fluorescing power with time is observed here also. Although the fall-off shown in Fig. 2 is semiloga-

rithmic, it may well be that this is not to be expected uniformly with various biological extracts. Such was the case with our previous observations on another (Slaunwhite-Engel) system of fluorescence analysis (4, 5). Goldzieher and co-workers (9) utilized a reaction mixture which contained sulfuric acid after dilution of about 15 cp viscosity; no loss of fluorescing power occurred in at least 24 hr. However, a similar mixture used by us, with a final sulfuric acid viscosity of about 9 cp, permitted fall-off of fluorescing power (4, 5).

It is of interest to note the equation of Heintz (10) which may be written as

$$\log_{10} \phi = \log_{10} \phi_0 - \frac{K_1}{\eta},$$

where ϕ is the specific fluorescence, K_1 is a constant, and η the viscosity.

Our observations of effect of temperature are an extension, under our conditions, of Ittrich's report concerning this variable (3).

We consider the Ittrich reaction to be a useful extension of methodology. Since it is based on the Kober reaction, fluorescing power is more uniform for the gamut of estrogens (for example 16-ketoestradiol) currently sought in addition to estrone, estradiol-17β, estriol, and 16-epiestriol.

The development of a rapid test for determining total estrogen levels permits wider study of the effects of various substances on estrogen-metabolizing tumors. We have placed emphasis on such development which would permit multiple determinations to be carried out with a minimum of time and labor, since much of our material to be tested is collected from columns or countercurrent machines. With the Ittrich procedure, some impurity is left behind in the aqueous (upper) layer. In some cases this may be sufficient to permit determination of estrogen levels without purification by columns or countercurrent distribution. It is expected, therefore, that this method may be increasingly adapted to various estrogen metabolism studies.

SUMMARY

1. The peak excitation and fluorescence wavelengths for acid-activated estrogens (Ittrich method) were determined with a powerful xenon arc lamp, a monochromator, and the Beckman DK-1 spectrophotometer. The values were respectively 537 and 550 mμ, close to Ittrich's values obtained with less suitable equipment.

2. Chloroform was found to be the solvent of choice.

3. On reading the fluorescence of such solutions at room temperature,

the fluorescing power of the estrogens was found to fall off rapidly with time, as was observed previously with the Slaunwhite-Engel system in this laboratory.

4. For routine determinations, the Farrand fluorimeter could be used with either micro- or macrocuvettes and the filter system primary 546 mμ (interference)/secondary 585 mμ (interference) + 600 (glass). Scattered light was negligible with this system.

5. Fluorescence readings by the time-lapse method were more specific than absorbance readings with the Allen correction applied on analyzing certain tissue extracts containing highly hydrophilic material.

6. Use of internal standards, to be added to an aliquot of the extract, is recommended.

7. The variation with temperatures noted by Ittrich was also observed and, in agreement with Breuer and Gertz, it is recommended that the experimental conditions be closely controlled.

8. The Ittrich procedure is a useful tool because of elimination of some impurities (held in the aqueous phase) and because it gives a more consistent fluorescence for the spectrum of known estrogens.

ADDENDUM

Barbara Williams of this Laboratory has found that 2-methoxyestrone can be determined by the Ittrich method with the same sensitivity as the other estrogens listed above. Since this compound gives little fluorescence with the Slaunwhite-Engel method, a differential assay is suggested.

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ANALYTICAL BIOCHEMISTRY 2, 497–501 (1961)

Detection and Identification of Fructose 1-Phosphate by Paper Chromatography

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INTRODUCTION

Paper chromatography has not been employed generally for the separation and identification of fructose 1-phosphate (F1P),[1] in part because there has not been a method for identifying F1P which was specific. Although various solvent systems have been employed (1), satisfactory separation of F1P, F6P, FDP, and other phosphorylated sugars has not been obtained. A satisfactory separation of F1P from other hexose phosphates can be obtained using the *tert*-butanol–water–picric acid system of Hanes and Isherwood (2) in the modification of Sidbury (3).[2] This system is slow, and the picric acid present obviates the possibility of detection other than by hydrolysis and identification of phosphate. Furthermore, satisfactory separation of the three phosphorylated fructose intermediates is not achieved; hence a modification of the technique which would permit identification of the individual fructose phosphates is desirable. This has been accomplished by using a solvent free of nonvolatile constituents, which permits greater latitude in the application of different color reactions. This paper reports a new solvent system and an ultraviolet fluorescence procedure which makes the detection and differentiation of F1P from other hexosephosphates possible.

While attempting to develop a method for selectively hydrolyzing the phosphate bond of F1P and then identifying the free fructose or its furfural derivative, we found that a strong ultraviolet fluorescence was produced by the F1P spot after dipping the chromatogram in a solution

[1] Abbreviations used: F1P, fructose 1-phosphate; F6P, fructose 6-phosphate; FDP, fructose 1,6-diphosphate; GIP, glucose 1-phosphate; G6P, glucose 6-phosphate; P_i, inorganic phosphorus.

[2] Also, personal communication from J. B. Sidbury, Jr.

containing 6% hydrochloric acid and glycerol, followed by heating to 100°C for 10 min. Glycerol had been added in order to retain moisture, but it turned out that its addition changed the intensity and color of the fluorescence. This fluorescence, after treatment with HCl–glycerol, is the basis for the identification of F1P. The ultraviolet fluorescence is masked when nonvolatile substances are used in the solvent system [e.g., picric acid (2)], but hydrochloric acid proved to be a satisfactory substitute.

METHODS

Standard samples of F1P, F6P, FDP, G1P, and G6P were chromatographed using S & S 2043b mgl paper with solvent systems containing *tert*-butanol and hydrochloric acid in different proportions and with certain additions (see Table 1). The chromatograms were dried in air

TABLE 1
R_F VALUES OF FRUCTOSE PHOSPHATES

Solvents[a]	G1P	G6P	F1P	FDP	F6P	P_i	
1	0.37	—	0.40	0.45	0.45	0.63	36–48 hr, descend.
2	0.59	—	0.61	0.61	0.69	0.87	20 hr, ascend.
3	0.37	—	0.44	0.50	0.51	0.65	16–20 hr, ascend.
4	0.48	0.51	0.59	0.65	0.64	1.0	18 hr, ascend.
5	0.48	—	0.53	0.60	0.60	0.72	18 hr, ascend.
6	0.08	—	0.12	0.03	0.12	0.50	48 hr, descend.

[a] Solvent 1, *tert*-butanol–water–picric acid, 80:20:2; solvent 2, *tert*-butanol–water–6*N* HCl, 80:20:2; solvent 3, *tert*-butanol–0.1 *N* HCl, 80:20; solvent 4, *tert*-butanol–0.1 *N* HCl–methanol, 60:20:20; solvent 5, *tert*-butanol–0.1 *N* HCl–acetone, 60:20:20; solvent 6, *tert*-butanol–water–formic acid, 70:10:20.

after they were run, and dipped in a freshly prepared solution containing 6 ml of conc. HCl, 3 ml glycerol, and 91 ml acetone (the glycerol–acetone mixture may be kept as stock, but the HCl should be added before use. The chromatograms were allowed to stand at room temperature until nearly dry and then heated to 100°C for 5 min. The chromatograms were then viewed with a filtered ultraviolet light source (3600-A region). The heating and ultraviolet scanning were repeated, and the fluorescent areas were marked with a pencil. The chromatograms could then be treated with the molybdate reagent as described by Burrows *et al.* (4), heated, and irradiated with unfiltered ultraviolet light (2500-A region) to obtain the position of the inorganic phosphate and phosphorylated esters as blue spots. Paper pretreated with HCl-glycerol reagent retains its white color for months after treatment with the

molybdate reagent, while paper treated only with molybdate reagent gets dark after a short time.

The following additional analytical techniques have been applied to chromatograms of the standard hexose phosphates:

1. Dipping in a freshly prepared solution of 6% conc. HCl in acetone, drying superficially, heating for 2–4 min at 100°C, and observing under ultraviolet light.
2. The quinine sulfate method of Rorem (5).
3. The sulfosalicylic method of Rorem (6).
4. The naphtoresorcinol dip-method as described by Smith (7).
5. The benzidine method as described by Smith (7).

RESULTS AND DISCUSSION

The R_F values found with the different solvents used are reported in Table 1. Solvent No. 3, which contains 80 ml *tert*-butanol and 20 ml of 0.1 *N* HCl, compares favorably with the solvent used by Hanes and Isherwood (2) and modified by Sidbury,[2] which due to its content of picric acid cannot be used for the detection of ultraviolet fluorescence. The R_F values are similar, and the F1P is clearly separated from F6P and FDP. Ascending development with this *tert*-butanol–HCl solvent is recommended as the method of choice. Barium, calcium, and magnesium salts of hexose phosphates may be applied directly at the starting line of the chromatogram, as the acidity of the solvent is sufficiently strong to promote migration [see Hinsberg and Lang (8)].

Solvent No. 6, containing formic acid, does not separate F1P from F6P and gives R_F values similar to solvents C and D used by Hanes and Isherwood (2), also containing formic acid.

A higher concentration of HCl (solvent No. 2) does not differentiate between F1P and FDP and partly hydrolyzes G1P. *tert*-Butanol with much stronger HCl has been used for the separation of nucleotides by Smith and Markham (9).

The addition of methanol (solvent No. 4) or of acetone (solvent No. 5) causes an increase of the movement of all phosphoric esters, but the distances between the spots remain essentially unchanged.

The results of the ultraviolet fluorescence after the application of the dip reagents are summarized in Table 2. With the HCl–glycerol reagent a typical strong white ultraviolet fluorescence is observed with F1P, and a pale red fluorescence with F6P. The lower limit of detection is about 0.02 μmole for F1P and 0.04 μmole for F6P. If the heating period is prolonged, the red fluorescence of F6P decreases in intensity while the white fluorescence of F1P remains stable for more than 10 min, and

TABLE 2

DIP REAGENTS FOR FRUCTOSE PHOSPHATES

	Dip reagent	Heating time and temp.	Ultraviolet reading	Background	F1P	F6P	FDP	G1P	G6P
1	6% HCl and 3% glycerol in acetone	5 min 100°	+	Gray–brown	White	Pale red	–	–	–
		10 min 100°	+	Gray–brown	White	Faint red	–	–	–
		15 min 100°	+	Gray	Faint white	Trace	–	–	–
2	6% HCl in acetone	2–4 min 100°	+	Gray–brown	Yellow	Yellow	–	–	–
3	Quinine sulfate (5)	–	+		+	+	+	–	–
4	Sulfosalicylic acid (6)	5 min 100°	+		–	Red	Faint gray–white	White	Trace
5	Naphthoresorcinol (7)	As original	–		–	Red	–	–	–
6	Benzidine (7)	As original	–		–	+	–	–	+

decreases only later. The fluorescent spots are stable and can be detected on the paper after weeks. No other reaction on paper chromatograms has been described which distinguishes the different fructose phosphates.

The HCl reagent without glycerol produces a yellow fluorescence with both F1P and F6P after a short heating. The spot of F6P has a weak reddish tinge, but the difference between the two spots is not obvious.

The other color reagents mentioned in Table 2 can be used for additional confirmation. The quinine sulfate reagent (7) gives a strong white fluorescence with all three fructose phosphates. The sulfosalicylic reagent (5) gives no color with F1P but a red fluorescence with F6P.

SUMMARY

A specific procedure is described for the selective detection of fructose 1-phosphate (F1P). The chromatograms are developed in *tert*-butanol–0.1 *N* HCl (4:1). After complete drying, the chromatograms are dipped in a solution of HCl and glycerol in acetone, dried, and heated for specified periods. Viewed under ultraviolet light, F1P shows a typical white and fructose 6-phosphate (F6P) a pale red fluorescence. After marking the spots, the chromatograms are treated with molybdate reagent and all phosphates are located. Other reagents for the detection of fructose phosphates are used for comparison.

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SHORT COMMUNICATIONS

The Application of Gas–Liquid Chromatography to the Analysis of Urinary 17-Ketosteroids[1]

Since the appearance of the first reports on the separation of steroidal molecules by gas–liquid chromatography (1–3), considerable interest has arisen with respect to the possible use of the technique as a clinical laboratory procedure. Recent investigations have shown that it is possible to separate certain specific 17-ketosteroids in synthetic mixtures (4, 5); however, no reports have appeared as yet regarding the separations obtainable in urinary extracts from various pathological origins.

Attempts were made to analyze urinary 17-ketosteroids in this laboratory to determine whether gross quantitative changes might be observed in pathological states and what differences in excretion pattern might be detected in pathological states in which the total 17-ketosteroid excretion lies within the normal range.

Urine extracts were prepared essentially according to the method of Drekter *et al.* (6), but modified to use sulfuric acid rather than hydrochloric acid for hydrolysis, and substituting dichloromethane for ethylene dichloride as the extracting solvent. In addition, the procedure was modified to include a backwash of the combined CH_2Cl_2 extracts with an equal volume of $3\,M$ H_2SO_4 prior to treatment with NaOH pellets. Following the base treatment the extracts were filtered through Whatman No. 1 filter paper and taken to dryness. The residue was taken up in about 2 ml. of $CHCl_3$ and placed on a Florosil column (7, 8). The column was washed with 5 ml $CHCl_3$, and the 17-ketosteroids were eluted with 45 ml of 2% (v/v) methanol in $CHCl_3$ (9). These extracts were taken to dryness, a known quantity of cholestane was added as an internal standard, and injected into the chromatograph in CH_2Cl_2 solution.

The gas chromatographic separations described in this publication were made with a Barber Coleman instrument (model 10) using either a 12-ft column of 1.5% SE-30 on 100–140 mesh Gas-chrom P or a 6-ft

[1] This investigation was supported in part by Research Grant A-4906 from the National Institute of Arthritis and Metabolic Diseases, United States Public Health Service.

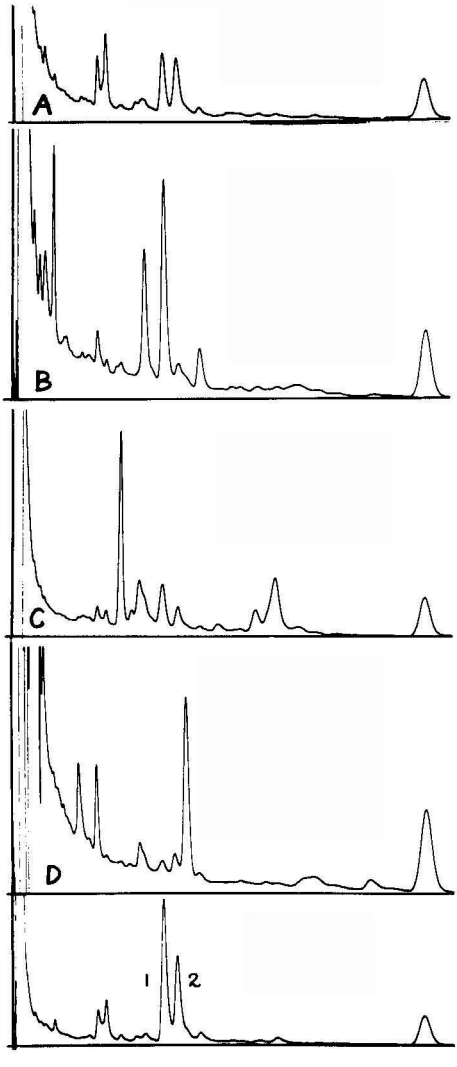

FIG. 1. Separation of urinary 17-ketosteroid extracts on a 12-ft column containing 1.5% SE-30 on Gas-chrom P at 222°C. Peak 1 contains etiocholanolone, while peak 2 contains both androstanolone and dehydroepiandrosterone. The final peak in the pattern is, in all cases, the internal standard, cholestane. *A*. Male castrate, +50 μg cholestane; *B*. Male infant with congenital adrenal hyperplasia, +110 μg cholestane; *C*. Pregnant female (4.5 months), +50 μg cholestane; *D*. Eight-year-old female dwarf, +10 μg cholestane; bottom. Normal male, +60 μg cholestane.

column of 0.75% neopentylglycol succinate polyester (NGS) on 100–140 mesh Gas-chrom P (10). Both columns were operated at 222°C.

The chromatographic tracings in Fig. 1 show that quite distinctive patterns were observed in the extracts reported. The extent to which these variations represent individual differences as opposed to clinical status is still in doubt. Further studies will indicate the degree of significance which may be placed on such variations for diagnostic purposes.

Previous attempts to utilize 17-ketosteroid distribution as a diagnostic tool have centered about the three principal members of the family: androstanolone (5α-androstane-3αol-17-one), etiocholanolone (5β-androstane-3α-ol-17-one), and dehydroepiandrosterone (Δ^5-androstene-3β-ol-17-one). Examination of Fig. 1 indicates that, although etiocholanolone appears as a separate peak, the androstanolone and dehydroepiandrosterone are not resolved on SE-30 columns. The problem of individual quantitation of these three compounds in synthetic mixture of standards may be handled by running the samples on an NGS column. This column fails to separate etiocholanolone from androstanolone, but does resolve dehydroepiandrosterone as a separate peak (10). Attempts to determine the dehydroepiandrosterone concentration in the urine extracts shown in Fig. 1 by means of an NGS column have been unsuccessful. Almost all of this compound is lost either by destruction or transformation during the acid treatment (11–18). In spite of the existence of these data, acid hydrolysis was chosen as a starting point in our studies with urine because of its simplicity. In addition, it was hoped that analysis of extracts, prepared in this way, by gas chromatography might yield some information with respect to the nature of action of the acid. However, it is now apparent that acid hydrolysis should not be the method of choice for studies involving gas chromatographic separations and particularly where interest is centered on determinations of dehydroepiandrosterone. When the pure compound is subjected to the procedure described above, 60% is destroyed within 10 min.[2] However, androstanolone and etiocholanolone withstood the acid treatment with only about 10% loss in 30 min. In each case definite and characteristic peaks appear on the gas chromatogram as a result of acid alteration, but their number and fluctuations in amount make it difficult to interpret the changes in structure which occur. It is obvious, however, that gas chromatography provides an excellent means of evaluating hydrolysis procedures, and this problem is currently under investigation. Additional difficulties arise when the concentration of the

[2] J. A. Cooper and B. G. Creech, unpublished data.

3β epimer of androstanolone is increased. This compound has a relative retention time of 4.3 on NGS, while the values for etiocholanolone and dehydroepiandrosterone are 4.0 and 5.0, respectively (Table 1). It is

TABLE 1
RETENTION TIMES, RELATIVE TO CHOLESTANE (222°C)

Compound	1.5% SE-30 on Gas-chrom P	0.75% NGS on Gas-chrom P
5α-Androstane	0.15	0.11
5α-Androstane-17-one	0.23	0.65
5α-Androstane-3α-ol-17-one	0.43	3.7
5α-Androstane-3β-ol-17-one	0.43	4.3
5β-Androstane-3α-ol-17-one	0.39	4.0
5β-Androstane-3β-ol-17-one	0.39	3.4
5α-Androstane-3α,17β-diol	0.44	
5α-Androstane-3,17-dione	0.45	5.1
Δ5-Androstene-3β-ol-17-one	0.42	5.0
5α-Cholestane	1.00	1.00

reported to be present in urine in only low concentration, but several samples have been found to contain enough to destroy the resolution of dehydroepiandrosterone from the androstanolone and etiocholanolone. Experiments are now in progress to determine if a high efficiency NGS can be prepared which will give adequate separation of all components in question.

The retention times, relative to cholestane, of a number of 17-keto-steroids and related compounds on both SE-30 and NGS columns are listed in Table 1. These data indicate that the separation on SE-30 is based primarily on molecular weight, while that obtained with NGS is essentially a polarity separation. Comparison of relative retention times for an unknown material on the two columns allows the investigator to make certain valuable inferences regarding structure. However, it should be pointed out that the use of columns having a low percentage of stationary phase results in considerable variation of relative retention time from column to column, and even from day to day under the same conditions. It is therefore essential that standard mixtures be chromatographed frequently to avoid error in identification of components.

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Measurement of C^{14} and S^{35} in a Single Sample

In studies involving protein metabolism, many investigators have found doubly radioactive labeled protein very helpful. Although a C^{14}-S^{35} labeled protein can be readily prepared, separation and determination of the activities is not too convenient. Fleischer *et al.* (1) counted the same C^{14}-S^{35} protein several times at different time intervals

with Geiger-Müller counters, and, by using a pair of simultaneous equations, they were able to calculate both C^{14} and S^{35} activity. Wiggans *et al.* (2) reported use of a similar technique, using a curve-fitting process after several countings of the same sample. Goldsworthy and Volwiler (3) oxidized duplicate samples using Pirie's reagent for the S^{35} and Van Slyke-Folch reagent for C^{14}. The S^{35} was collected as benzidine sulfate and the C^{14} as barium carbonate. Each salt was counted separately. However, correction for different counting efficiency due to self-absorption would be required in many instances.

Recently this laboratory has developed liquid scintillation methods for S^{35} (4) and C^{14} (5). The S^{35} method involves oxidation of the sample to magnesium sulfate, dissolving in glycerol, and diluting with a mixture of *N,N*-dimethylformamide–ethanol–toluene containing organic scintillators. The C^{14} method involves oxidation of the sample to carbon dioxide, trapping the gas in ethanolamine, and dilution with a methyl Cellosolve–toluene solution of a scintillator. This report will demonstrate that a combination of these methods permits a rapid, convenient, and accurate means of determining C^{14} and S^{35} in the same sample.

Solutions of ascorbic acid-1-C^{14} and serum albumin-S^{35} were prepared. One milliliter of each solution was analyzed by both the C^{14} and S^{35} method. A third solution, containing equal parts of the ascorbic acid and albumin solutions, was also prepared. Two milliliters of the C^{14}–S^{35} solution was also analyzed by both methods.

The results, seen in Table 1, clearly indicate that the C^{14} does not

TABLE 1
DETERMINATION OF C^{14} AND S^{35} IN THE SAME SAMPLE

	Observed counting rate (counts/min)		
	Solutions contained:		
Method of analysis	C^{14}	S^{35}	C^{14} and S^{35}
C^{14}	43,162	0	43,364
S^{35}	0	30,750	30,721

interfere with the determination of S^{35}, and S^{35} does not interfere with the determination of C^{14}. Since these methods employ a liquid scintillation counter, no correction for self-absorption, geometry, or difference in total C and S is required. Also, 2–3 times greater counting efficiency can be realized with this combination of method compared with Geiger-Müller counting.

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Liquid Scintillation Counting of $C^{14}O_2$ with Phenethylamine[1]

Carbonic-C^{14} has been determined by scintillation counting as gas (1) or as aqueous carbonate (2). Another technique is the incorporation into an organic scintillator solution by means of a compound that can "bind" carbon dioxide. Compounds suitable for this purpose should, in both the reacted and unreacted form, be soluble in a mixture in which toluene is the principal component and, at the same time, show little pulse quenching. The partial vapor pressure of the reactant should be low, and the reaction with carbon dioxide must be rapid and quantitative.

Thus far, this task has been solved with some success (3, 4) by the use of "Hyamine" hydroxide [*p*-(diisobutylcresoxyethoxyethyl)dimethylbenzylammonium hydroxide], a compound that can be prepared from the commercially available quaternary chloride, Hyamine 10-X (reg. trademark Rohm & Haas, Inc.). Upon reaction with CO_2, this base forms a toluene-soluble bicarbonate. The shortcomings in the use of this reagent are an appreciable quenching and high cost. A recent communication (5) reports the use of a tertiary alkyl primary amine (Primene 81-R; Rohm & Haas, Inc.). However, a still later paper (6) states that this compound "did not quantitatively absorb carbon diox-

[1] The work described in this report was supported by the U. S. Atomic Energy Commission.

ide." Consequently, we have turned our attention to other compounds.

We have observed that carbamates formed by the reaction of CO_2 with amines quench pulses only slightly. Of the unreacted amines, primary aliphatic compounds quench far less than secondary ones.[2] A series of available primary amines was then tested to determine which would give the best compromise with respect to the requirements stated in the first paragraph.[3]

The carbamate from phenethylamine (2-phenylethylamine) is very soluble in alcohols but much less so in toluene. With a sufficient stoichiometric excess (about 3 moles amine/mole CO_2), the reaction is quite rapid. The product formed in the presence of alcohol was found (by elemental analysis for C, H, and N) to have the empirical formula $C_{17}H_{22}N_2O_2$, which corresponds to phenethylammonium phenethylcarbamate. This compound decomposes near 75°.

Currently, the following preparation is employed: 27 ml of redistilled phenethylamine,[4] 27 ml of absolute methanol, 500 mg 2,5-diphenyloxazole (PPO), and 10 mg 1,4-bis-[2-(5-phenyloxazolyl)]benzene (POPOP). This mixture is diluted to 100 ml with toluene. The alcohol serves to increase the solubility of the product. At the same time, it lowers the partial vapor pressure of the amine, thus minimizing gas-phase reaction that leads to deposition of the carbamate at points removed from the amine solution. By the addition of some toluene at this point, the volume of solution is deliberately increased for more convenient handling, while the two phosphors are at a concentration equal to that of the stock toluene scintillator added later during sample preparation.

The apparatus used here consists of a diffusion system, with wide cross sections, in which the gas (from a cold trap or a generator) is released directly into a counting vial through an adapter. The vial contains the necessary amount of CO_2 reagent which is kept near 0° and agitated by a magnetic stirrer, and about 20 min is allowed for the gas uptake with an initial and final pressure of 100–150 mm.

We define a "millimole equivalent" of reagent as the quantity capable of reacting quantitatively with 1 mmole CO_2 under the conditions just described. This corresponds to 1.1 ml of the Hyamine base (1 *M* in methanol) or 1.5 ml of the phenethylamine–alcohol–toluene mixture.

[2] For example, for equal molar concentrations, quenching increases in the order *n*-butylamine $\ll$ di-*n*-butylamine $<$ tri-*n*-butylamine.

[3] Abandoned (mostly for reasons of insolubility of the carbamates) were: benzylamine, *t*-octylamine (kindly supplied by Rohm & Haas, Inc.), *n*-dodecylamine, and 4-(aminomethyl)cyclohexanemethanol.

[4] Matheson, Coleman & Bell Division, Cat. No. 6066; price $11.25 per kilogram.

Counting efficiencies were compared for preparations in which either 1 *M* Hyamine or the phenethylamine mixture were employed. All samples throughout were brought to 10 ml with toluene scintillator solution, and to final concentrations of 0.5% PPO and 0.01% POPOP. In a Packard Tri-Carb spectrometer at −10° (background 25 counts/min), 2 mmoles CO_2 could be counted with 60% efficiency when the phenethylamine mixture was used.[5] The "millimole equivalent" Hyamine sample gave less than 30% efficiency. In a custom-built spectrometer operating in coincidence at room temperature, 5 mmoles CO_2 could be introduced into 10 ml (containing 7.5 ml of phenethylamine mixture) and counted at 50% efficiency. At this temperature, part of the toluene can be replaced by naphthalene; this replacement results in even better efficiency.[6] The corresponding Hyamine sample (employing 5.5 ml of the 1 *M* solution) proved to have an efficiency close to zero. It is to be concluded that the phenethylamine mixture is superior to the "millimole equivalent" Hyamine preparations in regard to counting efficiency. From other experiments it appears reasonable to assume that the slight reduction in relative methanol content causes only part of this difference.

It should be pointed out that with both reagents the efficiency shows little dependence on the amount of gas reacted. For the most accurate work the internal standard method (addition of a known amount of C^{14}-labeled toluene) is recommended.

That the $C^{14}O_2$ is quantitatively absorbed into the phenethylamine solution was demonstrated by separately generating different known amounts of the gas (from accurately weighed $BaC^{14}O_3$ of known specific activity) into a fixed quantity of the amine. A straight-line relationship was established on plotting the liquid scintillation counting rate against the quantity of $C^{14}O_2$ used.

SUMMARY

Phenethylamine has been found to be superior to the widely used "Hydroxide of Hyamine 10-X" as a compound to convert $C^{14}O_2$ into a soluble form for liquid scintillation counting.

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[5] Samples containing CO_2 in excess of 2 mmoles/10 ml of total solution showed incipient crystallization when kept at −10° for prolonged periods.

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ANALYTICAL BIOCHEMISTRY 2, 513–520 (1961)

The Isolation of DNA-Containing Particles from Lyophilized Cells by a New Procedure[1]

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Received January 4, 1961

INTRODUCTION

Presently there are two methods for the separation of intracellular particulate matter from cells: (*a*) separation in aqueous media by means of differential centrifugation and (*b*) separation by flotation in non-aqueous organic media. It has been reported (1, 2) that these methods can result in losses of enzymes, lipids, and proteins as well as in undesirable adsorptions. The work reported herein is a new approach in that lyophilized cells were ground in a ball mill or in a fluid mill and used as such or made into an aerosol, from which point attempts were made to separate intracellular particulate matter by physical means without the use of organic or aqueous media.

The cells used were those of the $6C_3HED$ lymphosarcoma of Gardner (3) grown in ascites form in C_3H mice (4). The cells are obtainable 100% viable and over 96% pure after 150 hr of incubation within the mice.[2] From a smear stained with the Feulgen reagent and photographed at 440× with a Whipple disk in the field, size estimations were made of the nuclei and cell diameters from 42 intact whole cells. It was found that the nuclei ranged in diameter from 2.9 to 7.2 μ with an average of 4.6 μ. Whole cells ranged from 6.4 to 12.2 μ with an average of 8.1 μ.

METHOD FOR LYOPHILIZATION

After inoculation of mice with 0.1 ml ascites fluid each and incubation for 150 hr, cells were harvested from batches of 100 mice. The cells were then washed three times in saline from which they were separated by

[1] This investigation was supported (in part) by Public Health Service Special Research Grant C-3583 from the National Cancer Institute, U. S. Public Health Service.

[2] Private correspondence with Dr. G. A. LePage, University of Wisconsin.

centrifugation. The final sludge of cells, in a layer 1.0-mm thick spread on 0.35-mil aluminum foil, was quick-frozen by compression between two vertically stacked 40-lb cakes of Dry Ice, each previously chilled 30 min in liquid nitrogen. The disk of frozen cells was broken up with cold spatulas, placed into the chamber of a vacuum lyophilizer, and partially covered with liquid nitrogen. The system was placed under a pressure of 10^{-4} mm Hg for 48 hr to remove first nitrogen, then residual carbon dioxide, and finally water. The final sample averaged 4.3 gm dried weight, contained 5×10^{10} cells, and lost 1% of its weight when desiccated to constant weight over Drierite. It was ground to a coarse powder in a glass mortar for weighing purposes and stored in a desiccator. It is subject to the influence of static electricity. It is hygroscopic to the extent that, in an atmosphere which is held at 90% relative humidity at room temperature, it will absorb up to 45% of its weight of atmospheric moisture in 48 hr; absorption of atmospheric moisture under ordinary laboratory conditions is slow to the point that equilibrium may not be reached before several weeks, and absorption does not interfere with the weighing of samples for analysis.

METHODS FOR GRINDING

Three methods were used to grind the lyophilized cells: (*a*) for 52–350 hr by means of ¼-in. glass or steel balls at —25°C in a rotating steel bomb with a grinding medium of liquid carbon dioxide, (*b*) for 300–330 hr in a medium of liquid nitrogen by means of a single 1-in. steel ball activated by a rotating magnet, (*c*) in a fluid mill; the cells were fired through the deagglomerator head of the Sharples Micromerograph (5) at narrow settings of the annulus.

ATTEMPTS TO SEPARATE INTRACELLULAR PARTICULATES

Five methods were used in attempts to separate intact nuclei from lyophilized and ground cells, following the progress of each by means of microscopic size-frequency (6) counts and chemical analyses for deoxyribonucleic acid (DNA) by the Dische reaction: (*a*) centrifugation, using the Goetz Aerosol Spectrometer (7), of the aerosol produced by the firing of ground cells through the Sharples deagglomerator; (*b*) gravity settling of the particulates, according to the expressions of Stokes and Cunningham (8), from either stationary or moving aerosols; (*c*) segregation of particles in the aerosol by means of photophoresis (9), (*d*) gravity or centrifugal separation of particles suspended in a medium of liquid carbon dioxide at room temperature and at refrigerator temperatures; (*e*) separation of particles from a moving aerosol stream by means of electrostatic precipitation (10).

Separations Using the Goetz Aerosol Spectrometer (7)

Lyophilized cells ground in a ball mill were made into an aerosol by firing through the Sharples deagglomerator at wide settings of the annulus and were then drawn through the Goetz aerosol spectrometer running at various speeds. The DNA content rose somewhat in the fraction representing particle sizes less than 0.6 μ, indicating the friable nature of the nucleus. This is shown in Table 1. The Goetz instrument may be useful for the separation of submicron-sized particulates after

TABLE 1
SEGREGATION OF DNA IN AN AEROSOL BY MEANS OF THE GOETZ AEROSOL SPECTROMETER

Size (μ)[a]	Per cent DNA[b]
3.5	5.6
1.5	5.3
1.0	5.5
0.8	6.2
0.6	5.9
0.5	7.7

[a] Samples were taken from small areas down the channels of the rotor cone. The size reported is the average particle size for that area from the calibration tables accompanying the instrument.

[b] The DNA content of the intact untreated lyophilized cells was 6.7%. DNA determinations were reproducible to ±6.6% of the average value. For example, the range of the intact untreated lyophilized cells was 6.3–7.1% DNA.

the removal of intact and fragmented nuclei, but slow gas-flow rates through the instrument when it is adjusted for the collection of particles the size of intact nuclei probably disqualify it for that purpose in view of the rapid reagglomeration rates of aerosols (11).

Separations in Air or Other Gases under Gravity

Aerosols were made by firing the lyophilized cells at sonic velocity through the Sharples deagglomerator at narrow settings of the annulus. When used in this manner the Sharples instrument acts as a fluid mill which can be used to grind the cells to varying degrees of fineness, depending upon the annulus setting and firing pressure. The fall under gravity of the particles of such an aerosol follows the mathematical equations of Stokes and Cunningham (8). Particles smaller than 0.1 μ in diameter show Brownian movement. Because of the rapid rate of reagglomeration of aerosols (11), separations under gravity are more effective if the vertical-motion component is short and there is a long horizontal-motion component. Resolution is gained, with a horizontal

component, where there is a layer of aerosol-free gas moving at the same rate as the aerosol stream and lying between the aerosol and the collection plate. Attempts to effect separations by allowing the aerosol to fall vertically were failures. Attempts to impose a horizontal-motion component to the system by guiding the falling aerosol into the top 0.5 cm deep layer of a 3.0-cm deep stream of gas flowing through a horizontal channel 3.0 cm deep, 12 in. wide and 8 ft long at 2.4 cm/sec initially failed because of the instability of gas flow observable at such

TABLE 2

SEGREGATION OF DNA BY STOKES-CUNNINGHAM FALL IN AN AEROSOL MOVING HORIZONTALLY IN AN EIGHT-FOOT CHANNEL

Distance from entrance orifice (in.)	Per cent DNA[a]	Microscopic size-frequency estimations: ϕ(D) of particles[b]			
		Smaller than nuclei	Size of nuclei[c]	Size of cells	Larger than cells
10	8.2	0	0	4.0	84.0
15	8.6	5.0	0	20.0	64.0
20	8.1	6.0	2.0	53.0	46.0
25	7.1	2.0	15.0	53.0	30.0
30	6.3	0	36.0	46.0	17.0
35	5.9	1.0	52.0	38.0	10.0
40	5.3	3.0	62.0	30.0	5.0
50	5.4	8.0	74.0	19.0	1.0
60	5.5	16.0	68.0	13.0	0
70	5.7	24.0	67.0	8.0	0
80	—	32.0	65.0	6.0	0
90	—	38.0	56.0	3.0	0

[a] The DNA content of the intact untreated lyophilized cells was 7%. DNA analyses were reproducible to ±6.6% of the average value.

[b] ϕ(D) represents the per cent of the total number of visible particles that are of the size of the classification considered.

[c] The predicated area for the accumulation of particles the size of nuclei is 20–96 in. from the entrance orifice.

low Reynolds numbers (12). At the suggestion of Dr. Kenneth T. Whitby of the Dept. of Mechanical Engineering, a density gradient was obtained in the gas by layering, in 1.0-cm deep layers, nitrogen over carbon dioxide over methyl chloride. The aerosol was suspended in the top 0.5 cm of the nitrogen layer. Smoke streamers (13) indicated that a stable flow was obtained.

A row of clean microscope slides was laid down the center of the bottom of the eight-foot channel and one 10.0-mg shot of lyophilized cells was fired from the Sharples, at an annulus clearance of 200 μ and at a pressure of 400 lb/sq in., and allowed to flow as an aerosol through the

channel at 2.4 cm/sec. After the particles of the aerosol had either settled or passed from the channel as exhaust, 1000 particles on each slide were sized by means of a microscope equipped with a Whipple disk, and size-frequency curves (6) were determined for the points represented by each slide. After removal of the slides, additional shots were fired at 15-min intervals until enough material had collected on the channel bottom to permit analyses for DNA content. The data obtained are shown in Table 2. It can be seen that the DNA tends to concentrate in an area where large percentages of particles as large or larger than intact cells are found microscopically. This suggests that nuclei and nuclear fragments tend to reagglomerate more rapidly than do fragments of cytoplasm. The lowered DNA content of fractions further than 25 in. from the entrance orifice suggests friability of the nucleus, also observed from data obtained with the Goetz Aerosol Spectrometer.

Separation by Means of Photophoresis (8)

While photophoresis (lateral movement of falling aerosol particles away from and toward a beam of light) appeared to effect some separation according to microscopic count, there was no evident separation as determined by DNA analyses.

Separations under Gravity or by Centrifugation in Liquid Carbon Dioxide

Lyophilized cells were fired through the Sharples deagglomerator used, at narrow settings of the annulus, as a fluid mill, and collected in a plastic bag. The ground cells were dispersed in liquid carbon dioxide and allowed to settle through 3–10 in. of aerosol-free liquid carbon dioxide in steel bombs under gravity or by centrifugation at room temperature and at refrigerator temperatures (to elevate the density of the liquid). No consistent concentration of DNA was found in any fraction.

Separation by Electrostatic Means

A series of 10.0-mg aliquots of lyophilized cells were fired through the Sharples deagglomerator at an annulus clearance of 200 μ and at a pressure of 400 lb/sq in., and the resulting aerosol of fluid-milled cells was forced, by means of a stream of nitrogen metered from a tank, through the corona discharge of an electrostatic particle sizer built after the design of Langer (10). The charged aerosol was then driven between the charged plates of the particle sizer collector, with a layer of aerosol-free gas moving between the aerosol and the collection plates in order to increase resolution. All gas flows were metered at rates compatible

with stability of flow (12) as indicated by smoke streamers (13). Deposition was almost quantitative on the negatively charged collection plate. After the first shot the negative plate was removed and microscopic size-distribution counts (6) were made, using oblique light, at intervals along the plate. The plate was then replaced and shots were fired at 15-min intervals until enough material had collected to permit semimicro analyses of the DNA content of fractions collected at intervals along the length of the plate. The results are shown in Table 3. As with

TABLE 3
SEGREGATION OF DNA BY MEANS OF ELECTROSTATIC PRECIPITATION

Distance from entrance orifice (in.)	Yield (mg)	Per cent DNA[a]	Microscopic size-frequency estimations: ϕ(D) at (size range in μ)		
			0.5–1.5[b]	4.5–5.5[c]	9.5–10.5[d]
⅛ (0–⅛)	9.8	10.0	0	4.0	—
¼ (⅛–¼)	11.5	8.0	2.0	6.0	49.0
½ (¼–½)	5.8	6.0	5.0	15.0	8.0
¾	—	—	—	20.0	—
1.0 (½–1.0)	7.4	4.4	15.0	16.0	3.0
2.0 (1.0–2.0)	5.1	2.6	66.0	1.0	0
6.0 (2.0–9.75)	2.8	1.8	92.0	0	0

[a] The DNA content of the intact untreated lyophilized cells was 7%. The DNA analyses were reproducible to ±6.6% of the average value.

[b] Smaller in size than nuclei.

[c] The size of nuclei.

[d] Larger in size than nuclei.

separations under gravity in the horizontal channel, the DNA tends to collect in an area populated by particles larger than nuclei, suggesting rapid reagglomeration of nuclei and fragments of nuclei. The lowered DNA content of fractions more distant than ¼-in. from the entrance orifice again suggests the considerable friability of the nucleus. The size-distribution data from small particles in oblique light is somewhat in error because of the influence of scattered light on the particle size estimation.[3]

CONCLUSION

It has been shown that it is possible to concentrate the DNA of certain lyophilized cells from 7% in the intact cells to as high as 10% by electrostatic means and to a lesser extent by means of gravity settling. The nuclei of these cells appear to be more friable than does the cytoplasm. If the cells, averaging 8.1 μ in diameter, are spheres containing

[3] Private Correspondence with Dr. Gerhard Langer, Armour Research Foundation, Ill. Inst. Technol., Chicago, Ill.

spherical nuclei, averaging 4.6 μ in diameter, and if the DNA content of the intact lyophilized cells is 7%, then the DNA content of the intact isolated nuclei can be calculated to be 50.8%. According to this calculation, the electrostatic method has concentrated the DNA to approximately 20% of the theoretical content. This estimation would be elevated by consideration of the density differential existing between the nucleus and the cytoplasm.

While actual operation of the apparatus is not difficult, poor or no control of certain variables reveals the following areas for criticism:

1. Gas flow metered by hand as indicated by flowmeter readings can be irregular. An automatic feedback control should be designed for this purpose.

2. The Sharples deagglomerator fires samples in small batches, each of which must be discharged into a container, disconnected from the rest of the system, during the discharge. As a result there is a loss of about 0.9 of each sample on the walls of the chamber. Under design is a continuous fluid milling and deagglomeration system which will feed directly into the electrical system.

3. It has not been possible to devise a fluid mill which will produce more than 1 or 2% of the theoretical number of microscopically intact nuclei. This extremely low yield requires that grams of lyophilized cells must be used to obtain milligrams of what appear to be intact nuclei.

4. There is a question as to possible damage to cell constituents from the ionized gas molecules and radiation produced by the corona discharge. To circumvent this it is planned to convert the electrical part of the collection system to a nonuniform electric field, which will eliminate the necessity of precharging the aerosol particles.

5. The lyophilization process is destructive to cells.

SUMMARY

Attempts have been made to separate the nuclei from lyophilized cells by means of physical methods. After grinding the cells in a ball mill, converting them into an aerosol by blowing them at sonic velocity through a defined annulus, and effecting a particle size separation in an aerosol centrifuge, the deoxyribonucleic acid (DNA) is found to be slightly concentrated in the fraction representing particles less than 0.6 μ in size. After blowing the cells through a defined annulus at sonic velocity to grind them (fluid mill process) and to form them into an aerosol in one operation, it was found that the DNA could be concentrated by (*a*) gravity from a horizontally moving stream of aerosol and (*b*) differential precipitation on charged metal plates after precharging in a corona discharge. In the latter two cases it was found that the

DNA concentrated in the fraction representing particles larger than the nuclei, indicating rapid reagglomeration of particles containing DNA.

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Application of Different Colorimetric Tests to Cellodextrins

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INTRODUCTION

In experiments involving individual oligosaccharides or mixtures of oligosaccharides, it is often useful to be able to measure both monosaccharide content and molar concentration. Colorimetric procedures using orcinol–sulfuric acid (1–3) and anthrone–sulfuric acid (4–6) as reagents for measurement of monosaccharide content and those using dinitrosalicylic acid (7–9) as a reagent for measurement of molar concentration are investigated in the present study as they apply to the cellodextrin series. Results obtained with iodine–potassium iodide reagent, shown previously to measure molar concentration of cellodextrins (10), were used as a basis of reference.

MATERIALS

The cellodextrins used were prepared from acid hydrolyzates of cellulose by ethanol–water gradient elution from chromatographic columns composed of stearic acid-treated mixtures of charcoal and Celite (11). The cellotriose and -tetraose thus obtained were crystallized from aqueous ethanol; the -pentaose and -hexaose were used in the form of lyophilized preparations. Each cellodextrin appeared homogeneous by a microcolumn chromatographic test (12).

The cellobiose used was obtained from Eastman Kodak Company. The glucose, which was used as the standard sugar, was obtained from the Matheson Company.

PROCEDURES

Data which were obtained in the tests using the orcinol and anthrone reagents were converted to absorbancy by subtracting absorbances of

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blanks from absorbances of test samples; those obtained in the tests using the iodine–potassium iodide test, by subtracting absorbances of test samples from absorbances of blanks. Absorbancy data obtained with the various dinitrosalicylic acid reagents were calculated in terms of slopes of lines relating absorbance to quantity of test sample. For comparison of absorbancies of different sugars on a molar basis, values of absorbancy/mg of test sample were first calculated and were then converted to absorbancy/mole by multiplying absorbancy/mg by molecular weight.

Orcinol–Sulfuric Acid or Anthrone–Sulfuric Acid Reagents

Three milliliters of 0.2% orcinol (or anthrone) in 70% (v/v) sulfuric acid was added to 0.2 ml of test sample containing around 0.1 mg sugar, and the components were mixed. By the use of small volumes of the test samples, generation of heat during mixing was minimized, thus

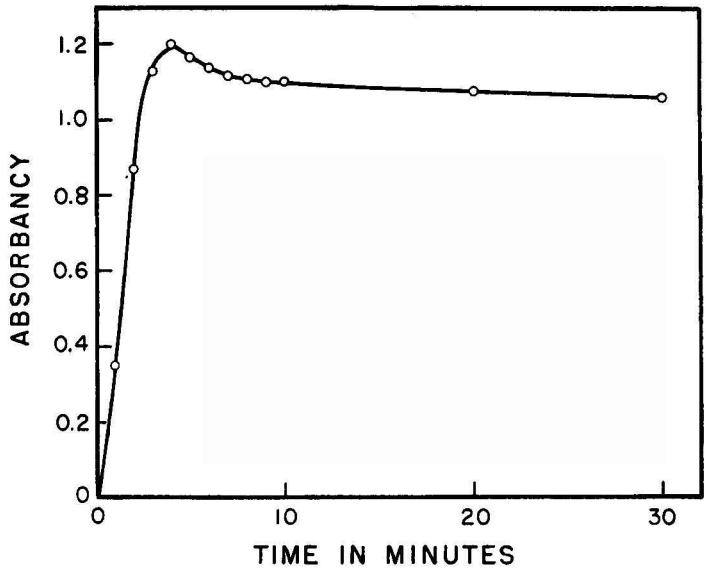

FIG. 1. Rate of color development in tests using orcinol reagent.

avoiding the premature development of color which may otherwise take place. The mixtures were heated for 20 min at 100°C and cooled rapidly to room temperature. Absorbances were measured in a colorimeter using a wavelength of 515 mμ for tests involving the orcinol reagent and at 550 mμ for those involving the anthrone reagent. Samples containing no sugar were treated similarly and used as blanks. The rates

of color development observed under these conditions are shown in Figs. 1 and 2. The maximum in the curve of Fig. 1, observed during the initial period of heating of the sugars with orcinol reagent, was characteristic; its explanation, however, is not known. Figure 2 shows that in the tests using the anthrone reagent greater stability of color

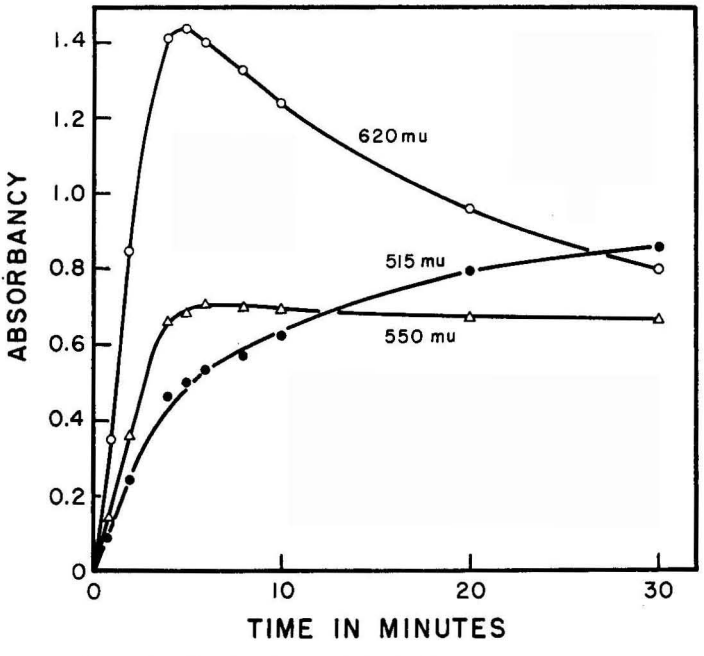

FIG. 2. Rate of color development in tests using anthrone reagent.

with time of heating was obtained when measurements were made at 550 mμ than when they were made at higher or lower wavelengths, an observation not made previously.

Results obtained with tests using either of the above reagents followed Beer's law over the ranges of concentration of sugars studied.

Iodine–Potassium Iodide Reagent

Procedures using the iodine–potassium iodide reagent are described elsewhere (10).

Dinitrosalicylic Acid Reagents

Sumner dinitrosalicylic acid reagent with Rochelle salts present (7, 8) contained 1% dinitrosalicylic acid, 20% Rochelle salts, 0.2%

phenol, 0.05% sodium sulfite, and 1% sodium hydroxide. Four milliliters of this reagent was added to 4 ml of test sample, and the components were mixed. The mixture was heated for 10 min at 100°C to produce maximum color and cooled rapidly to room temperature. Absorbance was measured at 550 mμ. To allow for destruction of sugar in the test (8), samples were made to contain 0.1, 0.2, and 0.3 mg sugar. Beer's law was obeyed following correction of the data for destruction of sugar.

Sumner reagent without Rochelle salts (8) possessed the same composition as the preceding except for the omission of the Rochelle salts. Further, the conditions of the test were the same except for the addition of 1 ml of 40% Rochelle salts to the mixtures after color development and before cooling (8).

The reagent of Meyer *et al.* (9) consisted of two components, namely, 1½% dinitrosalicylic acid and 6 *N* sodium hydroxide. Two milliliters of the dinitrosalicylic acid and 2 ml of the sodium hydroxide were added to 4 ml of test sample, and the components were mixed. Mixtures were heated for 30 min at 65°C and cooled rapidly to room temperature. Absorbance was measured at 550 mμ. Since the intensities of color produced under these conditions were comparatively low, test samples containing 0.5 and 1.0 mg glucose, or molar equivalents of the different oligosaccharides, were required. Again, Beer's law was obeyed following correction of the data for destruction of sugar.

A modification of Meyer's reagent consisted of 1½% dinitrosalicylic acid and 1 *N* sodium hydroxide. Tests using the modified reagent were carried out similarly to the above except that a temperature of 50°C was used, the test samples were made to contain 1, 2, and 3 mg glucose, or molar equivalents of the different oligosaccharides, and the period of heating was varied depending upon the particular requirements, as indicated below.

RESULTS

Results obtained with the orcinol–sulfuric acid and anthrone–sulfuric acid reagents are compared in Table 1 with those obtained with the iodine–potassium iodide reagent. While the iodine–potassium iodide reagent measured molar concentration, as demonstrated by approximately equal molar absorbancies for the different cellodextrins, the other reagents measured monosaccharide content, since the absorbancies in these instances were directly correlated with degrees of polymerization. Further studies showed that results obtained with the orcinol and anthrone reagents were highly reproducible. However, the orcinol reagent was found to be slightly preferable since it gave results more closely correlating with those obtained with the iodine–potassium reagent

while the anthrone reagent gave results which were, on the average, about 2% higher.

The failure of the molar absorbancies of some of the different cellodextrins to come quite up to the value for the glucose is attributable to

TABLE 1

RELATIVE MOLAR ABSORBANCIES OF DIFFERENT CELLODEXTRINS REACTING WITH IODINE–POTASSIUM IODIDE, ORCINOL–SULFURIC ACID OR ANTHRONE–SULFURIC ACID

Cellodextrin	D.P.[a]	Relative molar absorbancy (glucose = 1.00)		
		Iodine–potassium iodide	Orcinol–sulfuric acid	Anthrone–sulfuric acid
Cellobiose	2	1.00	1.97 (0.99)[b]	1.97 (0.99)[b]
Cellotriose	3	0.94	2.87 (0.96)	2.93 (0.98)
Cellotetraose	4	0.94	3.85 (0.96)	3.97 (0.99)
Cellopentaose	5	0.93	4.67 (0.93)	4.70 (0.94)
Cellohexaose	6	0.93	5.56 (0.93)	5.53 (0.92)

[a] Degree of polymerization.

[b] Data shown in parentheses are calculated in terms of molar absorbancy per mole of monosaccharide.

the presence of moisture and other inert contaminants in the preparations (11), for which corrections were not made in the present study. Results obtained in the tests which follow, using different dinitrosalicylic acid reagents, were corrected for the assumed presence of inert contaminants, based on data obtained with the orcinol reagent.

TABLE 2

RELATIVE MOLAR ABSORBANCIES OF DIFFERENT CELLODEXTRINS REACTING WITH DINITROSALICYLIC ACID UNDER VARIOUS CONDITIONS

Cellodextrins	D.P.[a]	Relative molar absorbancy (glucose = 1.00)			
		S-DNS-WO	S-DNS-W	M-DNS	M-DNS-M
Cellobiose	2	1.93	1.71	1.38	1.11
Cellotriose	3	3.00	2.42	1.60	1.02
Cellotetraose	4	4.33	3.19	1.88	0.95
Cellopentaose	5	5.31	3.74	1.92	0.95
Cellohexaose	6	5.97	4.15	1.84	1.01

[a] Degree of polymerization.

Sumner dinitrosalicylic acid reagent not containing Rochelle salts, S-DNS-WO, unexpectedly measured monosaccharide content (Table 2). Data obtained with this reagent were not, however, as reproducible or reliable for measurement of monosaccharide content as those obtained

with the orcinol and anthrone reagents (Table 1). Sumner dinitrosalicylic acid reagent containing Rochelle salts, S-DNS-W, measured neither monosaccharide content nor molar concentration (Table 2), suggesting partial correlations of the results with measurements of both. Results obtained with the reagent of Meyer, M-DNS, showed a closer correlation with molar concentration than with monosaccharide content (Table 2), but still were not satisfactory.

To determine whether the application of Meyer's reagent could be modified so as to yield a more satisfactory measurement of molar concentration, a preliminary study was made of the effect of time of heating. The results of this study showed that with a period of heating

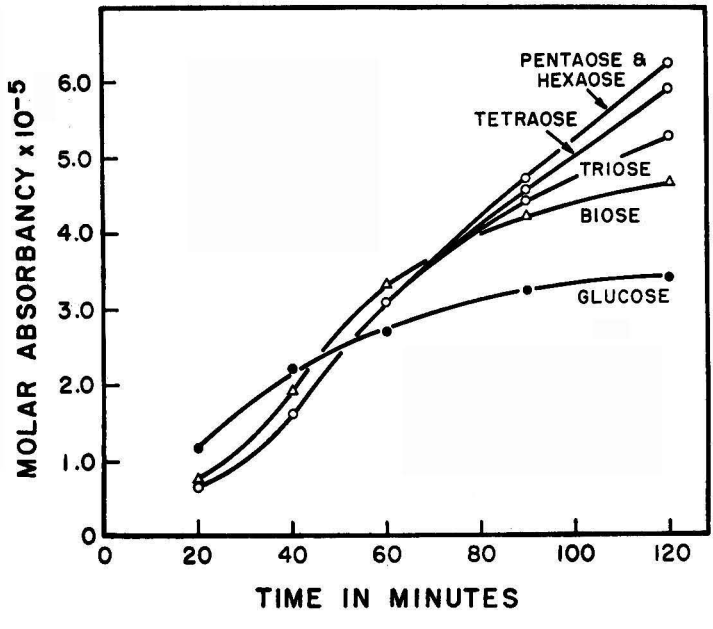

FIG. 3. Rate of change of molar absorbancy with time of heating in tests using modified Meyer reagent.

of only 3–4 min, the different cellodextrins produced absorbancies which were quite low but were approximately equivalent on the basis of molar concentration. With longer heating, however, the absorbancies increased, more rapidly for the higher than lower cellodextrins, so that the molar absorbancies became more and more indicative of monosaccharide content than of molar concentration.

Since the period of 3–4 min was inconveniently short, the temperature at which the test was carried out was lowered to 50°C and the concen-

tration of sodium hydroxide reagent was decreased to 1 *N*. With the modified Meyer reagent, M-DNS-M, the rates of change of molar absorbancy with time of heating were those shown in Fig. 3. It may be concluded from these data that for purposes of measurement of molar concentration the optimal time of heating is around 55 min when glucose is taken into consideration and around 70 min when glucose is excluded. When the different cellodextrins were retested with the modified Meyer reagent, using a heating time of 55 min, the results shown in the last column of Table 2 were obtained. Because of the marked and different effects of time of heating on color development of different cellodextrins in tests carried out with the modified Meyer reagent, results obtained with its use cannot be expected to be as precise as those obtained with the iodine–potassium iodide reagent.

DISCUSSION

The observation that orcinol–sulfuric acid and anthrone–sulfuric acid reagents measure monosaccharide content of the cellodextrins is explained on the basis of the hydrolysis of the cellodextrins by the acid in the reagents, as was to be expected from earlier work (2, 5). The close correspondence between the absorbancies per mole of monosaccharide obtained with orcinol and anthrone reagents and the absorbancies per mole of oligosaccharide obtained with the iodine–potassium iodide reagent indicates the absence of interfering side reactions. The fact that the Sumner dinitrosalicylic acid not containing Rochelle salts also measured mole concentration of monosaccharide may similarly be explained on the basis of hydrolysis of glucosidic linkages, or, perhaps more likely, on the basis of other types of degradative reactions which would produce quantities of reducing groups proportional to the degrees of polymerization.

Of the different methods using dinitrosalicylic acid reagents, the Sumner method and the original Meyer method cannot be applied to the measurement of either molar concentration or monosaccharide content of the cellodextrins. The modified Meyer method, described herein, can be applied, however, to the measurement of molar concentration, and, although it will not give as reproducible or reliable results as the iodometric method, it is more convenient than the latter. Furthermore, although the modified Meyer method is less sensitive than methods using the Sumner dinitrosalicylic acid reagent, results obtained with its use can be expected to have much more exact significance with regard to molar concentrations of the oligosaccharides. This advantage is particularly important in kinetic studies in which rates of hydrolysis of oligosaccharides or polysaccharides are measured. In systems containing both

cellodextrins and glucose, a heating period of 55 min is recommended, with glucose used as a standard of reference. In systems containing cellodextrins with little or no glucose present, as in cellulolytic digests of cellulose (13), a heating period of 70 min with cellobiose used as the standard of reference would be preferable. Where the modified Meyer method is used, it will be necessary to make provision for the partial destruction of sugar. This can be accomplished by the addition of extra glucose to the system, as has been done in tests using the Sumner method (8).

SUMMARY

Monosaccharide contents of cellodextrins can be measured with the aid of orcinol–sulfuric acid or anthrone–sulfuric acid reagents. Molar concentrations can be measured by a modification of Meyer's colorimetric procedure using dinitrosalicylic acid.

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The Determination of Gelatin in Blood and Urine

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INTRODUCTION

The use of gelatin and its chemical modifications as plasma expanders requires the study of the survival of these substances in the body. The present paper describes the determination of small quantities of gelatin in blood and urine. Rogers *et al.* (1) point out that the high hydroxyproline content of gelatin and its absence from normal plasma and urine can be used to determine gelatin in these fluids. There is some evidence, however, which suggests that small amounts of hydroxyproline are present in blood and urine. Troll and Cannan (2) consider the normal excretion of hydroxyproline to be about 0.5–2.5 mg/day, i.e., about 0.03–0.30 mg/100 ml urine. Müting (3) could not detect hydroxyproline in human serum protein (total). This worker (4) was able to detect 0.2 gm hydroxyproline/16 gm N in normal human serum albumin and a similar amount in normal human globin. Troll (5) found 0.06 gm hydroxyproline/16 gm N in normal bovine serum albumin. These very small amounts of hydroxyproline in blood and urine do not appear to have affected the method of Rogers *et al.* (1) which was as follows: The fluid was hydrolyzed to convert the proteins to amino acids, the hydroxyproline was separated chromatographically from proline, and its concentration was determined by their modification of the Troll and Cannan (2) colorimetric procedure.

The method described below depends on the chromatographic separation of hydroxyproline from tyrosine and its subsequent determination by a modification (6) of the Neuman and Logan (7) colorimetric procedure. Solutions of gelatin in blood are used as standards. The chromatographic separation of hydroxyproline and tyrosine is necessary as the latter yields 1.5% (7) and 2.0% (8) of the color yielded by the same weight of the former. Tryptophan, which yields 1.3% (8), is destroyed during acid hydrolysis. The effect of the small quantities of hydroxyproline already present in the urine and blood is readily eliminated by using the unadulterated blood or urine in a blank determina-

tion. In practice, samples of blood and urine would be taken from the subject before commencing the experiment. We have found this procedure more convenient for the lower concentrations of gelatin, and it also does not require a vibrator.

EXPERIMENTAL

Reagents

The reagents for the colorimetric procedure are described in Leach (6). Citrate buffer, pH 3.42, was prepared as described by Moore and Stein (9); no thiodiglycol, benzyl alcohol, or detergent was added.

Gelatin solutions were prepared by soaking the appropriate amount of gelatin in water for 1 hr, melting at 40°C, and diluting to the required volume.

Apparatus

Pasteur pipets and a centrifuge suitable for 20-ml samples of blood are required. The chromatography column consisted of a buret or glass tube 0.9 cm in diameter and approximately 15 cm long fitted with a tap or other suitable valve. At the bottom of the buret was placed a pad of finely broken glass wool onto which was poured a suspension of purified Dowex 50 (200–400 mesh, nominal 12% cross-linking) resin in pH 3.42 citrate buffer. The settled resin layer was 8 cm high; after washing with about 50 ml buffer it was ready to use. Regeneration was carried out with 25–50 ml of approximately 0.2 *M* NaOH followed by 25–50 ml buffer. An aspirator system or nitrogen cylinder with suitable reduction valve was used to supply a pressure equivalent to about a 3-ft head of water to give suitable flow rates through the column, i.e., 20 ml/hr during the addition of the hydrolyzate solution to the column. During the development of the column the above flow rate was achieved with a reservoir of buffer.

Preparation of Samples

The reference samples of gelatin in blood (outdated human blood) were prepared so that they were 0.0–0.07 gm gelatin (moisture- and ash-free) per 100 ml of whole blood. The cellular content of the blood was 43%. The samples were prepared by adding a given amount of gelatin in 1 ml solution to 20 ml blood. One milliliter of water was added to the unknown sample of blood containing gelatin, and 1 ml was added to 20 ml of the unadulterated blood. The gelatin used yielded 14.1 gm hydroxyproline/100 gm dry ash-free protein.

Centrifugation

The standards and unknown were centrifuged at moderate speeds to avoid hemolysis, and the plasma was removed by means of a Pasteur pipet.

Hydrolysis

Ten milliliters of the plasma was hydrolyzed with 10 ml conc. HCl in a sealed tube for 1½ hr at 145°C (1). If the tubes are heated in an autoclave at 145°C (i.e., 46 lb sq in. pressure) the possibility of explosion of the tubes under pressure is minimized. The tubes were cooled, shaken vigorously, and opened. The hydrolyzate was filtered, the first 2 ml (approx.) of filtrate being rejected, and the remainder collected. Five milliliters of the filtered hydrolyzate was evaporated to dryness on a steam bath to remove the acid.

Chromatographic Separation

Four milliliters of pH 3.42 citrate buffer was added to the hydrolyzate using successive deliveries from a 1-ml pipet. The hydrolyzate and buffer were thoroughly stirred with a small glass rod until the residue dissolved. The 1-ml pipet was rinsed with 0.5 ml of the solution, and finally 3 ml solution was placed on the column. As the method is a comparative one, the volume of the hydrolyzate residue was not considered in any of the calculations. A 25-ml graduated flask was placed to collect the eluate. When the hydrolyzate solution had sunk into the resin, aided by gas pressure, the hydrolyzate adhering to the glass was rinsed onto the resin with a further 1 ml buffer. When the solution had sunk into the resin, the column was filled up with buffer and the column developed. The 25-ml graduated flask was removed just before the solution reached the graduation, made up to volume with buffer, and thoroughly mixed. During the initial trials with this method two further 5-ml fractions were collected and analyzed to confirm that all the hydroxyproline had been collected.

Colorimetric Procedure

A. The hydroxyproline content of 1 ml eluate was determined as described by Leach (6) except that the blank was prepared using 1-ml buffer solution instead of water. No hydroxyproline standards were used as the method was comparative.

B. The following modification which gave an increase in sensitivity was also investigated and proved satisfactory. Two milliliters of the eluate or 2 ml of the buffer (blank) was used in the colorimetric pro-

cedure, and the increase in volume was eliminated by doubling the concentration of the $CuSO_4$ and NaOH and using 0.5-ml volumes of Table 1, and the results of the determination of unknowns are given in

TABLE 1

OPTICAL DENSITIES OBTAINED BY THE COLORIMETRIC PROCEDURE WITH GELATIN AND BLOOD ADMIXTURES AFTER HYDROLYSIS[a]

Grams gelatin/100 ml blood	1 ml of solution containing y mg gelatin added to 20 ml blood y	Optical density 555 mμ, 4 cm cell	
		Method A	Method B
0.0676	13.52	0.763	1.307
0.0507	10.14	0.582	1.008
0.0450	9.00	0.525	0.890
0.0406	8.12	0.468	0.840
0.0398	7.96	0.453	0.823
0.0380	7.60	0.433	0.787
0.0338	6.76	0.404	0.730
0.0290	5.80	0.359	0.671
0.0253	5.06	0.320	0.592
0.0225	4.50	0.289	0.515
0.0100	2.00	0.160	0.292
0	0	0.060	0.111

[a] Weights of gelatin are given on a moisture- and ash-free basis.

Table 2. The increase in sensitivity for method B is not quite twofold due to the effect of increased concentration of other amino acids as reported by Baker, Lampitt, and Brown (10).

TABLE 2

DETERMINATION OF GELATIN IN ADMIXTURES WITH BLOOD AND URINE

Fluid	Colorimetric method	Gelatin added (gm)	Gelatin determined (gm)	Difference (% of material added)
Blood	A	0.0636	0.0630	−0.9
	B	0.0636	0.0649	+2.0
	A	0.0406	0.0402	−1.0
	B	0.0406	0.0411	+1.2
	A	0.0290	0.0285	−1.7
	B	0.0290	0.0286	−1.4
	A	0.0113	0.0107	−5.3
	B	0.0113	0.0109	−3.5
Urine	A	0.0290	0.0285	−1.7
	B	0.0290	0.0286	−1.4
	A	0.0450	0.0439	−2.4
	B	0.0450	0.0438	−2.7

Determination of Gelatin in Urine

This was carried out as for gelatin in blood except that the centrifuging and filtration stages were not required.

RESULTS

The two 5-ml fractions following the 25-ml fraction which were collected in the preliminary experiments with gelatin–blood admixtures gave the same optical densities as those obtained with blood alone, showing that all the hydroxyproline had been recovered in the first fraction.

The optical densities for a range of gelatin concentrations are given in Table 1, and these were used to construct calibration graphs, which resulted in straight line plots. In the determination of gelatin in different samples of blood, allowance was made for variation of cellular content. Results of the determinations of known amounts of gelatin added to blood or urine are given in Table 2. In practice it was found that only one reference blood–gelatin solution and some unadulterated blood was required to determine the concentration in an unknown sample. The calculation used was as follows (d = optical density)

$$\text{conc. of unknown} = \text{conc. of reference solution } X \frac{d(\text{unknown}) - d(\text{blood alone})}{d(\text{reference}) - d(\text{blood alone})}$$

Similar results were obtained for the determination of gelatin in urine and the results for some unknowns are given in Table 2.

DISCUSSION

The method described has been employed for whole blood; however, it is equally applicable to plasma alone. In this case the standards would be prepared by adding known quantities of gelatin dissolved in a given volume of water to the plasma and an equal volume of water to the unknown plasmas. Miss D. Birch has suggested (private communication) the use of gelatin for the determination of blood and plasma volumes. The first ten concentrations of gelatin given in Table 1, approximately represent 20 ml of a 12½% w/v solution of air-dried gelatin in 3–9 l blood, which would be a convenient level for this work.

Higher concentrations of gelatin than those investigated in the present work can be determined simply by diluting the hydrolyzate with buffer before chromatography and carrying out the same procedure with the standards.

The hydroxyproline content of gelatins is rather variable (11) so that in any work involving the determination of gelatin via hydroxyproline

content one should either use a single batch of gelatin throughout the experiments or make allowance for the variation in composition of the different samples employed.

SUMMARY

A method for the determination of gelatin in blood (or plasma) and urine is described, and it depends on the presence of hydroxyproline in gelatin and its absence (or presence in very small quantities) from the two fluids. The hydroxyproline is separated chromatographically from tyrosine and subsequently determined by a modification of the Neuman and Logan colorimetric procedure. The determination of gelatin concentrations of 0.0–0.07 gm gelatin/100 ml of whole blood or urine is carried out. It is pointed out that the method can be used for higher concentrations of gelatin.

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Photophosphorylation as a Tool for the Synthesis of Specifically Labeled Nucleotides[1]

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INTRODUCTION

The wide variety of biochemical reactions in which the phosphates of adenosine triphosphate (ATP) participate make this substance, when labeled, an extremely valuable research tool. Many investigators have already resorted to the use of P^{32}-labeled ATP, and recently detailed procedures have been described for its chemical and enzymic synthesis (1–3). The usual method of synthesis utilized the mitochondrial oxidative-phosphorylative system (2).

We have previously reported (4) that chloroplasts, or chloroplast fragments isolated from swiss-chard leaves, could synthesize ATP very rapidly during photophosphorylation. These chloroplasts showed neither adenosinetriphosphatase (ATPase) activity nor phosphate–ATP exchange, had a very high affinity for adenosine diphosphate (ADP), and possessed no detectable adenylate–kinase activity. These properties, together with the simplicity and stability of the photophosphorylative system, suggested its use as an improved ATP^{32}-synthesizing system.

EXPERIMENTAL

Once-washed chloroplasts from swiss-chard leaves were prepared by the following procedure (4): A leaf (5 gm) whose midrib had been removed was cut into about 1×1 cm pieces and put within a 100-ml Virtis cup, placed in the ice-water bath of the Virtis "45" homogenizer (Virtis Co., 160 Ashburton Ave., Yonkers, N. Y.). Forty milliliters of chilled homogenizing medium (0.4 *M* sucrose, 0.05 *M* Tris-HCl, 0.01 *N* NaCl, 0.02 *N* sodium ascorbate, pH 7.8) was added, followed by a

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minimal addition of Dow-Corning Antifoam A spray. The homogenizer was run for 5 sec with its Variac set on 50, followed by 25 sec at 20 on the Variac. The slurry was squeezed through a double layer of gauze and centrifuged at 0°C for 90 sec at $500 \times g$. The supernatant was transferred and recentrifuged for 7 min at $1000 \times g$. The pellet was suspended in 40 ml of the homogenizing medium and centrifuged for 90 sec at $500 \times g$. The supernatant was transferred and centrifuged for 7 min at $2000 \times g$. The final pellet was resuspended in 1–2 ml of the homogenizing medium (once washed chloroplasts).

Chloroplast fragments were isolated by suspending once-washed chloroplasts, obtained from 5 gm leaves, in 35 ml of 10^{-3} *M* Tris buffer pH 7.8. The suspension was allowed to stand at 0°C for 10 min and was centrifuged at $1000 \times g$ for 5 min. The supernatant was recentrifuged at $20{,}000 \times g$ for 10 min, and the final pellet was suspended in a small volume of the homogenizing medium. Except where otherwise indicated, the reaction mixtures and assay were as previously described (4).

Labeled nucleotides were isolated from the reaction mixture as follows: The reaction was stopped by the addition of 30% $HClO_4$ to a final concentration of 3%. The precipitate was removed by centrifuging at $1000 \times g$ for 5 min at 0°C. The pH of the supernatant was adjusted to be between 7.5 and 8.5 with KOH, diluted at 100 ml, and passed through a column of 1×1 cm of Dowex 1 bicarbonate (5). The column was washed with 50 ml water. The inorganic phosphate and glucose 6-phosphate were eluted with 100 ml of 1.5% $KHCO_3$, and the ATP was eluted with 60 ml of 7% $KHCO_3$. To rid it of the $KHCO_3$, the ATP solution was then passed through a column of 3 cm height and 8 cm diameter of Dowex 50, hydrogen-ion form, and the column washed with 50 ml water. The combined eluates were evaporated to dryness with a rotating flash evaporator connected to an oil-vacuum pump through a liquid-air trap. The dry powder was suspended in a small volume of water and stored at —15°C. The Dowex resins used were analytical grade, X-8, purchased from California Foundation for Biochemical Research, Los Angeles, Calif. They were washed with 5 *N* HCl, converted to the form indicated, and thoroughly washed with water before use. Solutions were passed through the columns at a rate of less than 3 ml/min.

We have recently adopted a more simplified procedure for the isolation of the labeled nucleotides than the one described above (which was used in obtaining the results presented). The procedure consists of passing the supernatant of the acidified reaction mixture through a

charcoal–cellulose column (made within 6 mm I.D. glass tubing, plugged with glass wool and packed with 2.0 ml of a suspension of 1 gm Celite in 50 ml of 0.1 *N* HCl, followed by 2.0 ml of a suspension of 1 gm of acid-washed Norit A plus 2 gm of Whatman standard-grade cellulose powder in 50 ml of 0.1 *N* HCl and washed by 10 ml water). The column was washed with 5 ml of 0.1 *N* HCl, followed by 5 ml of 0.01 *N* HCl and 5 ml of water. The adsorbed nucleotides were eluted with 5.0 ml of 1% NH_3 in 60% ethanol. The solution was evaporated to dryness, dissolved, and stored as described above. This procedure yields only 50–70% of the synthesized nucleotide in the final preparation, but its simplicity and relative safety when using highly radioactive material made it useful for routine preparations.

Myosin was prepared from rabbit muscle according to the procedure of Kessler and Spicer (6). Adenylate kinase (Myokinase) was a commercial preparation purchased from Boehringer, Mannheim, W. Germany. It was dialyzed overnight against distilled water at 0°C before use, in order to remove $(NH_4)_2SO_4$ which uncouples photophosphorylation (7). Five-tenths milligram of the dialyzed preparation was added to a reaction mixture of 3.0 ml. The given specific activity of the preparation was 36 μmoles ADP formed/mg/min. Hexokinase type IV was purchased from Sigma Chemical Co., St. Louis, Mo. Carrier-free orthophosphate-P^{32} in very dilute HCl was obtained from the Radiochemical Centre, Amersham, England. It was treated before use as previously described (4) so as to decrease the amount of counts in the non-orthophosphate fraction, and was neutralized before addition to the reaction mixtures.

TABLE 1

ABSENCE OF ADENYLATE KINASE ACTIVITY IN CHLOROPLAST PREPARATIONS FROM SWISS-CHARD[a]

Preparation used	μmoles ATP synthesized/mg chl/hr	
	− Adenylate Kinase	+ Adenylate Kinase
Chloroplasts, unwashed	10	640
Chloroplasts, washed once	3	900
Chloroplasts, washed four times	0	750
Chloroplast fragments	0	500

[a] Reaction conditions and preparations as described under *Experimental*, except that the ADP in the reaction mixture was replaced by 12 μmoles AMP + 0.1 μmole ATP. The adenylate kinase was added as described under *Experimental*. Reaction time, 10 min. Light intensity, 140,000 lux. Temperature, 19°C. Chlorophyll content of chloroplast preparations in μg/ml of reaction mixture: unwashed, 9.0; washed once, 13.9; washed four times, 9.1; fragments, 26.0.

RESULTS

Determination of the Relevant Properties of the System

The apparent lack of adenylate kinase in chloroplasts from swiss-chard has been previously noted (4). Table 1 illustrates the complete lack of any adenylate kinase activity in chloroplast and chloroplast fragment

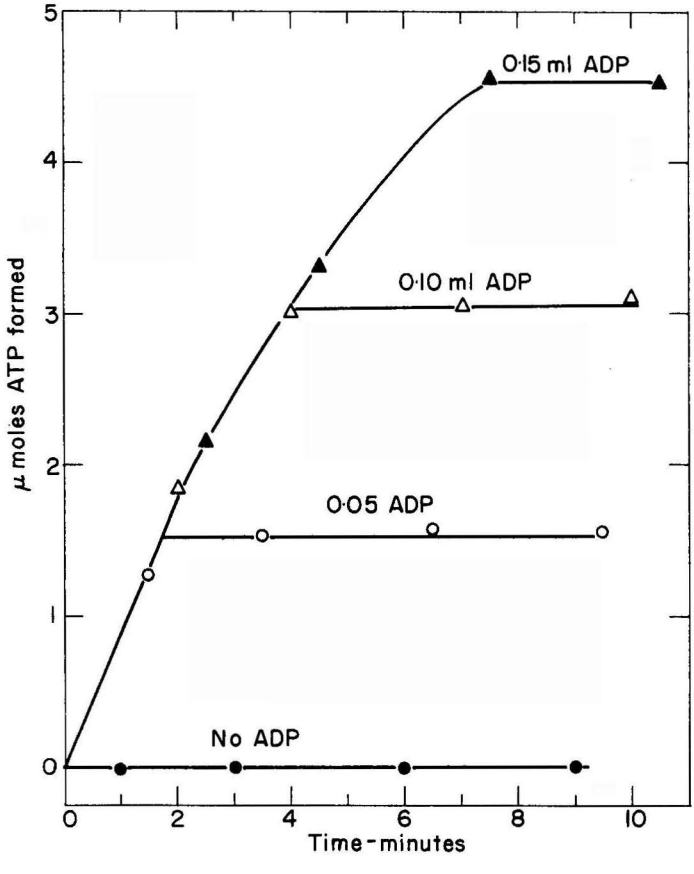

FIG. 1. The time course of utilization of several concentrations of ADP by fragmented chloroplasts. Reaction mixture as described under *Experimental*, except for variation in ADP content as indicated. The ADP solution added was 0.04 *M* with respect to its absorption in 260 mμ. Light intensity, 140,000 lux. Temperature, 19°C. Chloroplast fragments containing 15.9 μg chlorophyll/ml of reaction mixture.

preparations isolated from swiss-chard leaves. It also emphasizes the absolute specificity of the system for ADP, rather than adenylic acid (AMP), as the phosphate acceptor. These features make it possible to

synthesize γ-labeled ATP[2] (see below) as the product of the usual photophosphorylative reaction.

The complete esterification of ADP by swiss-chard chloroplasts is illustrated in Fig. 1. The figure also demonstrates the absolute requirement for ADP and the lack of any further incorporation of radioactivity

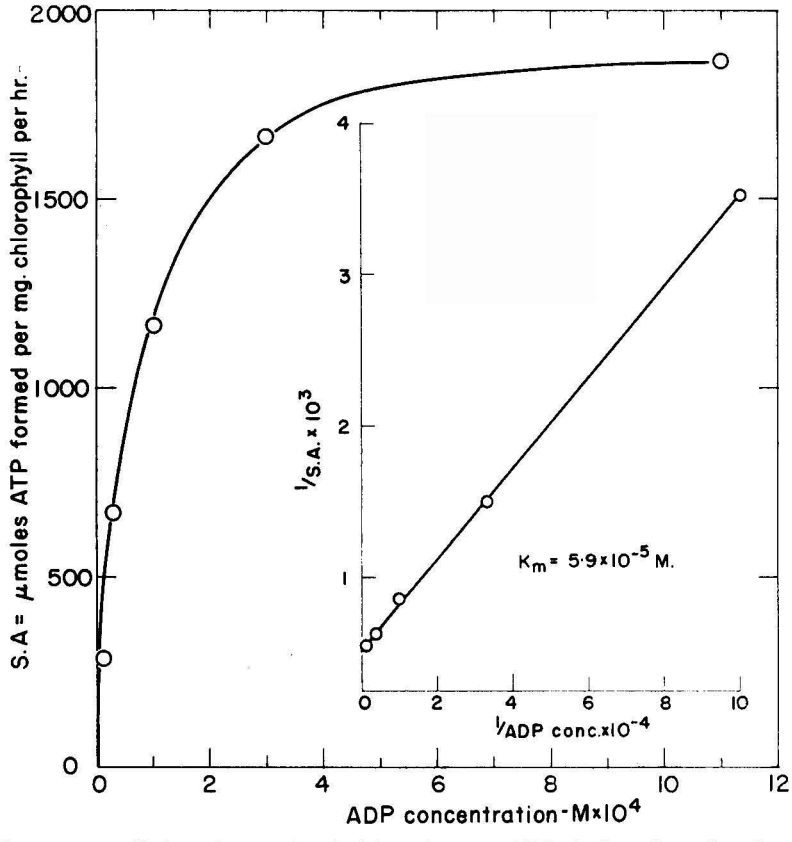

FIG. 2. The affinity of swiss-chard chloroplasts to ADP during photophosphorylation. Reaction mixture as described under *Experimental,* except for variation in ADP content as indicated, and the addition of 2 mg hexokinase and 60 μmoles glucose per cuvette. Reaction time, 2 min; light intensity, 140,000 lux; temperature, 17°C; once-washed chloroplasts containing 8.3 μg chlorophyll/ml of reaction mixture.

once the ADP has been esterified completely. In the presence of excess hexokinase and glucose, the affinity of the system for ADP could be determined (Fig. 2). The Michaelis constant varied in different experi-

[2] The phosphorus atoms of the polyphosphate chain are differentiated by the symbols α, β, γ, where α is the position of the phosphorus atom adjoining the sugar.

ments between 5 and 7×10^{-5} *M*. It is evident, therefore, that the ADP is almost totally converted to ATP by the photophosphorylative system. In separate experiments it was shown that with sufficient ADP the rate of ATP formation during 60 min of reaction was not affected by the addition of hexokinase and glucose. Competitive inhibition due to the accumulation of ATP as the reaction proceeded was therefore excluded.

The affinity of the system for inorganic phosphate is not high (8, 4). Determination of the Michaelis constant in ten separate experiments gave values around 1×10^{-3} *M* (range found: 5×10^{-4}–2×10^{-3}). It is, therefore, impractical to utilize more than about 50–75% of the total phosphate of the medium for the synthesis of labeled nucleotides.

The Synthesis and Analysis of Specifically Labeled ATP

This system makes possible the synthesis, by relatively simple procedures, of ATP labeled specifically in different positions. ATP labeled exclusively in the γ-position[2] was the product of the reaction when ADP was used as the phosphate acceptor. ATP labeled equally in the β and γ positions was isolated from a reaction mixture to which adenylate kinase was added, and in which the phosphate acceptor was AMP with a trace of ATP. ADP labeled in the β position was isolated after treatment of the latter ATP with hexokinase and glucose. The β-labeled ADP produced was rephotophosphorylated, in the presence of a large excess of cold inorganic phosphate, to provide β-labeled ATP. Each labeled nucleotide was isolated from the reaction mixture only at the end of the complete procedure.

For purposes of analysis, the final products were all treated with excess hexokinase and glucose. The reaction mixtures were separated on Dowex 1–formate columns, using gradient elution with ammonium formate as described by Hurlbert (9). The total counts/min in the glucose 6-phosphate (γ-phosphate of ATP), ADP ($\alpha + \beta$ phosphates of ATP), and inorganic phosphate were determined. The position of the peaks was determined by their 260-mμ absorbance (ADP), and by separation of organic (glucose 6-phosphate) from inorganic phosphate by the extraction procedure used for the determination of ATP (4). The radioactivity in the α-phosphate of the ATP was determined by hydrolyzing a sample of the ATP in 1 *N* HCl at 100°C for 10 min, and separating the resulting AMP from the inorganic phosphate by the same extraction procedure (4). The radioactivity in the AMP was then determined.

Table 2 shows the results, expressed as per cent of total radioactivity. It is evident that the γ-labeled ATP was almost exclusively labeled in its γ-phosphate. The isolation procedure used had removed all the inor-

TABLE 2
ANALYSIS OF SPECIFICALLY LABELED ADENOSINE POLYPHOSPHATES[a]

Desired product	Per cent of total radioactivity in			
	α-Phosphate	β-Phosphate	γ-Phosphate	Inorganic phosphate
$APPP^{32}$	0	3	97	0
$APP^{32}P^{32}$	0	49	51	0
$APP^{32}P$	0	99	1	0
APP^{32}	0	100	0	0

[a] Each reaction mixture contained, in μmoles: Tris, pH 7.8, 45; NaCl, 60; $MgCl_2$, 12; Na ascorbate, 30; Na, K phosphate, pH 7.8, 6.4 (containing 1.5×10^6 counts/min of P^{32}); phenazine methosulfate, 0.1; once-washed chloroplasts containing 51 μg chlorophyll, and water to a total volume of 3.0 ml. The following procedures were followed for the respective desired products: $APPP^{32}$, reaction mixture contained, in addition, 4 μmoles ADP. APP^{32}, $APP^{32}P$, $APP^{32}P^{32}$, reaction mixture contained, in addition, 6.4 μmoles phosphate, 4 μmoles AMP, 0.1 μmole ATP, and adenylate kinase as described under *Experimental.*

All reaction mixtures were placed in the light (140,000 lux), at 19°C for 10 min. Perchloric acid was then added to a final concentration of 3%, the suspension was centrifuged, and the $APPP^{32}$ and $APP^{32}P^{32}$ were isolated from the supernatant as described under *Experimental.* The supernatants from the cuvettes designed to produce APP^{32} and $APP^{32}P$ were adjusted to pH 7.8 with KOH, 20 μmoles glucose and 60 units hexokinase were added, and the reaction was allowed to proceed for 10 min. Perchloric acid was now added to a final concentration of 3%, the suspension centrifuged to remove the protein and $KClO_4$ precipitates, and the APP^{32} isolated as described under *Experimental.* The supernatant from the cuvette designed to produce $APP^{32}P$ was readjusted to pH 7.8 with KOH, and the $KClO_4$ precipitate was removed by centrifugation. To the supernatant were added 225 μmoles of Na,K phosphate, pH 7.8 (to dilute the specific activity of the inorganic phosphate approximately 1/50), 0.05 μmole of phenazine methosulfate, and a sample of the above chloroplasts containing 51 μg chlorophyll. The reaction mixture was placed in the light (140,000 lux) at 19°C for 10 min. Perchloric acid was added to a final concentration of 3% and the $APP^{32}P$ was isolated as described under *Experimental.*

For the analysis, a sample of each of the finally labeled adenine nucleotides was treated for 10 min at 20°C with 60 units hexokinase, 20 μmoles glucose, 15 μmoles Tris, pH 7.8; and 4 μmoles $MgCl_2$, in a total volume of 1.0 ml. The reaction was stopped by the addition of perchloric acid to a final concentration of 3%. The pH was adjusted to 6.0 with KOH, the precipitate was removed by centrifugation, and the supernatant was applied to the column for separation as described in the text.

ganic phosphate. The β- and γ-labeled ATP was equally labeled in its β- and γ-phosphates, as expected. The β-labeled ATP showed only 1% of the radioactivity located in the glucose 6-phosphate (γ-phosphate of ATP). The analytical procedure used was accurate to within approximately 1%.

In the case of the γ-labeled ATP, the product was rechecked for its radioactive distribution by hydrolyzing the γ-phosphate with a prepara-

tion of myosin ATPase and chromatographing the mixture on a Dowex 1–formate column as above. Ninety-six per cent of the radioactivity resided in the inorganic phosphate fraction and 4% in the ADP.

DISCUSSION

The method as presented provides for the synthesis of ATP labeled in its γ position, β position, or $\beta + \gamma$ positions, and of β-labeled ADP. With slight variations the method could also be used for the synthesis of a multitude of compounds which are produced by a reaction with ATP. We have synthesized, in this manner, glucose 6-phosphate^{32}, by adding to the reaction mixture ADP in catalytic amounts, with hexokinase and glucose. Many other sugar phosphates could be prepared in the identical manner due to the broad specificity exhibited by hexokinase. In general it can be said that many of the enzymes listed by Dixon and Webb (10) under the heading "Enzymes transferring phosphate groups" (*a*) and (*b*), could be used in conjunction with the photophosphorylative system to produce a large number of P^{32}-labeled substrates. It may be noted that even reactions which possess an unfavorable equilibrium constant could be used, since the reaction will be constantly "pushed" by the almost total removal of one of the products (ADP or AMP) from the reaction mixture to resynthesize a reactant (ATP).

Due to the relatively broad specificity of the reaction for the type of nucleoside–diphosphate supplied (4), we have also been able to synthesize P^{32}-labeled GTP, ITP, and UTP. CTP^{32} was also synthesized, but the yields were poor.

In a similar manner, the reaction was used for the synthesis of ATP labeled with O^{18} in its terminal phosphate[3] (11), and of glucose 6-phosphate labeled with O^{18} in the phosphate oxygen. Variations, in the manner discussed above, could again be used to yield other O^{18}-labeled compounds.

The synthesis of specifically labeled nucleotides could be carried out with all types of chloroplasts preparations (see Table 1). Also, the procedure described under *Experimental* for the preparation of the chloroplasts is not imperative, and simpler procedures, such as grinding the leaves with a mortar and pestle (8), can be employed. Ascorbate, however, has to be included in the homogenizing medium when swiss-chard chloroplasts are used. Chloroplasts prepared from spinach leaves may also be used, but since they contain a relatively large amount of adenylate kinase, they should be washed 5–6 times when used for the preparation of γ-labeled ATP.

[3] M. Avron and N. Sharon, unpublished observations.

The specific activity of the desired product is solely dependent on the specific activity of the inorganic phosphate added. Very high specific activities can, therefore, be obtained with relative ease. We have synthesized γ-labeled ATP with specific activities up to 200 μc/mole.

SUMMARY

A method is described for the direct synthesis of terminally labeled ATP^{32} with chloroplasts isolated from swiss-chard leaves.

By simple variations, both the central and terminal phosphates were equally labeled, or the central phosphate was exclusively labeled.

Coupling the reaction with other enzymes is suggested as a mode for the synthesis of many P^{32}-labeled compounds.

P^{32}-labeled GTP, ITP, and UTP were also synthesized by the same reaction. All the above compounds may also be synthesized labeled with O^{18} in their phosphate oxygens.

The advantages and disadvantages of the method, as compared with others, are discussed.

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A Study of Procedures for Isolation and Fractionation of Plant Cell Walls

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INTRODUCTION

A number of methods have been used for the preparation or isolation of plant cell walls for composition analysis (1–7). However, none of these procedures is entirely satisfactory for general application where quantitation of relations between cell walls and other cell components or structures is desirable. We have developed and studied alternative procedures for fractionation of plant cell materials to allow quantitative comparisons between cell wall constituents and other cellular components. These were (*a*) homogenizing the plant material in cold (0°) water with subsequent fractionation based on density gradient centrifugation; (*b*) homogenizing in anhydrous glycerol and isolation by fractional centrifugation; and (*c*) homogenizing in a mixture of ethylene glycol and glycerol (4:1) with subsequent isolation by fractional centrifugation. These procedures have been compared and evaluated for fractionation and isolation of components from small amounts of labeled plant material. Green leaves were chosen for the study since the green plastids could be used as a visual indicator of the efficacy of separation procedures and the leaves could be readily labeled in all parts through photosynthesis.

METHODS

The general outline of the evaluation procedure follows:

A. Preparation of a labeled plant material by photosynthesis in $C^{14}O_2$.

B. Coarse homogenization of the labeled plant in liquid nitrogen to obtain a uniform material which could be divided for the comparison treatments.

C. Further homogenization, extraction, and isolation of cell walls by each of four procedures described below.

D. Fractionation of each cell wall preparation through solubility characteristics.

E. Hydrolysis of cell wall fractions.

F. Chromatographic comparisons of activities in components of extracts and hydrolysates.

A complete description of each of these steps is given below.

Preparation of Labeled Plant Material

Oat plants (*Avena sativa,* var. *Siegeshafer*) were grown under constant light at 26°C until they attained a height of 80–100 mm. Forty leaves were harvested and weighed (1.8 gm). These were allowed to photosynthesize for 15 hr in a 1300-ml chamber containing $C^{14}O_2$ (12.5 mg $BaC^{14}O_3$ of 80 μc/mg activity). A single 150-w reflector flood lamp at a distance of 50 cm provided the illumination. The chamber was immersed in a water bath, maintained at 18–20°C, during the illumination.

Partial Homogenization and Sample Division

The labeled leaves were temporarily stored in an aluminum boat in a container of liquid nitrogen. They were transferred, a few at a time, into a glass, conical tissue grinder. The leaves were ground in this tube while suspended in a small amount of liquid nitrogen. As each addition of leaves was sufficiently compacted to a small volume by grinding, more leaves were added until half the leaves had been ground to a coarse powder. This homogenate was then roughly divided between four Potter type tissue homogenizing tubes of 5 ml capacity. The remaining leaves were similarly homogenized and distributed between a second set of four homogenizing tubes to provide duplicate samples for each of the four isolation treatments.

Isolation Treatments

1. *Hot Alcohol Extraction.* Since hot alcohol extraction prior to cell wall constituent analysis has been used frequently for plant materials (7, 8), this procedure was also included for comparison with the others.

The homogenizing tubes containing the ground samples were stored in Dry Ice until the liquid nitrogen had evaporated. Boiling 80% ethanol was then added to two tubes, and these samples were rapidly heated to boiling in a water bath. Each of these samples was thoroughly rehomogenized using close-fitting glass pestles. The extract was clarified by centrifuging and transferred to a volumetric flask through a capillary vacuum siphon. The samples were extracted a total of four times with boiling alcohol, each time rehomogenizing, centrifuging, and decanting by vacuum siphon. The alcohol extracts were combined and diluted to

volume for subsequent activity measurement and for chromatography. The sediment ("cell wall fraction") was finally suspended in absolute alcohol and retained for later treatment in the cell wall fractionation procedure.

2. *Cold Water, Density Gradient Isolation.* After evaporation of the liquid nitrogen from two samples in homogenizing tubes, 2 ml water at 0°C was added to each sample. Following a thorough rehomogenizing while cooling in an ice bath, 0.5 ml each of 1, 0.5, and 0.25 *M* sucrose solution was carefully layered under the homogenate. The cell wall fraction was obtained as a packed pellet by centrifuging in swingout cups at 700–1000 × *g* for 10–15 min. The remainder of the particulate material and the soluble fraction were separated from the cell-wall pellet by the full depth of the 0.5 *M* sucrose layer. After transferring the supernatant layers to a centrifuge tube by vacuum siphon, the pellet was rehomogenized with 2 ml of ice water, layered once more with sucrose solutions, and centrifuged. The supernatant layers were combined with the first extracts. The sediment was extracted three times with water, successively rehomogenizing, centrifuging without density gradient, and decanting by vacuum siphon. The sediment (cell walls) was suspended in absolute alcohol for later fractionation studies. The combined extracts and supernatant layers were centrifuged at 10,000 × *g* in plastic tubes to separate the particulate material from the water-soluble fraction. The plastid-particulate pellet was resuspended and diluted to a suitable volume for determination of activity. The water-soluble fraction was similarly diluted for determination of activity and for examination by chromatography.

3. *Glycerol Isolation.* For the glycerol procedure, tubes were chosen which had loosely fitting pestles. After the liquid nitrogen had evaporated, 2 ml glycerol was added. The material was rehomogenized as well as possible in the viscous medium while maintaining a temperature between 0 and 5°C. A slight twisting pressure was imparted to the tube during homogenizing to insure close contact at two points to aid in finer grinding. Considerably longer homogenizing time was required with glycerol than with the other solvents. The pestle was allowed to drain during several minutes, and the residual material was washed into the tube by allowing 1 ml glycerol to flow down over the slowly rotating shaft. Following centrifugation at 15,000 × *g* for 15 min (rubber adapter cups were used in an angle head), the supernatant material was carefully decanted by vacuum into a volumetric flask. The pellet was rehomogenized in glycerol, draining and washing the pestle as before. After centrifuging, the supernatant was combined with the first extract and diluted to a known volume with alcohol for subsequent chroma-

tography and activity determination. The high density of the glycerol medium did not allow direct separation of the plastid–particulate fraction although this could have been accomplished by dilution with water. The sediment was extracted three times with water at 0°, homogenizing, centrifuging, and vacuum decanting each time. The remaining sediment (cell walls) was suspended in absolute alcohol for later fractionation. The aqueous extracts were diluted to volume for activity determinations.

4. *Ethylene Glycol–Glycerol Isolation.* After evaporation of the liquid nitrogen, two tubes of the homogenate were each thoroughly rehomogenized at —20°C in 2 ml of a solvent consisting of four parts ethylene glycol and one part glycerol. Material adhering to the pestles was rinsed into the vessels with this solvent as described for the glycerol procedure. The suspension was centrifuged at $1500 \times g$ for 15 min, maintaining a temperature of —20°. Angle centrifuge heads were used. The supernatant solution was vacuum siphoned into a plastic centrifuge tube. The cell wall pellet was rehomogenized with more solvent and centrifuged once more. The second supernatant fraction was combined with the first and centrifuged at $8000 \times g$ to sediment the plastid–particulate fraction. The extract and the particulates were then diluted to convenient volumes for counting and chromatography. The cell wall fraction was extracted three times with water as in the glycerol procedure above. The aqueous extract was diluted for counting while the cell wall fraction was suspended in absolute ethanol for later fractionation.

Cell Wall Fractionation Procedure

The following procedure was adapted from previously published methods for plant cell wall fractionation (6, 9, 10). In this adaptation, the cell wall residue remained in its original container throughout the fractionation, and only solubilized material was removed for measurement.

1. *Lipid Extraction.* The cell wall residue, suspended in absolute alcohol in the homogenizing tubes and covered with glass marbles, was incubated for 10 min in an 80° water bath. After cooling, it was centrifuged and the alcohol was transferred to a graduated 12-ml centrifuge tube by suction. The pellet was resuspended in 2 ml of a 2:1 benzene–alcohol mixture and heated for 10 min in a 70° water bath. This extract was added to the alcohol extracts after centrifuging. The pellet was stirred with ethyl ether and centrifuged, and the ether extract was added to the previous extracts. The ether extraction was repeated, but the extract was discarded. The solvents were allowed to evaporate partially from the extracts before diluting to volume for activity determinations.

2. *Hot Water Extraction.* The pellet was extracted twice with 2 ml water in a boiling water bath during 30 min, centrifuging and vacuum decanting each time. The residue and tube were rinsed with 1 ml water The combined extracts, in a graduated tube, were retained for counting and hydrolysis.

3. *Dilute Acid Extraction.* The pellet was extracted three times with 2 ml of 0.05 *M* HCl, incubating for 20 min each time in a boiling water bath. The suspensions were centrifuged and decanted each time into a graduated tube as above. The residue was rinsed with 1 ml water, and the rinse was added to the acid extract.

4. *Sodium Chlorite Extraction.* The residue was treated with 2 ml water, 4 drops of 3% acetic acid and 15 mg $NaClO_2$ during 1 hr in a water bath at 75°C (7). Following centrifugal decantation of this extract, the residue was rinsed twice with 1 ml water, and the rinse was combined with the original extract.

5. *Dilute Alkali Extraction.* The pellet was extracted for 2 hr with 1.5 ml of 4% NaOH at room temperature. After centrifuging and decanting, the residue was rinsed with 0.5 ml of 4% NaOH.

6. *Four N Alkali Extraction.* The residue was extracted overnight with 1.5 ml of 17.5% NaOH at room temperature. After removing the alkali, the pellet was rinsed three times with 0.7 ml water each time. The rinses were added to the original extract. The residue was washed twice with 2 ml of absolute alcohol and once with 3 ml ether. These organic solvents were discarded, and the pellet was allowed to dry.

7. *Cellulose Extraction.* The pellet was extracted once with 1 ml and once with 0.5 ml of acetolyzing reagent, freshly prepared from 5 ml acetic anhydride, 5 ml of glacial acetic acid, and 0.25 ml of concentrated sulfuric acid (11). Each extraction was carried out for 20 min in a boiling water bath.

In the extraction procedures above, glass marbles were used to cover the tubes during extraction except in the alkali extractions where rubber stoppers were used. All the extracts were frozen until required for further processing.

Hydrolysis of Cell Wall Fractions

Several of the fractions above contained material which might interfere in the hydrolysis or subsequent chromatographic examination of the hydrolyzates. Accordingly, the alkali, chlorite, and cellulose fractions were dialyzed prior to hydrolysis. The aqueous extracts obtained in the glycerol or glycerol–glycol isolation procedures were also dialyzed. Duplicate 2-ml portions of each of these fractions (except the cellulose extract) were placed in ¼-in. diameter Visking dialysis tubing. One

milliliter of the cellulose extract was diluted by adding it to 1 ml water already in the dialysis bag. Each bag was dialyzed against three changes of 20 ml water in individual tubes, rocking the tubes for 4 hr on each change. The dialyzing medium was assayed for radioactive material in each case. The dialyzed fractions, and 2-ml aliquots in duplicate of the fractions which had not required dialysis, were transferred to 18-mm test tubes. An equal volume of concentrated hydrochloric acid was added to one tube from each fraction to give 6 *N* HCl, while 0.17 ml of concentrated hydrochloric acid was added to the duplicate samples in each case to give 1 *N* HCl. All tubes were sealed and heated in steam, the 1 *N* acid samples for 4 hr, the 6 *N* acid samples for 8 hr. The tubes were opened and dried *in vacuo* over KOH at room temperature. Each sample was taken up in 0.1 ml water for chromatographic analysis.

Chromatographic Procedures

All chromatography was carried out on S & S No. 589, White Ribbon, filter paper. Solvents used were butanol–acetic acid–water (52:13:34), ethyl acetate–pyridine–water (5:2:5), and water-saturated redistilled phenol.

The original solvent extracts obtained during the isolation procedures were chromatographed two-dimensionally using the butanol-acetic acid and the phenol solvents. All the cell wall hydrolyzates and other fractions were chromatographed one-dimensionally in two separate solvents, ethyl acetate–pyridine and butanol–acetic acid. Radioautograms were prepared from the chromatograms by exposure to 14 × 17 in. "no-screen" x-ray film.

Components of hydrolyzates were identified by position with respect to known standards applied in side strips on the chromatograms.

Activity Determinations

Aliquots of the extracts were placed directly on stainless steel planchets, dried, and counted using a Nuclear-Chicago D-47 gas-flow counter with a thin Mylar window. The high level of activity in the samples allowed counting to be carried out with infinitely thin layers, so no corrections were required other than for background and coincidence. The solubilized fractions from the cell walls were treated similarly by plating suitable amounts directly. Corrections were applied for self-absorption in the case of the alkali and cellulose extracts.

The total activity of the original samples was taken as the sum of the activities of all fractions since difficulties in taking aliquots from the original homogenates made it impossible to obtain comparable total activities of the original samples.

On the chromatograms, the area corresponding to the exposed spot on the radioautograms was cut out and counted on one or more planchets, depending on the size of the spot. Where two or more planchets were required for a single component, the activity was taken as the sum of the individual activities, after appropriate background and coincidence corrections.

Amino Acid Carboxyl Activity Determination

Comparisons of amino acid (protein) content of cell wall fractions were made by determining the activity in the carbon dioxide produced by reaction of the amino acids of the hydrolyzates with ninhydrin. The amino acid areas of the ethyl acetate–pyridine chromatograms of the hydrolyzates were cut out and placed in the bottom of a 20-ml vial. The ninhydrin solution, 0.2 ml of a 1% solution of ninhydrin in 0.05 *M* citric acid, was introduced. The vial was plugged with a special polyethylene cap which contained, in the lower portion, a packet of charcoal enclosed in filter paper and, in the upper part, a disk of filter paper wetted with NaOH solution. The lower part of the vial was placed in a boiling water bath and heated for 30 min. Excess pressure during heating was allowed to escape through pin holes in the top of the cap. The carbon dioxide was absorbed by hydroxide in the filter paper, and the charcoal prevented contamination of the filter paper by the activity of volatile aldehydes.

The filter paper disk was allowed to dry and counted immediately as described above.

RESULTS

The effect of different isolation procedures on the distribution of activity into various cell fractions is shown in Table 1. Three procedures give rise to similar amounts of activity in the isolated cell wall fraction, while the fourth, the hot alcohol treatment, is at great variance with the others. The lack of agreement in the hot alcohol treatment might be expected since the "cell wall" fraction, in this case, would also contain all polysaccharide and protein material from cytoplasm, plastids, and other particulates.

Good agreement is found for three treatments in the results of solubility fractionation of the cell walls (Table 2), the hot alcohol isolation again being an exception. From such results it appears that the cell walls isolated by the three procedures, cold water extraction, glycerol extraction, or glycerol–glycol extraction, might be comparable in nature and composition.

Figure 1 is a graphic representation of the relative carbohydrate com-

TABLE 1
EFFECT OF CELL WALL ISOLATION PROCEDURES ON DISTRIBUTION OF ACTIVITY IN OAT LEAF CELL FRACTIONS

Isolation treatment	Fraction activities[a]				
	Solvent soluble (%)	Plastid–particulate (%)	Cold-water soluble (%)	Lipids (%)	Cell walls[b] (%)
Alcohol	73.2	—	—	0.19	26.4
	76.2	—	—	0.13	23.6
Aqueous, gradient	—	13.0	75.2	0.60	9.8
	—	13.6	76.6	0.51	9.4
Glycerol	87.1[c]	—	3.06	0.51	9.3
	85.6	—	3.32	0.65	10.4
Glycol–glycerol	74.3	16.4	1.8	0.13	7.5
	69.9	16.1	4.4	0.22	9.4

[a] Activity as per cent of total found in all fractions.
[b] Cell wall activity is sum of all cell wall fractions after alcohol extraction.
[c] Glycerol-soluble fraction also contains plastid–particulate material.

position of the cell wall fractions. The activities of the four sugars, xylose, arabinose, glucose, and galactose, are plotted as per cent of the sum of the activities of these sugars. The other major sugar component, galacturonic acid, was not included in this chart since its activity could not be quantitatively distinguished from the activity of amino acids which appeared in the same areas on the chromatograms. Only sugar activities were plotted since the large amount of amino acid activity in the alcohol system tended to obscure comparisons of the carbohydrates otherwise. The amino acid (protein) content is treated separately in Table 3. The sugar composition of the hydrolyzates for the cold water

TABLE 2
DISTRIBUTION OF ACTIVITY IN FRACTIONS FROM CELL WALLS

Isolation procedure	Cell wall fractions[a]					
	Hot-water soluble (%)	Dilute HCl soluble (%)	Chlorite soluble (%)	Dilute NaOH soluble (%)	4 *N* NaOH soluble (%)	Cellulose (%)
Alcohol	18.2	33.7	8.3	19.7	6.4	13.6
	20.6	33.4	8.1	18.2	7.6	11.9
Aqueous	3.3	41.7	5.0	10.1	11.2	27.5
	3.3	40.5	4.9	8.6	13.8	26.6
Glycerol	4.0	45.2	4.7	10.7	6.7	29.1
	4.4	44.2	5.3	12.5	5.7	27.9
Glycol–glycerol	3.9	41.3	5.1	11.1	11.1	26.7
	4.2	42.6	5.2	10.3	10.2	27.7

[a] Activity as per cent of total activity found in cell wall preparation.

and the glycol isolations show reasonable agreement for most fractions. Some differences appear in glucose content of the dilute acid-soluble fraction. Glycerol-isolated cell walls differ from the others in that the hot water-soluble fraction is higher in xylose and arabinose, lower in glucose. They also differ from the cell walls prepared in cold water in

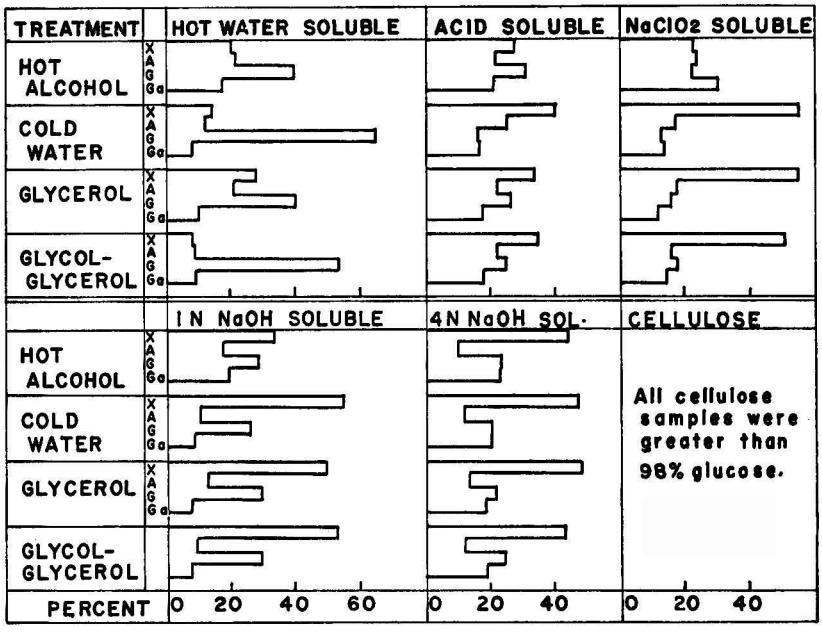

FIG. 1. Distribution of activity in four sugars from cell wall fraction hydrolyzates. The activities of the four sugars, xylose, arabinose, glucose, and galactose, in each fraction hydrolyzate were added to give a total activity. The length of the bar for each sugar represents the per cent of this total activity. Activity due to amino acids could not be completely separated from galacturonic acid, so the galacturonic acid was omitted from these data.

X = xylose; A = arabinose; G = glucose; Ga = galactose.

the composition of the dilute acid-soluble fraction but correspond closely to the glycol-isolated cell walls in this same fraction. Otherwise, the three systems result in cell wall fractions having very similar sugar compositions. The alcohol isolation system again gives results differing greatly from the others except in the 4 *N* alkali-soluble fraction and in the cellulose (glucose was the only sugar showing significant activity in all cellulose preparations).

The relative amount of protein in the various fractions was studied by determining the amount of activity in carbon dioxide obtained by decarboxylation of the amino acids in fraction hydrolyzates (Table 3).

TABLE 3
AMINO ACID CONTENT OF CELL WALL HYDROLYZATES

Treatment	Ninhydrin CO_2 Per cent of total fraction activity			
	Alcohol (%)	Water (%)	Glycerol (%)	Glycol–glycerol (%)
Fraction				
Hot water soluble	3.9	0.73	0.53	0.15
0.05 *N* HCl soluble	9.1	1.1	2.7	1.4
$NaClO_2$ soluble	10.5	3.7	3.4	1.4
1 *N* NaOH soluble	13.3	0.90	2.9	1.8
4 *N* NaOH soluble	2.3	0.33	0.68	0.49

Differences in amino acid activity do occur among the treatments. The differences occurring in the chlorite and alkali fractions among the three better systems might be significant. Thus the glycerol and the glycol isolation treatments tend to give cell wall preparations which contain greater amounts of difficultly soluble protein.

Another area in which some differences occur is demonstrated in Table 4 which shows the distribution of activity in a few of the com-

TABLE 4
DISTRIBUTION OF ACTIVITY IN SOME OF THE COMPOUNDS SOLUBLE IN THE ISOLATION MEDIA

Treatment	Per cent of activity[a]		
	Alcohol	Cold water	Glycol–glycerol
Sucrose	17.6	18.1	19.8
Glucose	30.8	41.6	33.3
Fructose	1.1	2.6	0.9
Phosphate esters	2.3	0.9	2.1
Unidentified[b]	—	0.3	—
Origin	0.6	9.6	6.6

[a] Per cent of total activity in soluble fraction. Values are averages of the activity distribution found in the duplicate isolation systems.

[b] An unidentified active component which was present only in the aqueous system extracts. Its chromatographic position was close to but not identical with that of glutamine.

ponents which were soluble in the media used for the isolations. The extracts from the glycerol isolation system were omitted here since the large quantity of glycerol on the chromatograms caused streaking and interfered with proper determination of activity of the individual components. It may be seen that fructose and glucose are somewhat higher in the cold water sample than in the others. The phosphate ester activity is considerably lower in this sample, however. The cold water extract

also contained small amounts of activity in a component which could not be found at all in any of the other samples. All these factors suggest that the cold water procedure allows some of the hydrolytic enzymes to operate during the isolation, and sucrose, phosphate esters, and possibly some soluble glucosans have been hydrolyzed to some extent. This is in line with previous experience (12) which shows that phosphatases may be active at very low temperatures.

Both the cold water and the glycol systems contained relatively large amounts of activity in material which did not move from the origin upon chromatography. These substances are probably polysaccharide or protein in nature, and their presence in the glycol medium suggests that this solvent system tended to retain the soluble cytoplasmic proteins and polysaccharides in solution rather than precipitating them on the particulate materials during the preparation.

Chromatographic examination of the plastid–particulate fractions from the cold water and the glycol isolations revealed little other than nonmobile polysaccharide or protein activity. Very small amounts of glucose and sucrose were also found, to the extent of about 5% of the total activity. The cold aqueous extract of the glycol-isolated cell walls contained only polysaccharide–protein activity in one case, but in the other, having a higher total activity (Table 1), glucose and sucrose were found. This indicates that the second sample had not been properly extracted in the original glycol solvent.

DISCUSSION

It is quite apparent from these results that the use of hot alcohol extraction in the preparation of material for the study of cell wall composition is open to severe question. This is not a novel conclusion, yet such a procedure has a fairly extensive history of application for just this purpose. Such use would have to be based on the premise that only the carbohydrate (polysaccharide) composition of the isolated material would be studied, and the coprecipitated material would not add significantly to the polysaccharides of the cell wall nor affect the solubility characteristics of these polysaccharides during fractionation. However, it is obvious from the activity distributions in the hydrolyzates of cell wall fractions (Fig. 1) that, in oat leaf tissue at least, the carbohydrate composition of the fractions may be profoundly affected by the adherent material. This might not be so in all tissues but one should not lose sight of the possibility.

Most workers in the field of cell wall metabolism have turned to isolations based on disruption and extraction of the tissues in ice-cold water. This procedure is adequate for most purposes, but it also has

potential drawbacks. There is the possibility, even at low temperatures, that enzyme activity may alter composition in some manner. Further, the usual procedures with aqueous isolations do not result in recovery of the entire cell wall fraction. Thus, the quantitative relation with the remainder of the cellular constituents is destroyed. The latter objection is apparently largely overcome by the gradient centrifugation technique of cell wall isolation used here. Despite the use of a grinding technique which resulted in the formation of a large proportion of very small cell wall fragments, microscopic examination indicated that these were found along with the larger pieces while plastids were not sedimented. The total activity found in the cell wall fraction isolated by gradient centrifugation checked very well with the activity in the fraction obtained by two other procedures, giving further indication that this procedure is potentially quantitative.

Evidence presented here, however, does suggest that limited hydrolysis of several components may occur during aqueous isolation. Sucrase activity is indicated by the higher activity found in free fructose in the extracts from aqueous isolation (Table 4). Phosphate ester activity is reduced in the aqueous extracts compared with alcohol or glycol extracts, indicating some phosphatase activity. This is in agreement with previous studies which have shown that phosphate esters may be hydrolyzed even at low temperatures in alcoholic solutions (12). Whether the limited hydrolysis observed here would be of importance depends on the nature of the investigation. The aqueous isolation procedure is advantageous because of its relative simplicity.

The use of anhydrous media (1) has the advantage of minimizing enzyme activities during the isolation. Anhydrous solvents may, however, cause protein denaturation to the extent that proteins will precipitate on particulate material or even cause agglomeration of various particulates. Experiments preliminary to the work above showed that egg albumin solutions, introduced into ice-cooled glycerol, precipitated only slowly, more rapidly in ethylene glycol, and very rapidly in ethanol. Cooling the glycol further to —20° and adding ¼ to ⅕ vol glycerol greatly slowed or stopped completely the precipitation of the albumin The data presented here indicate that coprecipitation of proteins on the cell walls during isolation in the glycerol–glycol medium is not a serious problem. The composition of the cell walls isolated in this medium compares quite favorably with the composition of cell walls isolated from an aqueous system. Enzyme activity is apparently largely suppressed by the use of this system. Within certain limits, it should be possible to vary the glycerol content of this medium to give a density most favorable for the material being fractionated. No study was made

of this aspect since the initial medium gave good results with the oat leaves.

Pure cell walls have been isolated previously in a glycerol medium (1). This earlier study is supported by the results here, in that cell walls of good definition may be obtained. The glycerol-isolated cell walls have compositions quite similar to those isolated either in water or in ethylene glycol. However, the quantitative analysis is made much more difficult when glycerol is used because of the problems which arise in connection with its high viscosity. It seems best to reserve its use for studies in which quantitative recovery of fractions is not essential.

The ethylene glycol procedure has been applied successfully to cell wall studies in oat coleoptiles since the investigations above were carried out.

SUMMARY

Three procedures have been developed and tested for quantitative isolation and fractionation of plant cell walls in tracer experiments using relatively small amounts of plant material. *Avena* seedlings photosynthetically labeled with C^{14} were used as the test material.

A cold water extraction at 0°C with an isolation based on a sucrose density gradient centrifugation was found to give well-defined fractions but appeared to allow slight hydrolysis of some components during isolation. Extraction in anhydrous glycerol at 0–5°C with differential centrifuging also gave rise to well-defined cell wall fractions but was subject to many technical difficulties arising from the high viscosity of the isolating medium. Isolation carried out in a solution of glycerol in ethylene glycol (1:4) at —20°C with differential centrifuging allowed excellent fractionation without apparent hydrolysis.

Isolations and fractionations of the cell wall materials were carried out entirely by centrifugation techniques in the original homogenizing tubes, thereby avoiding transfer losses. Thus the procedures are well adapted for use in tracer experiments where limited amounts of plant material are used.

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A Method for Measuring the Microbiological Activity of Tetrahydrofolic Acid and Other Labile Reduced Folic Acid Derivatives

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INTRODUCTION

The usual folic acid microbiological assay is not suitable for the determination of tetrahydrofolic acid (THFA) or for a number of other reduced derivatives of folic acid. Many of these compounds are very unstable and, if unprotected, are almost completely inactivated during the autoclaving and incubation steps. Since THFA is present in or may be liberated from natural materials and since metabolic studies of folic acid often involve this reduced form, it would be of value to achieve a satisfactory microbiological assay for THFA.

Silverman and co-workers (1) were able to determine anhydrocitrovorum factor and N^{10}-formyl-THFA in urine microbiologically by protecting them with ascorbic acid. Furthermore, they have shown that THFA may be detected in metabolic reaction mixtures by using "aseptic" assay conditions and a suitable reducing agent such as ascorbate (2). The present paper deals with the development of a method for the measurement of the microbiological activity of THFA and the effect of various factors on this activity.

METHOD

Except for the indicated modifications for the "aseptic plus ascorbate" assay, standard routine microbiological assay procedures were used. The assay organisms were *Pediococcus cerevisiae* (*Leuconostoc citrovorum*) ATCC 8081, *Streptococcus faecalis* ATCC 8043, and *Lactobacillus casei* ATCC 7469. Bacterial growth was measured turbidimetrically (660 mμ filter) after 16–20 hr incubation (20–24 hr for *L. casei*). The media used for *L. casei* and *S. faecalis* were essentially those described by Teply and Elvehjem (3). For *S. faecalis* the glucose level was halved

and the sodium citrate was replaced by sodium acetate at a level of 6.65 gm/l of double-strength medium. The medium used for *P. cerevisiae* is a modification of that used by Snell *et al.* (4). The concentration of salts and Tween is reduced by half, the casamino acids are present at double the concentration, xanthine and folic acid are present at concentrations of 20 mg and 20 mμg, respectively, per liter of double-strength medium, and oleic acid is omitted.[1]

The stock solution of citrovorum factor[2] (CF; 5-formyl-5,6,7,8-tetrahydrofolic acid) used as reference standard contained 10 μg Ca citrovorum factor/ml in 30% ethanol made alkaline with ammonia (0.1 ml concentrated NH_4OH/100 ml) and was stored at 5°C.

The THFA used for most of the work was kindly supplied by Dr. Herbert Tabor. It was prepared by the catalytic reduction of folic acid in glacial acetic acid (5). Dr. Tabor assayed it by converting it enzymically to 5,10-methenyltetrahydrofolic acid and measuring the optical density at 350 mμ (6).

A stock solution of this sample, containing 75 μg THFA and 50 mg ascorbate[3]/ml was used as reference standard for the microbiological assays. It was stored at —15°C. Although DL-THFA was used throughout, values for the concentration of THFA refer to the amount of L-THFA present, since this is the isomer which is active microbiologically.

The assays were set up in one of two ways: "aseptic plus ascorbate" and "regular." For the former the standard solutions may be either CF or THFA, depending on the nature of the samples to be assayed. The working standards are prepared by diluting the stock solutions to contain 5 mμg/ml. Aliquots may be stored safely at —15°C for as long as 1 month. When diluting the working standard and also the samples to be assayed, ascorbate is added to provide a final concentration of 6 mg/ml.

Five milliliters of assay medium and 4 ml water are added to each

[1] Commercial sources of CF medium should be suitable for the measurement of THFA, provided the initial pH is adjusted to 6.3. One was tried (Difco) and gave satisfactory results although activity of THFA relative to CF was less than in the medium described above.

[2] Isolated from horse liver and kindly supplied by Dr. John C. Keresztesy and Dr. Milton Silverman.

[3] Throughout this paper the term "ascorbate" shall refer to a solution of potassium ascorbate at pH 6.0 and, unless otherwise indicated, at a concentration of 6 mg/ml (calculated as the free acid). It is prepared by neutralizing a solution of ascorbic acid with potassium hydroxide to pH 6.0 and diluting with water to a concentration of 100 mg ascorbic acid/ml. Portions are stored in test tubes in the freezer.

assay tube and autoclaved. After cooling, standard and samples are added aseptically, together with enough ascorbate, 6 mg/ml, to bring the total volume of solutions added to each tube after autoclaving to 1 ml. Thus each assay tube will contain 6 mg ascorbate in a total volume of 10 ml. It is important to add the ascorbate to the assay tubes before adding the standard or samples. The assay is then inoculated. Unless otherwise indicated, samples of THFA were assayed by this procedure using *P. cerevisiae.*

For the "regular" assay the ascorbate was omitted and the assay tubes were autoclaved following addition of the CF reference standard and samples.

EXPERIMENTAL RESULTS

THFA vs. CF for P. cerevisiae

When a solution of THFA, unprotected by reducing agents, is assayed on a regular CF assay, it has very little activity for *P. cerevisiae.* A solution containing 6 mμg THFA/ml possessed less than 0.5 mμg of CF activity/ml using CF as a standard. However, when THFA is substituted for CF in the presence of a reducing agent as in the "aseptic plus ascorbate" assay, it is active for *P. cerevisiae* (2). The THFA has less

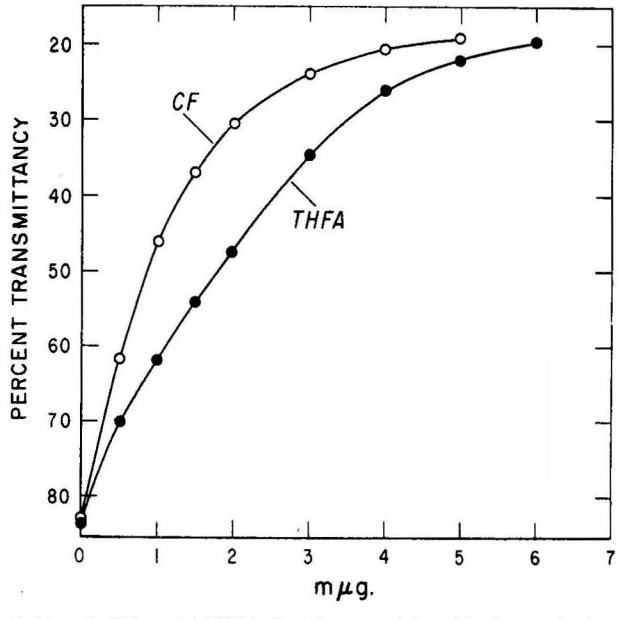

FIG. 1. Activity of CF and THFA for *P. cerevisiae.* Evelyn colorimeter (660-mμ filter).

relative activity with respect to CF at the lower levels than at the higher levels ("positive drift") (see Fig. 1). This makes it difficult to get quantitative results in the measurement of THFA when CF is used as a standard. When the folic acid activity of the samples is due only to THFA and when THFA is used as a standard, drift does not occur. Thus when samples contain either THFA or CF, good quantitation is possible by using as the standard the same folic derivative as is present in the sample. When both THFA and CF are present in the same sample, neither CF nor THFA is suitable as a standard. One way of circumventing the difficulty would be to convert the THFA to CF. This was

TABLE 1

ACTIVITY OF THFA BEFORE AND AFTER CHEMICAL CONVERSION TO CF

Before conversion to CF			After conversion to CF		
THFA added per tube (mμg)	CF activity found[a] (mμg)	CF activity/mμg THFA	Reaction product added (mμg/tube)[b]	CF activity found[a] (mμg)	CF activity/mμg reaction product
1.2	.50	0.42	0.92	0.90	0.98
2.4	1.35	0.56	1.84	1.80	0.98
3.6	2.25	0.63	2.76	2.80	1.02
4.8	3.50	0.73	3.68	3.75	1.02
6.0	4.60	0.77	4.60	4.60	1.00

[a] Values are for "aseptic plus ascorbate" assay using *P. cerevisiae* with CF as the standard.

[b] Assuming complete conversion to CF.

done by formylating THFA with formic acid to form N^{10}-formyl-THFA followed by conversion to N^5-formyl-THFA (CF) by heating in the presence of ascorbic acid (7). For assay the solution was diluted to contain the equivalent of 4.33 mμg THFA/ml. This, together with a solution of the original unformylated THFA diluted to contain 6 mμg THFA/ml, was assayed in duplicate four different times on the "aseptic plus ascorbate" assay using CF as a standard. Conversion to CF was essentially quantitative (103% of theoretical). The results of a representative assay are presented in Table 1.

Effect of Modifying Assay Conditions

Although the conversion of THFA to CF enables one to achieve good quantitation with samples containing both THFA and CF, this would not be feasible for samples of low concentration and is not practicable for routine assays. Thus a study was made to find conditions under which the growth response of *P. cerevisiae* was equivalent for CF and THFA. In the following series of experiments a solution containing

6 mμg THFA/ml was assayed in duplicate against CF as a standard at five different levels of sample: 0.2, 0.4, 0.6, 0.8, and 1.0 ml. The "aseptic plus ascorbate" method was used, with assay conditions modified as indicated below. Values are expressed as mμg of CF activity/ml obtained by averaging the values obtained for the five levels.

Effect of pH of the Medium. The pH of the assay medium, which is ordinarily 6.3 for *P. cerevisiae*, was adjusted to several pH values with the following assay results:

pH of medium:	7.1	6.3	5.8	5.3	4.8
CF activity (mμg/ml):	1.4 0.9	3.2 3.2	4.7 4.6	4.9 4.8	4.4 4.4
"Positive drift":	—	Marked	Slight	None	None

Although "positive drift" was absent and equimolecular activity was approached at pH 5.3, the dose–response curve for CF at that pH was not as steep as at 6.3. At the latter pH the greatest slope was obtained. pH levels of 5.0, 5.2, 5.4, and 5.5 gave values approximately the same as those for pH 5.3. Examination of all assays indicates that a pH of 5.5 results in the best compromise with respect to absence of drift, equimolecular activity, and slope of dose–response curve for CF, yet does not result in equivalent growth with CF and THFA.

Effect of Temperature of Incubation. To determine the effect of temperature of incubation (ordinarily 37°C for *P. cerevisiae*), various temperatures were tried with the following results:

Temperature of incubation (°C):	30	37	42
CF activity (mμg/ml):	4.4 4.5	3.7 3.7	2.3 2.1
"Positive drift":	Marked	Marked	Marked

Although the relative activity of the THFA is greatest at 30°C, the dose–response curve for CF at that temperature is not as steep as at 37°C; i.e., the lower temperature of incubation depresses the growth in the tubes containing CF.

An increase in the ascorbate concentration to 12 mg/tube or incubation of the assay tubes in an atmosphere of helium did not increase the relative activity of THFA with respect to CF.

Since, under the experimental conditions investigated, equivalence could not be obtained for *P. cerevisiae* with CF and THFA, it is not desirable to use CF as the standard exclusively.

Stability of THFA

Since THFA is very unstable, it was necessary to determine whether a sample of THFA used as a standard for assay could be stored so that

it would retain its microbiological activity. Solutions containing various concentrations of THFA and ascorbate were stored at —15°C for 15 months and assayed from time to time. Those containing 1.5 μg/ml or more of THFA protected by 6 mg/ml or more of ascorbate had full activity at the end of the 15-month period. Very dilute concentrations of THFA (as little as 6 mμg/ml) were stable after 1 month at —15° when protected with 6 mg/ml ascorbate. As shown in Table 2, the same concentration of ascorbate will protect THFA for a period of 6 hr at 5°C and also at 23°C. The THFA solution employed as a reference standard retained its activity relative to CF during the course of the 15-month period.

TABLE 2
AMOUNT OF ASCORBATE NECESSARY TO PROTECT THFA[a]

	Time and temperature of storage							
	6 hr 5°C		6 hr 23°C		24 hr 5°C		24 hr 23°C	
Ascorbate (mg/ml)	THFA activity (mμg/ml)	% loss	THFA activity (mμg/ml)	% loss	THFA activity (mμg/ml)	% loss	THFA activity (mμg/ml)	% loss
0	0.6	90	<0.6	>90	<0.6	>90	<0.6	>90
1	1.0	83	1.2	80	0.8	87	0.8	87
2	2.6	57	2.2	63	1.3	78	0.9	85
3	3.6	40	3.4	43	2.2	63	1.7	72
6	5.9	2	5.7	5	4.7	22	3.5	42

[a] THFA solution containing 6 mμg/ml.

The effect of various types of treatment on the stability of a solution of THFA containing 6 mμg/ml in ascorbate, 6 mg/ml, was studied by subjecting portions of it to the following: (*a*) storage for 2 hr at room temperature and also at 37°C; (*b*) shaking the solution in a test tube for 10 sec in the presence of air; (*c*) heating in a 100°C water bath for 3, 10, and 20 min; (*d*) heating in flowing steam for 20, 40, 80, and 120 min. There was no loss of microbiological activity for *P. cerevisiae*. Indeed, those solutions which were subjected to the heat treatments had greater stability on storage than the untreated samples. Two aliquots of the THFA solution which had been heated in flowing steam for 80 min and two which had been unheated were stored at 5°C and assayed after 1 day and again after 5 days. After 1 day's storage the unheated sample showed a 32% loss in activity, whereas the heated sample had a 5% loss. After 5 days at 5°C the losses in activity were 82 and 32%, respectively. A possible explanation for the greater stability of the THFA after heating of the solution is that dissolved oxygen is lost and therefore the rate of oxidation of the THFA is reduced.

The stability of THFA in ascorbate to repeated thawing and freezing was tested at relatively high concentrations of THFA and ascorbate (300 mμg/ml and 80 mg/ml, respectively) and also at relatively low concentrations (6 mμg THFA/ml and 6 mg ascorbate/ml). Aliquots in test tubes were thawed and refrozen daily for 5 days. After thawing, the contents of each tube were mixed (by inverting) and kept for several minutes at room temperature before refreezing. They were assayed at the end of the 5-day period. At the higher concentration there was no detectable loss in activity. At the lower concentration there was an 8% loss in activity. A second trial showed no detectable loss at the higher concentration and a 14% loss at the lower concentration.

A problem resulting from the marked instability of THFA is indicated by the following. Three samples of THFA obtained at different times from a commercial source were assayed. They were labeled as containing 250 mg DL-THFA/ml stabilized with 0.1% mercaptoethanol and sealed under nitrogen. The microbiological activity present in each of the solutions was only one-fourth of that expected from the designated value. The low value found was not due to the presence of inhibitory substances. This was checked by adding the reference THFA to the THFA from the commercial source and assaying the combination.

Response of Other Organisms

In order to determine the relative activity of THFA and CF for other organisms, a solution containing 5 mμg THFA/ml was assayed with "aseptic plus ascorbate" technique against CF as a standard using

TABLE 3

ACTIVITY OF THFA FOR *P. cerevisiae*, *L. casei*, AND *S. faecalis*[a]

THFA added (mμg/tube)	CF activity found[b] (mμg)			mμg CF activity per mμg THFA		
	cerevisiae	*casei*	*faecalis*	*cerevisiae*	*casei*	faecalis
1	0.49	0.79	0.61	0.49	0.79	0.61
2	0.96	1.34	1.26	0.48	0.67	0.63
3	1.82	2.34	1.91	0.61	0.78	0.64
4	2.82	3.01	2.66	0.70	0.75	0.66
5	3.78	3.74	3.20	0.76	0.75	0.64

[a] Initial pH of medium was 6.3, 6.8, and 6.8, respectively; temperature of incubation was 37, 37, and 30°C, respectively. CF was used as the reference standard.

[b] Each value represents average of four replicates.

P. cerevisiae, *L. casei*, and *S. faecalis*. The indicated pH of the medium and temperature of incubation are those ordinarily used with these organisms. As shown in Table 3, there was no "positive drift" with *L. casei* and *S. faecalis*. However, equimolecular activity of CF and

THFA still was not obtained. In comparing results of any two organisms consideration should be given to the other variables involved (pH and composition of the medium and temperature of incubation). Results would be affected by these factors as well as by the difference in organisms. *S. faecalis* and *L. casei* respond not only to CF and THFA, but also to other forms of folic not reduced to the tetrahydro level.

DISCUSSION

In 1951 Broquist *et al.* (8) tested the activity of THFA for *P. cerevisiae* and found it only 2.5% as active as leucovorin. In 1954 Steinman *et al.* (9) found DL-THFA only 5% as active as L-leucovorin for *Treponema pallidum* (strain S-60). Although they added the THFA aseptically after the assay tubes had been autoclaved, no reducing agent was present. This would account for the low activities found.

Samples for the "aseptic plus ascorbate" assay with gross bacterial contamination should be heated in flowing steam for 30 min before addition to the assay tubes. The steaming (in the presence of ascorbate) does not affect the THFA activity of the samples, but does prevent interference by the bacteria. Although a reasonable amount of care has to be exercised in these "aseptic" additions, it is not necessary to use a strictly aseptic technique. In making up the dilutions for the standard and for those samples which contain relatively few bacteria,[4] the distilled water and glassware are used without prior sterilization. Apparently the number of vegetating bacteria left on the glassware following the routine cleaning procedure is too small to influence the assay results during the course of the short period of incubation.

It should be emphasized that when the folic acid activity of the samples is due only to THFA and when THFA is used as a standard, there is no problem of "drift" or of quantitation in the "aseptic plus ascorbate" assay. Samples containing various known amounts of THFA can be assayed with an accuracy of $\pm 10\%$. With randomization and increase in number of replicates, greater accuracy is possible.

The conditions under which the assay is carried out (organism, pH of the medium, temperature of incubation, presence of reducing agent) markedly affect the response to THFA, CF, and other forms of folic acid. By setting up a "regular" assay simultaneously with an "aseptic plus ascorbate" assay, one can determine whether a given sample contains a labile form of folic acid. The use of *L. casei* and *S. faecalis* as the assay organisms, since they respond to many folic derivatives which are inert for *P. cerevisiae* [*L. casei:* folic acid, N^{10}-formylfolic acid,

[4] The presence in the samples of enough bacteria to interfere with the assay may be determined by including tubes containing sample to which no inoculum is added.

pteroyltriglutamic acid; *S. faecalis:* folic acid, N^{10}-formylfolic acid, pteroic acid, N^{10}-formylpteroic acid (10)] will give additional information as to the form of folic acid present.

It is not suggested that the assay herein described is specific for THFA. In the "aseptic plus ascorbate" assay, CF, N^{10}-formyltetrahydrofolic acid and THFA will all have activity for *P. cerevisiae.* In the "regular" assay, since they are not protected by a reducing agent, these folic derivatives (with the exception of CF, which is fully active) have very little activity for *P. cerevisiae.* When used in conjunction with information obtained by other means, it is possible to use the "aseptic plus ascorbate" method as an aid in the isolation of folic derivatives from natural materials and in studying metabolic reactions involving the above compounds. This method is also of value in determining the potency of solutions of THFA and affords a sensitive means of detecting loss in activity.

Donaldson and Keresztesy (11) prepared a concentrate from horse liver containing a precursor of THFA which they designated "prefolic A." This precursor was enzymically converted to THFA and the latter to CF under various experimental conditions. THFA was assayed by the "aseptic plus ascorbate" method described in the present paper using CF as a reference standard. Using this method, they were able to get satisfactory quantitation of the THFA content.

Silverman and Noronha (12) made use of the "aseptic plus ascorbate" assay in devising a method for the preparation of THFA from folic acid and sodium hydrosulfite and in the chromatographic separation of various forms of folic acid.

SUMMARY

A procedure is described for the microbiological determination of tetrahydrofolic acid. The method is applicable to its measurement in complex reaction mixtures under conditions in which spectrophotometric methods are unsuitable. Because of its instability, routine microbiological assay is not feasible for tetrahydrofolic acid. However, by modifying the assay procedure and protecting with ascorbate, the activity may be measured satisfactorily. Its stability properties and its activity for *P. cerevisiae, S. faecalis,* and *L. casei* are reported. The activity of tetrahydrofolic acid relative to citrovorum factor for *P. cerevisiae* varies with concentration, temperature of incubation, and initial pH of the medium.

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ANALYTICAL BIOCHEMISTRY 2, 568–575 (1961)

Single Sample Analysis with the Sodium Electrode[1]

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The determination of sodium by standard flame photometry is ordinarily limited to a precision of the order of ±1%, to measurement of concentration rather than activity, and to the use of diluted samples. In 1957, Eisenman, Rudin, and Casby demonstrated that the specific affinity of glasses of the sodium aluminosilicate series for the alkali-metal cations is a systematic function of their composition (1). Their general conclusions also applied to glass systems in which lithium or potassium replaces sodium, boron replaces aluminum, or germanium replaces silicon. In turn, the affinity of these glasses for specific cations is a function of ionic activity rather than concentration.

In cooperation with these authors, we proceeded with the development of electrodes suitable for use in biological systems. In this work we were at first particularly concerned with the continuous monitoring of cationic activity in a moving stream (2, 3). The present report is concerned specifically with the determination of (Na^+) in single samples. We propose to show that the sample need not be diluted and that greater precision than that of flame photometry can be achieved.

METHODS

Sodium Electrode

A cannula-type electrode measuring approximately 2 mm internal diameter and 1.5 cm long was used throughout. The cannula was blown from NAS_{11-18} glass,[2] then silvered and copper plated at the central membrane area to make a metal-connected electrode. The method of fabrication has been described in detail elsewhere (4). The size is not critical. A calomel electrode in the line served as reference.

[1] This work was carried out with the aid of grants from the B. C. Heart Foundation and the National Research Council of Canada.

[2] Corning Glass Works, Corning, N. Y.

Electrometer

A Vibron electrometer was used.[3] A calibrated bucking voltage (calibrator) was interposed in the reference lead in order to back off the electrode potential to mid-scale on the electrometer. Thus the amplifier was essentially used as a null-point indicator, and readings were made on the calibrator.

Shielding

The electrode assembly was enclosed in a metal case which was solidly connected to the shield of the electrode cable. The shield was taken to earth, and the calibrator case was also taken to the same point. The apparatus is shown in Fig. 1.

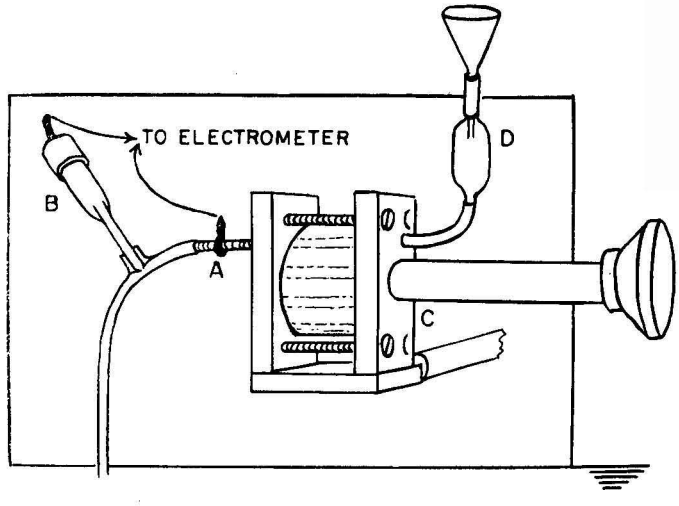

FIG. 1. Electrode assembly for measurement of sodium in single samples. The grounded enclosure contains the cannula electrode. (*A*) and calomel reference (*B*) in line. Samples can be loaded into the rotary sample chamber (*C*) or run in singly through an air gap (*D*).

Sample Delivery

Samples with a volume greater than 2 ml (diluted or undiluted) were fed into the electrode directly through a funnel. Samples with a volume less than this were first loaded into a sample changer devised for this work. The sample changer carried 12 samples of 0.2 ml each, and after loading was connected into the electrode path. The sample delivery components are shown in Fig. 2.

[3] Electronic Instruments Ltd., Richmond, Surrey, England.

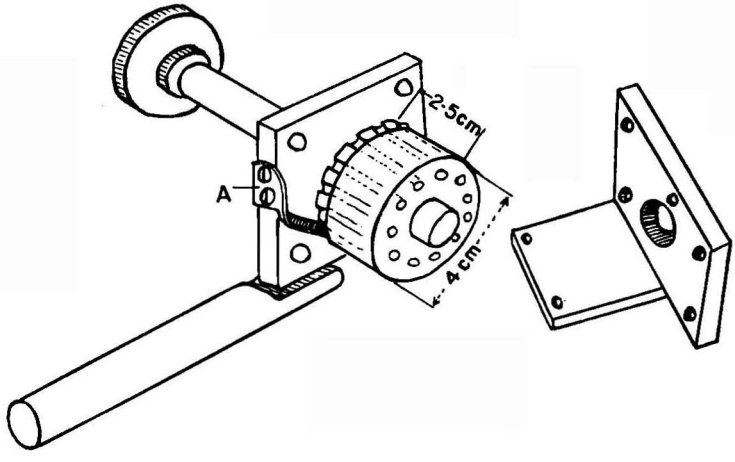

FIG. 2. Exploded view of rotary sample chamber. Capacity of each chamber is approximately 0.2 ml.

Analytical Measurements

NAS_{11-18}, the sodium-sensitive glass used, is insensitive at equilibrium to H^+ change above pH 5–6. All electrolyte solutions were thus prepared in a mixture of 0.1 *N* HCl and 0.2 *N* Tris [2-amino-2-(hydroxymethyl)-1,3,propanediol] buffered to pH 7. The plasma samples studied are, of course, biologically buffered.

Under these conditions,

$$E = E_0' + \frac{RT}{F} \ln [(Na^+) + k_{NaK}(K^+)]$$

where E is the measured electromotive force (E.M.F.), E_0' is a constant standard potential for the assembly, (Na^+) and (K^+) are the activities of sodium and potassium, and k_{NaK} is the empirically observed selectivity constant of the electrode for K^+ relative to Na^+. Since k_{NaK} for NAS_{11-18} is of the order of 0.004, it follows that in buffered solutions of a high Na^+ to K^+ ratio the electrode will be substantially unaffected by K^+ and will act as a pure Na^+ electrode at equilibrium.

Ideally, RT/F ln can be converted to 58 $\log_{10}$ in millivolts. In practice, we determine the actual value of this slope, S, by measuring E in two calibrating solutions of sodium:

$$\Delta E = S \log_{10} (Na_1 - Na_2)$$

The value of unknowns is then obtained directly from the measured value ΔE (relative to one standard) and the calibrated value of S.

All meter readings were taken after allowing a uniform period of

equilibration of 2 min after changing samples. This interval was selected since it is about twice the time necessary for transient potentials to disappear (3). In all of the experiments which follow, the standard solution was first used to center the meter at mid-scale. The unknown solution was then fed in and ΔE measured. This procedure avoids any error due to instrumental drift.

RESULTS

Measurement of Sodium in Diluted Samples

The electrode was calibrated by measuring S in two buffered solutions of NaCl containing 120 and 160 meq/l Na, respectively. The value of S was usually about 60 mv for the particular electrode assembly used and the ambient temperature of the laboratory. A series of NaCl samples from buffered solutions containing 130, 135, 140, 145 and 150 meq/l, respectively, diluted 1:20 in distilled water, was then analyzed by the simple run-through method. Six separate runs were carried out. Exact values corresponding to the above known concentrations were obtained. In no case did the standard error of the mean exceed ± 0.25 meq/l.

Measurement of Sodium in Undiluted Samples

The electrode was calibrated as before. A series of undiluted samples from buffered NaCl solutions 2 meq/l apart in Na^+ concentration in the range of 140–160 meq/l was analyzed. For purposes of method comparison, samples from the same solutions were analyzed by flame photometry in two ways. In one case the samples were diluted 1:20 before flame analysis; in the other the samples were undiluted so that their content of buffer was the same as that submitted to the electrode. Six separate trials, some on different days, were carried out. The results are shown in Fig. 3.

The precision of the electrode analysis measured over this range of concentrations was compared with the standard flame photometric results (Table 1). The electrode is evidently at least twice as precise.

Measurement of Sodium Added to Blood

Standard amounts of NaCl calculated to produce approximately 5 meq/l step rises in the plasma were added to aliquots taken from a pool of whole blood. Since this addition upsets the osmotic equilibrium between cells and plasma and since some sodium moves into cells, it is not possible to calculate the absolute values expected. Accordingly, plasma [Na] was analyzed by flame photometry and these results were used as a base for the electrode data. The electrode analyses were made

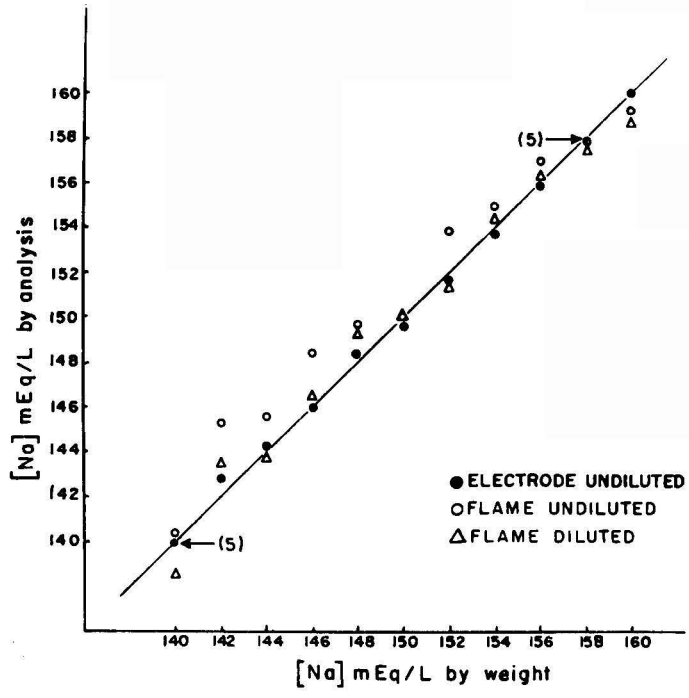

FIG. 3. Comparison of sodium analysis by flame photometry and glass electrode. Test NaCl solutions prepared by weight.

TABLE 1

COMPARISON OF ELECTRODE AND FLAME PHOTOMETRIC ANALYTICAL PRECISION[a]

	Sodium concentration			
	Electrode (0.2 ml)		Flame (diluted)	
Sample (meq/l)	Average	Standard error	Average	Standard error
140.0	140.0	±0.1	138.5	±1.20
142.0	142.8	±0.4	143.5	±0.72
144.0	143.3	±0.3	143.8	±1.35
146.0	146.0	±0.4	146.4	±0.84
148.0	148.4	±0.5	149.3	±1.56
150.0	149.6	±0.4	150.0	±0.01
152.0	151.6	±0.3	151.4	±1.20
154.0	153.7	±0.6	154.4	±1.34
156.0	155.9	±0.1	156.3	±1.26
158.0	157.9	±0.8	157.4	±0.90
160.0	160.0	±0.0	158.7	±0.81
Average % error	0.20	±0.06	0.43	±0.10

[a] Samples of NaCl at pH 7.

using 0.2-ml samples of whole blood in the sample changer. The results are shown in Fig. 4.

The electrode values fall on a straight line comparable to that of flame analysis and with about the same variability of results. Most important, however, is the clear indication that these lines diverge by

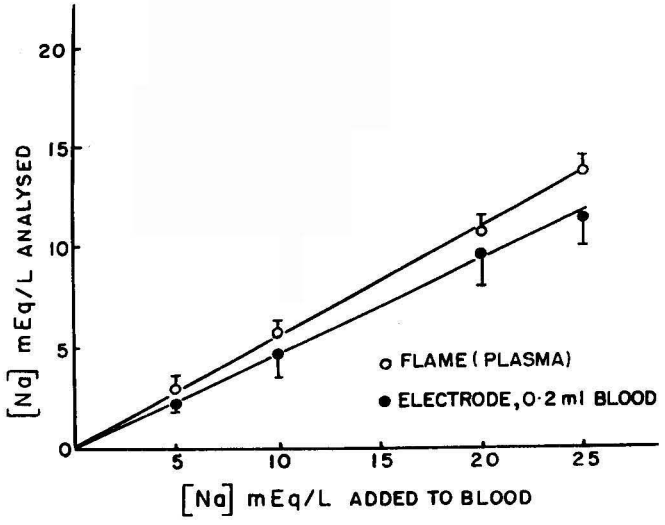

FIG. 4. Electrode analysis of sodium concentration in whole blood after addition of weighed amounts of NaCl. The actual plasma Na concentration achieved was measured by flame photometry.

a small constant. This strongly suggests that some proportion of the sodium in the plasma is inaccessible to the electrode; i.e., it is bound.

Measurement of Sodium Added to Plasma

The question of plasma binding of sodium is open to direct experimentation since, in contrast to the case with blood, it is possible to add NaCl to plasma in precisely known amounts. For this, NaCl was added to plasma aliquots from one original pool to form a series of samples of increasing [Na] in 5 meq/l steps. Samples from these were then analyzed by flame and electrode after 1:20 dilution. Any plasma binding should be reduced to vanishing by such dilution. A third set of samples was analyzed without dilution using the electrode sample changer. The results are shown in Fig. 5.

The results obtained on diluted samples analyzed either by flame or electrode evidently lie on the same line which, in turn, follows the anticipated 5 meq/l step. By contrast. the values for the undiluted electrode

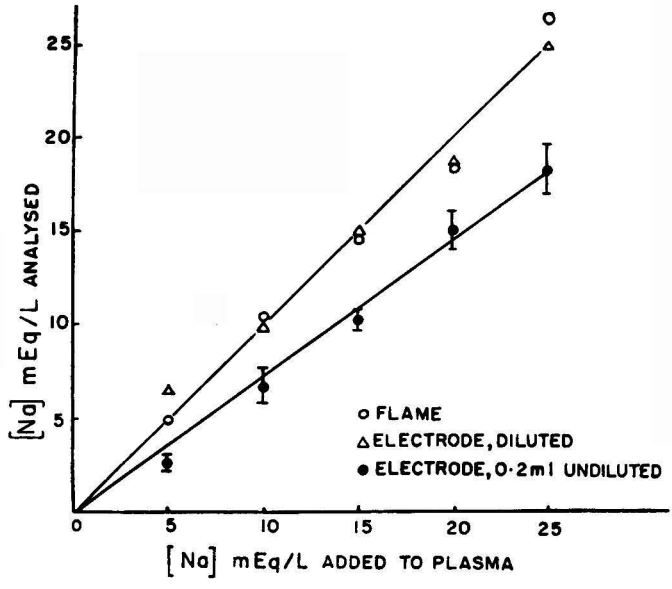

FIG. 5. Electrode analysis of sodium concentration in plasma after addition of weighed amounts of NaCl. The actual plasma Na concentration achieved was measured by flame photometry. Note that electrode analyses yield the same line as flame when 1:20 dilution is used but fall away when sample is not diluted.

samples lie on a line which diverges by a constant from that of diluted samples. This again suggests that some sodium in plasma is inaccessible to the electrode.

DISCUSSION

The sodium electrode of NAS_{11-18} glass is a practical instrument for the measurement of Na^+ in single samples of biological materials. It can be used alone provided the (H^+) of the solution is above pH 6. If precise measurements are to be obtained the (H^+) of the test solution should remain reasonably constant. The electrode does respond to K^+, but the affinity is very much lower than that to Na^+ and can be disregarded provided the test solution contains a high Na/K ratio. For practical purposes it is unresponsive to all other ions. The empirical formula of Eisenman *et al.* (1) given above permits one to assess beforehand whether K^+ is apt to present a problem in a particular application. For the routine analysis of Na^+ in blood and urine the electrode seems very useful.

The analysis of Na^+ can be carried out most simply using a run-through device requiring a sample of 2–3 ml. It can also be made on a sample as small as 0.2 ml using a sample changer. This latter amount

can probably be still further decreased. In either procedure, the sample used may be full strength or diluted, and a wide latitude in degree of dilution is permissible. It is important, however, that the calibrating solutions should be made up the same way as the samples to be tested and should cover the particular range of concentration under study. If solutions containing molecules potentially capable of binding Na^+ are used without dilution, the results may not necessarily agree with those obtained by chemical or flame photometric methods. For most purposes the calibrating solutions should be made in terms of concentrations and the results of analysis then expressed in the same terms. Greater accuracy will be obtained, of course, if activities are used for both calibration and analysis. This desideratum will perhaps be more practicable when the electrode comes into more general use.

A dip-type electrode can obtain precision equal to the cannula-type described here only if it remains immersed in the sample. In the analysis of separate samples the make–break contact potentials occasioned by sample changes make stable high precision measurement impractical. The cannula electrode, by contrast, allows the separate solutions to be exposed to the electrode without such contact potentials.

Automation of the sample feed is quite practical and we are studying this. Direct readout of linearized sodium values as milliequivalents per liter (meq/l) rather than as millivolts on a logarithmic scale also seems eminently practical.

SUMMARY

The cannula-type metal-connected electrode is a practical device for the measurement of sodium in single aliquots of biological materials such as blood or urine. Measurements can be carried out with either diluted or undiluted samples using simple devices to present the solutions to the sensing membrane. Since the electrode responds to activity rather than concentration, ion binding has an important bearing on the analytical result. Evidence suggesting significant sodium binding in plasma is presented.

ACKNOWLEDGMENT

We are indebted to Mr. John Lees for his skill in blowing the glass electrodes.

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ANALYTICAL BIOCHEMISTRY 2, 576–588 (1961)

Modification of the Beckman Model DU Spectrophotometer for Recording Linearly in Absorbancy at Fixed Wavelength[1]

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The rates of many chemical reactions can be determined by following the formation or disappearance of a chromogen. The Beckman DU spectrophotometer has been used extensively to assay pyridine nucleotide dehydrogenases as well as other enzymes for which a substrate or product absorbs in the ultraviolet. Several methods have been devised to adapt this instrument, designed for measurement by potentiometric null balance, for single-beam recording at a fixed wavelength (1–4). If the kinetics of an enzymic reaction are zero order, absorbancy is a linear function of time. Thus, for maximum utility, the recording should be of absorbancy rather than transmittancy.

This paper describes the adaptation of the Beckman DU spectrophotometer for recording with a commercial direct-current (dc) amplifier and a simple network of biased diodes to convert the output, which is proportional to transmittancy, to absorbancy. This adaptation is entirely compatible with the manual use of the spectrophotometer.

APPARATUS

Spectrophotometer

A Beckman DU spectrophotometer equipped with 156 and 2342B diode phototubes was used. The standard tungsten source was powered from a 6-v storage battery of 330 amp-hr capacity which was charged at a net rate of 0.5–1.0 amp during operation to improve stability. The Beckman ultraviolet source (hydrogen lamp and regulated current supply) was sufficiently stable without modification. The sample compartment was maintained at a fixed temperature by pumping water from a water bath through a heat exchanger (Beckman part #2180).

[1] This work was supported by grants-in-aid 4261 and 9860 from the National Science Foundation.

DC Amplifier

The resident, single-ended dc amplifier of the spectrophotometer was neither sufficiently stable nor sufficiently linear for direct recording. Furthermore the output was inadequate to drive the absorbancy network to be described. Chance and Legallais (1) found that the electrometer stage alone could be used for short-term recording; the linearity was adequate if the change in plate current was kept small. Nielson (2) applied servo feedback to the input of the amplifier. This method overcomes the nonlinearity of the resident amplifier; however, instability resulting from drift in offset of the amplifier is still a problem, and the generation of the absorbancy function would be complicated. The modification by Halperin and Braner (3) substitutes a balanced electrometer. Although this amplifier may be adequate, we chose a commercial amplifier of sufficient input impedence to permit the use of large load resistors required with diode phototubes and of sufficient stability that the drift of the amplifier was less than the variation of the light source. The Keithley 610 dc amplifier satisfies these requirements and delivers 10 v at approximately 1 ma to the absorbancy network. The input of the amplifier is attached through the central conductor of a RG-58A/U coaxial cable soldered to the clip of the 2000-megohm load resistor which is common to the cathode of the phototube; the shield of the cable is attached to the chassis of the phototube housing of the spectrophotometer. The vacuum tubes and leads to the grid caps were removed from the phototube housing. No alteration was made of the standard anode voltage of the phototube (+20 v) supplied from the battery pack in the monochromator.

Absorbancy Network

The transformation of the amplifier output of the phototube to absorbancy electrically rather than by the use of nonlinear potentiometers or cams offers the distinct advantage of scale expansion. Wood (4) has used the circuit of Sweet (5, 6) in which the output of a multiplier phototube is used to control its dynode voltage; the dynode voltage varies approximately as the absorbancy.

The network (Fig. 1) is similar in principle to the circuit designed by Yang (4), the thermionic tubes of which are replaced with IN210 silicon diodes. The diodes are under progressively higher reverse bias supplied by a 12-v storage battery through the series resistances R_{15} through R_{24}. When the signal voltage exceeds the bias of a given diode it begins to conduct; the current from the individual elements of the network are summed by resistance R_{25-27} to provide the function illus-

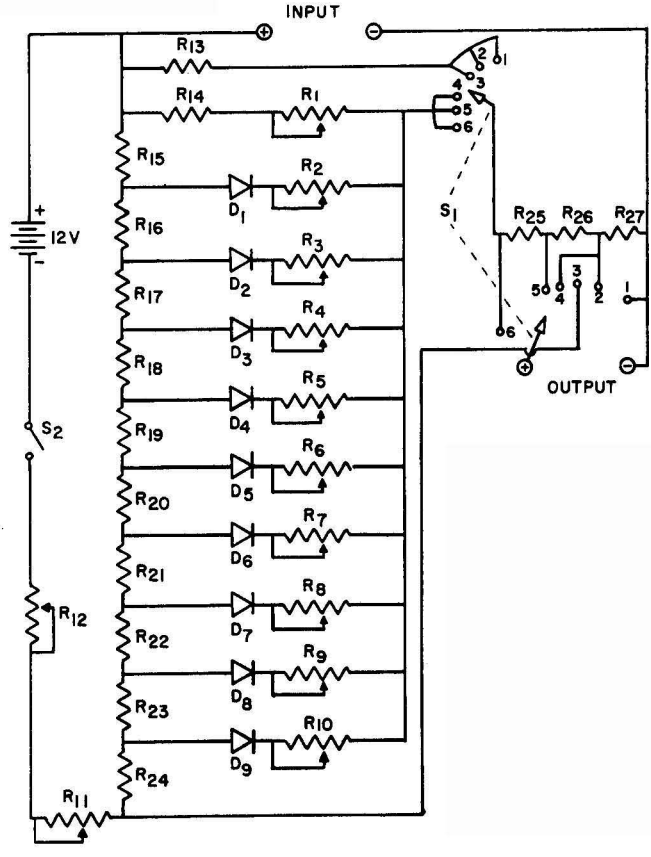

FIG. 1. Circuit diagram for the diode network used for the generation of the absorbancy function. R_1, 5 kiloohms; R_2, 1.0 megohm; R_3, R_4, R_5, and R_6, 500 kiloohms; R_7, R_8, and R_{10}, 100 kiloohms; R_9, 50 kiloohms; are Clarostat type RV4LAY 2W carbon potentiometers. R_{11}, 10 ohms; R_{12}, 500 ohms, are wire-wound potentiometers. R_{13}, 10 kiloohms; R_{14}, 20 kiloohms; R_{15}, R_{17}, R_{18}, R_{19}, R_{20}, and R_{25}, 100 ohms; R_{16}, 125 ohms; R_{21} and R_{22}, 75 ohms; R_{23}, R_{26}, and R_{27}, 50 ohms; R_{24}, 225 ohms, are 1% 1 watt deposited-carbon resistors. D_1–D_9 are 1N210 silicon diodes. S_1 is a 2-section, 6-position rotary switch and S_2 is a toggle switch.

trated in Fig. 2. The circuit was designed for maximum accuracy from 0 to 0.5 absorbancy; seven of the nine diodes are used in this region. Silicon diodes were selected to minimize reverse current; however, we have also used 1N67A and 1N34 germanium diodes. The reverse current of the germanium diodes significantly offsets the zero point. Otherwise, the less expensive germanium diodes are satisfactory. Semiconductor diodes offer a distinct advantage over thermionic diodes in this application. The nonlinear characteristic of the semiconductor diodes elim-

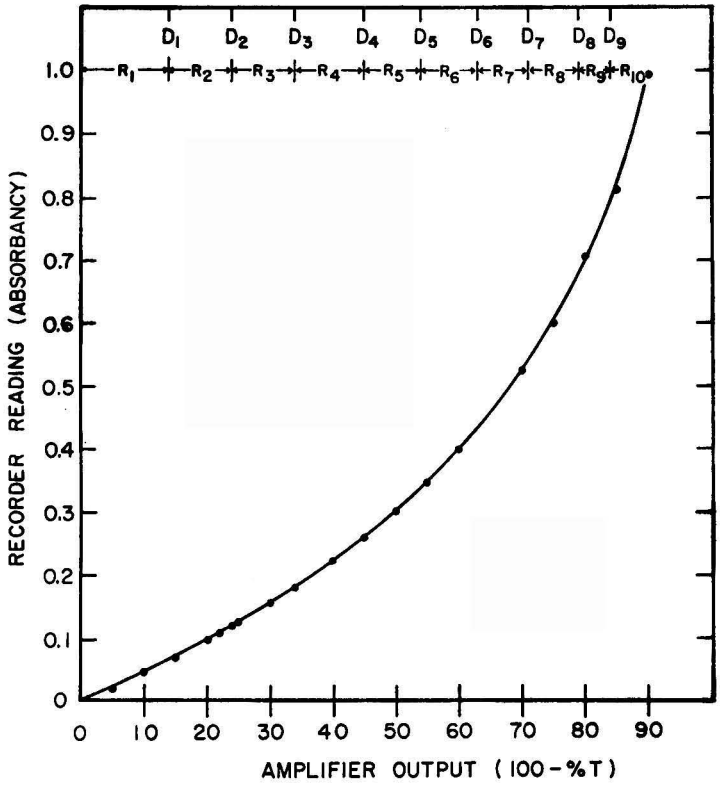

FIG. 2. Relationship between absorbancy and transmittancy. The solid line is calculated from the function, $y = -\log_{10}(1 - x/100)$. The abscissa corresponds to the input of the absorbancy network and the ordinate to the output. Individual points are positions of the recorder pen corresponding to various outputs of the amplifier. The output of the amplifier at which each diode begins to conduct is illustrated at the top of the graph. The variable resistance (R_1–R_{10}) which controls each segment of the function is indicated.

inates abrupt transitions in slope at the turnover points generating a smooth curve rather than a series of linear segments.

The absorbancy network was constructed in a metal box 13 × 5¼ × 2⅝ in. with the operating controls (S_1, S_2, and R_{11}) mounted on the front and the input and output terminals at the rear. The potentiometers which control the slope of the individual segments shown in Fig. 2 (R_1–R_{10}) and the coarse bias adjustment (R_{12}) are mounted through the top of the box. Position No. 1 of the selector switch, S_1, shorts the input to the recorder; position No. 2 gives an output of transmittancy (inverse scale); position No. 3 is used to adjust the bias voltage of the

network, and positions Nos. 4, 5, and 6 give 1, 2, and 4× absorbancy, respectively.

The amplifier, recorder, and battery charger were connected to the ac main through a constant-voltage transformer; this precaution is not essential but does reduce the effect of transients from the high-current switching of other equipment common in our laboratory.

Adjustment of the Absorbancy Function

The output of the amplifier is connected with the diode network; the output of the network in turn is coupled to the recorder, the span of which is set at 50 mv. With the selector switch, S_1, of the network at position No. 2, the output of the amplifier is adjusted such that the

TABLE 1

ADJUSTMENT OF THE ABSORBANCY FUNCTION[a]

Amplifier output[b]	Adjustment	Absorbancy scale[c]		
		4X	2X	1X
0	—	0		
15	R_1	30.3		
25	R_2	50.0		
35	R_3	74.8		
45	R_4	—	51.9	
55	R_5		69.4	
62	R_6		84.0	
70	R_7		—	52.3
80	R_8			69.9
85	R_9			82.4
90	R_{10}			100

[a] The function is computed as $y = -\log_{10}(1 - x/100)$, in which y = absorbancy and x = amplifier output.

[b] Output meter reading. A reading of 100 (10 v) is equivalent to 0% transmittancy; a reading of 0 is equivalent to 100% transmittancy.

[c] The values given are recorder readings (full scale = 100).

output meter coincides with the span of the recorder; this may be checked by varying the zero (offset) adjustment of the amplifier. For full-scale deflection of the recorder the output of the amplifier should be 10 v.

With the recorder reading full scale on position No. 2, the selector switch of the network is advanced to position No. 3. The coarse and fine bias controls (R_{12} and R_{11}) are adjusted to return the pen of recorder to zero. This adjustment sets the voltage over R_{15}–R_{24} equal to the voltage across R_{13} at zero transmittancy.

The selector switch of the diode network is advanced to position

No. 6 (the 4× absorbancy scale), the output of the amplifier is set to zero, and the recorder should read zero. The output of the amplifier is increased to a meter reading of 15; R_1 is adjusted to move the pen of the recorder to 30.3. This establishes the slope of the first segment in Fig. 2. The output of the amplifier is increased to 25; R_2 is adjusted to move the pen of the recorder to 50.0. This establishes the slope of the second segment. The adjustment is continued as indicated in Table 1.

Operation

The output of the spectrophotometer is coupled to the input of the amplifier (see *Apparatus*). The polarity of the amplifier output meter is switched to the negative position, and the gain is set to 0.1 v for full-scale deflection of the output meter (gain of 100). The internal shunt is selected in accordance with the slit opening and response time required for the particular measurement. Lower shunt resistance decreases the response time of the measuring circuit but increases the required slit opening of the monochromator. We usually use a 10^{-8}- or 10^{-9}-a setting of the function switch of the amplifier which correspond with shunt resistances of 100 and 1000 megohms, respectively.

The selector switch of the spectrophotometer is set at position No. 1, and the slide-wire is set to the counterclockwise limit (0% transmittancy). This setting removes the bias of the slide-wire from the phototube circuit and renders the "sensitivity adjustment" of the spectrophotometer inoperative. This setting of the electrical controls of the spectrophotometer must be observed when the instrument is used for recording.

The steps in routine operation of the instrument are as follows:

1. The selector switch (S_1) of the diode network is set to position No. 2. The shutter is closed, and the dark-current adjustment of the spectrophotometer is rotated to give a full-scale reading of the recorder.

2. The absorption cell which serves as the reference is placed in the optical path; the shutter is opened, and the slit is adjusted to return the pen of the recorder to zero. Alternate closed and open positions of the shutter should position the pen of the recorder at 100 and zero, respectively.

3. With the shutter switch off (recorder at 100), S_1 of the diode network is moved to position No. 3; the bias controls, R_{11} and R_{12}, are adjusted to return the recorder pen to zero.

4. S_1 of the diode network is set to the appropriate absorbancy scale (position Nos. 4, 5, and 6 giving 1, 2, and 4×, respectively). The absorption cell containing the sample is placed in the optical path; the shutter is turned on, and the absorbancy is recorded.

Routine operation requires only steps 1, 2, and 4. The adjustment of the diode bias (step 3) is necessary only once each day to compensate for slight changes in the voltage of the 12-v storage battery.

For manual use of the instrument the amplifier function switch is ordinarily set to "volts" to remove the shunt of the load resistor, and the meter is set to zero-center with the dark-current adjustment. The remainder of the operation is conventional except that the output meter of the amplifier serves as the null indicator. The gain of the amplifier may be increased to 0.03 or to 0.01 v full scale if maximum photometric accuracy is desired. The high voltage gain and stability provided by the amplifier grealty improve the manual photometric accuracy of the spectrophotometer at small slit openings. Also, the only dry battery required is one to provide the minute current of the photodiodes for which the service is the shelf life of the battery.

EVALUATION

Function Generation

The accuracy of the diode network in generation of the absorbancy function is indicated by the experimental measurements indicated as points in Fig. 2. Over the range of absorbancy of 0–0.5 the maximum deviation from the desired function (solid line) was within the dead-band of the recorder. Significant departures resulting from the line-segment approximation are evident above an absorbancy of 0.7. The absorbancy may be held within the region of maximum accuracy by the use of absorption cells of path length less than 1 cm.

Photometric Accuracy

The absorbancies of solutions of varying concentrations of alkaline K_2CrO_4 (7) were measured at 370 mμ both by null-balance and by recording device. The results (Fig. 3) show an excellent agreement to an absorbancy of 0.5 with a small error evident at higher absorbancy.

Stability

The stability of dark current and 100% transmission are illustrated in Fig. 4. Over a period of 12 min the dark current did not vary measurably. A slight variation in the tracing of the 100% line is detectable, but less than 1%. Since the apparatus was designed for measurement of moderately rapid reactions in which the absorbancy change exceeds 0.05, no further effort was made to improve the stability.

The stability of the absorbancy function is excellent. Several models

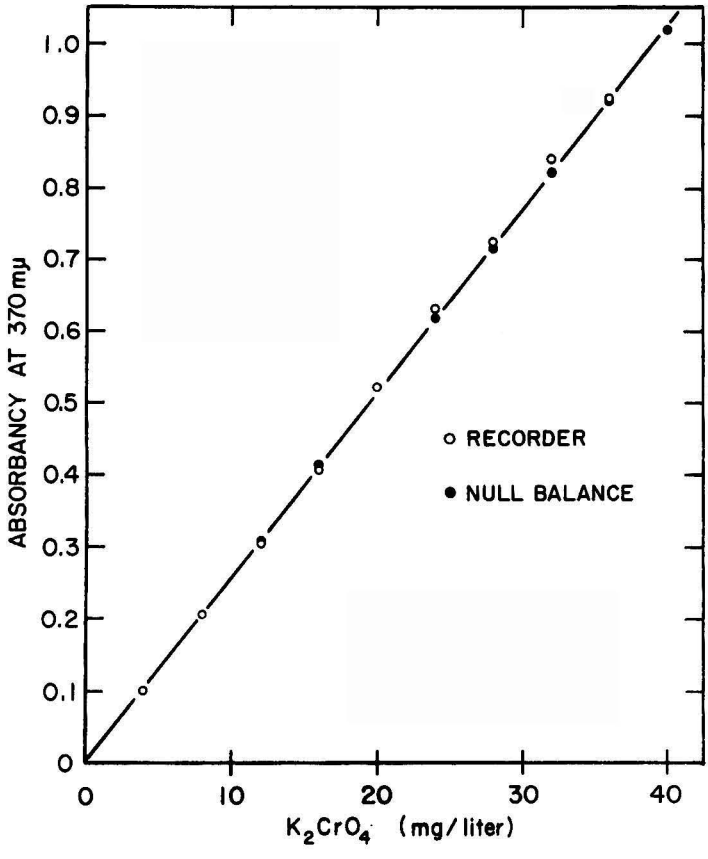

FIG. 3. A comparison of the absorbancy at 370 mμ of solutions of K_2CrO_4. Each solution was measured both by null balance and by direct recording. If only one symbol is evident, the measurements by the two methods differed by less than 0.00 absorbancy units. (Optical path, 1.00 cm; slit width, 0.01 mm (manual) or 0.14 (recorder); temperature, 30°; tungsten lamp.)

of the diode network have been in operation for more than 2 years without need for readjustment or repair. Also, the amplifier has proved to be trouble-free.

Response Time

Figure 5 illustrates the response of the instrument to a step change in absorbancy. The response time of less than 1 sec was limited only by the balancing time of the recorder. Larger load resistors in the phototube circuit reduce the slit width but increase the response time.

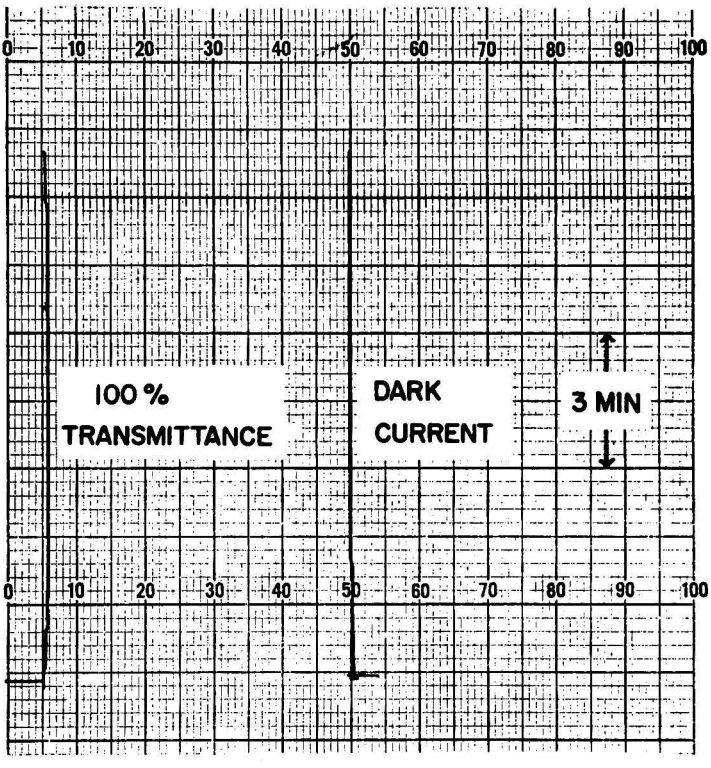

FIG. 4. Stability of dark current and 100% transmittance. Each scale division is 1% transmittance. (Phototube load resistor, 100 megohms; amplifier gain, 100×; λ, 340 mμ; slit width, 0.24 mm; tungsten lamp.)

Assay of Enzymes

Figure 6 illustrates the use of the recording attachment for the assay of D-mannitol dehydrogenase (7). The change in absorbancy results from the formation of DPNH. One tracing was made at each of the three absorbancy scales, 1, 2, and 4×. The enzyme gives almost zero-order kinetics under the conditions of the assay. The slopes estimated over the range from 0 to 0.25 are 0.130, 0.133, and 0.131 absorbancy/min, respectively. The repeatability in many replicate assays has been found to be ±1%.

Since the kinetics of oxidation of D-mannitol are nearly zero order, the slope of the recording is directly proportional to the amount of enzyme, which simplifies the calculations and improves the accuracy. The recording of reaction rate makes possible a precise assay in 3 min or less; thus, one operator may make over 100 assays each day. The

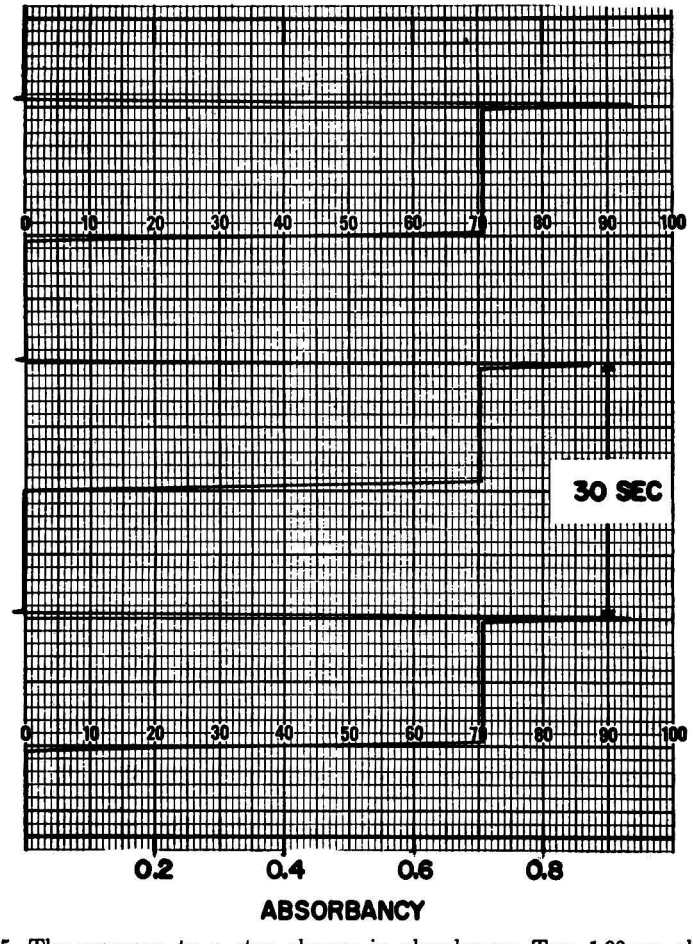

FIG. 5. The response to a step change in absorbancy. Two 1.00-cm absorption cells, one filled with water and one filled with a solution of DPNH of 0.7 absorbancy, were alternated in the optical path by quickly moving the cell holder. (Load resistor, 100 megohms; amplifier gain, 100×; slit width, 0.15 mm; λ, 340 mμ; tungsten lamp.)

advantage of electrical function generation is evident from the ease of scale expansion which is not possible with mechanical function generation or nonlinear potentiometers that are commonly used for generating the absorbancy function.

Figure 7 is an assay of DPNH oxidase. The rate of change of absorbancy is initially constant but decreases as the concentration of DPNH decreases. The minimum concentration of DPNH required to saturate the enzyme is equivalent to an absorbancy of at least 0.6/cm.

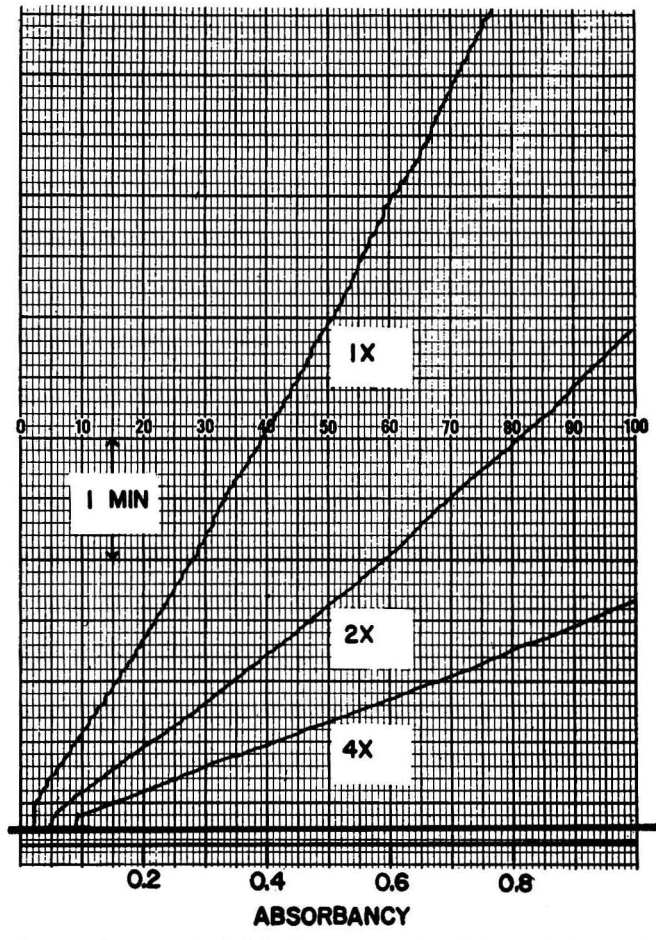

FIG. 6. Assay of D-mannitol dehydrogenase using 1, 2, and 4× scale expansion. The 1.00-cm absorption cell contained 2.26 ml of 0.05 *M* Tris chloride buffer, pH 8.6; 0.50 ml of 0.5 *M* D-mannitol; and 0.20 ml of 0.02 *M* DPN. This mixture was used as the reference. The reactants were held at 30° before mixing, and the sample compartment was 30°. The reaction was started by discharging 0.040 ml of a preparation of D-mannitol dehydrogenase from a microsyringe into the absorption cell, the contents of which were mixed by inverting the cell over a Parafilm cap. Each tracing is an identical reaction using 1, 2, and 4× absorbancy scales. (Photo-tube load resistor, 100 megohms; slit width, 0.25 mm; λ, 340 mμ; amplifier gain, 100×; tungsten lamp.)

Since this is beyond the range of maximum accuracy of the diode analog, the optical path length was reduced from 1.0 to 0.5 cm which allowed the reaction to be measured over a more accurate range.

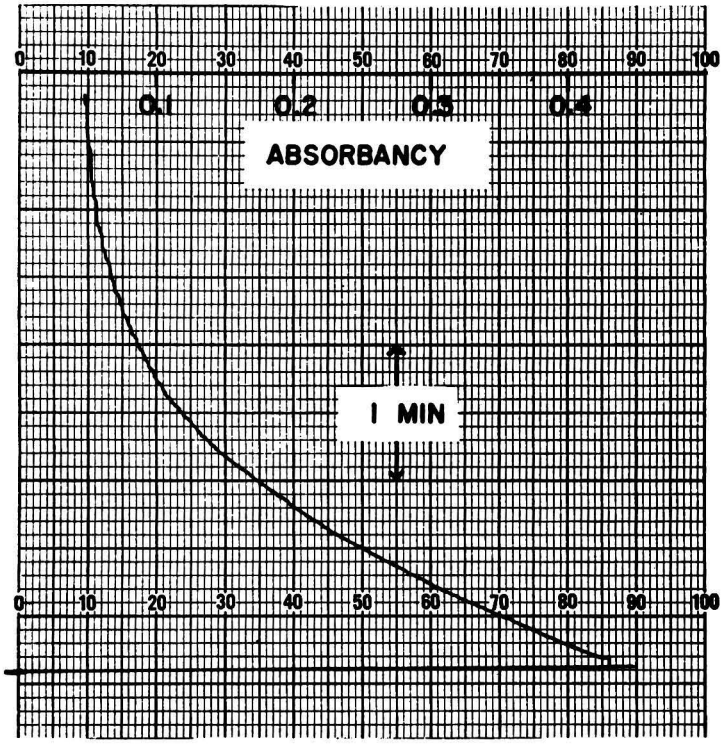

FIG. 7. Assay of DPNH oxidase. The 0.50 cm absorption cell contained 1.35 ml of 0.05 *M* Tris chloride buffer, pH 8.6, and 0.05 ml of a preparation of DPNH oxidase. This mixture was used as the reference. The reaction was started by adding 0.10 ml of a solution containing 2 mg DPNH/ml. Photometric conditions were the same as those given in the legend of Fig. 6.

SUMMARY

The Beckman DU spectrophotometer has been adapted for recording with a commercial direct-current amplifier and a network of biased silicon diodes to generate the absorbancy function from 0 to 1. The network was designed to provide maximum accuracy between 0 and 0.5 absorbancy to permit the use of expanded scales. This adaptation is not only compatible with but improves the precision of photometric measurement manually by null balance.

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ANALYTICAL BIOCHEMISTRY 2, 589–600 (1961)

A System for Automatic Recording of Absorbancy and Its Application to Enzyme-Catalyzed Reactions

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INTRODUCTION

The standard method for determining the velocity of enzyme-catalyzed reactions which utilize pyridine nucleotides involves measuring the associated changes in light absorbancy at 340 μ as a function of time. This spectrophotometric procedure requires successive measurement of absorbancy using a stop watch to establish precise intervals. The data obtained are plotted and the slope and shape of the curve are used to establish the reaction velocity and other characteristics of the reaction. Frequently these determinations are made on multiple samples in which a second parameter such as concentration of enzyme or substrate may be varied in the different samples.

There are numerous advantages to employing automatic recording systems for obtaining this type of data directly. Since a recorder would follow the reaction as it proceeds, it would be possible to measure rates which could not be followed by manual measurement. Greater accuracy could be achieved with the elimination of human errors which tend to appear after a long series of measurements. The substitution of a recorder time scale would also add to the accuracy. Additional errors in transcription and replotting of the data would be eliminated. The complete shape of the curve would be seen from the start of the reaction permitting changes to be made if desired.

While there are available a number of recording spectrophotometers, most are designed specifically for recording absorption spectra. These instruments go considerably beyond the requirements for a reaction velocity measurement at a single wavelength that is under discussion, and because of their general lack of availability are not often used for this purpose. The spectrophotometers common to most laboratories such as the Beckman DU are not directly convertible to automatic recording

[1] Contribution No. 2824 of the Michigan Agricultural Experiment Station.

because they do not have the requisite long-term stability for this type of operation. Further, the output of such systems are generally linear functions of transmittancy instead of the desired function absorbancy and they generally have a range limited to about 1.0 absorbancy unit. Special recorders are available with logarithmic response, but these are usually more expensive and these still do not overcome the other shortcomings of conventional spectrophotometers.

The instrument to be described in this paper is a special photometer system which can be attached to any spectrophotometer and makes possible direct recording of absorbancy over a range of 3.0–4.0 absorbancy units with excellent long- and short-term stability. This unit has adequate sensitivity and stability to permit full-scale recordings of 0.1 absorbancy unit. One per cent full-scale accuracy can be attained with the system when carefully adjusted. The circuit employed uses low resistances thus avoiding the instabilities and attendant need for humidity control. The unit provides great flexibility of operation and has been very useful in recording reaction velocities.

The basic photometer circuit was originally described by Sweet (1). It employs a photomultiplier tube in a feedback arrangement which permits operation of the phototube at a constant and extremely small anode output current, regardless of the amount of radiant flux impinging on the photocathode. This type of operation is achieved by automatically adjusting the tube sensitivity in accordance with the light flux reaching the cathode by altering the dynode potential of the photomultiplier. Thus, for high light intensities the sensitivity is decreased, while with low light levels it is increased. Since the feedback signal which sets the sensitivity originates from the phototube anode, the net effect of such operation is to maintain essentially constant anode current. This type of operation results in extremely stable operation which is virtually free of power supply fluctuations and fatigue in the phototube and other components.

The output signal in such a constant anode current photometer system is derived from the changing dynode voltage through suitable voltage dividers. The derivation of the output signal from this point has several extremely important advantages: (*a*) large signals are available for recording without the need for additional amplification; (*b*) of even greater importance—the relationship between light intensity and voltage is essentially logarithmic over an extremely wide range. Only a simple correction network is required to make output exactly logarithmic and hence directly and linearly proportional to absorbancy.

The same principles have been previously utilized by Sweet (1) in a densitometer system for photographic use, by Gilford *et al.* (2) in the measurement of dye concentration in blood for special diagnostic pro-

cedures, and by Malmstadt and Roberts (3) for recording end points of titrations in dense solutions. In this application to spectrophotometry, we have yet another demonstration of the accuracy, stability, and flexibility of this unique photometer system.

DESCRIPTION OF PHOTOMETER CIRCUIT

Reference to the circuit schematic in Fig. 1 will show the system to consist of three basic elements: power supplies, the feedback and control network for the photomultiplier, and the output circuits.

There are two power supplies in the instrument. A high-voltage supply furnishes potential for the photomultiplier and consists of a conventional voltage doubler using 2X2A rectifiers. A low-voltage supply stabilized by voltage regulator tubes furnishes +225, +150, +75, and −105 for operation of the rest of the system.

The voltage applied to the photomultiplier tube is controlled by a feedback system which derives its signals from the load resistance of the phototube anode. This signal is amplified in the cathode-coupled cathode follower and amplifier combination of V_{10} before being applied in the proper phase to the 6AU5 series control tube grid.

Output signals are derived from the variable dynode potential through a voltage divider. For the specific initial conditions determined by (*a*) the intensity of the light source, (*b*) the efficiency of the monochromator, (*c*) the setting of the slit width, and (*d*) a sample of zero absorbancy in the light path, an equilibrium dynode voltage is established. At this zero absorbancy point the output of the system is made to equal zero by matching a portion of the dynode voltage (from a voltage divider) with another voltage of equal but opposite polarity. This voltage, supplied by the "Background Balance" control, is adjusted to give zero output for zero absorbancy.

Since the variation of the dynode potential is not exactly logarithmic and hence not directly proportional to absorbancy, a correction network is introduced to make the relationship between absorbancy and dynode potential conform exactly. Since the uncorrected characteristic of the system is a curve which gives greater output than required with increasing absorbancy, a series of biased diodes are used to progressively shunt the output by exactly the amount required to give the linear absorbancy relationship. This calibration of the instrument or "linearization" must be done with great care if the ultimate accuracy of the system is to be achieved. It has been found that five diodes are generally sufficient to correct the system over 4.0 absorbance units to within an accuracy of 1%.

The dynode voltage, first adjusted for zero absorbancy and then

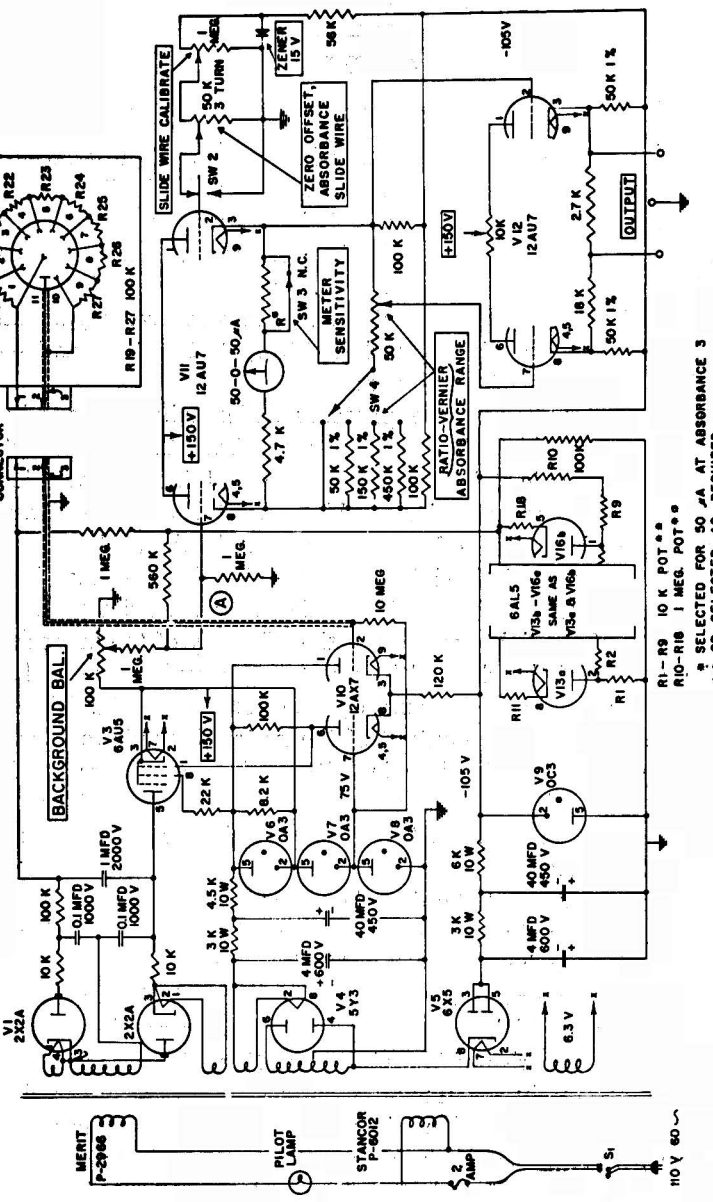

Fig. 1. Schematic diagram of photometer system.

corrected to be proportional to absorbancy, is applied to the output circuits. In some of the earlier units a small fraction of this voltage was applied directly to a recorder through a filter and a variable potentiometer which was used as a sensitivity control (4). Subsequent units have made use of pairs of cathode followers to give greater flexibility in operation and permit lower impedance recorders to be used. In the circuit schematic (Fig. 1) the signal from the photometer system is fed to one side of a balanced cathode-follower pair while the reference side is connected to a stabilized variable voltage source. With the reference source set at zero, the output of the pair of cathode followers will be zero when light intensity striking the phototube corresponds to zero absorbancy. A sensitive microammeter connected across the cathode-follower outputs permits the operator to adjust the output for zero absorbancy by changing the slit width of the monochromator until the meter reads zero.

The variable voltage source on the reference side of the cathode follower can be adjusted by means of a calibrated potentiometer to present reference voltages corresponding to a blank of any absorbancy from 0 to 3.0 units. This makes it possible to neutralize or balance out the background absorbancy of the sample and to expand the recording to any full-scale sensitivity regardless of the initial background absorbancy. The calibrated dial of the "Optical Density Off-Set" can be established with considerable accuracy by means of an internal calibration control which is used to set the voltage across the dial against an absorbancy standard.

The same calibrated control can be used to make single determinations of absorbancy in the absence of an automatic recorder in the usual manner for conventional spectrophotometers. With a sample of unknown absorbancy in the system and after establishment of the zero absorbancy with a blank, the "Optical Density Off-Set" control is adjusted until the null meter reads zero. The dial will then indicate directly the absorbancy of the unknown.

Across the same cathode-follower pair that drives the meter is connected a precision attenuator. The attenuator feeds a second balanced pair of cathode followers whose output is then fed to the recording system. A vernier control associated with the precision stepped attenuator permits the recorder to be set for any full scale value within the range of the instrument. The stepped attenuator permits switch selection of full-scale values that are one-half, one-quarter, or one-tenth of the original full-scale setting. The low impedance of the output cathode follower permits the use of the low-impedance potentiometer recording system.

OTHER COMPONENTS

In setting up a complete system, in addition to the photometer circuit described, some form of self-balancing recording system is required. These are offered in a variety of widths of chart, chart-drive mechanisms, and other refinements. Generally, however, any self-balancing potentiometer having a sensitivity of about 100 mv could be used. This is connected to the output of the photometer system.

A Beckman DU spectrophotometer was used throughout this study, although the system is applicable to any spectrophotometer. The Zeiss PMQII monochromator also functions satisfactorily. Only the monochromator was used together with the incandescent and hydrogen lamp and their respective supplies; the electronic components of the original instrument were not energized. It should be pointed out that it is extremely important to have these sources in excellent operating condition as the stability of the over-all system is highly dependent upon the stability of the light sources. The 1P28 photomultiplier of the special photometer was mounted in a simple light-tight box having the same mounting arrangement as the multiplier system supplied with the DU (earlier versions of the system made use of the DU phototube housing with the photomultiplier tube reconnected). No shutter arrangements have been found necessary with the new photometer since it automatically safeguards itself against excessive light by decreasing its sensitivity drastically.

PERFORMANCE

The relationship between voltage or chart deflection and light flux under normal operating conditions, i.e., without pinhole diaphragm and with 3-ml quartz cells ($b = 1$), is shown in Fig. 2. These data were obtained as follows: two cuvettes, one containing water, and the second a solution of absorbancy about 0.2 were employed. After setting the water cuvette to give zero deflection, the 0.2 absorbancy was introduced and its deflection recorded. By adjusting the monochromator slit, water was set to give the same absorbancy as the sample, and then the 0.2 absorbancy sample was introduced again, to give another recorder pen deflection. By repeating this process, successive slit width settings equal to the absorbancy of the second cuvette were made and the output recorded. The plot of these successive deflections show that as the light flux diminishes, there is an increasingly greater pen deflection at each step (Fig. 2). This deviation from linearity was corrected by setting the diodes to conduct at the voltages indicated by the arrows, these being the points where the first departure from linearity occurred in

each successive stage of correction. The corrected response is shown by the lower line. Although five diode stages have been found sufficient for linearity, eight stages were employed in this case. Also, it has been found that the response can be linearized for light-flux values equivalent

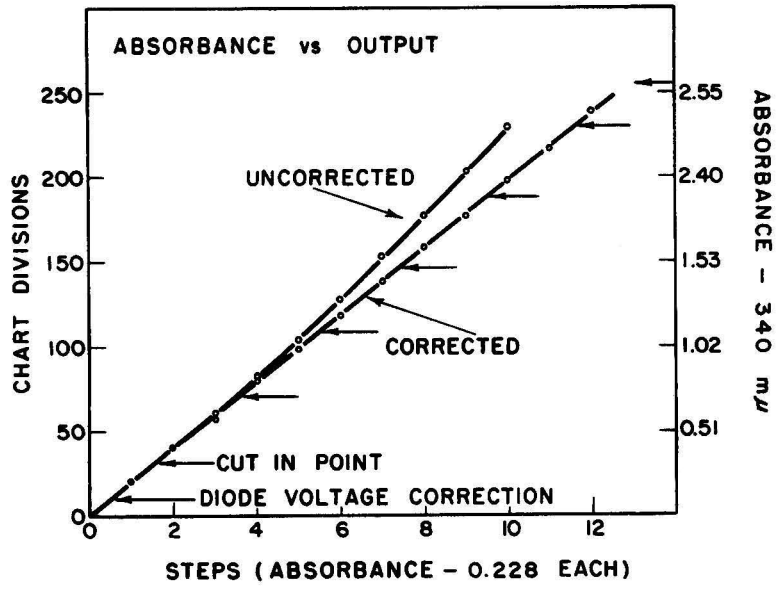

FIG. 2. Output of photomultiplier system as a function of absorbancy.

to absorbancy range of 0 to 3 or 4. Similar observations have been made with the microcuvettes ($b = 1$, fluid vol. = 0.1–0.35 ml). However, a pinhole diaphragm (*ca.* 1 mm dia.) is required for accurate positioning. The decrease in incident light thus sustained corresponded to about 1.1 absorbancy units. The slit width settings required for balance at zero absorbancy at selected wavelengths are shown in Table 1. The

TABLE 1

SLIT WIDTH SETTINGS FOR ZERO ABSORBANCY AT VARIOUS WAVELENGTHS[a]

Pinhole size	Slit width (mm)			
	220 mμ[b]	260 mμ[b]	340 mμ[b]	540 mμ[c]
None	0.28	0.19	0.14	0.24
X	0.70	0.47	0.34	0.07
½X	1.25	0.83	0.61	0.16
¼X	>2.0	1.70	1.21	0.25

[a] Water in a 1-cm silica cell was taken as zero absorbancy.
[b] Hydrogen lamp.
[c] Tungsten lamp.

settings are also shown for the pinhole diaphragm of Lowry and Bessey (5).

By means of the ratio and vernier settings, a range of 0–4 absorbancy units or as little as 0.1 absorbancy unit could be made to occupy the full chart width. At the very high optical density values or with high sensitivity affording a full-scale deflection with 0.1–0.2 absorbancy unit, the photomultiplier noise was appreciable and was displayed by a pen vibration which increased the width of the tracing. Much of this component, however, can be removed by a filter in the output. The maximum sensitivity, therefore, is limited only by the phototube noise, sensitivity of the recorder, and stability of the various components in the optical and electrical systems. An accuracy of the same order as the chart resolution or between 0.5 and 1% has been achieved. Reproducibility also is of this order of magnitude. Zero drift varies considerably with the performance of the light source employed, but has been measured to be within 1% over a 35-min period. For high reproducibility with microcuvettes an accurate positioning cuvette changer, accurately collimated optics, and pinhole installed close to the cuvette are required. Calibration has been stable over long periods of time.

The response recorded by solutions of adenosine at 260 mμ over a wide range of absorbancy values is shown in Table 2. A stock solution of nominal absorbancy of 5 was prepared using the published extinction

TABLE 2

ABSORBANCY MEASUREMENTS AT 260 Mμ USING STANDARD ADENOSINE SOLUTIONS

Absorbancy			Transmittancy		
Nominal	Observed	Error (%)	Nominal	Observed	Stray light
0	0	—	100	100	—
0.5	0.48	−4	32	33	—
1.0	1.00	0	10	10	—
2.0	2.06	+3.3	1	0.87	—
3.0	3.07	+2.3	0.1	0.085	—
4.0	3.72	−7.0	0.01	0.019	0.9:1
5.0	3.94	−21.2	0.001	0.0115	11.5:1

coefficient of adenosine. Solutions of lower absorbancy values were then prepared by dilution. The absorbancy readings therefore contain the errors sustained in all of the operations involved. It can be seen that within the limits of these errors, stray light is not a discernible factor at absorbancy 3. However, at absorbancy 4 and 5 this factor contributes errors of 7 and 21%, respectively. These values are in line with the stray light expected from the Beckman DU monochromator. Further,

this table illustrates that the spectrophotometric measurement in the region of absorbancy 3 are quite feasible insofar as stray-light errors are concerned.

APPLICATION TO ENZYME MEASUREMENTS

Figures 3–6 are unretouched records showing the performance of the instrument. Figure 3 shows the reduction of triphosphopyridine nucleotide by purified glucose-6-phosphate dehydrogenase from yeast, as meas-

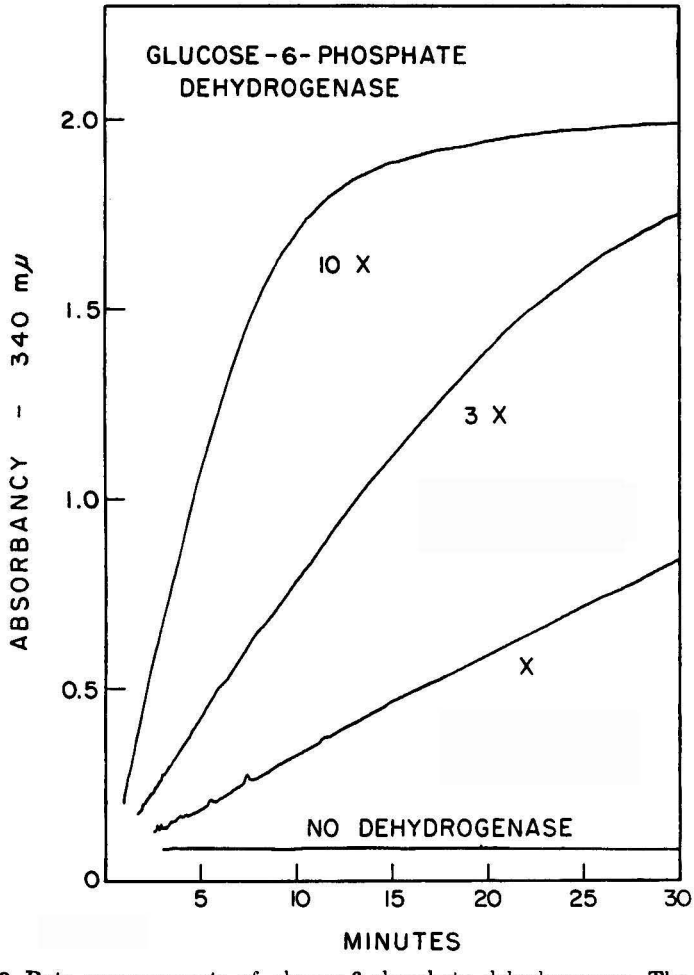

FIG. 3. Rate measurements of glucose-6-phosphate dehydrogenase. The reaction mixture (0.35 ml) in 0.5-ml microcuvettes ($b = 1$) contained 25 μmoles glycylglycine-HCl buffer, pH 7.5, 5 μmoles $MgCl_2$, 0.75 ml TPN, 1 μmole glucose-6-P, and 0, 0.035, 0.105, or 0.35 unit of purified glucose-6-P dehydrogenase as indicated.

ured by increased absorbancy at 340 mμ. In the reaction mixture, the only component limiting the velocity was the amount of dehydrogenase. Variation in the dehydrogenase content thus changed the reduction rate as shown. However, the velocities were too rapid for the rate to be precisely proportional to the amount of the catalyst added. These tracings were

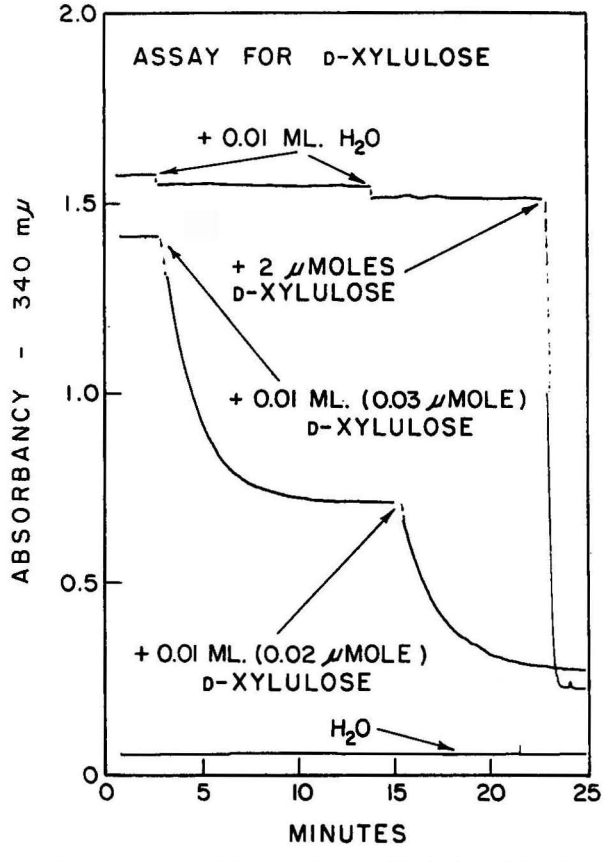

FIG. 4. Determination of D-xylulose with D-arabitol (D-xylulose) dehydrogenase. The reaction mixture contained, in 0.35 ml, 25 μmoles potassium phosphate buffer, pH 7.0, *ca.* 0.5 μmole DPNH, 1.3 units dehydrogenase, and D-xylulose as indicated.

made in single cuvettes, and the chart paper was returned to the starting point for the next run. The aggregate of the drift, noise, convection currents in the cuvette, etc., can be seen from the bottom lines where the enzyme was omitted.

A similar sequence in which reduced diphosphopyridine nucleotide is oxidized in the presence of purified D-arabitol (D-xylulose) dehydro-

genase from *Aerobacter aerogenes*, and D-xylulose is shown in Fig. 4. In these tests the amount of D-xylulose was limiting whereas the amount of dehydrogenase was in excess. The decrease in absorbancy can be used to calculate the amount of D-xylulose added.

Figure 5 shows traces of the course of color development in the

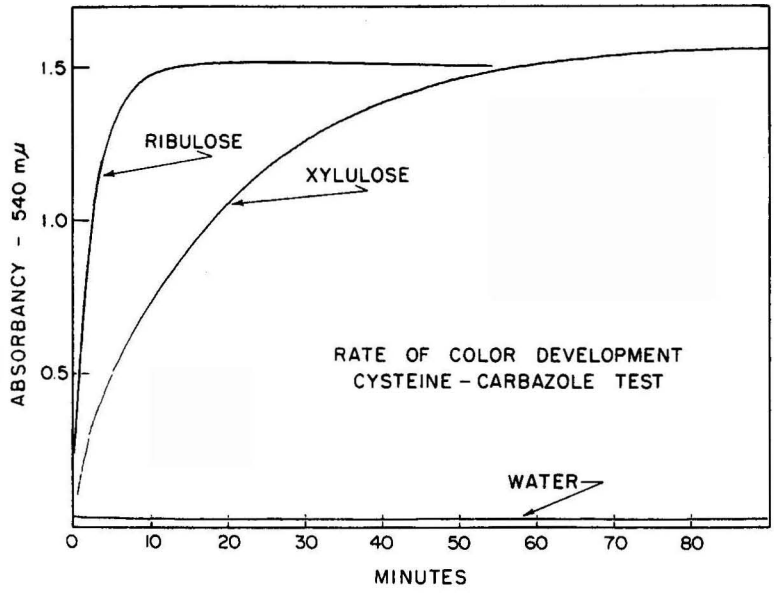

FIG. 5. The rate of color development of ribulose and xylulose in the cysteine–carbazole test. The test was performed as described by Dische and Borenfreund (6) using approx. 0.3 μmole ketopentose. Temperature of color development, 37°.

cysteine–carbazole test for ketopentoses. After the reagents are mixed the rate of color development at 540 mμ is characteristic of either ribulose or xylulose.

SUMMARY

A photometer of high stability and linear absorbancy characteristics is described. Attached to a conventional laboratory spectrophotometer like the Beckman DU, it permits direct recording of absorbancy changes with time. The device uses a photomultiplier in a special feedback system which permits flexible operation at full scale values from 0.1 to 3.0 or 4.0 absorbancy units. Linearity and reproducibility are of the order of 1% with a high order of base line stability. Detailed performance of the unit is described and typical velocity recordings of enzyme and chemical reactions are presented.

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ANALYTICAL BIOCHEMISTRY 2, 601–609 (1961)

Recording of Several Simultaneous Enzyme Reactions Facilitated by an Automatic Cuvette Positioning Attachment for the Spectrophotometer[1]

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INTRODUCTION

In the preceding paper (1), a photomultiplier system was described which provided a means for continuous recording of absorbancy changes at one wavelength over a wide range of absorbancy values. While the value of this system was clearly evident, it became apparent that its use need not be limited to observation of single reactions. Systematic changing among two to four cuvettes, each being observed for a period of time, yielded a series of interrupted lines equivalent to segments of a continuous tracing. These are interconnected by vertical lines representing the pen travel (and associated transients) from the absorbancy level of one cuvette to that of the next. By use of a stop watch and manual cuvette positioning, very satisfactory multiple tracings by single-pen recorder were achieved. However, an operator was needed to perform the shifting operation, a requirement out of proportion to the function supplied. This paper reports on the considerations involved in development of an automatic cuvette positioner and provides a demonstration of successful application to recording of multiple simultaneous spectrophotometric enzyme assays. The combination of the photomultiplier system previously described (1), the automatic cuvette positioner, and a commercially available recorder accomplishes the complete "automation" of spectrophotometric enzyme assays.

DESCRIPTION OF APPARATUS

From a mechanical standpoint, a cuvette positioner must provide a precise amount of lateral motion equivalent to the distance between the

[1] Reported in part before the American Society of Biological Chemists, Chicago, April, 1960.

[2] Contribution No. 2825 of the Michigan Agricultural Experiment Station.

vertical lines of center of the cuvettes and provide a return to the starting point. Although many systems may accomplish this function, a mechanism employing a horizontal rack and pinion connected through a magnetic clutch to a reversible motor was chosen initially to test the possibilities of automatic positioning. Since microcuvettes and a 1-mm pinhole were to be employed, precise positioning was necessary. This was provided by a cuvette carriage sold by A. S. Aloe and Company which has a latching arrangement for exact positioning.

Figure 1 shows the external portion of the cuvette positioner. The motor with forward and reverse field windings and gear train was connected through a magnetic clutch to a rack and pinion. The shaft of the cuvette changer was modified to provide a hook which fastened over the last gear tooth of the rack. Thus the lateral motion of the rack provided the positioning. The stop point in the travel was established by contact of a lug on the rack with one of four microswitches. In order to unlatch the cuvette changer, the shaft must be raised to disengage the positioning groove in the shaft from the positioning edge on the carriage housing. The lift was provided by a solenoid-actuated lever under the carriage shaft. A pulse to the solenoid unlatched the shaft; upon relaxing the solenoid, the shaft fell on the positioning edge and rode to the next positioning slot where it stopped. At the same time power to the magnetic clutch was removed by the corresponding microswitch. In normal operation the drive motor functioned continuously and the torque was provided to the pinion gear by actuation of the magnetic clutch.

The mechanical unit was controlled by a "programmer" which provided (*a*) clock or manual actuation of transport, (*b*) selection of the number of cuvettes to be observed (for the Beckman DU spectrophotometer 2 to 4 cuvettes), (*c*) a disconnect of the recorder servo motor during cuvette position, and (*d*) an indication of which cuvette was under observation. In addition, activation of forward or reverse motor, clutch, and unlatching solenoid were provided. These functions were provided by the microswitches shown in Fig. 1 and by a programmer containing nine multicontact relays.

The linear motion system performed very satisfactorily, but an alternate design was developed which had a number of advantages over the rack and pinion drive. The new design[3] is based on a cam operated push-rod mechanism which provides the required precise positioning with a much simplified control system. This unit was built and tested and found to be equal in performance in all respects to the above linear motion system.

[3] Patent pending.

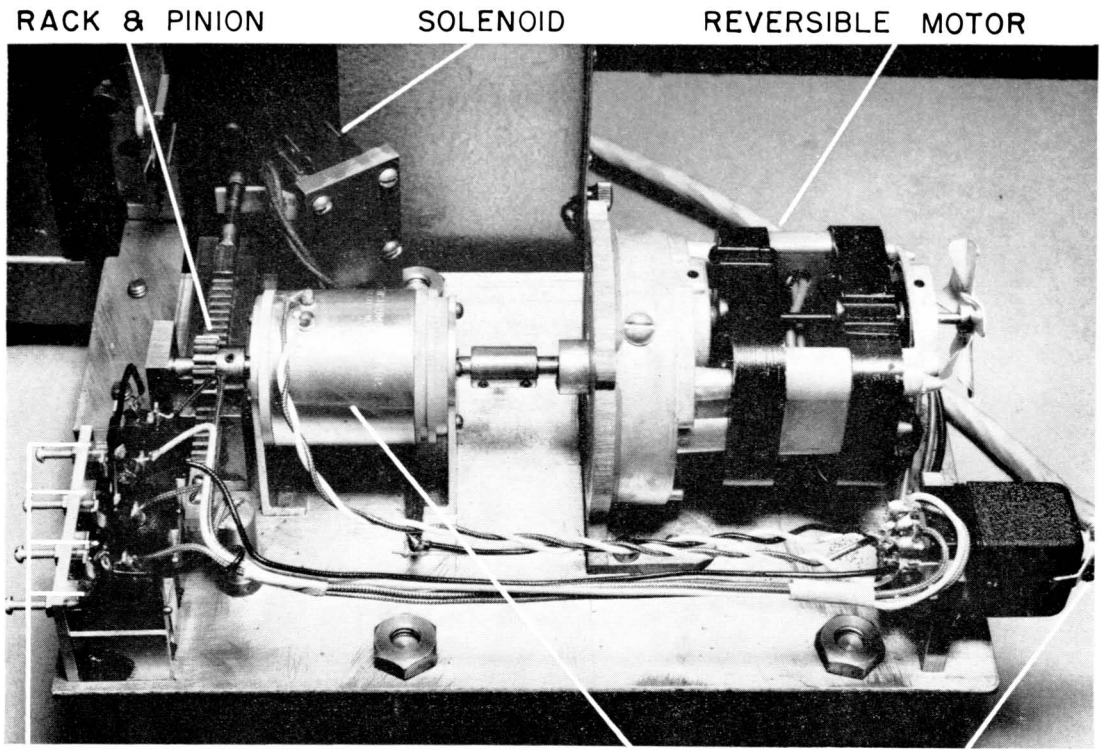

FIG. 1. Rack and pinion automatic cuvette positioner, mechanical unit.

The cam provides the required lateral motion and positioning accuracy by means of four radical segments with radii equivalent to the lateral displacement required. These are located at 90° intervals around the cam. A second cam lever, attached to the main cam, operates a set of associated microswitches which stop the cam-driving motor on the radial segment. Figure 2 shows a functional diagram of the cam, switch

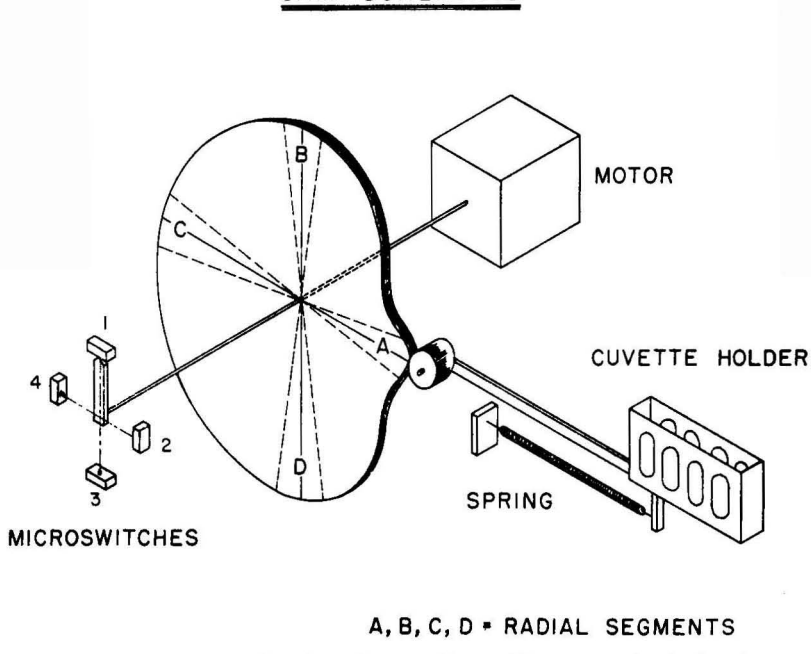

FIG. 2. Cam-operated automatic cuvette positioner, mechanical unit.

mechanism, the cam push rod, and spring-loaded carriage. Since a rest point anywhere on the radial segment produces the same amount of lateral displacement, precise stopping of the cam and attendant accurate adjustment of the microswitches is unnecessary. Further, a reversing operation is unnecessary since on the cam, position 1 follows position 4.

By the wiring arrangement outlined in abbreviated form in Fig. 3, it is possible to select any desired cuvette position by opening the appropriate manual selector switch. For automatic programming, the desired selector switches are opened and the timer switch starts the cam motor by shorting the motor driving contact on the relay. Once the motor has started, it will stop only when it strikes an unbypassed cam switch. When the cam motor is stopped, the timer starts and runs until

its rotation again shorts the motor-driving switch and the cam motor goes through the next cycle.

The above description serves only to illustrate the basic principle of operation. In the actual circuit, provision must be made for dynamic braking to the cam motor. Also, there are additional circuit elements

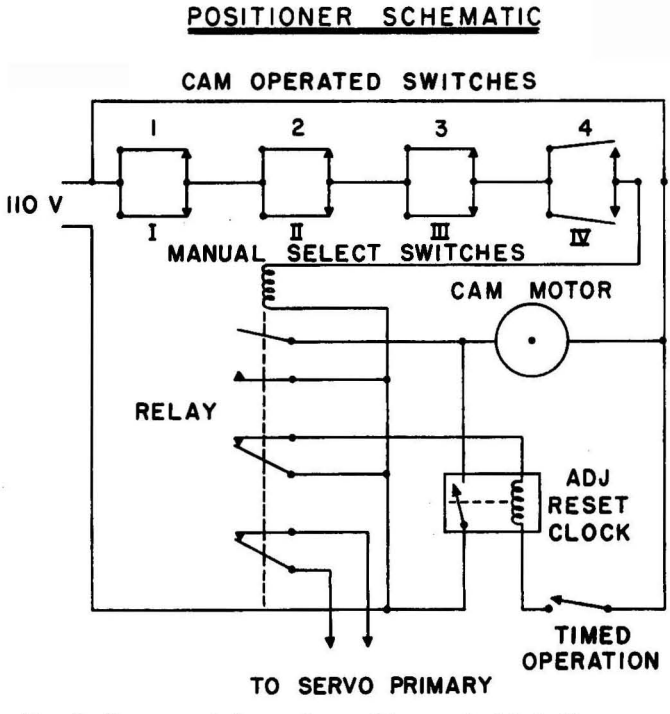

FIG. 3. Cam-operated cuvette positioner, electrical diagram.

such as cuvette position-indicating lights and a pair of contacts on the relay which open the primary winding of the servo motor on the recorder during the period of cuvette transport or of cam motor operation.

RESULTS

Figure 4 illustrates the type of tracings obtained with the automatic cuvette positioner in conjunction with the photomultiplier system (1), Sargent recorder and Beckman DU spectrophotometer. Pyrocell cuvettes were employed ($b = 1$ cm) which contained a 0.35-ml reaction volume. In these reactions, the oxidation of glucose 6-phosphate, coupled to the reduction of triphosphopyridine nucleotide and catalyzed by purified glucose-6-phosphate dehydrogenase, is accompanied by increased absorbancy at 340 mμ. Full chart width equals an absorbancy of 2.5. The

velocity of reaction in each cuvette is accurately recorded even though each cuvette is observed for only 15 sec of the total cycle. The increase in absorbancy is a measure of the amount of glucose 6-phosphate added

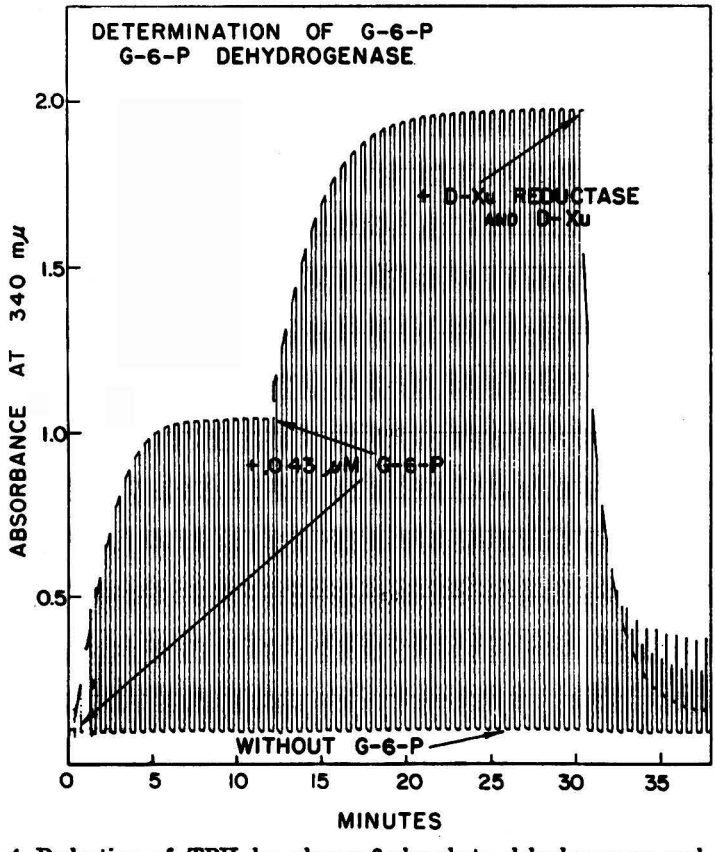

FIG. 4. Reduction of TPH by glucose-6-phosphate dehydrogenase and glucose 6-phosphate. The cuvette contained in 0.35 ml, 0.043 μmole glucose 6-phosphate (added twice), 1 μmole triphosphopyridine nucleotide, 2.5 μmoles glycylglycine, pH 7.5, purified glucose-6-phosphate dehydrogenase, and water. After approximately 31 min, 1 μmole L-xylulose and purified xylitol (L-xylulose) dehydrogenase from guinea-pig liver were added.

(two additions). It can be seen that after oxidation of the second sample of glucose 6-phosphate, addition of D-arabitol (D-xylulose) dehydrogenase and D-xylulose[4] resulted in rapid reoxidation of reduced triphosphopyridine nucleotide.

[4] Abbreviations used: Xu, xylulose; TPN and TPNH, oxidized and reduced triphosphopyridine nucleotide, respectively; G-6-P, glucose 6-phosphate.

Figure 5 shows the oxidation of TPNH by L-xylulose as catalyzed by xylitol (L-xylulose) dehydrogenase (2) and observed at the same wavelength. Three cuvettes were studied. The slope of these lines, which represents the information to be derived from the assays, is easily

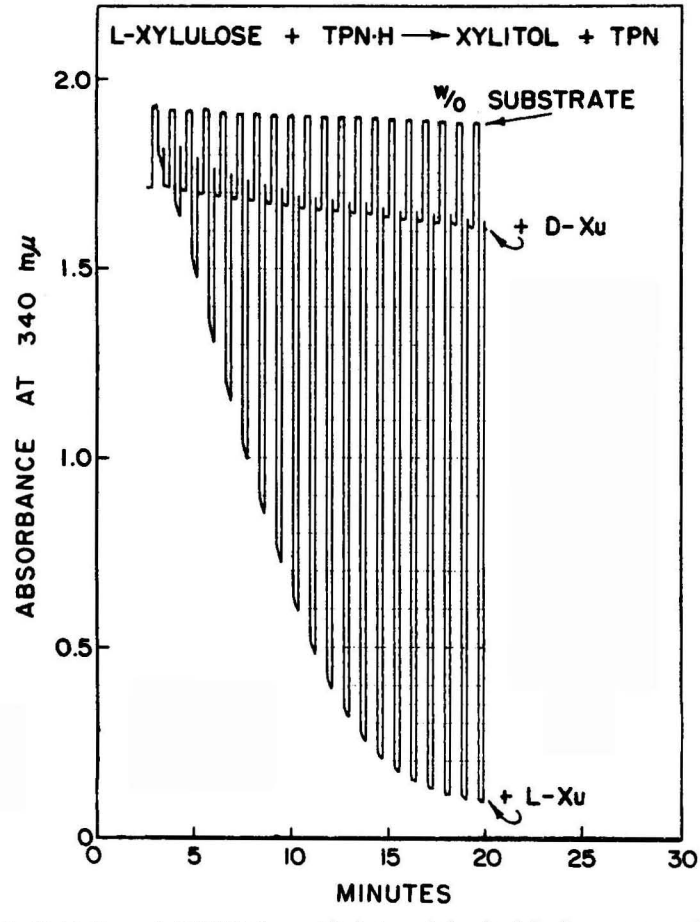

FIG. 5. Oxidation of TPNH by xylitol (L-xylulose) dehydrogenase and L-xylulose. The assay was carried out in 0.35-ml under the conditions described by Hickman and Ashwell (2). Xylulose additions were made as indicated.

obtained from these tracings. By variation of chart speed and observation time, very rapid and very slow reaction velocities also can be accommodated. In addition, these measurements show (*a*) the high reproducibility of the positioning function (variations appear as differences in heighth between adjacent observations of the same cuvette), and (*b*)

total drift and other irregularity of the intact system. These include variations of light source, monochromator, photomultiplier system, recorder and cuvette positioner, and photochemical decomposition of the light-absorbing material, dihydrodiphosphopyridine nucleotide. The positioning error is approximately one part in 250 (the resolution of the paper used), and the drift over 36 min was a maximum two to three parts in 250 for certain of the cuvettes.

Figure 6 shows the use of the automatic cuvette positioner in determination of the absorption spectrum of a ribose–ribulose mixture in

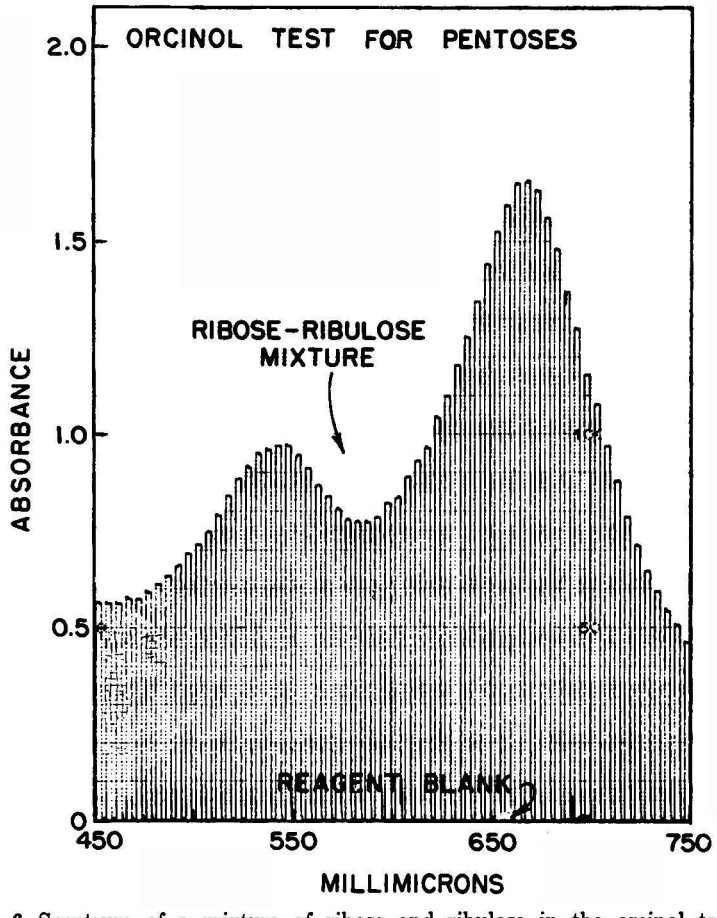

FIG. 6. Spectrum of a mixture of ribose and ribulose in the orcinol test. The test was performed as described by Horecker (3). Automatic changing between blank and the ribose–ribulose solution was set up. When the blank entered the light beam in each cycle, the slit was adjusted manually to give zero absorbancy. The absorbancy of the test was then automatically plotted.

the orcinol procedure for pentose (3). The cuvette positioner alternately positioned the reagent blank and test solution every 15 sec and the operator set the wavelength (5-mμ intervals) and zeroed the reagent blank with the slit. In this way a spectral record is obtained without reading and plotting absorbancy values. The dual peaks with maxima at 540 and 670 mμ are characteristic of ribulose. The increased absorbancy at 670 mμ is due to the additional color produced by ribose which absorbs maximally at 670 mμ, but not at 540 mμ.

SUMMARY

Two clock-operated devices for accurate sequential positioning of a cuvette carriage for the Beckman DU spectrophotometer is described. Through their use with associated equipment absorbancy changes resulting from reactions occurring in two to four cuvettes simultaneously can be recorded with a single-pen recorder. Data on reproducibility of positioning and typical recordings of enzyme-catalyzed reactions are provided.

ACKNOWLEDGMENTS

The author is indebted to Mr. Levin J. Peek and Mr. Heinrich Martens for their valuable aid in development of this equipment. An initial phase of this work was performed at the University of Illinois.

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ANALYTICAL BIOCHEMISTRY 2, 610–616 (1961)

Quantitative Determination of Esterase Activities after Starch Gel Electrophoresis[1]

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INTRODUCTION

The use of starch gel electrophoresis for the separation of molecular varieties of enzymes (isozymes) (1) has found widespread use, mainly because of the high resolving power and simplicity of this technique. However, one of the disadvantages of this technique has been the lack of a suitable method to quantitate the separated enzymes. Several attempts of quantitation have already been reported. Dixon and Smithies (2) have cut the starch strip into a sequence of transverse sections, broken the gel structure by freeze-thawing, and then added the substrate to the sponge-like mass obtained. C. L. Markert (personal communication) used a similar procedure, but located the desired enzyme bands first by subjecting a small portion of the strip to histochemical reactions; the enzyme was then eluted from the rest of the starch strip and its activity determined colorimetrically by using a dye coupler which yielded a soluble azo dye. This method gave fluctuating results, probably due to incomplete elution of the enzyme from the starch. Plagemann, Gregory, and Wróblewski (3) degraded the starch with α-amylase and recovered 55% of the inserted lactic dehydrogenase in the supernatant after centrifugation, and a total of 85% of this enzyme with the starch residue included. Allen (4) has described a semiquantitative approach; densitometer tracings were made along the translucent starch strips, and the recorded maxima of the absorbance were taken for a rough estimation of the esterases of *Tetrahymena*.

All the described techniques suffer from the disadvantage that either the maximum resolving power of the starch gel electrophoresis is lost or accuracy and reproducibility are unsatisfactory.

[1] This investigation was supported in part by a National Science Foundation research grant, G-11328 to Theodore R. F. Wright, and in part by a research grant of the U. S. Public Health Service, RG-6846 to Konrad Keck.

In the present paper a satisfactory and useful technique for quantitation is described. It was found that the water-insoluble azo dye, final product of esterase action, can be eluted from starch with a mixture of *n*-amyl acetate–ethanol in the ratio of 1:1, and that the amount of the extracted dye, as determined colorimetrically, is correlated with satisfactory accuracy to the amount of enzyme present.

The quantitative assay for esterase activities does not require any fundamental changes in the procedures commonly applied to starch gel electrophoresis of enzymes, although certain modifications are necessary in order to achieve a high degree of reproducibility of the results obtained. Thus a known amount of material must be inserted in the starch, necessitating the use of micropipets. For accurate delivery and because of the small bore of such pipets, tissue homogenates should not be applied directly. Instead, it is recommended that the cell debris first be removed by high-speed centrifugation. A known volume of the supernatant can then be adsorbed to a piece of filter paper which is inserted in the starch. The inserted paper should not contact the bottom or sides of the Lucite tray in which the starch is contained. The ratio between the volume of liquid adsorbed to the paper and the area of the inserted paper is rather critical. Any excess of liquid will be squeezed out since tight contact between the inserted filter paper and the starch gel is obligatory; a rather "dry" insertion, on the other hand, introduces a high electrical resistance and, because of the likelihood of uneven application of the material, may lead to distorted bands. For optimum results approximately 1 μl liquid is required to properly moisten 4–4½ sq mm of Whatman 3MM filter paper.

The determination of esterase activities is based upon the amount of colored water-insoluble azo dye precipitated in the starch during a given period of incubation. α-Naphthyl acetate serves as substrate, and Fast Blue BB (Dajac Laboratories, Philadelphia) is used as dye coupler. The possibility must be considered that in thick starch strips the distribution of the enzyme (narrow or wide bands) may significantly influence the reaction. The rate of enzyme action at a particular point within the starch could be limited by a suboptimum local concentration of substrate, caused by slow diffusion of the substrate and its rapid hydrolysis by the enzyme. Furthermore, the precipitate formed during the reaction could impose an additional barrier to the substrate. In order to minimize these uncontrollable factors, it is suggested that the thickness of the incubated starch strips be no greater than 1½ mm. Strips of the commonly used dimensions, $6 \times 25 \times 300$ mm, should thus be cut into four parallel horizontal slices, each 1½ mm thick, prior to the incubating in the histochemical reagent. Each slice is placed in a separate vial with

reaction mixture, enforcing a vertical position of the strip which permits penetration of the reagents from both sides. Reproducibility of the results is furthermore assured by strictly controlling the temperature and time of incubation.

After development of the bands, the strips are quickly placed in distilled water. The unreacted reagents are removed by several rinses with water, each of 20 min duration, until all yellow background color has disappeared from the starch. The strips are then placed in a shallow glass tray in distilled water, and the desired colored bands are carefully cut out with a scalpel; excess water is removed by blotting with filter paper, and the cut sections are transferred to small tubes which have been calibrated to hold a certain volume. A 1:1 (v/v) mixture of *n*-amyl acetate–ethanol is added to fill the tube slightly below the calibration mark. The dye is completely extracted from the starch fragments after several hours of shaking at room temperature. The water originally present in the starch pieces is readily taken up by the solvent mixture under these conditions; however, if agitation is omitted or when too narrow tubes are used, water from the starch will collect at the bottom of the tube and interfere with the elution. The amount of eluant to be used for the elution of a given starch fragment depends on the size of the piece as well as on the over-all intensity of the bands. The total volume should not be less than 10 times the volume of the starch to be eluted. On completion of the elution, more solvent is added to fill the tubes to the predetermined mark. After thorough mixing the solvent can be withdrawn for colorimetric determination of the dye content without further centrifugation.

We have originally tried to develop the above technique for the determination of enzyme activities in individual slices, thus being able to determine more than one enzyme type per run. In comparing corresponding bands from four parallel horizontal slices of a 6-mm thick starch strip, which were treated identically, we encountered significant deviations in the ratio of different bands within one slice, as well as between the amount of dye in corresponding bands of parallel slices. The fluctuations could be attributed to imperfections of the slicing technique, because the slices of starch varied in their thickness. A satisfactory relationship could be obtained between the amount of enzyme applied to the origin at the beginning of electrophoresis and the absorbance of the eluted dye, when the absorbances of corresponding bands from all slices were combined.

The absorption spectrum taken from the gray-colored eluates of esterase bands shows a broad peak at 450 mμ, another one at approximately 600 mμ, and very little decrease of absorption between these

peaks. Thus any wavelength within the visible range could be used for the colorimetric assay. Since, however, the reagent mixture (Fast Blue BB) absorbs very strongly below 450 mμ, we have chosen to read our eluates at 500 mμ in order to avoid any possible error in the determination that might be produced by traces of the reagent left in the starch.

RESULTS AND DISCUSSION

As concrete examples of these techniques we will describe in detail esterase assays of a dilution series, made both on a macro and also on a micro scale. In these experiments homogenates of male flies of a highly inbred Oregon-R strain of *Drosophila melanogaster* were used as a source for esterases. A homogenate was prepared by grinding up 300 flies in 1.5 ml of 0.5 *M* tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.7, and the cell debris was removed by spinning the homogenate for 5 min at 12,000 rpm (Intern. Micro-Capillary Centrifuge, model MB, head #905). The opalescent supernatant served directly as the undiluted enzyme solution. From this supernatant dilutions with concentrations equal to 0.8, 0.6, 0.4, 0.2, and 0.1 of the original solution were prepared.

For the large-scale assay 20 μl of these solutions was adsorbed to 5×17 mm large Whatman 3MM filter papers, and these were inserted in $6 \times 25 \times 300$ mm starch strips. Connaught (Toronto) partially hydrolyzed starch was used, and the starch gel was prepared with 0.05 *M* Tris buffer, pH 8.7 in the concentration recommended by the supplier. Tris buffer of the same pH, but 0.25 *M* was used for the electric bridge. The samples were exposed for 5 hr at room temperature to a voltage gradient of 5 v/cm; the starch strips were then sliced horizontally into four parallel slices, which were individually incubated in vials with a solution of 300 mg Fast Blue BB and 30 mg α-naphthyl acetate in 100 ml of 0.1 *M* phosphate buffer, pH 6.5. The strips were incubated for exactly 20 min at 22°C, then subjected to several rinses with distilled water, and finally transferred to a shallow glass dish. Two esterase bands (Nos. 5 and 6)[2] were cut out and eluted with 2 or 1 ml, respectively, of the *n*-amyl acetate-ethanol mixture. The eluates of the corresponding bands from all four slices of a given run were first read individually at 500 mμ in a Beckman DU spectrophotometer; the corresponding samples were then pooled and the mixture read. Starch blanks—esterase-free portions of equal dimensions—were cut out from each slice and extracted in the same way; the values measured were subtracted from the esterase values. The values obtained from the com-

[2] T. R. F. Wright, in preparation, 1961.

bined eluates of esterase bands 5 and 6—corrected for the blanks—were plotted against the concentration of the insertion sample (Fig. 1).

A similar concentration series was analyzed on a micro scale. Small starch strips with the dimensions $1 \times 10 \times 30$ mm were prepared in trays assembled on microscope slides. Two microliters of the sample solution were adsorbed to a 1×10 mm Whatman 3MM filter paper and

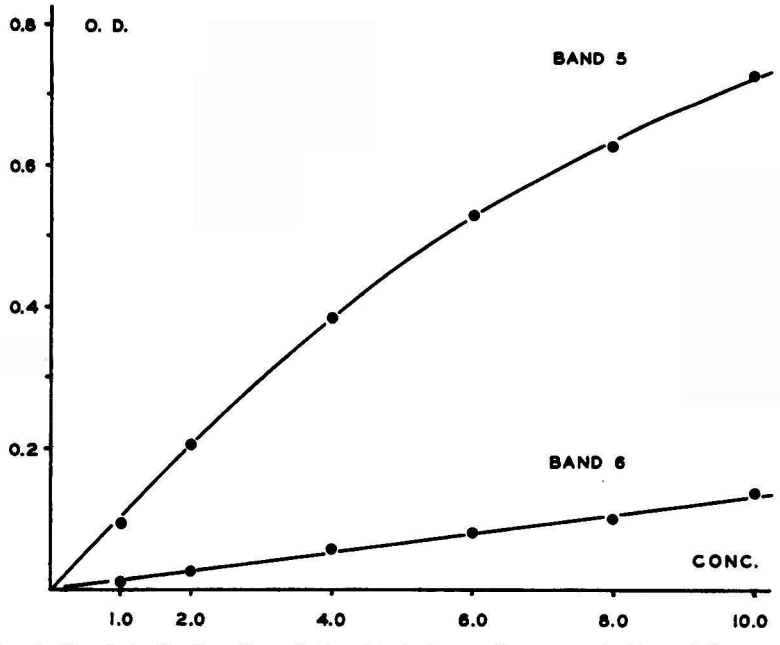

FIG. 1. Graph indicating the relationship between the concentration of the enzyme and optical density at 500 mμ of the azo dye eluted from esterase bands 5 and 6, respectively, of *Drosophila melanogaster*. The eluates of the corresponding bands from four parallel slices were combined and the values read corrected for the starch blanks.

inserted in the starch. Electrophoresis was carried out for 3 hr at room temperature in a voltage gradient of 3 v/cm. Since no slicing is required in this procedure, the total strips of 1 mm thickness were incubated for esterases as described above. The dye was eluted with 300 μl of the *n*-amyl acetate–ethanol mixture, and the optical densities were read in a microspectrophotometer. The instrument and the microcuvettes used here have been described elsewhere (5) (Fig. 2).

Both these techniques appear equally suitable for the quantitative determination of esterase activities after starch gel electrophoresis. The concentration–optical density relation is linear for lower enzyme concen-

trations, and shows a gradual slope off toward higher concentrations, which is probably due to diffusion factors as discussed earlier. Using a particular volume of eluant, the enzyme concentration may be varied 20-fold, but this range can be extended toward lower concentrations by decreasing the volume of the solvent, at least until the lowest possible volume, 10× the volume of the starch, is reached.

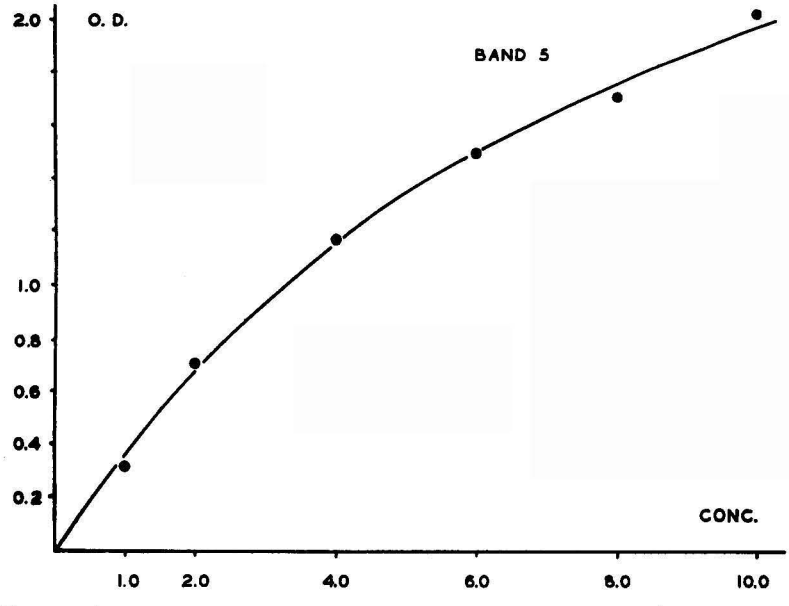

FIG. 2. The enzyme concentration–optical density relationship of band 5 of *Drosophila melanogaster* esterase, as assayed on the micro scale.

The histochemical esterase reaction in the starch does not proceed at a constant rate. In Fig. 3 we have plotted the amounts of azo dye precipitated by esterase band 5 during 80 min of incubation of the starch slices. With this enzyme the initial rate slows down appreciably after 20 min of incubation, probably due to the build-up of the insoluble azo dye within the starch which may progressively limit the accessibility of substrate and dye coupler to the site of enzyme action. Therefore we have chosen an incubation time of 20 min, a period which does not extend into the plateau region of the curve.

Differences in the kinetic characteristics between esterases from various sources as well as between the multiple molecular forms of one source must be anticipated, and it is therefore suggested that both a suitable incubation period be determined and a dilution series be assayed in each of these cases. It should also be possible to extend the

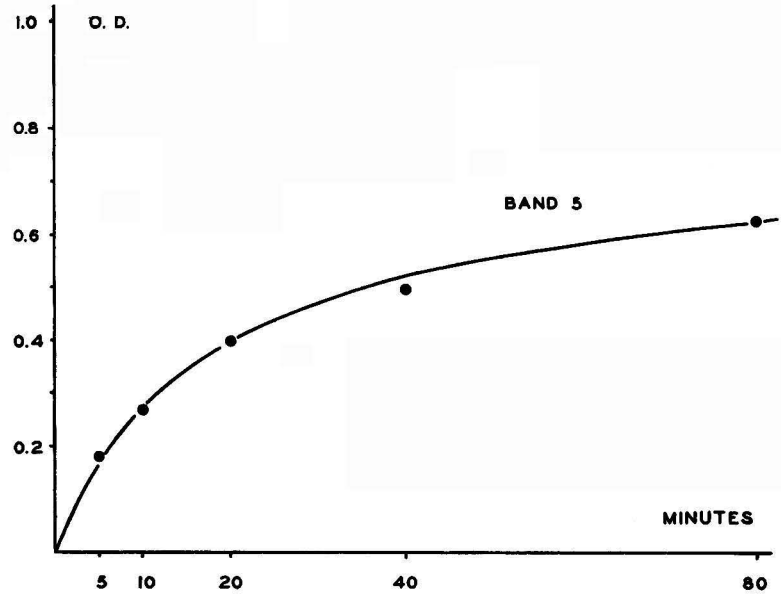

FIG. 3. The amount of azo dye deposited by the esterase of band 5 after various periods of incubation of the starch slices. Each of the corresponding bands of four parallel slices was eluted with 2 ml of the extraction mixture, and the eluates were pooled for the colorimetric determination.

described methods to other enzymes which split naphthyl esters, for instance, phosphatases and cholinesterases.

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ANALYTICAL BIOCHEMISTRY 2, 617–618 (1961)

SHORT COMMUNICATION

Determination of Urinary Corticoids

Certain critical comments appear to be in order regarding the publication of a method for urinary corticoids by Kingsley and Getchell (1).

There is a large body of evidence to indicate that the major secretory product of the human adrenal cortex, within the "corticoid" genera, is hydrocortisone. Therefore the aim of any method, such as the one under question, should seek to reflect the quantity of the metabolites of this steroid in the urine. That hydrocortisone is excreted in the urine, primarily as a reduced product, tetrahydrocortisone, conjugated as the glucuronoside is consonant with current evidence (2). Through the generosity of Doctor John Schneider, we have been able to study the hydrolysis of this particular (and admittedly, virtually unobtainable) conjugate which had been isolated from urine and purified. We have recorded that this substance is poorly, if at all, hydrolyzed by acid under various conditions (3, 4). More recently we have attempted to hydrolyze this steroidal conjugate by the precise method described by Kingsley and Getchell, without success. Approximately 25 μg of the conjugate was dissolved in 10 ml water and submitted to the procedure described by the latter author. This failure does not necessarily appear to be attributable to destruction by acid, since the conjugated steroid can be recovered in its original form—or subsequently hydrolyzed by enzymes—following such treatment. It is apparent that the good recoveries of free cortisone and cortisone acetate, reported by Kingsley and Getchell, do not answer the question. These standards are not representative of urinary components. In the absence of evidence that other important acid-hydrolyzable corticoid conjugates are present in urine, it must be assumed that the final extract measured with blue tetrazolium contains reactive compounds which are *not* those ideally sought, and must be regarded as nonspecific until they are characterized. Even were the authors to consider "corticoids" and their metabolic urinary products, other than hydrocortisone, to be of prime importance, the overwhelming evidence indicates that

most occur as glucuronosides. Therefore these remarks concerning hydrolysis cannot be overlooked.

Another point is worthy of note. A comparison between substances reacting with blue tetrazolium and those designated as 17-ketogenic following bismuthate oxidation (5) is not strictly in order and therefore should not be employed as a means of substantiation. Thus, it is now well known that normal urine contains steroids which would not react with blue tetrazolium, but which are indeed 17-ketogenic (6–8). The fact that the reverse is also true, namely, that blue tetrazolium may measure steroids which are not 17-ketogenic (e.g., corticosterone), may account for the agreement between the two methods in Table 5. But this must be regarded as coincidental. Under "abnormal circumstances" (and after all such tests are employed to detect disease), the discrepancy may be very great between the two methods. The classical example wherein a great difference in results between the two methods occurs is congenital adrenal hyperplasia due to a deficiency of 21-hydroxylase (9), and the very large difference which is to be anticipated has been noted in this laboratory (10). Under these circumstances, attempts to substantiate either method by establishing agreement would obviously fail.

These points do not clarify the issue of the most convenient method for urinary corticoids and are not intended to answer this question. Rather, they are noted to emphasize the precautions required for the interpretation of the method in question.

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Sexuality and the Genetics of Bacteria

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