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ANALYTICAL BIOCHEMISTRY 13, 1–5 (1965)

Rapid Conversion of Organic Nitrogen to N_2 for Mass Spectrometry: an Automated Dumas Procedure[1]

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Received November 27, 1964

A disproportionately large share of the time required to obtain an N^{15}/N^{14} isotope ratio for a sample of organic material is consumed in converting the contained nitrogen into N_2 with the commonly used Rittenberg Method (4), which requires the following sequential steps: (*a*) micro-Kjeldahl digestion at elevated temperature for a prolonged period, (*b*) distillation, and (*c*) oxidation *in vacuo* of the ammonia to nitrogen gas by the addition of alkaline hypobromite. The procedure is laborious and time consuming, and the many steps required pose a finite hazard with regard to the loss of samples. This paper describes the use of an automated Dumas combustion system combined with an improved sample collection procedure. The method reduces both the time required and the measures necessary to prevent the inclusion of hydrocarbon contaminants in the final sample and allows the collection of smaller amounts of gas. The Dumas method has been adapted to allow the preparation of samples for mass spectrometry by Holt and Hughes (2, 3). The appearance of the Coleman Nitrogen Analyzer, an automated Dumas system, provided the basic instrumentation necessary for a rapid procedure. Sternglanz and Kollig (5) have evaluated the machine, suggested procedures to ensure the accurate determination of nitrogen content in tractable compounds, and indicated modifications in technique for refractory compounds.

Briefly, the Dumas method as applied to the Coleman Nitrogen Analyzer involves oxidation at 700°–1100°C of a sample packed with cupric oxide in a quartz combustion tube. An atmosphere of high-purity CO_2 is introduced to the system; following combustion, additional CO_2 is furnished to sweep the sample first through a postheater tube packed

[1] Work supported by Public Health Service and the National Science Foundation under grants WP 00422 and GB 24, respectively. Contribution No. 8 from the Institute of Marine Science.

with copper and cupric oxide and then into the nitrometer. The latter contains a strong KOH solution in which the CO_2 sweep gas is absorbed, leaving a bubble of N_2 which then is measured volumetrically.

EXPERIMENTAL

We abandoned an effort to collect the bubble of N_2 from the nitrometer, because of the danger of exchange between successive samples through solution of small quantities of N_2 in the KOH and due to the difficulty encountered in the stopcock manipulations required to separate samples as small as 25 μl from the underlying KOH. We decided instead to remove the sweep CO_2 with a trap cooled by liquid nitrogen. Several techniques were tried, all designed to prevent the occurrence of below-atmospheric pressures in the nitrogen analyzer, a condition certain to produce air contamination. The procedure described below has been in routine use in our laboratory for over a year without modification.

Sample System

In Fig. 1, the right-hand portion or sample pumping system is identical to that of Rittenberg (4). The installation of a Pirani gage in the

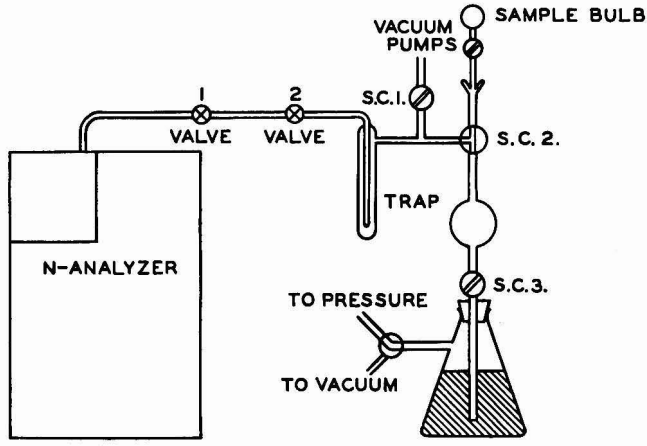

FIG. 1. Conversion apparatus and sample system.

vacuum side of the system is highly desirable. Valve 1 regulates the gas flow rate into the vacuum system, and valve 2 isolates the nitrogen analyzer from the vacuum system without disturbing valve 1. In the nitrogen analyzer a "T" is installed in the stainless-steel tubing which leads from the combustion train to the nitrometer, thereby allowing the use of the machine in its intended manner simply by closing valve 2. The

vacuum system should be arranged so that the CO_2 can be removed from the trap by means of a high capacity mechanical pump.

Operating Procedure

(*I*) *Initial adjustment of valve 1:* Adjust the sweep rate control on the nitrogen analyzer to the far open (CCW) position. Place analyzer cycle control in sweep position, turn on cycle delay, and open valve 2. Adjust the sweep rate to 30 cc/min with valve 1. Allow CO_2 to sweep into the vacuum system for 5 or more minutes, depending upon the length of time the machine has remained idle. Close valve 2, and return cycle delay and cycle control to off.

(*II*) *Pumpdown:* With valve 2 closed, open stopcock (S.C.) 1, and pump until a limiting pressure is obtained. Finish pumping with liquid nitrogen cooling the trap.

(*III*) *Sample combustion and collection:* Begin combustion cycle by turning cycle control to start. Near the end of the purge cycle a solenoid will be heard opening, and the flow indicator on the analyzer panel will fall from the top of the scale to 5 cc/min. Now open valve 2; the flowmeter should indicate about 30 cc/min. This is a purge of the entire combustion train and should be lengthened by turning on the cycle delay as the furnaces come into place around the combustion tube. After 1 min turn off cycle delay, and close valve 2. Allow the analyzer to complete its combustion cycle normally; close S.C. 1 near the end of final combustion; when the sweep cycle begins, open valve 2 and allow the sample to enter the vacuum system. Entrance of N_2 can be observed on the Pirani gage. Close valve 2 promptly at the end of sweep and raise the Dewar several inches. Pump the sample N_2 into the sample bulb or directly into the mass spectrometer sample system by appropriate manipulations of S.C. 2 and S.C. 3.

(*IV*) *Trap warming:* Open S.C. 1 and lower the Dewar flask from the trap. By-pass the mercury diffusion pump with the large capacity pump, and warm the trap to hasten evaporation of the CO_2.

DISCUSSION

In addition to the short conversion time other improvements are incorporated in this method: (*a*) the vacuum system need not be exposed to the atmosphere with subsequent difficulty in ridding the system of water vapor, and (*b*) the system encompasses virtually all the procedures recommended by Hoering (1) for the preparation of N_2 samples of the highest purity for mass spectrometry. The presence of the liquid nitrogen cooled trap prevents the entrance into the sample system of the small quantities of hydrocarbons encountered in the Dumas method

and eliminates the need for the special combustion tube packing procedures used by Holt and Hughes. Peaks at mass 27 never are observed on these conversions, although they had been a problem in our earlier efforts to collect the N_2 sample over KOH.

RESULTS

The 28, 29, and 30 mass peaks read from organic samples converted by this method indicate random pairing of the atoms in the nitrogen molecule, although, according to Holt and Hughes (2), this would not necessarily occur if N—N bonds were present.

Replicate samples agree within the precision of our mass spectrometer, a Bendix Time-of-Flight Model 17-210, i.e., within about 0.01 atom % N^{15} at low N^{15} enrichment levels. While the Time-of-Flight mass spectrometer is not as precise as the isotope ratio machines it is adequate for all but the most demanding tracer analyses and being quite sensitive is useful in the measurement of small samples. With the TOF spectrometer and the Dumas conversion, it has been feasible to run samples at the 10-μl level. The series of samples noted in Table 1 was run immediately following a number of highly enriched ($>$ 15 atom % N^{15}) standards. No nitrogen hold-over was evident, and indeed no memory effects ever have been observed with this system. A drop in isotope ratio with extremely small sample size is in evidence in Table 1. Possible causes are atmospheric nitrogen or traces of CO_2, either of which will produce an inflated 28 peak.

TABLE 1
CONVERSION OF SMALL QUANTITIES OF LOW-ENRICHMENT NITROGEN SAMPLES[a]

Sample no.	Volume, μl	Ratio of peak intensities 28/29	Atom % N^{15}
5/6/65 −27	14	62.10	0.80
−28	14	62.41	0.79
−29	34	62.07	0.80
−30	4	72.10	0.69
−31	6	65.34	0.76
−32	34	61.98	0.80

[a] Ammonium chloride containing 0.8 atom % N^{15}.

The reliability of the system is demonstrated by a run in which over 200 isotope ratios were obtained with the loss of only a single sample using a direct connection from the sample preparation Toepler pump to the mass spectrometer inlet. On a sustained basis, five samples per hour can be run by two persons, one operating the mass spectrometer while

the other performs the conversions and associated vacuum frame manipulations.

SUMMARY

Existing methods for the automated conversion of organic nitrogen to N_2 and for the preparation of N_2 for mass spectrometry have been modified to reduce the time and effort required for N^{15}/N^{14} isotope ratio determination. Detailed procedures are given for the operation of the nitrogen analyzer and the vacuum system. The technique is in practice free of atmospheric contamination, permits the trapping of residual traces of hydrocarbons, and does not require venting of the vacuum system to the atmosphere.

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ANALYTICAL BIOCHEMISTRY 13, 6–10 (1965)

Mixing Device for Generating Simple Chromatographic Gradients

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The "stand-pipe" principle has been extensively used in the design of gradient elution systems for ion-exchange chromatography (1–7). The cheapest and least complicated devices for producing simple linear, concave, or convex gradients (1–4) have utilized hydrostatic leveling between a limiting eluent reservoir (containing the final concentration desired) and a mixing chamber (initially filled with starting eluent). Eluent withdrawn from the system via the mixing chamber, which must be provided with mechanical stirring, follows a concentration gradient which is a function of the area ratio of the two vessels. Any desired simple gradient may be obtained either by selecting reservoir containers of appropriate cross section or by introducing objects of uniform cross section into the containers to adjust the area ratio.

Most of these devices employ siphons or tubing interconnections and are therefore cumbersome to position and assemble. Extensive modification of geometries is sometimes involved in producing different types of gradients, and frequently the introduction of objects into the mixing vessel obstructs efficient stirring. We describe here a general arrangement of two fixed reservoirs which can be constructed to deliver a linear gradient but is easily adapted for concave or convex gradients, or for a logarithmic gradient.

Construction of Linear Gradient Device

Reservoirs of equal area may be fashioned compactly and inexpensively from several combinations of concentrically arranged acrylic cylinders (Fig. 1). The portion of the larger cylinder not occupied by the smaller one constitutes the outer (limiting eluent) reservoir, containing the same volume as the smaller cylindrical mixing chamber at hydrostatic equilibrium. Interconnection of the reservoirs is made by a narrow U-channel (E, Fig. 1), which effectively eliminates diffusion between the

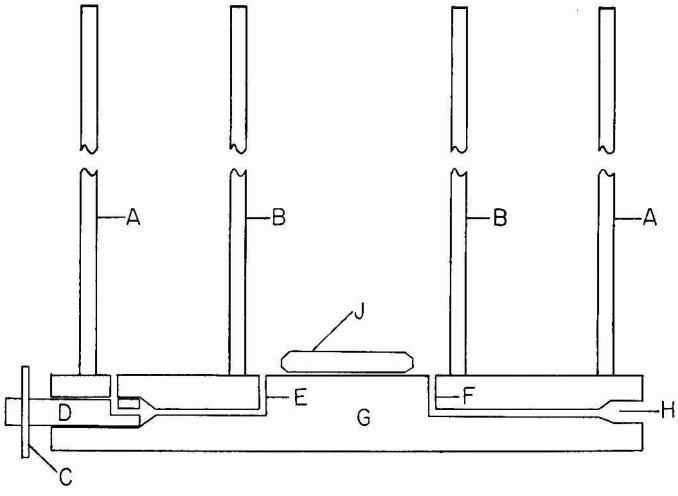

FIG. 1. Cross-sectional diagram of gradient device showing relative positions of essential ingredients. Outer and inner cylinders A and B are glued to ½" acrylic plate (G) to form fixed concentric reservoirs of equal area. Plastic rod C allows rotation of a snugly fitting stopcock of ¼" Teflon rod D, which is drilled to match configuration of the 5/64" U-channel (E) connecting outer and inner reservoirs. The central mixing chamber, shown with magnetic stirring bar J in place, is provided with narrow outflow channel F. Connection is made to a chromatographic column with plastic tubing, which may be fit directly into undersized expansion H.

two chambers and allows sufficiently rapid equilibration to accommodate fast chromatographic flow rates. At one portion of the channel, a "stopcock" (D, Fig. 1) is interposed to allow convenient priming and to permit independent filling of the reservoirs.

A large variety of cylinders is commercially available, and the selection of appropriate sizes to produce "matching area" combinations is tedious. Table 1 provides a convenient listing of available "matching area" cylinder combinations[1] with less than 2% difference in the reservoir areas as calculated from the nominal diameters of the two cylinders.

Modification for Nonlinear Gradients

Concave or Convex Gradients. The area ratio of many of the reservoir combinations of Table 1 may be altered systematically for the production of a variety of convex and concave gradients by nesting different numbers of close-fitting acrylic cylinders around or inside the fixed

[1] Specifications for "Cadco" cast acrylic resin tubes, Teflon rod, and cementing materials available from: Cadillac Plastic and Chemical Co., 15111 Second Ave., Detroit 3, Michigan. Uniform inventories prevail among many manufacturers.

TABLE 1

SUGGESTED COMBINATIONS OF COMMERCIALLY AVAILABLE CYLINDERS FOR THE CONSTRUCTION OF FIXED "MATCHING AREA" RESERVOIRS TO GENERATE A LINEAR GRADIENT

A_i, A_o = areas computed from inner and outer diameters of cylinders. A_I, A_O = areas of reservoirs (mixing chamber and limiting eluent reservoir, respectively) resulting from the combination of these cylinders. $A_O = A_i$ (outer cylinder $- A_o$ (inner cylinder). Diameters and areas in inches to conform to manufacturers' specifications (1 in.^{2} = 6.45 cm^2).

Outer cylinder		Inner cylinder					Ratio of reservoir areas, A_I/A_O
i.d.	A_i	o.d.	i.d.	A_o	$A_i(A_I)$	A_O	
1.875	2.761	1.500	1.125	1.767	0.994	0.994	1.000
2.125	3.547	1.625	1.375	2.074	1.485	1.473	1.008
2.500	4.909	2.000	1.500	3.142	1.767	1.767	1.000
3.000	7.069	2.250	2.000	3.976	3.142	3.093	1.016
3.375	8.946	2.500	2.250	4.909	3.976	4.037	0.985
3.625	10.321	2.750	2.375	5.940	4.430	4.381	1.011
4.000	12.566	3.000	2.625	7.069	5.412	5.497	0.984
4.250	14.186	3.125	2.875	7.670	6.492	6.516	0.996
4.500	15.904	3.375	3.000	8.946	7.069	6.958	1.016
4.875	18.666	3.625	3.250	10.321	8.296	8.345	0.994
5.125	20.629	3.750	3.500	11.045	9.621	9.584	1.004
5.500	23.758	4.000	3.750	12.566	11.045	11.192	0.987
5.750	25.967	4.250	3.875	14.186	11.793	11.781	1.001
7.250	41.283	5.250	5.000	21.648	19.635	19.635	1.000

center cylinder to reduce the area of the outer or inner reservoirs, or both. Figure 2 shows an assortment of gradients that could be produced if combinations of a series of 8 cylinders were nested into one of the linear gradient generators of Table 1. These gradients were calculated from the general formula (1):

$$1 - C = (1 - v)^{A_o/A_i}$$

where C = fraction of the limiting concentration attained when a fraction, v, of the total volume has been withdrawn from the mixer, and A_o, A_i = areas of the outer reservoir and inner mixing chamber, respectively.

Logarithmic Gradient. When the central mixing chamber of any of the linear gradient generators of Table 1 is converted to a constant-volume reservoir by capping it tightly with a rubber stopper, a logarithmic gradient (8) can be generated having characteristics circumscribed by the formula (1):

$$1 - C = e^{-v}$$

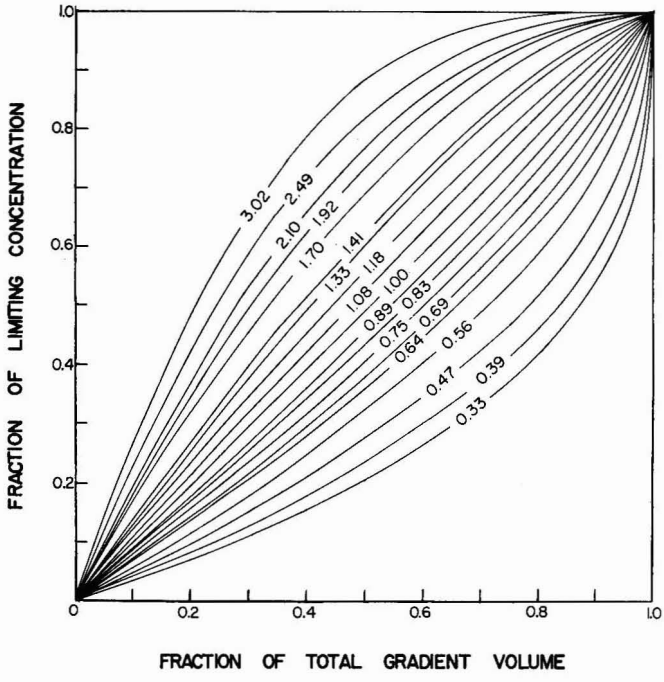

FIG. 2. Assortment of gradients possible when one of the linear gradient generators selected from Table 1 is modified with different combinations of 8 nesting cylinders. The area ratio (outer reservoir/inner mixing chamber) generating each curve is indicated.

where C = fraction of the limiting concentration attained when a fraction, v, of the fixed volume in the closed reservoir has been withdrawn from the mixer. Note that attainment of a satisfactory end point for such a gradient may require that an excess of limiting eluent in the outer reservoir be provided by refilling or initial adjustment.

DISCUSSION

The gradients generated by our device have been in excellent agreement with those calculated for different combinations of cylinders, although some deviation has been detected, probably attributable to the error inherent in some of the "matching area" combinations and the error introduced by manufacturing tolerances. These considerations do not apply if the actual area ratio is determined by measuring the volumes required to bring the solutions in the reservoirs to the same level. This method also provides direct visualization of the volume corrections that may be necessary to achieve equal hydrostatic pressures

in the two reservoirs with eluent solutions of appreciably different densities.

The compactness, simple design, and low cost (under $25) of this device recommend it for the production of many frequently required gradients.

SUMMARY

A linear gradient device consisting of two concentric reservoirs of equal cross-sectional area, in which the initial and limiting eluent solutions are maintained in hydrostatic equilibrium, may be constructed from several combinations of commercial acrylic resin cylinders. By the use of nesting cylinders, the areas of the reservoirs may be reduced independently, allowing an assortment of nonlinear gradients to be produced by simple adjustment of the area ratio of the two reservoirs.

ACKNOWLEDGMENTS

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ANALYTICAL BIOCHEMISTRY 13, 11–18 (1965)

Use of Cholinesterase for Hydrolysis of Steroid Acetates[1]

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Received February 15, 1965

Different methods have been outlined for the hydrolysis of steroid acetates. Velluz *et al.* (1) demonstrated that saponification with alcoholic potassium hydroxide not only hydrolyzed the acetates from the C-21 position of 11-deoxycorticosterone and 21-acetoxy-Δ^5-pregnen-3-ol-20-one, but also oxidized the side chain of the steroids to produce the corresponding etiocholenic acid. Herzig and Ehrenstein (2) observed the 6β-hydroxyl group of a 3-keto-Δ^4-steroid to be unstable in alkali, whereas the 6α-hydroxyl analog was stable (2). The 6β-acetate can, however, be saponified in alcoholic base without artifact formation if oxygen is excluded from the reaction mixture (2).

Hydrolysis of steroid acetates with enzymes has been employed to avoid artifact formation. Zaffaroni (3) used a 250-fold purified horse liver esterase. If less than 500 μg of a steroid acetate was used as substrate, the hydrolysis went to 85% completion without detectable formation of artifictitious compounds (3). An acetyl esterase isolated from orange peel converted 90% of a steroid acetate to the corresponding free steroid, but the remaining 10% of substrate was lost (4). A porcine renal acylase has been employed in the characterization of steroid radiometabolites obtained from incubations of ovarian minces with progesterone-4-C^{14} (5), and aldosterone diacetate was converted to both the monoacetate (in 18–25% yield) and the parent compound (in 20% yield) with the enzyme acetylcholinesterase (6). Fevold and Eik-Nes (7) successfully applied the cholinesterase method to the saponification of cortisone-4-C^{14} acetate.

It, therefore, appeared of interest to study various procedures of hydrolysis of steroid acetates because: (*1*) acetylation is a routine procedure in the identification of steroids, (*2*) it would be advantageous

[1] This investigation was aided by grants T1 GM 152-05 and T4 CA 5000 from the United States Public Health Service.

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to apply one hydrolytic method to the acetates of C_{18}, C_{19}, and C_{21} steroids, and (*3*) adequate yield of hydrolysis and minimal artifact formation are desirable and of special concern when identifying small amounts of a steroid like those encountered when studying the metabolism of radioactive steroids by endocrine tissues.

METHODS AND MATERIALS

Radioactive steroids were purchased from New England Nuclear Corporation and acetylcholinesterase with a stated specific activity of 50 units per mg was obtained from Nutritional Biochemicals Corporation as were also the eserine preparations.

Dehydroepiandrosterone-4-C^{14} acetate was purchased as the acetate. Other steroid acetates were prepared by adding 200 μl of an acetic anhydride-pyridine mixture (1:4, v/v) to the dried steroid contained in a small tube. The tube was tightly stoppered, and incubated in the dark at room temperature overnight. Methanol-chloroform (1:1) was then added and the mixture evaporated to dryness under a stream of nitrogen at 45°C. The residue was dissolved in a known volume of methanol and portions were transferred to glass-stoppered conical tubes. The samples were evaporated to dryness, and the steroid acetate was concentrated at the tip of the conical tube with repeated methanol rinses and with drying under nitrogen at 45°C. Duplicate samples were used for each analysis, and samples of steroid acetates incubated in the absence of the hydrolytic agent served as controls.

The methods of hydrolysis were:

Method I (*Sodium Carbonate*): The acetylated steroids were dissolved in 0.5 ml ethanol, and 0.5 ml aqueous 2.5% sodium carbonate was added. The reaction mixture was incubated for 12 hr at 30°C. At the end of this period, 5 ml water was added, and the mixture extracted 2 times with 10 ml of ethyl acetate saturated with water. The combined extract was evaporated to dryness.

Method II (*Sodium Hydroxide*): 1 ml of methanolic 0.1 *N* sodium hydroxide was added to the acetylated steroid. After 0.5 hr incubation at 37°C, 5 ml water was added to each tube, and the samples were processed as described under Method I.

Method III (*Acid*): 1 ml of ethanol was placed in each tube containing acetylated steroids, and then 1 ml of aqueous 0.1 *N* acetic acid was added. The mixture was kept at 37°C for 0.5 hr. The mixture was extracted as described under Method I after the addition of 5 ml water to each tube.

Method IV (*Enzyme*): The steroid acetates were dissolved in 0.1 ml methanol. Five hundred units of cholinesterase, dissolved in 4 ml of

phosphate buffer (8), was added and the mixture was incubated at 30°C for 6 hr. The samples were then extracted as described under Method I.

The residues from these extractions were chromatographed on paper in an appropriate solvent system(s) which provided suitable separation of the free compound and the corresponding acetate(s). Quantification of C^{14} steroids on the chromatogram was done by calculating the area of radioactivity under the curves of the tracings of a gas-flow strip scanner (9, 10).

RESULTS AND DISCUSSION

1. *Comparison of Different Saponification Methods:* Testosterone-4-C^{14} acetate was used as substrate in these investigations since preliminary results suggested that it gave lower yields of free compound after cholinesterase hydrolysis as compared with other steroid acetates. The sodium carbonate and enzymic methods appeared to be the most adequate (Table 1). Longer incubations with sodium hydroxide would result in a greater per cent conversion of the ester to the alcohol but may also promote a greater formation of artifacts with some steroid acetates (1, 2).

TABLE 1

COMPARISON OF DIFFERENT HYDROLYTIC METHODS

Isolation and quantification of testosterone-4-C^{14} and its acetate after hydrolysis have been described in the text of this paper.

Method used for hydrolysis	Free steroid, dpm	Acetate, dpm	Conversion,[a] %	% steroid recovered[b]
Sodium carbonate	14,900	1,200	92	94
	12,900	2,100	86	88
	15,000	1,500	91	96
Sodium hydroxide	9,600	5,100	65	86
	9,100	5,700	61	87
	10,000	4,200	69	83
Acid	"0"[c]	18,600	0	109
	"0"	14,700	0	86
Cholinesterase	16,000	2,100	88	100
	14,200	2,400	86	97
	14,200	2,300	86	96

[a] Per cent conversion to steroid alcohol

$$= \frac{\text{free steroid (dpm)}}{\text{free steroid (dpm)} + \text{steroid acetate (dpm)}} \times 100$$

[b] This figure represents the radioactivity accounted for as the free steroid and its acetate; 17,000 dpm of testosterone-4-C^{14} acetate was used as substrate in each experiment.

[c] Below limit of detection on a gas-flow Geiger tube strip scanner; the lower limit of detection was approximately 300–400 dpm/cm^2 (10).

TABLE 2

COMPARISON OF TWO METHODS FOR HYDROLYSIS OF STEROID ACETATES

Isolation and quantification of steroids after hydrolysis have been described in the text of this paper.

Acetylated substrate	Sodium carbonate			Cholinesterase			Control[b]		
	Acetate, dpm	Free steroid, dpm	Conversion,[a] %	Acetate, dpm	Free steroid, dpm	Conversion, %	Acetate, dpm	Free steroid, dpm	Conversion, %
11-Deoxycorticosterone-4-C^{14}	12,400	2,200	15	700	28,400	98	27,900	TA[d]	0
	9,700	1,700	15	1,200	27,800	96	27,700	TA	0
Corticosterone-4-C^{14}	0[c]	8,600	100	0	22,400	100	24,800	TA	0
	0	10,400	100	0	22,900	100	20,800	TA	0
Dehydroepiandrosterone-4-C^{14}	0	20,600	100	600	35,100	98	37,300	2,100	5
	0	19,300	100	500	40,300	99	34,600	1,800	5

[a] See Table 1.

[b] Same conditions were used as in the cholinesterase incubations except that no enzyme was added.

[c] See Table 1.

[d] Trace amounts not sufficient for accurate quantification.

2. *Specificity:* The sodium carbonate (Method I) and cholinesterase (Method IV) methods were compared for their ability to hydrolyze various acetylated steroids (Table 2). Hydrolysis of primary and secondary esters was achieved with the use of either method. Also compounds with a double bond at either position 4 or position 5 appeared equally susceptible to hydrolysis. The enzyme had a wide range of specificity with regard to the steroid configuration and location of the ester group(s) on the steroid skeleton. The enzyme would hydrolyze the acetates of compounds like cortisol, Δ^5-pregnenolone and 17α-hydroxypregnenolone to the free steroids in yields exceeding 85%. Kinetic studies are, however, required before the substrate specificity of the enzyme can be defined in more detail.

The monoacetate of estradiol was obtained in large yields when the diacetate of estradiol was hydrolyzed with the enzyme under the conditions described (Table 3). The products of enzymic hydrolysis of estra-

TABLE 3

HYDROLYSIS OF ESTRADIOL-4-C^{14} DIACETATE BY DIFFERENT METHODS

Isolation and quantification of steroids after hydrolysis have been described in the text of this paper.

	Compounds isolated after hydrolysis					
	Estradiol		Estradiol monoacetate		Estradiol diacetate	
Method	dpm	%[a]	dpm	%	dpm	%
Sodium carbonate	6,100	28	15,400	72	0[b]	0
	8,100	30	18,700	70	0	0
Cholinesterase	4,400	14	24,700	81	1,400	5
	4,400	15	22,900	81	1,100	4
Control[c]	0	0	900	3	29,600	97
	0	0	0	0	37,100	100

[a] Per cent of the original amount of substrate converted to this compound.

[b] Below limit of detection on a gas-flow Geiger tube strip scanner; the lower limit of detection was approximately 300–400 dpm/cm^2 (10).

[c] Same conditions were used as in the cholinesterase incubations except that no enzyme was added.

diol diacetate were chromatographed on paper in several different solvent systems and compared with the chromatographic mobility of estradiol, estradiol diacetate, and estradiol monoacetate, prepared by partial acetylation (11). In this manner it was determined that the phenolic acetyl group was removed first by enzyme hydrolysis. Since selective acetylation of estradiol yields primarily 3-acetoxyestradiol (11), and cholinesterase hydrolysis of estradiol diacetate yields predominately 17β-

acetoxyestradiol, both monoacetates of estradiol can be readily synthesized.

3. *Time Study:* Testosterone-4-C^{14} acetate was incubated with 500 units cholinesterase for varying periods of time (Fig. 1). The results

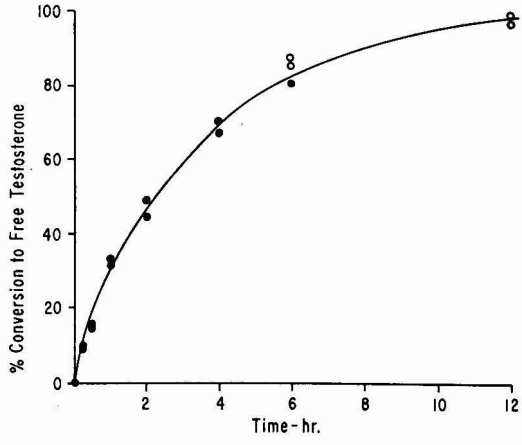

FIG. 1. Hydrolysis of testosterone-4-C^{14} acetate by cholinesterase as a function of time: 35,000 dpm testosterone-4-C^{14} acetate was incubated with 500 units cholinesterase at 30°C for various time periods; isolation and quantification of radioactive steroids following hydrolysis have been described in the text of this paper, and quantitation of the substrate testosterone-4-C^{14} acetate was done by liquid scintillation spectrometry (10). Open circles: experiments done on November 30, 1963. Dark circles: experiments done on November 24, 1963.

indicate that, even with relatively large concentrations of enzyme and microgram quantities of substrate (0.1–0.2 μg), relatively long incubation times are required to obtain good yields of hydrolysis. Reducing the incubation volume and increasing the reaction temperature should shorten the necessary incubation time. Nevertheless, the high yields, the simplicity of the procedure, the reliability of the method, and the ease of isolation and quantification of the products make the enzyme method attractive.

4. *Inhibition of the Enzyme:* To test whether or not the enzymic hydrolysis of the steroid acetates could be attributed to a cholinesterase activity, incubations with the enzyme were performed in the presence of low concentrations ($1 \times 10^{-5} M$) of eserine (8), a classical inhibitor of cholinesterase. Eserine inhibited the hydrolysis of the steroid acetate to the free compound (Table 4). Thus, the enzymic activity of this preparation is attributed to a cholinesterase.

TABLE 4

ESERINE INHIBITION OF CHOLINESTERASE HYDROLYSIS OF STEROID ACETATES

Isolation and quantification of steroids after hydrolysis have been described in the text of this paper.

Incubation condition	Substrate	Conversion products, dpm		Conversion to steroid alcohol, %
		Free	Acetate	
500 units cholinesterase	Cortisol-4-C^{14} acetate	15,300	2,000	88
		16,500	1,500	92
500 units cholinesterase and 1×10^{-5} *M* eserine salicylate		900	21,100	4
		1,000	20,600	5
500 units cholinesterase	Testosterone-4-C^{14} acetate	27,400	6,200	81
		26,600	6,100	81
500 units cholinesterase and 1×10^{-5} *M* eserine sulfate		1,000	31,100	0
		7,700	32,000	19

Because of the expense of cholinesterase, the sodium carbonate method is preferred in routine work. The cholinesterase method, however, is to be recommended when isolating "unknown" compounds where artifact product may result from exposure of the compound to alkaline conditions. The enzymic procedure has also proved useful in our hands when low conversions were obtained by the carbonate method of hydrolysis, e.g., 11-deoxycortisol acetate and 11-deoxycorticosterone acetate.

ACKNOWLEDGMENT

It is a pleasure to acknowledge discussions with Dr. H. R. Fevold at the initiation of this study.

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ANALYTICAL BIOCHEMISTRY 13, 19–27 (1965)

Pyridoxal Phosphate Determination in Isolated Leucocytes and Tissue by *E. coli* Apotryptophanase[1]

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A reliable method for the determination of vitamin B_6 content of tissues was needed as part of a plan to study the effects of vitamin B_6 deficiency in human neoplastic diseases. Wada *et al.* (1) described a simple enzymic method for the determination of pyridoxal phosphate (PLP) in blood and tissues using *Escherichia coli* apotryptophanase. In the presence of an excess of the apoenzyme and tryptophan, PLP is a limiting factor for the catalytic degradation of tryptophan to indole, pyruvic acid, and ammonia. The amount of indole produced is quantitatively dependent, under specified conditions, on the amount of PLP available in the assay system. Difficulties were encountered in applying the above method (1) to the extraction of PLP from tissues. The metaphosphoric acid solution used for extraction had a definite inhibitory effect on the enzyme system. This observation was also made by others (2, 10). A sensitive reproducible modification of the apotryptophanase method is reported in this paper.

MATERIALS AND METHODS

Preparation of Apotryptophanase. E. coli (Crookes strain) was used. The culture medium, incubation time, harvesting technique, and lyophilization of the preparation followed the procedure of McCormick *et al.* (3). The lyophilized preparation was stored at −10° to −15°C. To prepare a cell-free extract of the bacteria, 0.5 gm of the lyophilized preparation was suspended in 50 ml of 0.02 *M* potassium phosphate buffer, pH 7. All operations were carried out at 0° to 4°C. The suspension was sonicated for 10 to 15 min with the Biosonick Ultrasonic probe. Debris was removed by centrifugation at 18,000 rpm for 30 min. The supernatant was adjusted to pH 4.7 with 1 *M* acetic acid and centrifuged

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at 9,000 rpm for 10 min. The precipitate was suspended in 50 ml of water and dialyzed against 4 liters of water for 4 hr. The dialyzed preparation was then suspended in 100 ml distilled water and stored in convenient volumes at −10° to −15°C. Immediately before use, a tube of the frozen material was thawed and any precipitated material was resuspended by brief homogenization.

Separation of White Blood Cells and Preparation of Extract. The entire procedure was carried out at about 4°C, and the preparation was shaded from the light as much as possible. Syringes and glassware were dry, clean, and siliconized. Ten to thirty milliliters of blood was drawn to provide about 10^8 leucocytes. The blood was immediately transferred to a 40-ml conical centrifuge tube containing 1 ml of 0.1 *M* EDTA, pH 7.3. The tube was gently inverted over parafilm a few times to assure proper mixing of the blood with the EDTA. All bubbles were removed with a capillary pipet. In patients with rapid erythrocyte sedimentation associated with anemia or disease, the blood was left standing for 10 to 20 min to allow sedimentation. Some samples were centrifuged at 200 rpm for 3 min. In subjects with normal erythrocyte sedimentation, erythrocyte isoagglutinins were used to promote rapid sedimentation of the red cells (4). The leucocyte-rich plasma, which was usually about one-third the volume of the blood drawn, was transferred to another graduated centrifuge tube and, after gentle mixing by inversion over parafilm, a leucocyte count was performed. The plasma was then centrifuged at 1,500 rpm for 10 min. The excess supernatant plasma was removed, leaving approximately 2 ml. The reddish white pellet was suspended in the plasma by the Vortex Jr. Mixer. Hypotonic hemolysis was effected with 5 ml of distilled water added to the tube with gentle mixing for 30 to 60 sec. The preparation was returned to isotonicity by adding 5 ml of double-strength Eagle medium (5) lacking calcium and vitamin B_6. The leucocytes were collected as a clear white pellet after centrifugation at 1,500 rpm for 10 min. The leucocytes were suspended in 4 ml of 0.02 *M* potassium phosphate buffer, pH 7, and sonicated in ice for 0.5 min intervals for a total of 3 min. The sonicate was adjusted to pH 4 with 1 *M* acetic acid. It was heated for 10 min in a boiling water bath, allowed to cool to room temperature in the dark, and then centrifuged at 20,000 rpm for 30 min. The sediment was discarded and the supernatant adjusted to pH 7 with a measured quantity of a 1 *M* potassium phosphate solution, pH 8.3.

Preparation of Tissue Extracts. The tissue was weighed and then homogenized for 3 min in 4 ml of 0.02 *M* potassium phosphate buffer in a Potter-Elvehjem homogenizer. The preparation was then sonicated in ice for 0.5-min intervals for a total of 3 min. The other steps in the

extraction were identical to those for the extraction of white blood cells.

Preparation of Blank and Standard Solution. A fresh standard preparation of PLP was prepared for each assay from a stock solution containing 0.5 mg/ml. The stock solution retained its potency for more than 2 months if it was kept in the dark in a refrigerator. Measured quantities of PLP (usually 0–0.2 μg) dissolved in 4 ml of the 0.02 *M* potassium phosphate buffer were treated exactly the same way as the tissue homogenates, by acidification, heating, and adjustment to pH 7 with 1 *M* potassium phosphate solution. The volume of all preparations was brought to 5 ml with equal quantities of 0.02 *M* potassium phosphate buffer, pH 7. It was essential that all preparations be brought to the same volume and pH and that they contained the same amount of potassium ion.

Determination of PLP. The procedure was based on the method described by McCormick *et al.* (3) modified so as to increase its sensitivity. The standard solutions and tissue preparations were transferred to 50-ml Er'lenmeyer flasks. To each of the flasks 5 ml of toluene was added, followed by 1.4 ml of a solution containing 1 ml of apotryptophanase preparation, 0.2 ml of 1 *M* reduced glutathione, and 0.2 ml 1 *M* potassium phosphate solution pH 8.3.

The flasks were stoppered and incubated for 30 min with moderate shaking (about 100 cycles per minute) in a water bath at 37°C to allow association of the apoenzyme with PLP. One-half milliliter of 0.05 *M* solution of L-tryptophan was then added and the flasks were incubated with shaking for an additional 20 min. The reaction was stopped by the addition of 0.2 ml of 100% (weight per volume) trichloroacetic acid solution. The flasks were then shaken vigorously by hand to extract the indole into the toluene layer. To 0.5 to 3 ml of the toluene layer was added 0.5 ml of 5% *p*-dimethylaminobenzaldehyde in 95% ethanol and 5 ml of sulfuric acid-ethanol reagent (80 ml of concentrated sulfuric acid per liter of 95% ethanol); at least 10 min was allowed for the pink color to develop before reading the absorbancy at 540 mμ in a spectrophotometer. The color was stable up to 24 hr.

RESULTS AND DISCUSSION

Apotryptophanase specificially uses PLP as a cofactor [pyridoxine, pyridoxal, and pyridoxamine were ineffective as cofactors (1)] and it has been demonstrated that the enzyme preparation used in this assay is free of pyridoxal phosphokinase (1) (see also Table 5). It is therefore essential that the method used for PLP extraction from tissue should not transform it into other forms of vitamin B_6. Very low values for PLP were obtained in samples of rat liver extracted by heating the

tissue in metaphosphoric acid solution as described by Wada *et al.* (1). Similarly, pure solutions of PLP heated with the metaphosphoric acid solution retained less than 60% of their activity in comparison to the same concentration of PLP used in the assay without previous exposure to metaphosphoric acid. A similar experience was reported by Donald (2), who attributed the inhibitory effect of metaphosphoric acid to the high sodium content of the metaphosphoric acid used [sodium is known to be inhibitory to apotryptophanase (6) and the metaphosphoric acid Donald used contained 60% sodium phosphate as a preservative].

Extraction of pyridoxal phosphate from tissues with 0.055 *N* hydrochloric acid, a concentration used for vitamin B_6 extraction for the microbiological method (7), was tried. It was found that the longer the tissue was heated in this acid the lower the spectrophotometric reading obtained when the sample was assayed for PLP by apotryptophanase (Table 1). Similarly, there was almost 50% loss of activity when

TABLE 1
EFFECT OF HEATING AT pH 1.5 ON PLP EXTRACTED FROM TISSUES

25 mg mouse liver samples homogenized in 5 cc of 0.055 *N* HCl	OD at 540 mμ
Heated at 100°C for 15 min	0.066
Heated at 100°C for 30 min	0.052
Autoclaved at 15 lb for 30 min	0.024

Three samples of mouse liver (25 mg each) were homogenized and heated in 5 ml of 0.055 *N* HCl. The pH was brought back to 7 with equal amounts of 0.5 *N* KOH solution. The samples were assayed for PLP by apotryptophanase.

standard PLP preparation was treated with 4% trichloroacetic acid solution, even though the trichloroacetic acid was extracted three times with ether prior to assay. These data suggested possible dephosphorylation of PLP by heating at this acid concentration (pH 1.2–1.5). Then the effect of heating at various concentrations of acid on standard preparations of PLP was studied. The data indicate that heating for 10 min at pH 3 or lower gives lower results than heating at pH 7 (Fig. 1). Heating at pH 4 gives almost identical results to heating at pH 4.5, 5.5, or 7.

For PLP extraction from tissue homogenates, heating at pH 4 for 10 min is definitely superior to heating at pH 7 (Table 2). PLP extractions from tissues by alkaline hydrolysis, as described by Boxer *et al.* (8) and by Donald (2), was compared to heating homogenates at pH 4. Both methods gave comparable results when applied to isolated leucocytes, but alkaline hydrolysis of liver tissue gave lower and less reproducible

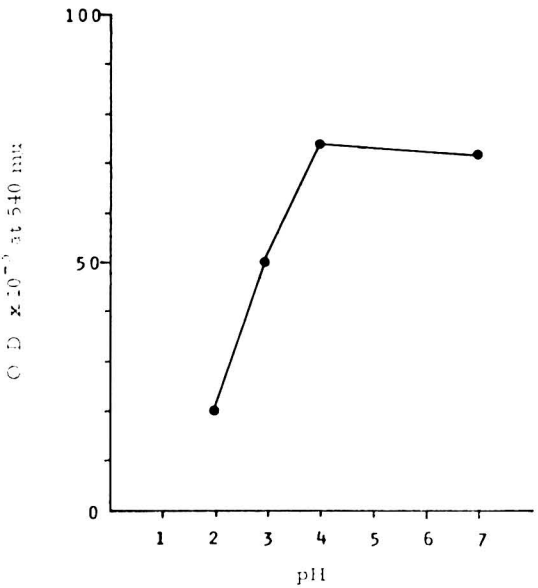

FIG. 1. Effect of heating at 100° for 10 min on 0.2 μg PLP solution assayed under standard conditions by apotryptophanase.

TABLE 2

COMPARISON OF PLP EXTRACTION FROM TISSUE BY HEATING AT pH 7 AND pH 4

Sample	OD at 540 mμ	
	Heated at pH 7	Heated at pH 4
22 mg mouse liver	0.056	0.095
35 mg mouse spleen	0.038	0.077
35 mg mouse skin	0.047	0.069

Mouse tissue assayed for PLP by apotryptophanase. Extraction was done by immersing tube containing tissue homogenate in boiling water for 10 min. Samples for each of the specimens were identical except for the pH at which they were heated.

results (Table 3). Similar low results were reported by Wachstein and Moore (9) when they applied alkaline hydrolysis for PLP extraction from rat tissues (average of 0.86 μg PLP/gm of rat liver compared to a reported 7.7 μg vitamin B_6/gm of liver by the microbiological method).

Recovery of PLP added to the tissue homogenate before heating at pH 4 varies between 80 and 90% of the added quantity (Table 4). This is much higher than the 45% recovery reported by Donald using alkaline hydrolysis (2). On the other hand, addition of pyridoxal hydrochloride to the tissue homogenate after heating caused no increase in the absorbancy (Table 5), a proof of complete inactivation of the pyridoxal phosphokinase in the assayed tissue.

TABLE 3
COMPARISON OF ACID AND ALKALINE HYDROLYSIS FOR PLP EXTRACTION FROM TISSUES

Date	μg PLP per gram liver	
	Heating at pH 4 for 10 min	Heating in 0.5 *N* KOH for 5 min
4/8/64	8.2	5.46
4/10/64	8.68	4.4

Samples of rat liver assayed for PLP by apotryptophanase using both acid hydrolysis and alkaline hydrolysis as described by Donald (2). Acid hydrolysis gave higher and more consistent results.

TABLE 4
RECOVERY OF PLP ADDED TO TISSUE HOMOGENATES

10 mg mouse liver	OD at 540 mμ	μg PLP content	Per cent recovery
Sample A	0.058	0.098	
Sample A + 0.1 μg PLP	0.108	0.188	90%
Sample B	0.032	0.053	
Sample B + 0.1 μg PLP	0.076	0.133	80%

TABLE 5
EVIDENCE FOR COMPLETE INACTIVATION OF TISSUE PYRIDOXAL PHOSPHOKINASE DURING ASSAY PROCEDURE

10 mg mouse liver	OD at 540 mμ
Sample A	0.068
Sample A + 1 μg PAL·HCl[a]	0.070
Sample B	0.070
Sample B + 1 μg PAL·HCl	0.068
Sample C	0.088
Sample C + 1 μg PAL·HCl	0.085

[a] PAL·HCl = pyridoxal hydrochloride.

Heating leucocytes in acid or alkaline solution results in the formation of sticky material which adheres to the wall of the tubes and the pipets. Ultrasonic disruption of the cells overcomes the difficulty by evenly dispersing the material with less loss during transfers. This was reflected by better and more reproducible results. The supernatant fluid of the acid hydrolyzate gives equal or higher readings than the whole hydrolyzate. At no time did the whole hydrolyzate give higher readings. These results led to centrifugation of the hydrolyzate at 20,000 rpm for 30 min. The assay was done on the supernatant.

The method is fairly reproducible: duplicate samples give results within 10% of each other. It is also fairly sensitive: 0.01 μg of standard PLP preparation could always be measured and most of the time 0.005 μg could be measured (Fig. 2). The method was applied for measure-

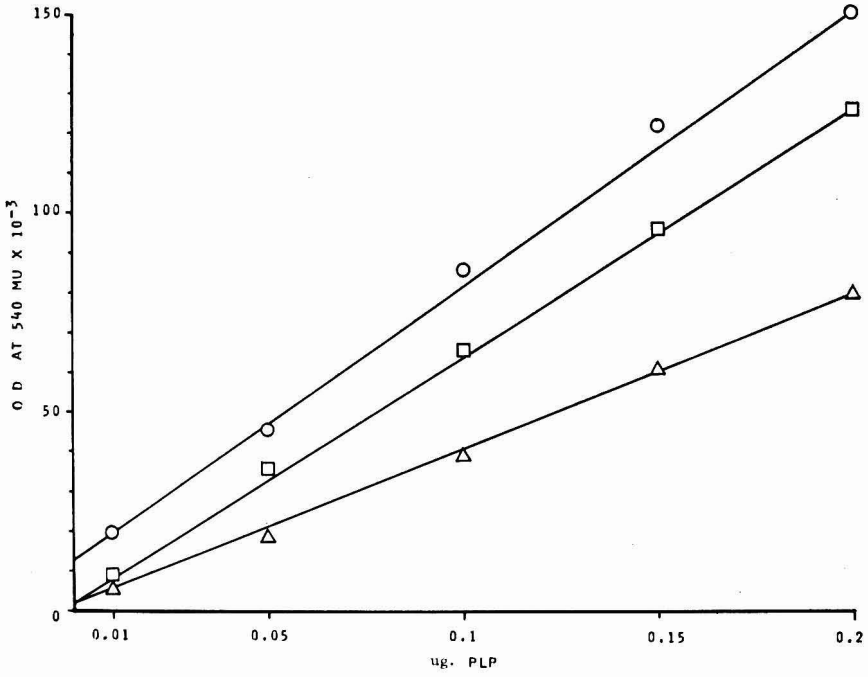

FIG. 2. Three representative standard curves showing sensitivity of the method and linearity of response.

ment of PLP in isolated human leucocytes in eighteen adult cancer patients—detectable PLP activity was found in all but one case if more than fifty million leucocytes was isolated. The range of PLP in isolated leucocytes was 0 to 0.055 μg with a mean of 0.021 μg per hundred million leucocytes (Fig. 3). This compares favorably with an average of 0.015 μg per hundred million in normal adults reported by Boxer *et al* (8). PLP in eight liver samples obtained by needle biopsy was 3 to 12.5 μg per gram of liver tissue.

When eleven patients with advanced neoplastic disease were fed vitamin B_6-deficient liquid formula diet (0.028 mg/1,000 cal) for periods ranging from 11 to 65 days, there was a progressive drop in PLP content of the isolated leucocytes from a mean of 0.021 μg per million cells to unobtainable levels by the end of the fifth week at the latest. Serial

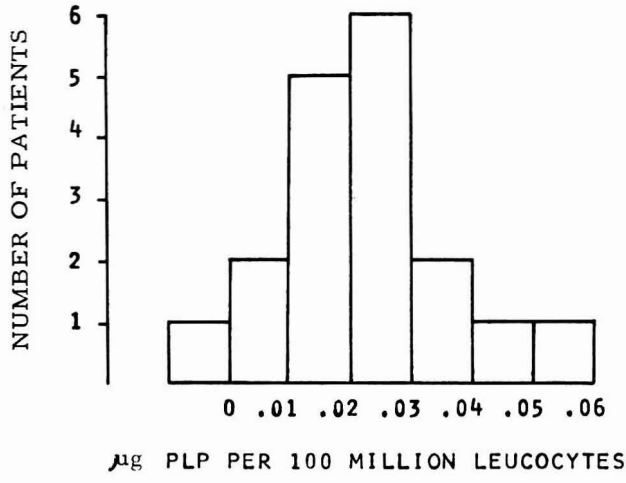

FIG. 3. PLP levels in isolated leucocytes.

liver biopsies were done on five of these patients and four of them showed progressive decline in PLP content of the liver (Fig. 4). One of these patients, after receiving the deficient diet for fifty-five days, was given

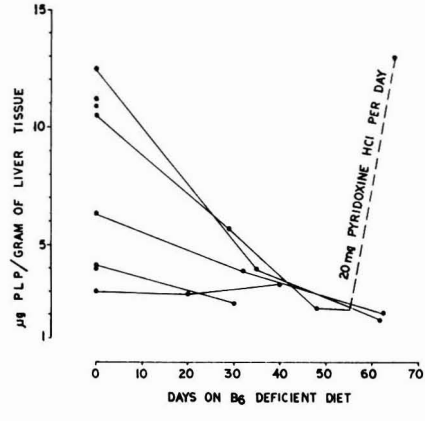

FIG. 4. Effect of vitamin B_6 deprivation and administration on PLP levels in liver.

20 mg of pyridoxine hydrochloride per day for seven days. The PLP content of his liver rose from 2.3 μg/gm on the forty-eighth day of the deficiency period up to 13 μg/gm after the seven days of pyridoxine administration. During the same period the PLP content of the isolated

leucocytes also rose from an unobtainable level up to 0.054 μg per hundred million cells.

SUMMARY

A reproducible enzymic method for determination of PLP in isolated leucocytes and tissue using *E. coli* apotryptophanase is described. PLP extraction was achieved by heating at pH 4. Heating at lower pH values gives lower results, most probably due to dephosphorylation of PLP. PLP levels in human isolated leucocytes and liver obtained by needle biopsy, and the effect of vitamin B_6 depletion and repletion on liver PLP levels, are reported.

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ANALYTICAL BIOCHEMISTRY 13, 28–42 (1965)

Identification of Deoxyribonucleases in Polyacrylamide Gel Following Their Separation by Disk Electrophoresis[1]

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Disk electrophoresis and its predecessor starch gel electrophoresis have both been extremely valuable tools for the separation and identification of enzymes and their multiple molecular forms (1, 2). Although a large number of enzyme systems have been studied using these techniques, the range of the investigations has been limited by the fact that most histochemical methods, which have been adapted for localization of the enzymes in the gel, require rapidly diffusible substrates of low molecular weight. An exception to this is the modification of the agar gel plate technique of Jeffries *et al.* (3) for the detection of DNases[2] which have been separated by agar gel electrophoresis (4, 5).

The present communication describes a more direct method for detecting DNases which have been separated in acrylamide gel by disk electrophoresis. This method is more sensitive and gives better resolution than the earlier indirect detection methods. The procedure differs from previous methods in that the substrate is immobilized in the gel during polymerization and is present in the gel during all subsequent steps. The method, therefore, requires that care be taken to prevent enzymic activity during the initial electrophoretic separation. With this accomplished, the enzyme is incubated at its final resting position with the substrate found at that position. The remaining or modified substrate is

[1] This investigation was supported in part by Public Health Service fellowship 5-F1-GM-20,700-02 from the National Institute of General Medical Sciences and Grant No. GB-2215 from the National Science Foundation.

[2] The following abbreviations are used: Bis, *N,N'*-methylenebisacrylamide; BSA, bovine serum albumen, fraction V powder; DNA, deoxyribonucleic acid; DNase, deoxyribonuclease; EDTA, ethylenediaminetetraacetic acid, disodium salt; R_f, [distance traveled by nuclease]/[distance traveled by marker (BSA)]; TEMED, *N,N,N',N'*-tetramethylethylenediamine; tris, tris(hydroxymethyl)aminomethane.

then revealed by staining, and the enzyme positions are identified by noting the discontinuities in the substrate background.

This same concept was recently applied to amylases, another class of enzymes which attacks a high molecular weight substrate (6, 7). In the first report, all of the electrophoretic supporting medium is substrate, while, in the second, starch is polymerized into acrylamide as is done here with DNA. Such applications indicate that this approach can extend the range of gel electrophoresis to still other important enzyme classes.

MATERIALS

Unless otherwise indicated the materials and methods used here are those originally described by Ornstein and Davis (8–11).

Apparatus and Instruments

Electrophoresis cylinders. Pyrex tubing, i.d. 5 mm cut in 8.5-cm lengths, and lightly fire polished.

Protein elution cylinders. Soft glass, i.d. 6 mm in 7.0-cm lengths, lightly fire polished, and constricted at one end. When these cylinders are constructed by removing both ends from Bacteriological Disposable Capillary Pipettes,[3] they fit the same buffer reservoirs used for electrophoresis.

Buffer reservoirs. Upper reservoirs are constructed with eight electrical grommets per polyethylene refrigerator dish. Both reservoirs are fitted with platinum electodes (9).

Power supply. Duostat, Model RD-2.[4]

Camera. Polaroid MP-3 Industrial View Land Camera[5] fitted for transmitted fluorescent illumination.

Densitometer. Automatic Recording Microdensitometer Mark III Model.[6]

Cuvet for densitometry. Glass stoppered quartz cuvet $1 \times 1 \times 5.8$ cm.[7]

Compensating polar planimeter. Model 4236M.[8]

Reagents

Stock solutions (see Table 1). The DNA solutions are prepared by dissolving highly polymerized calf thymus DNA (sodium salt, type I)[9]

[3] Aloe Scientific, St. Louis, Missouri.
[4] Beckman Instruments, Inc., Fullerton, California.
[5] Polaroid Corporation, Cambridge, Massachusetts.
[6] Joyce, Loebl & Co., Ltd., Newcastle upon Tyne, England.
[7] Pyrocell Manufacturing Co., Westwood, New Jersey.
[8] Keuffel and Esser Co., New York, New York.
[9] Sigma Chemical Co., St. Louis, Missouri.

in the appropriate solvent at 4°C over 0.5 ml $CHCl_3$. The solutions are stirred once each day with a glass rod to break up the clumps. All solutions except stock solution (I) (Table 1) are stable at 4°C for at least a month.

Electrophoresis buffer stock. Used at 10 fold dilution. Tris 6.0 gm, glycine 28.8 gm, H_2O to 1 liter.

Elution buffers. Although the buffers are used at 4°C, the pH measurements are made at room temperature:

a. Upper reservoir (anodic) buffer. Electrophoresis stock buffer diluted 5-fold, 0.001 *M* EDTA, titrated to pH 9.4 with 1.0 *N* NaOH.

b. Lower reservoir (cathodic) buffer. Electrophoresis stock buffer diluted 10-fold, 0.001 *M* EDTA, titrated to pH 8.5 with 0.1 *N* NaOH.

c. Gel soaking buffer. Electrophoresis stock buffer diluted 20-fold, 0.001 *M* EDTA, titrated to pH 9.2 with 1.0 *N* NaOH.

DNA Staining Solutions

Native DNA stain. Methyl green stain is prepared according to Kurnick (12) with 77% dye, C.I. No. 42590.[10] The solution can be used repeatedly, but it is discarded when the pH falls below 4.5.

Denatured DNA stain modified from Kurnick (13). A 2% solution of pyronin B (40% dye content, C.I. No. 45010)[10] is prepared in water and extracted exhaustively with $CHCl_3$. The aqueous phase is stored at 4°C in the dark and diluted 10-fold with 0.2 *M* acetate buffer, pH 4.5, before use. The diluted stain is discarded after one use.

Enzyme Preparations

DNase I. To test the method crystallized and lyophilized DNase I from bovine pancreas (Lot No. 103B-0180, 1 Kurnick methyl green unit/mg)[9] was dissolved in buffer 1 (see below) at a concentration of 0.1 mg/ml. This solution was then diluted into buffer 2 just before use to give a final concentration of 10^{-7} to 10^{-8} g/ml. *Buffer 1:* 0.1% (w/v) BSA, 0.25 *M* ammonium sulfate, in 0.05 *M* tris, pH 7.0. *Buffer 2:* 15% (w/v) sucrose, 0.05% (w/v) BSA, 0.25 *M* ammonium sulfate, in electrophoresis stock buffer diluted 10-fold.

DNase II. Amorphous powder from bovine spleen, 500 units/mg (Lot No. 73B-1960).[9]

METHODS

General. Figure 1 is a flow diagram presenting the basic sequence of operations employed for detecting enzymes with DNase activity after their separation in a DNA-polyacrylamide supporting medium. After

[10] National Aniline Division, Allied Chemical Corp., New York, New York.

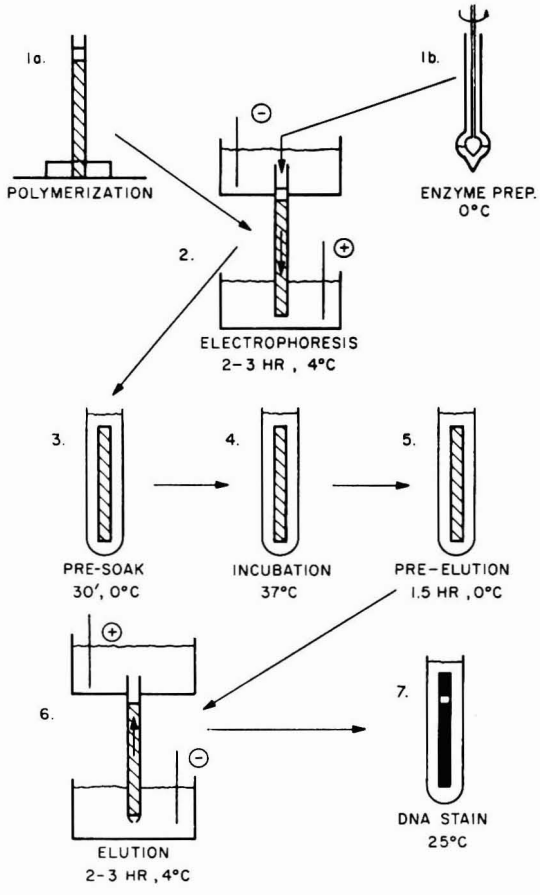

FIG. 1. Schematic representation of steps involved in detecting DNase activity following electrophoresis of the enzyme into an acrylamide gel containing trapped DNA.

DNA has been trapped in the acrylamide gel matrix, the gel is utilized for separation of the enzymes by the standard disk electrophoresis procedures (9, 11). The gel containing the separated enzymes is then incubated under conditions appropriate for DNase activity. Incubation is followed by electrophoretic removal of the proteins and the DNA fragments which have been freed from the matrix by enzymic action. Both the incubation and elution stages are preceded by a presoaking step to establish the appropriate buffering conditions in the gel. Finally, the gel is stained for residual DNA, and the resulting nuclease pattern is recorded by photography or densitometry.

Preparation of the Gel. Gels containing native DNA or denatured DNA are prepared in a similar manner with one exception. Just prior to mixing the stock solutions for polymerization, the DNA containing solutions must be pretreated and in different fashions for the two substrates. Native substrate is pretreated by degassing solution (G) over a period of about 20 min as it warms to room temperature under a moderately strong vacuum provided by a water pump. Denatured substrate is prepared by heating solution (H) in a boiling water bath for 15 min. Heating is followed by quick cooling in an ice bath.

TABLE 1

STOCK SOLUTIONS—MODIFIED FROM DAVIS (9)

	(A)			(F)	
1 *N* HCl		48 ml	Solution (A)		25 ml
Tris		36.6 gm	H_2O		75 ml
TEMED		0.23 ml		(G)	
H_2O	to	100 ml (pH 8.9)	DNA		50 mg
			Solution (F)		25 ml
	(B)		$CHCl_3$		0.5 ml
1 *N* HCl		48 ml		(H)	
Tris		5.98 gm	DNA		50 mg
H_2O	to	100 ml	H_2O		25 ml
	(C)		$CHCl_3$		0.5 ml
Acrylamide		30.0 gm		(I)	
Bis		0.8 gm	Ammonium persulfate		0.28 gm
H_2O	to	100 ml	H_2O		100 ml
	(D)		(discard after one week)		
Acrylamide		10.0 gm		(J)	
Bis		2.5 gm	Sucrose		30 gm
H_2O	to	100 ml	H_2O		70 ml
	(E)		BSA	at	2 mg/ml (optional)
Riboflavin		4.0 mg			
H_2O	to	100 ml			

Room temperature solutions are used to form the mixtures prescribed in Table 2. Mixing is done in 40-ml conical centrifuge tubes to facilitate the removal of air bubbles by centrifugation immediately after mixing. Acid-washed glass electrophoresis columns are filled to a height of 6.2 cm with the gel solution, which is in turn overlayered with water.

After polymerization of the DNA-containing gel, 0.2 ml of the spacer gel solution containing 1 μl TEMED/ml of gel is layered on the DNA gel. This solution is overlayered with water and photopolymerized according to the original instructions of Davis (9).

Electrophoresis. All operations between gel formation and staining,

TABLE 2
FORMULAS FOR FINAL GEL SOLUTIONS

Gels containing native DNA as substrate (1.0 mg DNA/ml gel[a])	Gels containing denatured DNA as substrate (0.75 mg DNA/ml gel)	Large-pore spacer gel
2 parts (G)	3 parts (H)	1 part (B)
1 part (C)	1 part (A)	2 parts (D)
1 part (I)	2 parts (C)	1 part (E)
	2 parts (I)	4 parts H_2O

[a] For good densitometry tracings the native substrate containing gels are made up at a concentration of 0.67 mg DNA/ml gel by appropriately diluting (G) with (F) before final mixing.

with the exception of incubation, are carried out at either 0° or 4°C. The gels are, therefore, chilled for 15 min at 4° before use. An enzyme preparation of 25 to 100 μl can be conveniently layered under the buffer solution and over the spacer gel with lambda pipets (14). Before layering, the enzyme solution is generally diluted 1:1 with stock solution (J). The BSA in combination with excess bromphenol blue in the upper (cathodic) reservoir provides a convenient internal marker when it is necessary to run the fast moving bromphenol blue dye marker off the lower end of the gel. Electrophoresis is performed at 1.5 ma/gel for 2 to 3 hr.

Incubation. At the completion of electrophoresis the gels are removed from the glass cylinders by forcing a stream of water between the gel and the glass (14). Before placing the gels in the 0°C incubation buffer they are stabbed completely through with a No. 11 scalpel blade at either of the two visible dye markers, and the spacer gel is discarded. All operations between the electrophoresis and elution steps are performed with each gel remaining in a single test tube (25 ml). Solutions are quickly changed by inverting a rack of tubes over a wire screen.

After the 30-min preincubation at 0° the incubation buffer is replaced with 25 ml of fresh cold incubation buffer. These buffers should be about 0.1 *M* and well buffered in order to overcome the buffer already present in the gel (see "Discussion"). Incubation is commenced by transferring the tubes from the ice bath to a rapidly circulating water bath at 37°.

Elution. Prior to electrophoretic elution the gels are soaked at 0°C for 1.5 hr in the "Gel Soaking Buffer." Halfway through this soaking the buffer is replaced with fresh cold buffer. The gels are then placed in the protein elution cylinders with the origin end up. Electrophoretic elution is carried out for 2.5 hr at 3 ma/gel and 4° with the electrodes reversed from the original orientation used for electrophoresis.

Staining and Removal of Excess Stain. These and subsequent operations are carried out at room temperature. Gels which contain native DNA as a substrate are stained directly in the methyl green stain solution for 1 hr or more. The excess stain is removed by soaking the gels in several changes of 0.2 *M* acetate buffer, pH 4.0, over a period of 1 to 3 days.

Gels containing denatured DNA must first be soaked in 0.2 *M* acetate (pH 4.5) for 1 or more hours before they can be stained. They are then stained in the diluted pyronin B stain for one day, after which the excess stain is removed by again soaking in the pH 4.5 buffer.

Photography of Stained Gels. Prior to photography or densitometry the portion of the gel below the scalpel slash is usually broken off and discarded. The end of the gel then serves as an internal mobility marker in the recording. Polaroid 55/PN film 4 × 5 in. is used to photograph gels which have been covered with buffer in an open flat-bottom glass dish. Transmitted fluorescent light is the only source of illumination. By placing a No. 25 A Kodak Wratten gelatin filter in front of the camera lens it is possible to obtain pictures of the methyl green stained gels which are far clearer than direct observation of the gel. Photographs of pyronin B stained gels, for which no satisfactory filter has been found, fail to show quite as much detail as can be seen directly with the eye.

Densitometry of Stained Gels. Scanning is performed by placing the cuvet containing the gel in a horizontal position on the densitometer scanning table. The gel is completely immersed in the appropriate final soaking buffer. In order to focus this particular instrument properly it is necessary to "lever up" the condensor optics as described in the instructions accompanying the instrument. Methyl green stained gels are scanned using a Joyce Loebl red filter in the light path between the gel and the photocell. A 1.0 OD neutral filter is used with pyronin stained gels. Before centering the beam in the gel the gel is aligned parallel to the direction of scan by following the outside edge of the gel with the cross-hairs. The width of the scanning light beam is 50 μ. R_f values can be measured from the scans relative to the dye marker at which the gel was broken off.

RESULTS

DNase I

The two basic methods for recording an experiment are illustrated in Figs. 2 and 3. Densitometry tracings of both native and denatured DNA-containing gels are presented in Fig. 3 together with recordings of parallel controls. Although these controls did contain DNase I, their densitometry

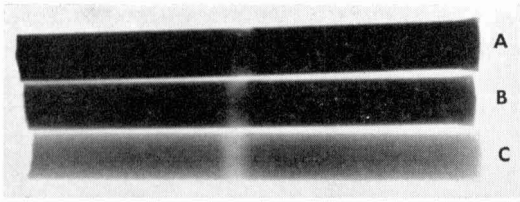

FIG. 2. Polaroid photograph of DNase I patterns in native DNA-containing gels. Incubation was carried out for 1-hr at 37°C in 0.1 *M* tris, 0.005 *M* $MgCl_2$, and at pH 7.0. Gels A, B, and C contained DNA at concentrations of 1, 0.67, and 0.33 mg/ml gel, respectively. Each gel contained 10^{-9} gm DNase I.

tracings are identical to those of control gels which did not contain any enzyme.

The fact that the controls show no indication of enzymic activity demonstrates that all such activity occurs during the 37°C incubation. This means that the enzyme does not degrade the DNA through which it passes during electrophoresis at 4°. These backgrounds are constant enough to permit quantitative measurements of the areas under the peaks obtained from incubated gels. An artifact which is always present at the origin of gels containing native DNA (Fig. 3) can probably be

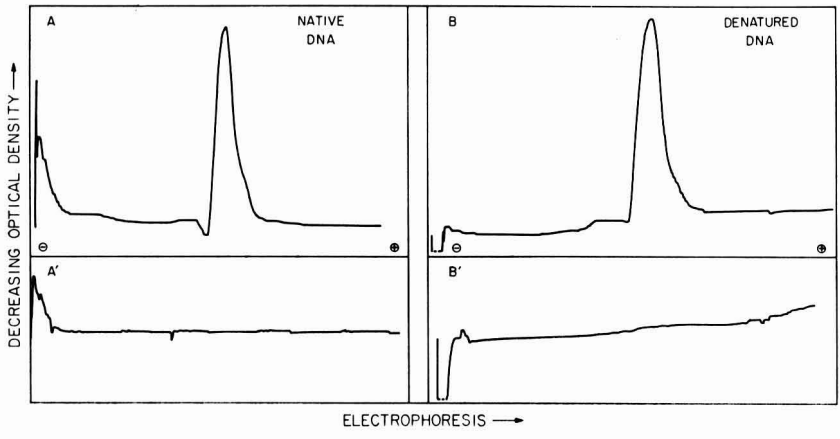

FIG. 3. Densitometry tracings of DNA-containing acrylamide gels after electrophoresis and incubation with DNase I. Electrode orientations are indicated by + and —. Gel A which contained 0.5×10^{-9} gm DNase I and 0.67 mg native DNA/ml was incubated for 0.5 hour. Control A′ contained 10 times as much DNase I. Both gels were treated identically except that A′ was placed in elution buffer at 0°C after the 35-min preincubation soak and, therefore, received no 37° incubation. Gel B, which contained 3×10^{-9} gm DNase I and 0.75 mg denatured DNA/ml was

explained by the observations of Davis (11). Since a thin layer of gel at this position seems to be more porous than the bulk of the gel, some of the DNA may be free to move out of the gel leaving a small bleached area at this position. This explanation is substantiated by the fact that this artifact is magnified when acrylamide concentrations less than 7.5% are used to trap DNA.

Figure 4 was constructed from data obtained by using a planimeter

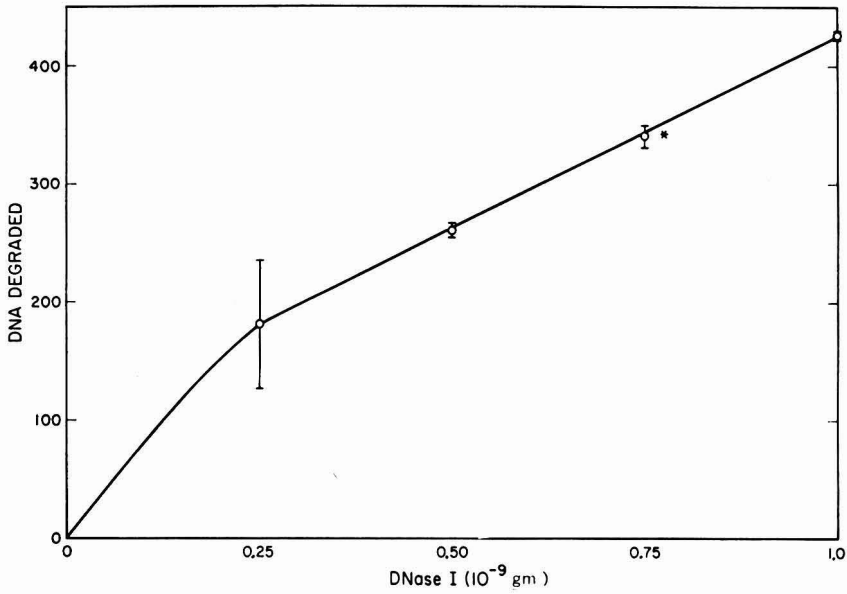

FIG. 4. Native DNA degraded by DNase I during 1-hr incubation at 37°C. Ordinate represents relative areas measured from peaks of densitometry tracings. Each point represents average value obtained from three separate gels unless otherwise indicated. Error range is one standard deviation. * Average from two gels.

to measure the areas under curves such as those in Fig. 3. This curve first of all provides an indication of the sensitivity of the system. Although 0.25 nanogram is the least amount of DNase I yet to be applied to such a gel, it is clear that the sensitivity lies well below this value and it could be still further increased. Second, the data in Fig. 4 provide an estimate of the reproducibility of the method. The average of the percentage errors (coefficient of variation) of seven points (19 gels) above 0.25 nanogram of DNase I and half hour incubation is 3.1%. Since the reproducibility of the combined densitometry and planimetry steps is 2.5%, the remainder of the error can be accounted for by

pipetting and incubation time errors. Below these concentration and time limits the variation has been considerably higher. This error analysis applies only to data within one experiment.

A curve very similar to that of Fig. 4 is obtained when "DNA degraded" is plotted against "time of incubation." Such kinetic data provides strong support for the contention that the observed gel bleaching is due to a catalyzed reaction rather than to an inhibition of the DNA staining. That the "hole" in the DNA background is due to DNase I activity is further indicated by the failure of the "hole" to appear when a gel containing the enzyme is stained directly after electrophoresis in a solution containing EDTA. A corresponding gel stained in a Mg^{++}-containing solution develops a steadily widening "hole."

The data in Fig. 4 demonstrate that for DNase I, and probably for other nucleases, it is possible to find a range of activity for which the results are both reproducible and concentration dependent. This then makes it possible to assay quantitatively for one enzyme in the presence of a mixture of enzymes which are separable from each other by disk electrophoresis. It should be pointed out that the concentration dependence exhibited in Fig. 4 is most probably due to a concentration-dependent diffusion process. This is implied from the fact that the peaks from which these data were taken are all essentially the same height. The increase in area is due rather to a broadening of the peaks. It therefore appears that the biphasic nature of the curve is a result of a rapid degradation of the substrate at the initial position of the enzyme. This phase is then followed by a slower diffusion of the enzyme into the adjacent substrate. Unpublished data further indicate that a peak does not begin to broaden significantly until much of the substrate has been degraded at the initial resting position of the enzyme.

When gels with and without substrate are stained for protein following electrophoresis, the resulting patterns demonstrate that the mobilities of proteins in a crude mixture are only slightly retarded by the presence of either native or denatured DNA in the gel. This question must be further investigated for the nucleases, because with them there is a greater likelihood that an enzyme-substrate interaction will occur during electrophoresis. Data such as that found in Fig. 3 may be used to determine what effect the state of the DNA has on the relative mobilities of nucleases. R_f measurements from such scans show that there is no significant difference between the mobility (standard deviation$_{R_f}$ = 0.002) of DNase I in native DNA and its mobility in denatured DNA. Thus, if there is any interaction between the enzyme and the substrate during electrophoresis, it is of the same order of magnitude in both substrates. This is an important question which must be resolved for each enzyme

if a satisfactory determination of the substrate specificity of an enzyme is to be obtained from an enzyme mixture.

DNase II

Unlike DNase I, DNase II will not move as a discrete band through a gel containing native DNA. It apparently interacts strongly with the DNA under the conditions of electrophoresis and binds to the DNA at the gel origin. At higher enzyme concentrations it progressively clears the gel of DNA from the origin downward. This behavior of an important DNase does not limit studies of crude enzyme mixtures as much as it might appear. In all three of the organisms which have been investigated only a small percentage of the total enzymic activity behaves in this manner.

Crude Enzyme Preparations

The densitometry tracings in Fig. 5 are presented to demonstrate the type of information this method can contribute to biochemical and developmental studies. In particular, this series of scans demonstrates some of the great diversity of enzymic activities which can be revealed with this method. The simplicity of disk electrophoresis further makes it possible to follow closely the dynamic changes such patterns undergo during growth and differentiation. As Kaplan (15) has clearly pointed out, however, this type of information by itself is not sufficient to establish the identity of an enzyme. Other techniques and experiments are required to determine to what extent such peaks represent complexes, aggregates, and isozymes of other components. The need for such caution is emphasized by the fact that up to four minor peaks appear when DNase I is assayed at high concentration. Nevertheless, the added degrees of sensitivity and resolution of this method are of considerable help in outlining and resolving these problems.

Biochemical genetics presents another broad area for the potential application of this method. As an example, we have demonstrated the appearance of a new DNase activity upon the infection of *Escherichia coli* with the phage T4. The speed and sensitivity of this procedure have made it feasible to scan the large number of mutants of the phage which potentially have a deficiency or alteration of the normal enzyme. The identification of such mutants should contribute to our understanding of the function of some of these enzymes.

DISCUSSION

The general procedure described here has been designed to detect the majority of DNase activities which might be found in a crude homog-

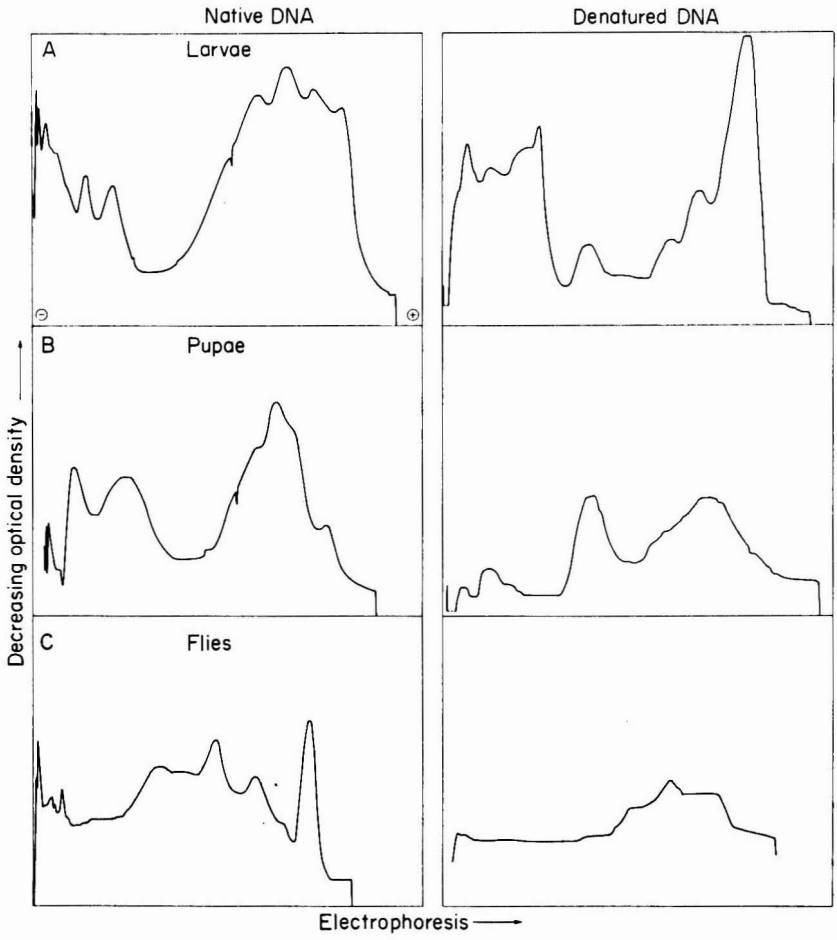

FIG. 5. Densitometry tracings of DNase patterns obtained from homogenates of *Drosophila melanogaster*. Animals were cultured at 25°C. Larvae were collected 61 hr after egg laying, pupae 55 hr after pupation, and flies less than 1 day after emergence. Homogenates were diluted and submitted to electrophoresis. Incubation was conducted for 35 min at 37° in 0.1 *M* acetate, 0.005 *M* $MgCl_2$, and at pH 4.0.

enate. Since no one set of conditions will be optimum for all enzymes in such a wide spectrum of activities, the procedure must frequently be modified to suit a particular problem. For example, the conditions presented here give good results with *Drosophila* homogenates but are not the best for similar preparations from microorganisms. The following discussion defines the parameters of the method in more detail in

order to indicate possible approaches for improvement in a given situation.

Trapping the DNA. As long as the DNA is *not* dissolved in a stock solution containing the acrylamide monomers, it appears to have little effect on the polymerization of the gel. With the stains described here it is best to keep the DNA concentration at 0.5 mg/ml of gel or higher. A concentration of 1.0 mg/ml is best for photographic recordings while a value somewhere intermediate between these two is most suitable for densitometry.

It is often useful to run the proteins of low mobility further into the gel in order to attain better resolution. This can be accomplished either by decreasing the gel concentration or by running the electrophoresis for a longer period of time. The latter approach is preferable because of the possibility that the DNA will migrate along with the protein when the acrylamide concentration is decreased.

Incubation pH. The question of the efficiency of buffer replacement by the half hour preincubation period described here has not been investigated extensively. However, it may be noted that a fairly rapid exchange of ions between gel and surrounding buffer probably occurs, since some enzymes which are active in a Mg^{++}-containing buffer show no activity in one containing EDTA. On the other hand, longer preincubation soakings are probably required to change completely the pH within a gel, and this matter must be examined further if it is desired to determine the pH optimum of an enzyme contained in a gel.

Elution. There are three principal reasons for eluting the gels following incubation: First, removal of the oligonucleotides which have been freed from the gel by enzymic action greatly increases the sensitivity of the method. Second, protein removal provides a superior background, because the DNA stains also stain protein to some extent. And, finally, the nucleases must be removed, as they often continue to act in the stain solution, eventually clearing the entire gel of DNA.

The present procedure has been designed to remove greater than 95% of the protein in a crude homogenate from gels which have been incubated at any pH from pH 4 to 10. Although this requires that the conditions of the presoak and electrophoresis steps be excessive for gels incubated at higher pH, it does make it possible to handle all such gels in a single operation. Also in the elution step it is particularly important that the pH in the upper reservoir (anodic) does not drop during the course of elution. When this occurs the gels swell and split prohibitively.

Staining. Of the several stains which have been tried, including the Feulgen stain, methyl green and pyronin B have given the most reproducible results and are the easiest to use. Under standard staining

conditions neither stain binds to acrylamide itself, as is indicated by the complete removal of color from gels which contain no DNA. Neither, however, is the binding of the dyes to DNA absolutely permanent. It is, therefore, recommended that the nuclease patterns be recorded sometime in the month following staining. An additional advantage in using methyl green is that it is fairly specific for highly polymerized native DNA (12). This characteristic eliminates any ambiguity which might arise from contamination of native DNA with denatured DNA.

Recording. While photography provides a rapid method for qualitatively scanning a large number of gels, the densitometer has proved itself invaluable as a source of finer resolution and semiquantitative data. These data are not limited by the error which results from the different affinities of proteins for a given stain in protein stained gels. Nevertheless, much work remains to be done with enzymes having different mechanisms of action before the significance of quantitative nuclease determinations can be fully evaluated.

Background Considerations. It is important to realize that, unlike DNase I, which acts only during the 37°C incubation, many enzymes exhibit considerable activity during the soakings at 0°C. This is to be expected from a consideration of the wide spectrum of requirements exhibited by enzymes of this class. Depending upon the enzymes under study this "background" can be eliminated or reduced by certain procedural modifications. These include decreasing the enzyme concentration and increasing the incubation time or the addition of specific inhibitors before the completion of incubation.

Applications. The results shown in Fig. 5 demonstrate the potential of this method for the rapid preliminary identification and study of DNases. The quantitative aspects of the procedure should further facilitate isolation of the enzymes once they have been identified. In addition, it may be possible in crude mixtures tentatively to characterize DNases with respect to their pH optimums, substrate specificities, and even their mechanisms of action.

SUMMARY

A method is described for detecting and measuring deoxyribonucleases in polyacrylamide gels after their separation by disc electrophoresis. The procedure is based on an initial trapping of either native or denatured deoxyribonucleic acid in the gel matrix. Using crystallized deoxyribonuclease I, the quantitative reproducibility has been shown to be within 5%. The method is sensitive to less than 0.25 nanogram of this enzyme. Application of the method to crude enzyme preparations is demonstrated in examples using homogenates of *Drosophila melanogaster*.

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ANALYTICAL BIOCHEMISTRY 13, 43–65 (1965)

The Spectrophotometric Constants of Di- and Trinucleotides in Pancreatic Ribonuclease Digests of Ribonucleic Acid[1]

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Current investigations into the base sequences of polynucleotides underway in several laboratories (1–4) are of special interest because of the high probability that the biological activities of these macromolecules are determined by the sequences of the purine and pyrimidine bases. The quantitative estimation of oligonucleotides derived from polymers by partial hydrolysis, either chemical or enzymic, is usually through the determination of their absorption in the ultraviolet (1, 5–7), although this parameter has also been used in conjunction with radioactive labels (2, 4, 8). In either case, accurate estimations are dependent upon a knowledge of the molar extinction coefficients. Heretofore, in the absence of these data, investigators have ignored the hypochromicity of oligonucleotides (4, 8), have employed extinction coefficients determined upon closely related compounds (5, 6), or have converted the oligonucleotide fractions to mononucleotides and nucleosides with known spectral properties. Because of the value of complete spectra for the identification and quantitation of oligonucleotides arising through the action of pancreatic ribonuclease, the present study was undertaken.

MATERIALS AND METHODS

RNA[3] was isolated from the ribosomes of *Escherichia coli,* strain W 3101, by phenol extraction of the protein in the presence of sodium

[1] Taken from the dissertation submitted by W. M. Stanley, Jr., in partial fulfillment of the requirements for the Ph.D. degree at the University of Wisconsin, Madison, Wisconsin.

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[3] Abbreviations used in this work: RNA, ribonucleic acid; RNase, ribonuclease;

dodecyl sulfate and Macaloid, a purified hectorite. The physical characterization of this RNA was described earlier (11).

Chromatographically purified crystalline pancreatic RNase (EC 2.7.7.16) was obtained from Mann Research Laboratories. Hydrolyses of the RNA were carried out at 37° for 18 hr in 0.05 *M* tris-HCl, pH 7.5, at an

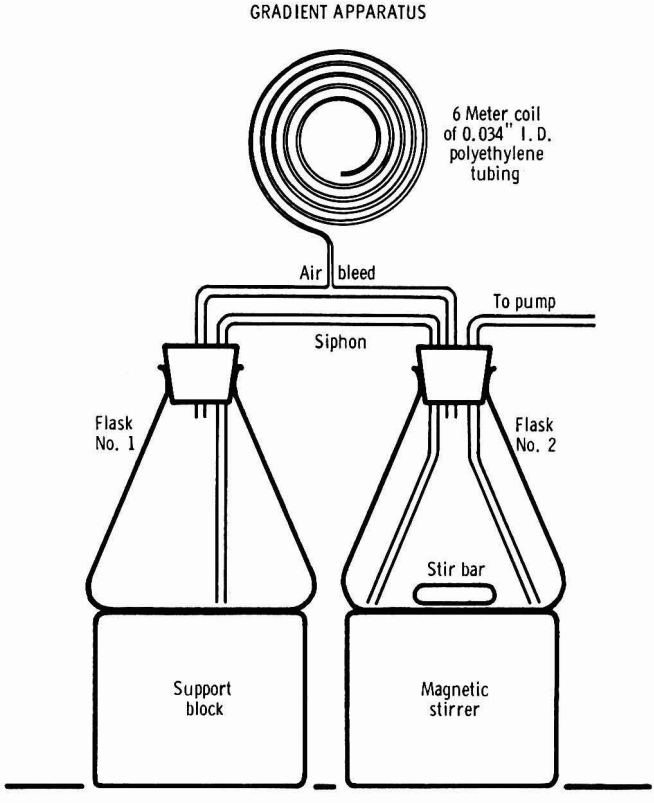

FIG. 1. Gradient apparatus for maintenance of pH stability during production of linear concentration gradients of volatile buffer systems. Flask No. 1 contains the more concentrated solution and flask No. 2, the more dilute. The gradient produced is linear provided the cross sections of the two flasks are equal at equal heights. The 6-meter coil of polyethylene tubing allows air to enter the system as liquid is removed and also serves as a kinetic barrier to diffusion of volatile components of the buffer system. The apparatus will maintain the pH of an 0.2 *M* ammonium bicarbonate solution of initial pH 8.6 to within 0.02 pH unit for at least 1 week.

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tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; C, cytosine; U, uracil; A, adenine; G, guanine. For the designation of oligonucleotides the system of Markam and Smith (9) and Heppel *et al.* (10) has been used.

RNA concentration of 7 mg/ml and a substrate:enzyme ratio (w/w) of 100:1. The pH was maintained by periodic additions of 1 *M* KOH and the final hydrolyzate was diluted 5-fold with distilled water and adjusted to pH 8.6 prior to column chromatography.

DEAE-cellulose (Selectacel, type DEAE, 1.0 meq/gm) was purchased from Brown Co., and was washed and prepared according to Staehelin (12). Suspensions of DEAE-cellulose in the dilute buffer of the gradient to be employed were packed into columns at room temperature under pressure increasing from 5 to 10 psi. The columns were washed with several bed volumes of the dilute buffer before use.

Columns were operated at 20° at flow rates of 20 ml/hr/cm^2 maintained with a miniPump (Milton Roy Co.). Linear gradients of constant pH were produced by the gradient device illustrated in Fig. 1. The effluent of each column was passed through a quartz flow cell of either 1- or 10-mm optical path mounted in a Beckman DB spectrophotometer operated at 260 mμ. The output of the spectrophotometer was recorded automatically as a function of time. Because of the constancy of the delivery from the miniPump, these traces were valid records of absorbancy at 260 mμ versus volume of liquid delivered. Oligonucleotide fractions were recovered and freed of water and salt by lyophilization.

Preparative paper chromatography was carried out at room temperature by streaking oligonucleotide fractions in water on Whatman No. 3MM paper and developing the paper by descending chromatography in solvent A (see below). Oligonucleotide compounds were located by their quenching in the ultraviolet and areas containing the compounds were cut out, eluted with water, diluted, and adsorbed to small DEAE columns. The columns were washed with 20 bed volumes of 0.01 *M* ammonium bicarbonate, pH 8.6, and the oligonucleotide material was eluted with 0.7 *M* ammonium bicarbonate, pH 8.6. Water and salt were removed by lyophilization and the compounds were stored as approximately 10 mg/ml solutions in water at —20°.

The identification and base sequences of the oligonucleotides were determined by their relative positions in the DEAE column effluent at pH 8.6 (12), by their base compositions plus the known specificity of pancreatic RNase, and by their migrations in solvent systems A (13) and B (14). The sequences of the isomeric pairs ApGpCp-GpApCp and ApGpUp-GpApUp were checked by paper chromatography in solvent A of the products arising from digestion with RNase T_1 (EC 3.1.4.8).

The base compositions of the oligonucleotides were determined by paper chromatography in solvent C following hydrolysis in 1 *M* HCl for 1 hr at 100°. The purine bases and the pyrimidine nucleotides were located by their quenching in the ultraviolet and were cut out along with

blank areas of corresponding location. The paper disks were eluted with 0.01 *M* HCl and the spectra of the eluted compounds recorded with reference to the corresponding blank in a Cary model 11 recording spectrophotometer.

Analytical paper chromatography was performed on Whatman No. 1 at room temperature by descending development in the following solvents. *Solvent A:* 40 gm of ammonium sulfate dissolved in 100 ml of potassium phosphate adjusted to pH 6.9 (14). *Solvent B:* 1 vol of 3 ml 98% formic acid per liter of water plus 1 vol of *tert*-butanol adjusted to pH 4.8 with ammonium hydroxide (14). *Solvent C:* isopropanol, 179 ml; concentrated HCl, 41 ml; water to 250 ml (15).

Spectra of the oligonucleotides were determined at 20° in a Cary model 15 recording spectrophotometer calibrated in both wavelength and absorbancy scale by a representative of Applied Physics Co. The spectral band width did not exceed 2 A except below 200 mμ. The spectra were reproduced photographically from the original spectra or from traces of the spectra. The latter process became necessary when the Cary 15 developed a tendency to produce "blips" at certain wavelengths. These "blips" did not affect the accuracy of the spectra.

Stock solutions of the oligonucleotides were prepared in water and dilutions with the same pipet were made for the spectral curves at acid, neutral, and alkaline pH. The buffers employed were 0.10 *M* HCl, 0.02 *M* sodium phosphate, and 0.002 *M* NaOH for pH 1, 7, and 11, respectively. Following the recording of each spectrum, the sample cell was removed from the spectrophotometer and the pH of the contents checked. The curves were recorded with reference to a blank cell containing the same buffer solution as the sample cell, both cells having previously been matched to within ±0.002 absorbancy unit.

The spectra of the alkaline hydrolyzates were obtained upon aliquots of the stock solutions which had been hydrolyzed in sealed tubes in 0.3 *M* KOH at 37° for 18 hr. They were neutralized with HCl and diluted into 0.02 *M* sodium phosphate, pH 7, and the spectra recorded. The pH of each sample was checked following the recording of the spectrum. Since Lane and Butler (16) have reported that certain nucleotide sequences are relatively resistant to alkaline hydrolysis, aliquots of the hydrolyzates were subjected to paper chromatography in solvent A to make sure that the hydrolysis had been complete.

Spectral constants at 20° were calculated from the hyperchromic shifts at pH 7 following hydrolysis utilizing the extinction coefficients for the component mononucleotides given in Table 1.

The dependencies of the absorption at 260 mμ of various oligonucleotides upon temperature (melting curves) were determined in 0.05 *M*

TABLE 1
MONONUCLEOTIDE MOLAR EXTINCTION COEFFICIENTS EMPLOYED FOR THE CALCULATION OF OLIGONUCLEOTIDE SPECTROPHOTOMETRIC CONSTANTS

Compound	$a_m \times 10^{-3}$ at 260 mμ, pH 7
2′(3′)-Cytidylic acid	7.3
2′(3′)-Uridylic acid	9.9
2′(3′)-Adenylic acid	15.4
2′(3′)-Guanylic acid	11.6

Na_2HPO_4, 0.05 *M* NaH_2PO_4 in a Beckman DB spectrophotometer. Temperatures of the contents of the sample cell were continuously monitored by a thermister probe positioned directly above the light beam. Details of the apparatus have been described elsewhere (17). All data were corrected for the thermal expansion of the solution.

All pH determinations were performed with a Leeds and Northrup pH meter, catalog No. 7664, equipped with the miniature pH electrode assembly, catalog No. 124138.

RESULTS

A DEAE cellulose column chromatograph at pH 8.6 of a RNase digest is reproduced in Fig. 2. The improved resolution over similar chromatograms (2–4, 7, 12) published prior to this work is undoubtedly due to: (*1*) operation at 20°; (*2*) columns of 250-cm length; (*3*) linear gradients; and (*4*) control of the pH by the device illustrated in Fig. 1. For preparative runs, columns of 2.4 × 110 cm were employed and were developed with an 8-liter linear gradient of from 0.01 to 0.4 *M* ammonium bicarbonate, pH 8.6. Fractions corresponding to ApCp, ApUp, GpCp, GpUp, ApApCp, ApApUp, GpApCp + ApGpCp, GpApUp + ApGpUp, GpGpCp, and GpGpUp were collected and recovered. These fractions were individually rechromatographed upon DEAE cellulose columns (1.2 × 250 cm) at pH 6.5 with linear gradients of ammonium formate according to the schedules outlined in Table 2. Oligonucleotides not well separated at pH 8.6 are satisfactorily resolved at this pH (6, 18). The individual oligonucleotides were recovered and further purified by preparative paper chromatography in solvent A as described under "Materials and Methods."

The final preparations were found to give single spots upon analytical paper chromatography in solvents A and B. The base ratios determined after acid hydrolysis are given in Table 3. From these data it is probable that each fraction is greater than 95% homogeneous.

The spectra obtained from the oligonucleotides are reproduced in Fig. 3 and the spectrophotometric constants calculated from the spectra

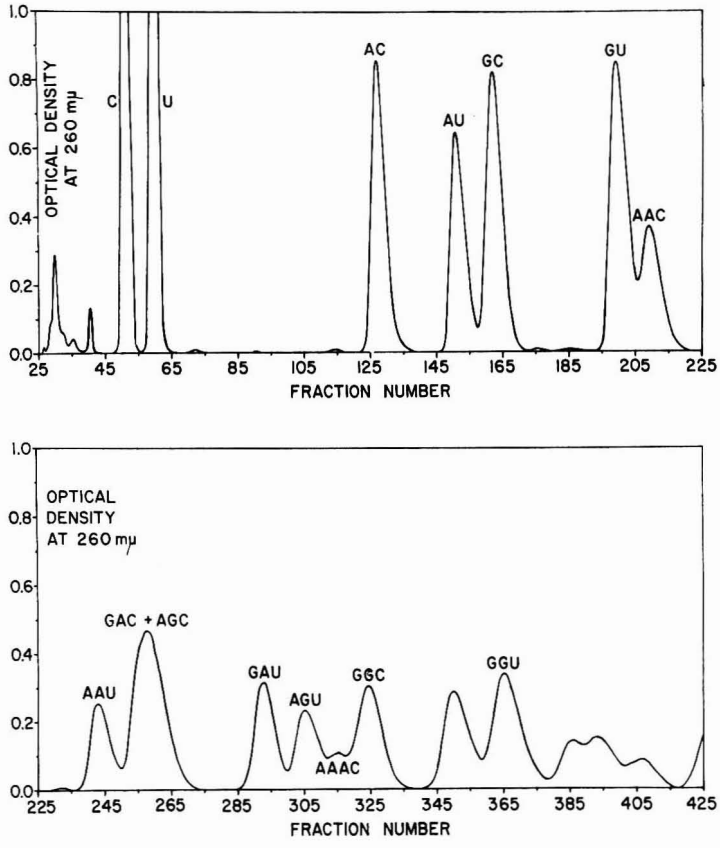

FIG. 2. Chromatogram of a pancreatic RNase digest of 22.4 mg RNA on a DEAE cellulose column (1.2 × 250 cm) eluted with a 2.44-liter linear gradient of from 0.01 to 0.344 *M* ammonium bicarbonate, pH 8.6. Fraction size was 5.75 ml; further details are given under "Materials and Methods." The large peak eluted between GpGpCp and GpGpUp was composed of three tetranucleotides, ApApApUp, (ApApGp)Cp, and (ApApGp)Cp. The parentheses indicate unknown sequences.

are summarized in Table 4. It was observed that the optical density of the oligonucleotides was a function of temperature. Representative melting curves at 260 mμ are illustrated in Fig. 4.

DISCUSSION

The necessity for the experimental determination of the molar extinction coefficients of oligonucleotides arises because of the fact that the coefficients are not simply the sum of the coefficients of the constituent mononucleotides. This phenomenon, known as *hypochromicity*,

TABLE 2

AMMONIUM FORMATE GRADIENTS FOR RECHROMATOGRAPHY OF OLIGONUCLEOTIDE FRACTIONS AT pH 6.5[a]

Oligonucleotide fraction	Gradient volume, liters	Ammonium formate concentration, *M*	
		Initial	Final
ApCp	2	0.05	0.15
ApUp	2	0.06	0.16
GpCp	2	0.08	0.18
GpUp	2	0.10	0.22
ApApCp	2	0.18	0.28
ApApUp	2	0.19	0.28
GpApCp + ApGpCp	2	0.20	0.30
GpApUp + ApGpUp	3	0.15	0.35
GpGpCp	2	0.22	0.32
GpGpUp	2	0.23	0.33

[a] Oligonucleotide fractions corresponding to those listed in the above table were obtained from a single DEAE cellulose column developed at pH 8.6 with ammonium bicarbonate as described under "Results" (see also Fig. 2). These fractions were individually rechromatographed at 20° upon columns (1.2 × 250 cm) of DEAE cellulose (1.0 meq/gm) developed at a flow rate of 20 ml/hr/cm² with linear gradients of ammonium formate, pH 6.5, according to the schedule given in the above table. Oligonucleotides not completely resolved at pH 8.6 are well separated at pH 6.5. The di- and trinucleotides were recovered from the column eluates by lyophilization and subjected to further purification by preparative paper chromatography as described under "Materials and Methods."

TABLE 3

BASE RATIOS OF DI- AND TRINUCLEOTIDES

Compound	Composition[a]			
	U	C	G	A
ApCp	—[b]	1.00	—[b]	1.04
ApUp	1.00	—[b]	—[b]	1.02
GpCp	—[b]	1.00	1.01	—[b]
GpUp	1.00	—[b]	1.04	—[b]
ApApCp	—[b]	1.00	—[b]	2.02
ApApUp	1.00	—[b]	—[b]	1.88
ApGpCp	—[b]	1.00	1.03	1.07
GpApCp	—[b]	1.00	1.08	1.04
ApGpUp	1.00	—[b]	1.06	1.03
GpApUp	1.00	—[b]	0.98	0.97
GpGpCp	—[b]	1.00	1.95	—[b]
GpGpUp	1.00	—[b]	2.00	—[b]

[a] Base ratios were determined as described under "Materials and Methods" and are expressed relative to the pyrimidine.

[b] Not detected.

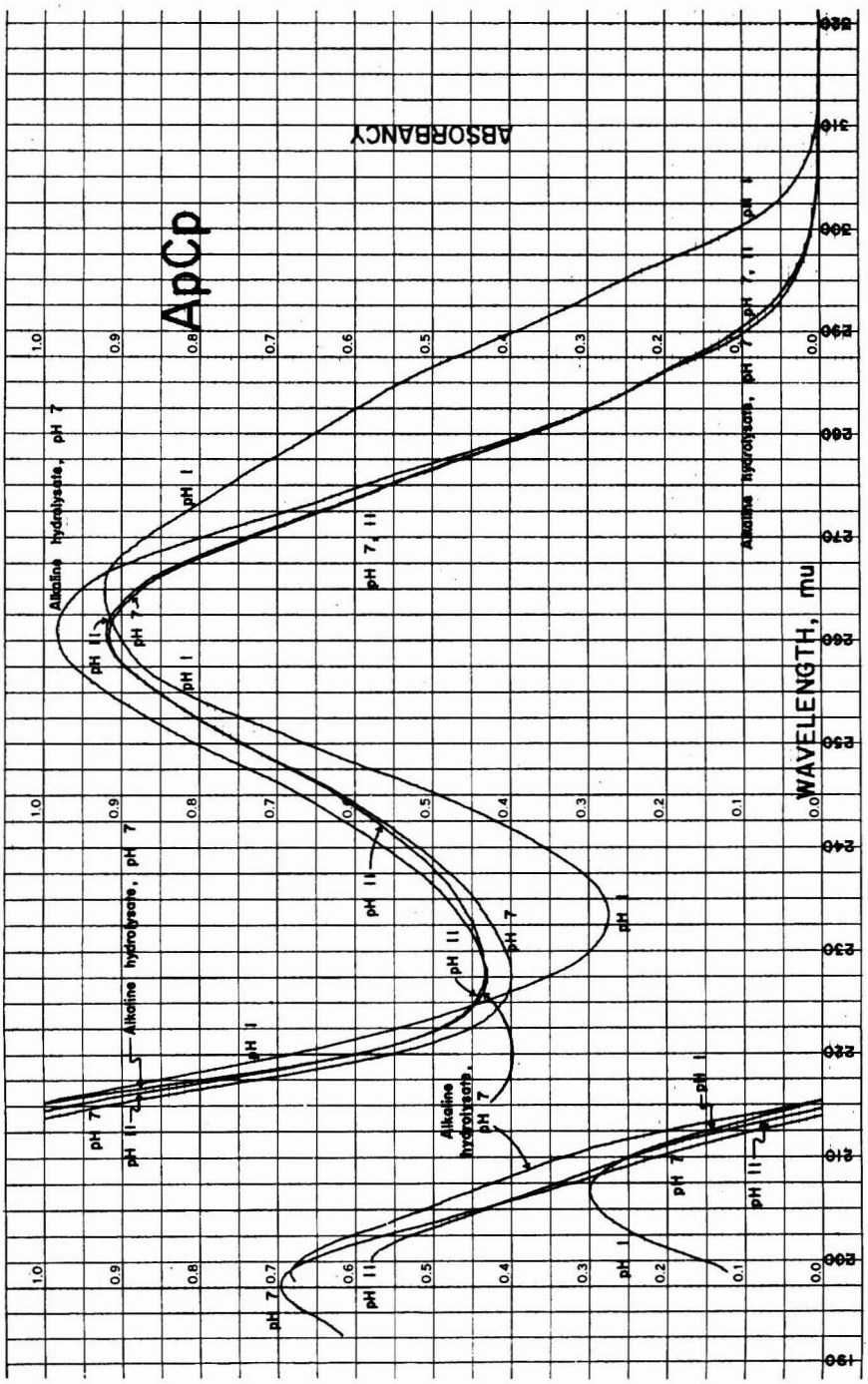

FIG. 3(1).

FIG. 3. Spectra of di- and trinucleotides from a RNase digest of RNA at pH 1, 7, and 11 and the spectra of their alkaline hydrolysates at pH 7. Experimental details are given under "Materials and Methods."

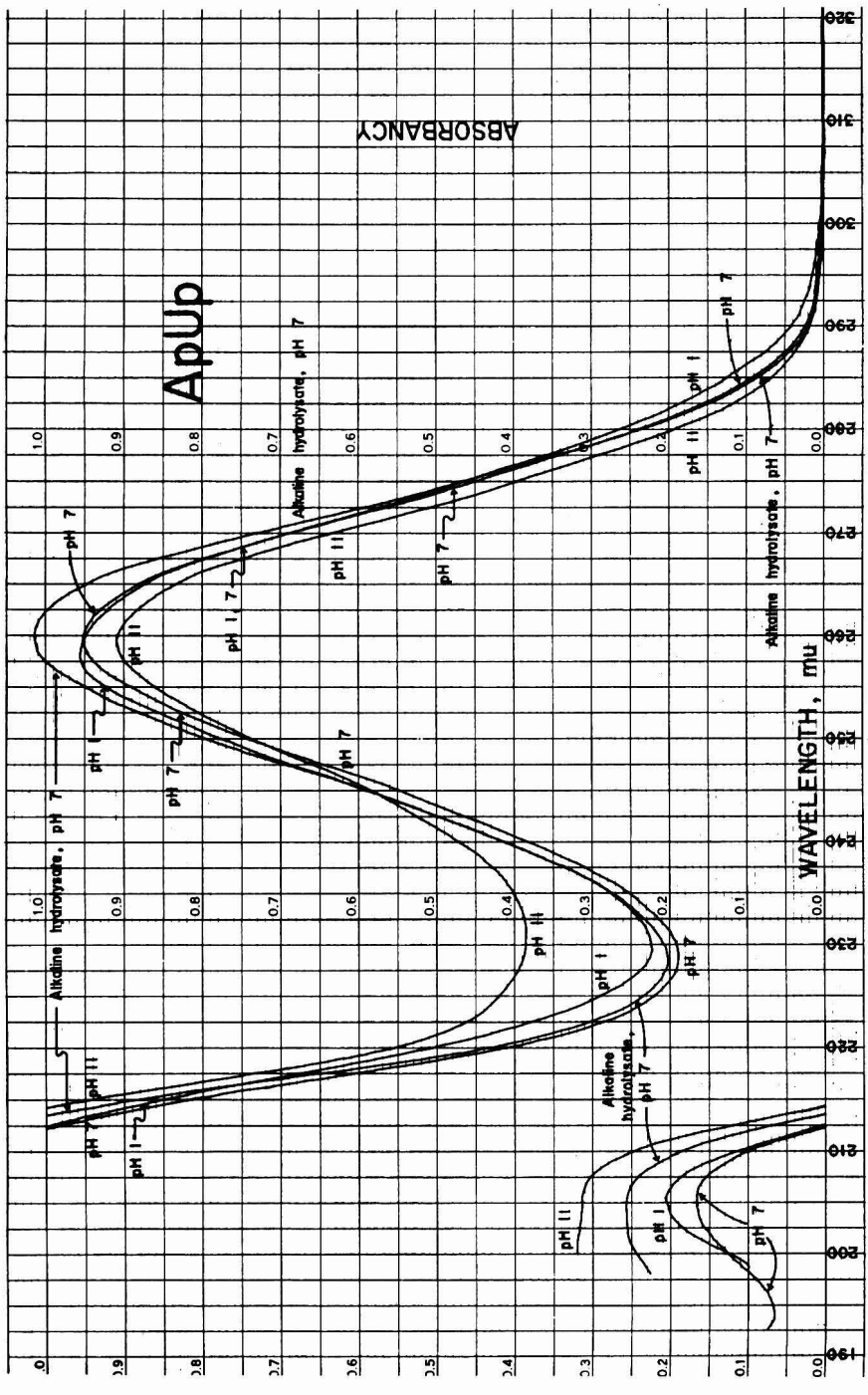
ApUp
ABSORBANCY
WAVELENGTH, mu
Alkaline hydrolysate, pH 7
pH 7
pH I
pH II
pH III
Alkaline hydrolysate, pH 7
pH I
pH II
pH 7
Alkaline hydrolysate, pH 7
pH I
pH II
pH 7
Alkaline hydrolysate, pH 7
pH I
pH II
pH 7
Alkaline hydrolysate, pH 7
pH I
pH II
pH 7

FIG. 3(2).

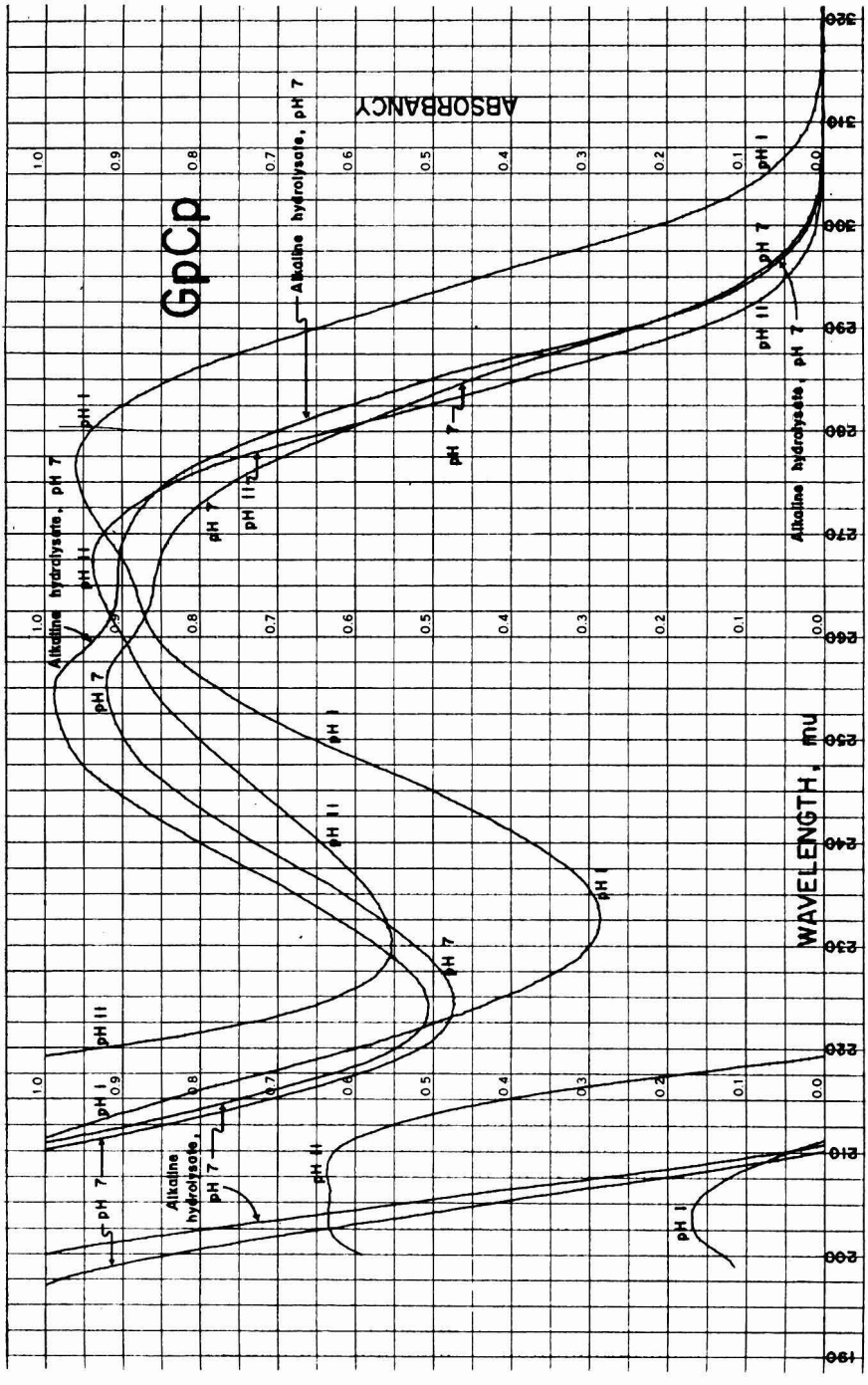
GpCp
ABSORBANCY
WAVELENGTH, mμ
Alkaline hydrolysate, pH 7
pH I
pH II
pH 7
320
310
300
290
280
270
260
250
240
230
220
210
200
190
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0

FIG. 3(3).

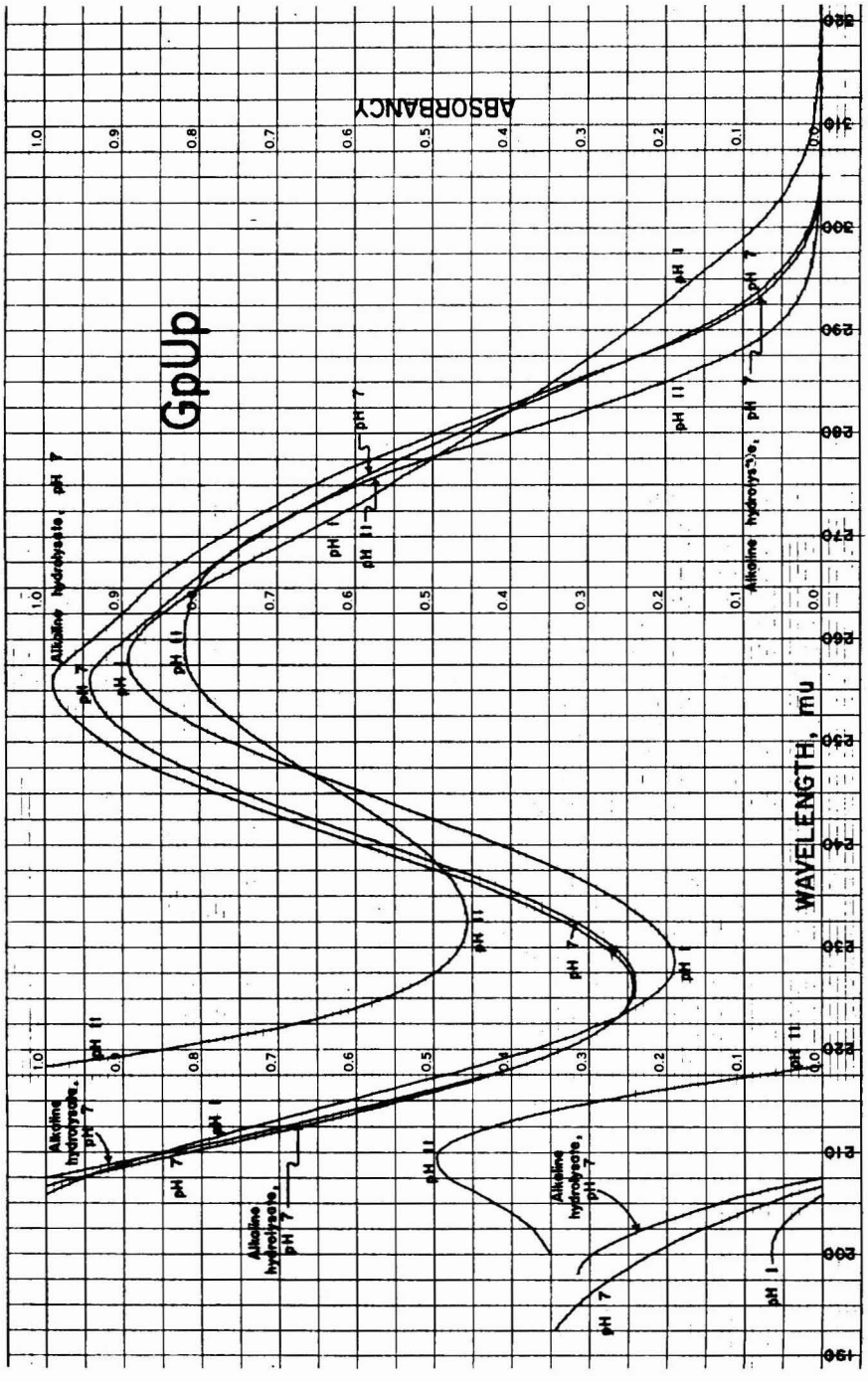
GpUp
ABSORBANCY
WAVELENGTH, mμ
Alkaline hydrolysate, pH 7
pH 1
pH 7
pH 11
Alkaline hydrolysate, pH 7
pH 1
pH 7
pH 11
Alkaline hydrolysate, pH 7
pH 1
pH 7
pH 11
Alkaline hydrolysate, pH 7
pH 1
pH 7
pH 11
Alkaline hydrolysate, pH 7
pH 1
pH 7
pH 11

FIG. 3(4).

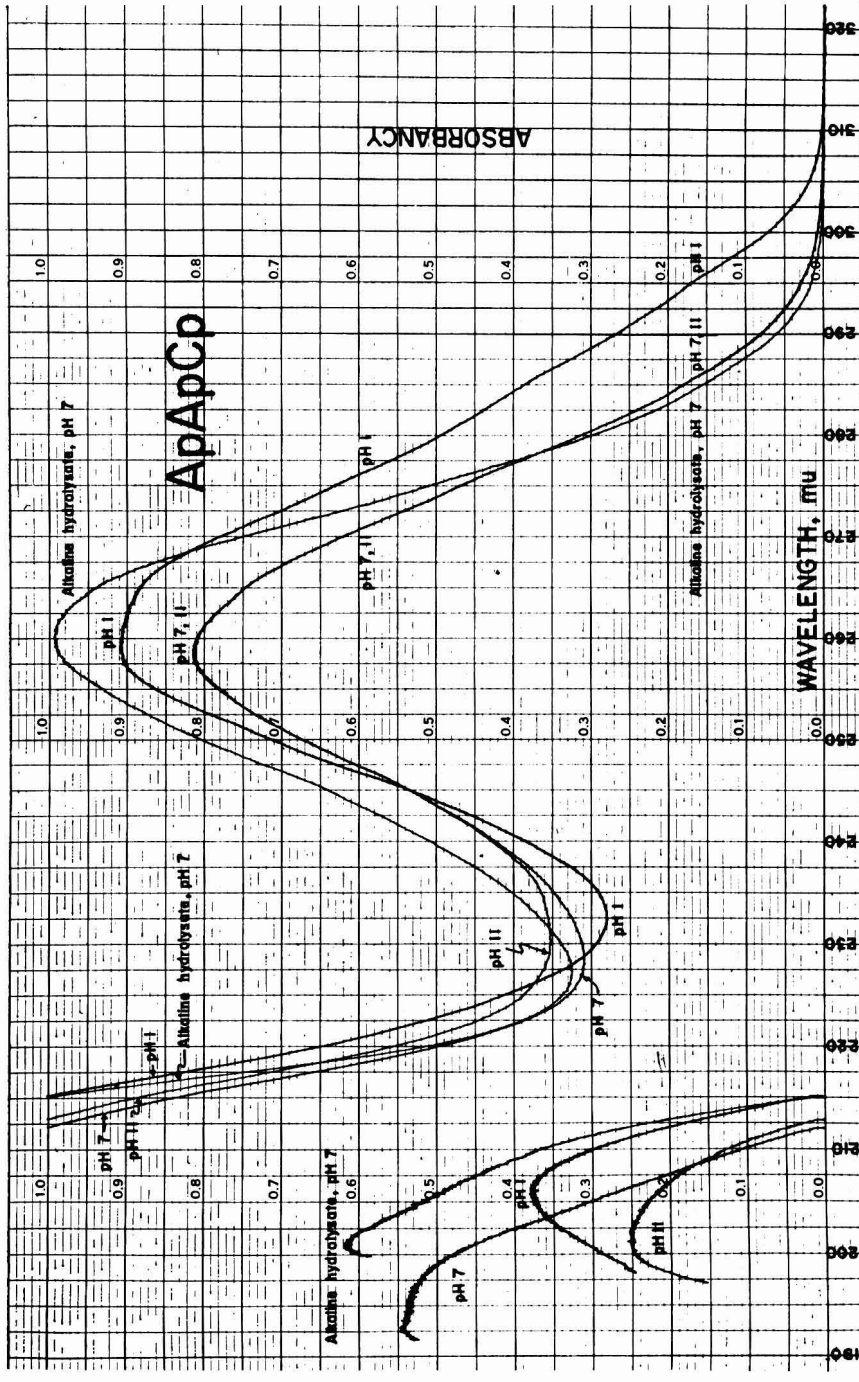
ApApCp
ABSORBANCY
WAVELENGTH, mμ
Alkaline hydrolysate, pH 7
pH 1
pH 7, II
pH 7
pH II
Alkaline hydrolysate, pH 7
pH 1
pH 7, II
pH 7
pH II
Alkaline hydrolysate, pH 7
pH 1
pH 7, II
pH 7
pH II
Alkaline hydrolysate, pH 7
pH 1
pH 7, II
pH 7
pH II

FIG. 3(5).

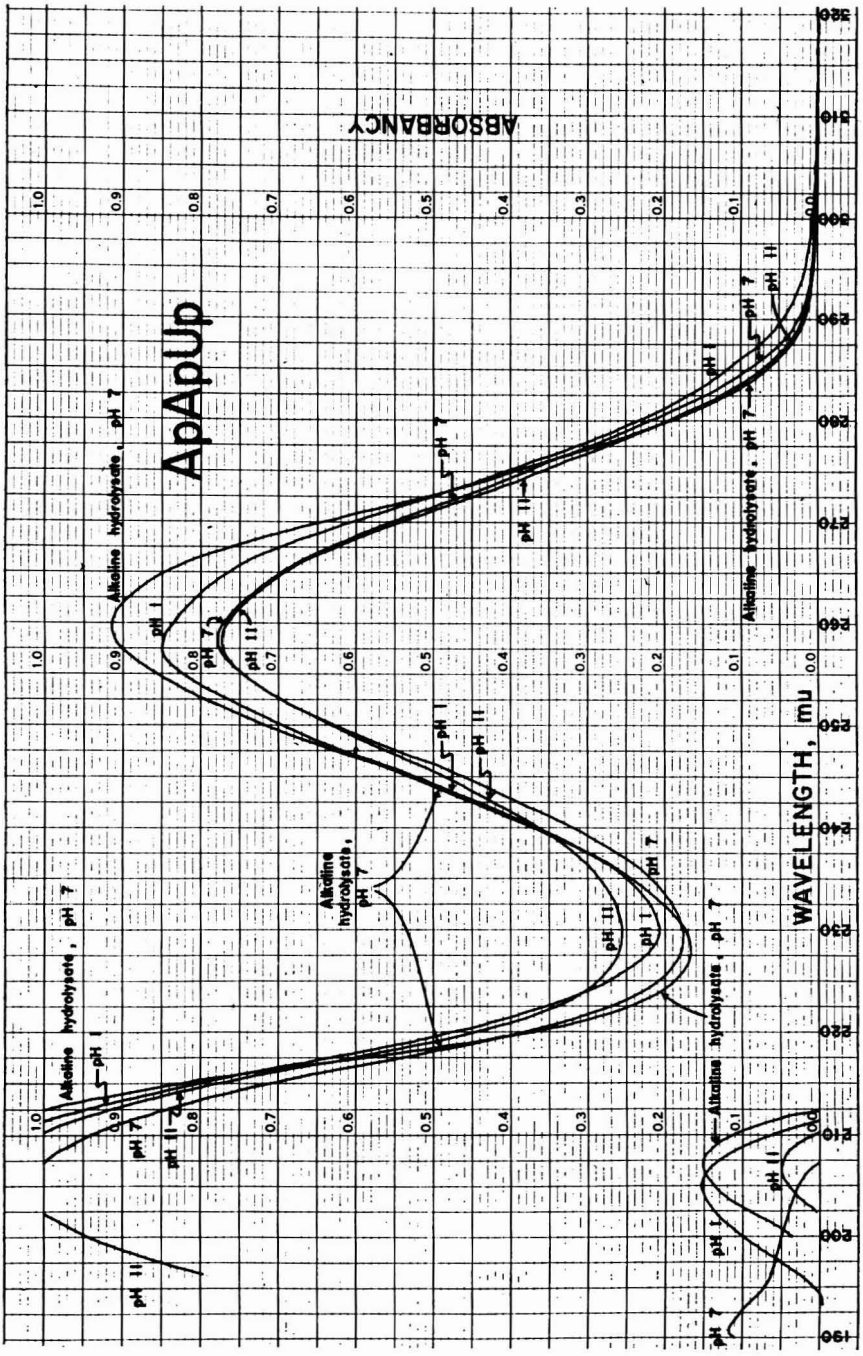
ApApUp
ABSORBANCY
WAVELENGTH, mμ
Alkaline hydrolysate, pH 7
pH 1
pH 7
pH 11
Alkaline hydrolysate, pH 7
pH 1
pH 7
pH 11
Alkaline hydrolysate, pH 7
pH 1
pH 7
pH 11
Alkaline hydrolysate, pH 7
pH 1
pH 7
pH 11
Alkaline hydrolysate, pH 7
pH 1
pH 7
pH 11

FIG. 3(6).

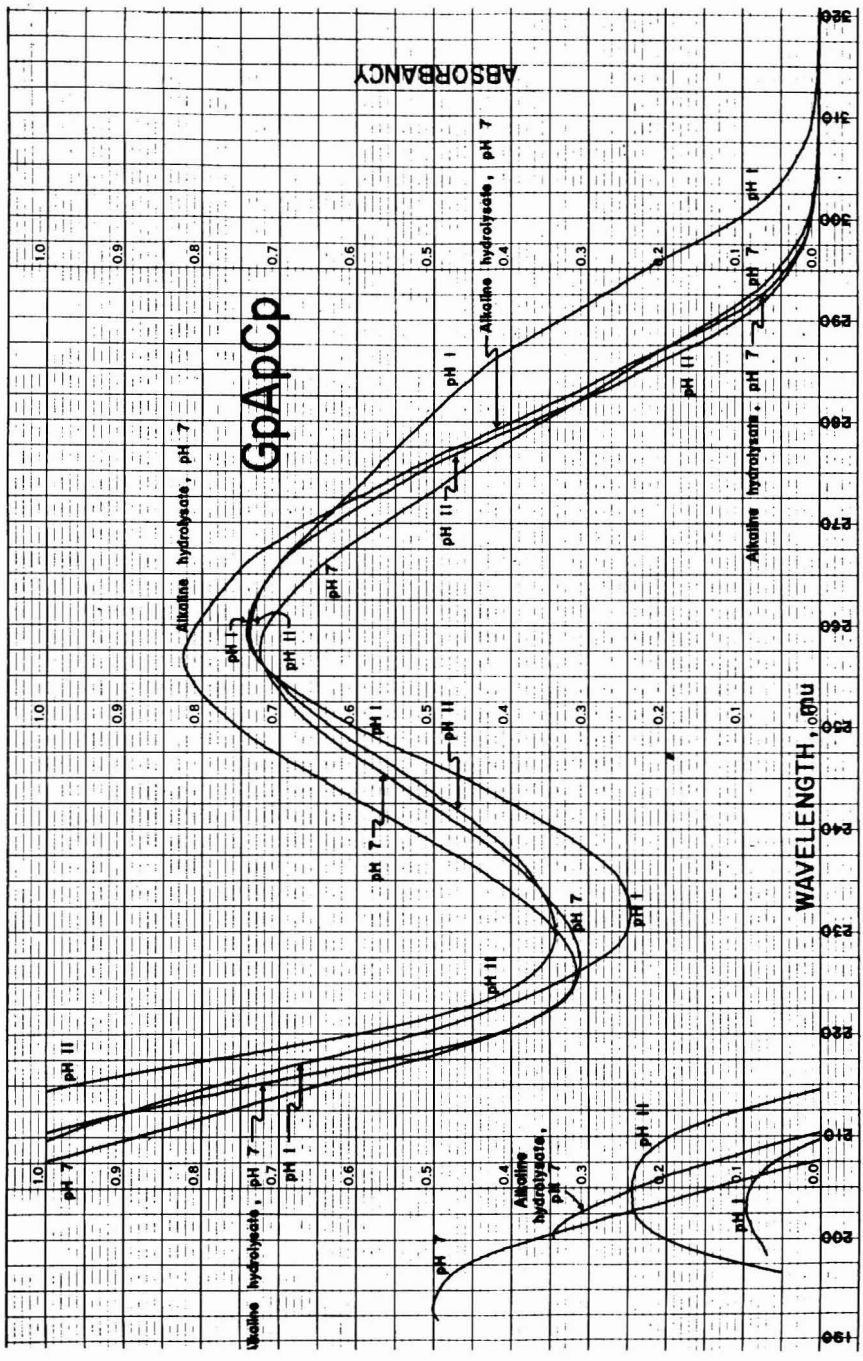
GpApCp
ABSORBANCY
WAVELENGTH, mμ
Alkaline hydrolysate, pH 7
pH I
pH II
pH 7

FIG. 3(7).

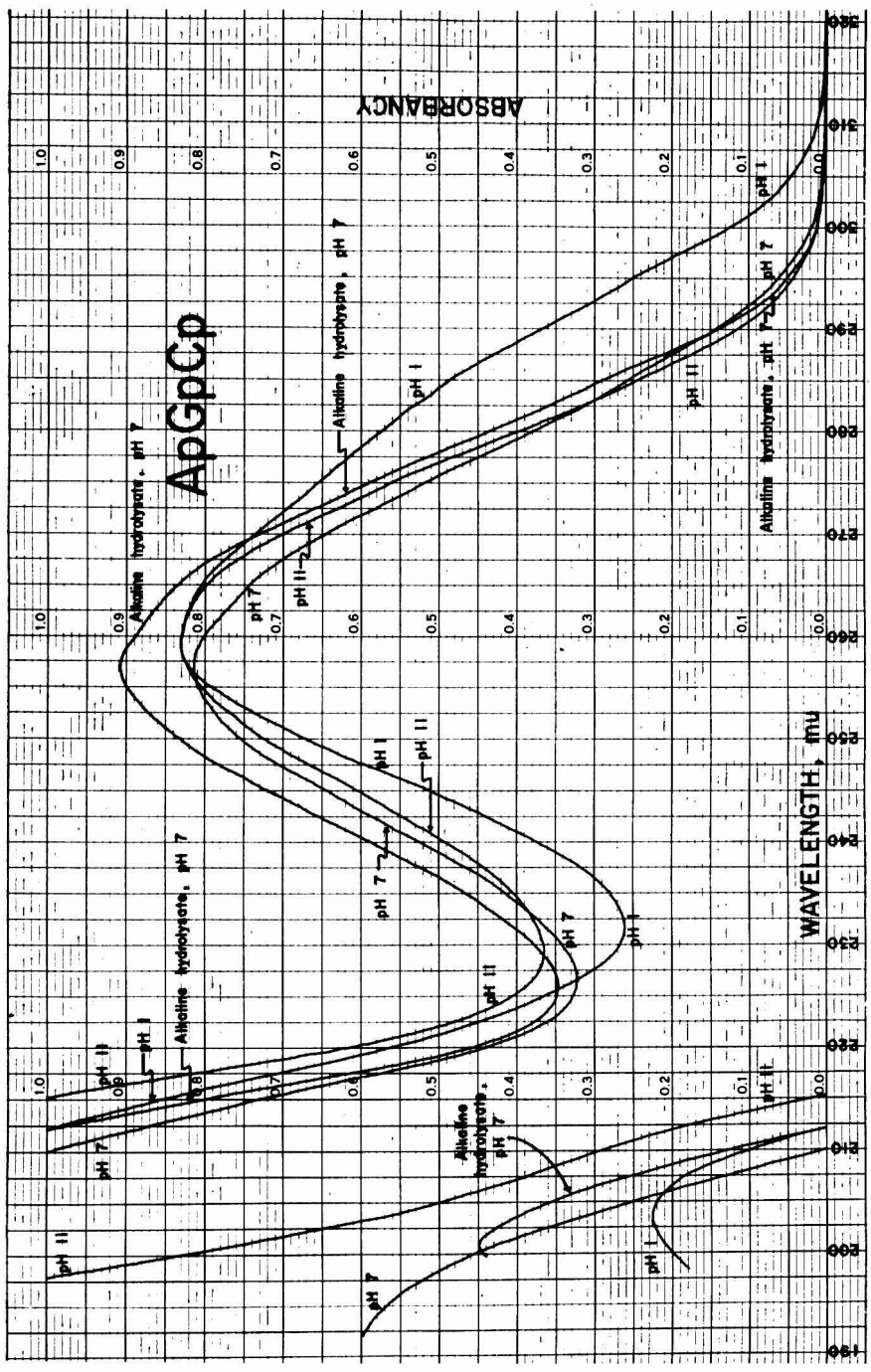
ApGpCp
ABSORBANCY
WAVELENGTH, mμ
Alkaline hydrolysate, pH 7
pH 1
pH 7
pH 11

FIG. 3(8).

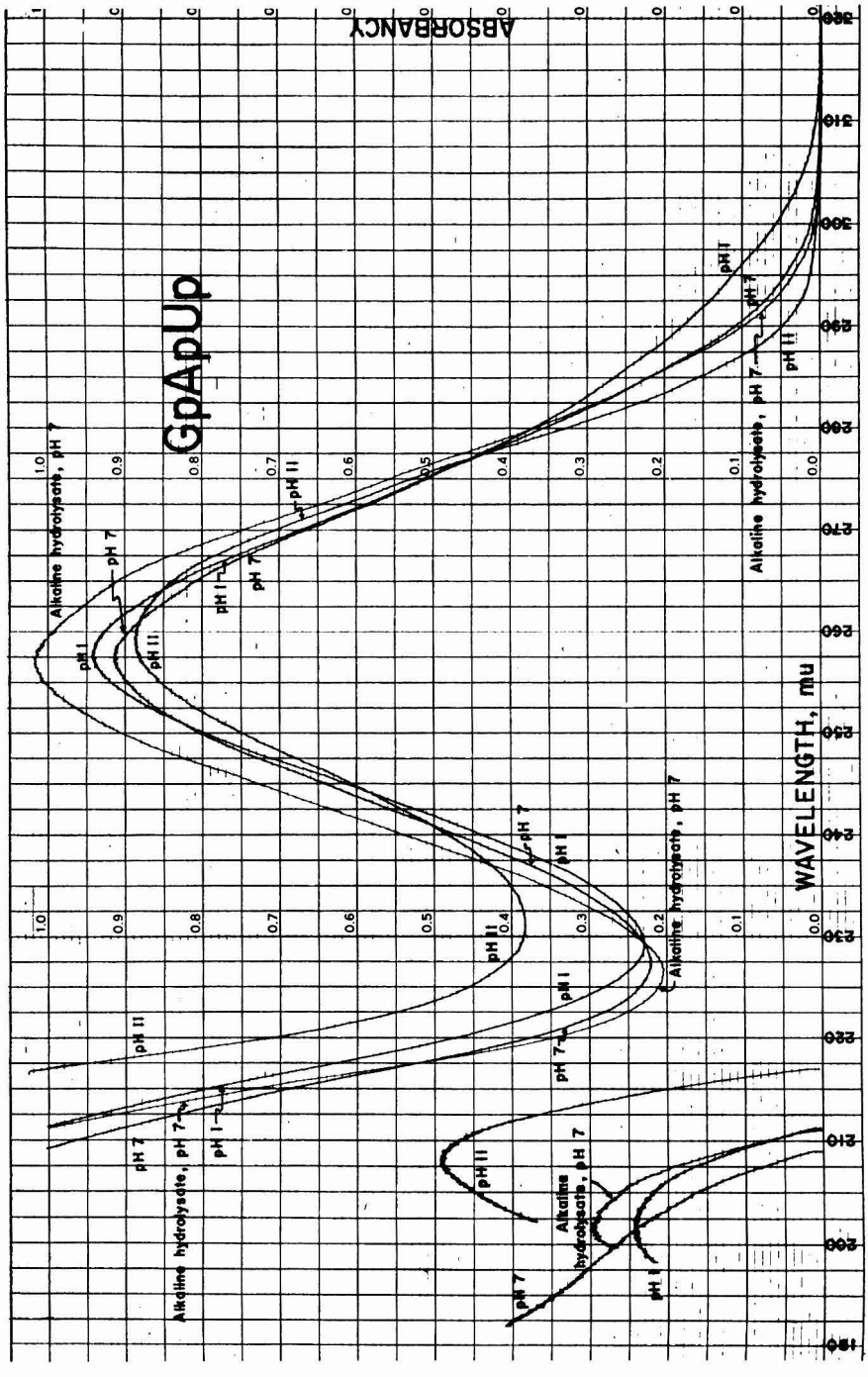
GpApUp
ABSORBANCY
WAVELENGTH, mu
Alkaline hydrolysate, pH 7
pH I
pH 7
pH II
Alkaline hydrolysate, pH 7
pH I
pH 7
pH II
Alkaline hydrolysate, pH 7
pH I
pH 7
pH II
Alkaline hydrolysate, pH 7
pH I
pH 7
pH II
Alkaline hydrolysate, pH 7
pH I
pH 7
pH II
Alkaline hydrolysate, pH 7
pH I
pH 7
pH II

FIG. 3(9).

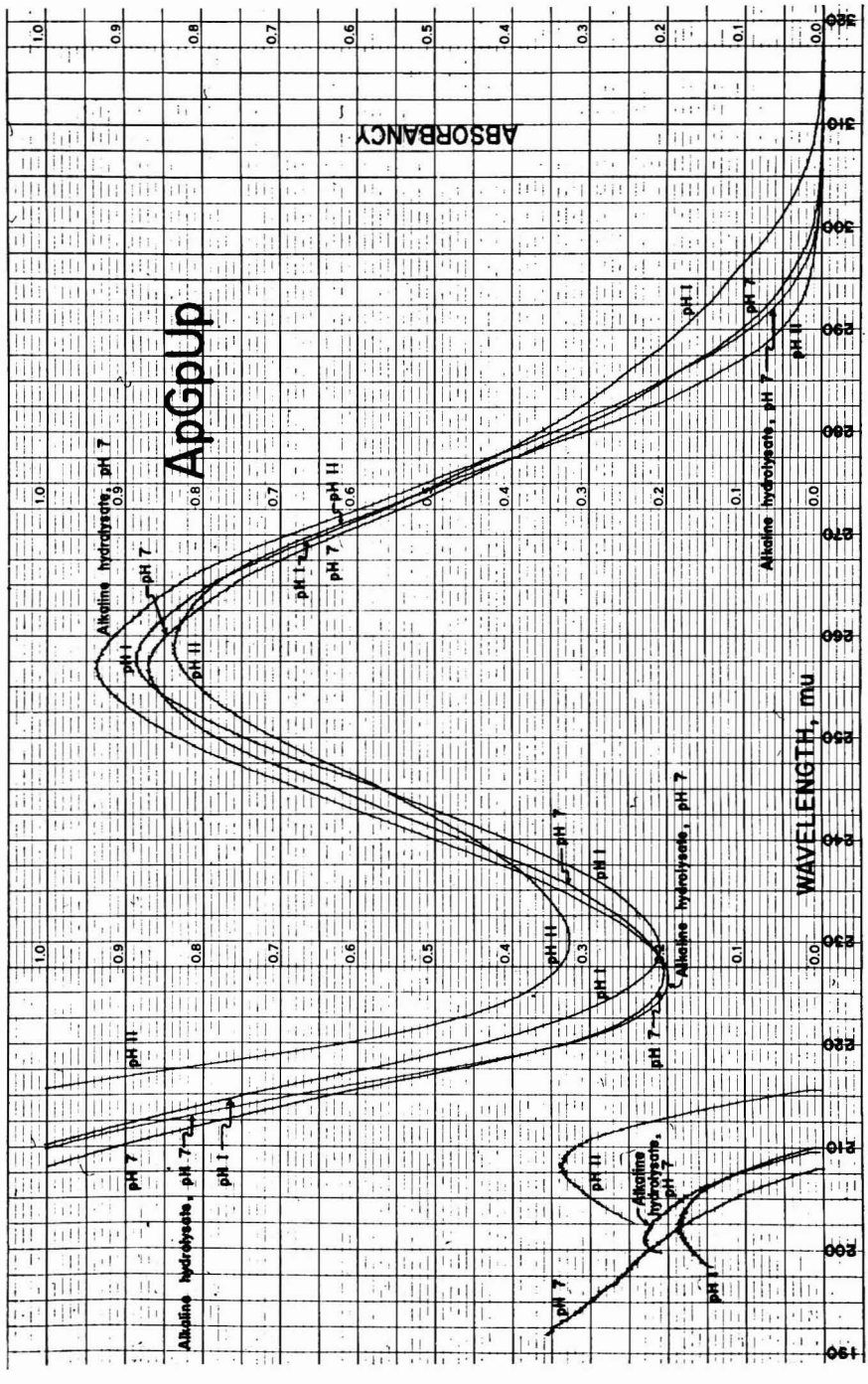
ApGpUp
ABSORBANCY
WAVELENGTH, mu
Alkaline hydrolysate, pH 7
pH I
pH 7
pH II
Alkaline hydrolysate, pH ?
Alkaline hydrolysate, pH 7
Alkaline hydrolysate, pH ?

FIG. 3(10).

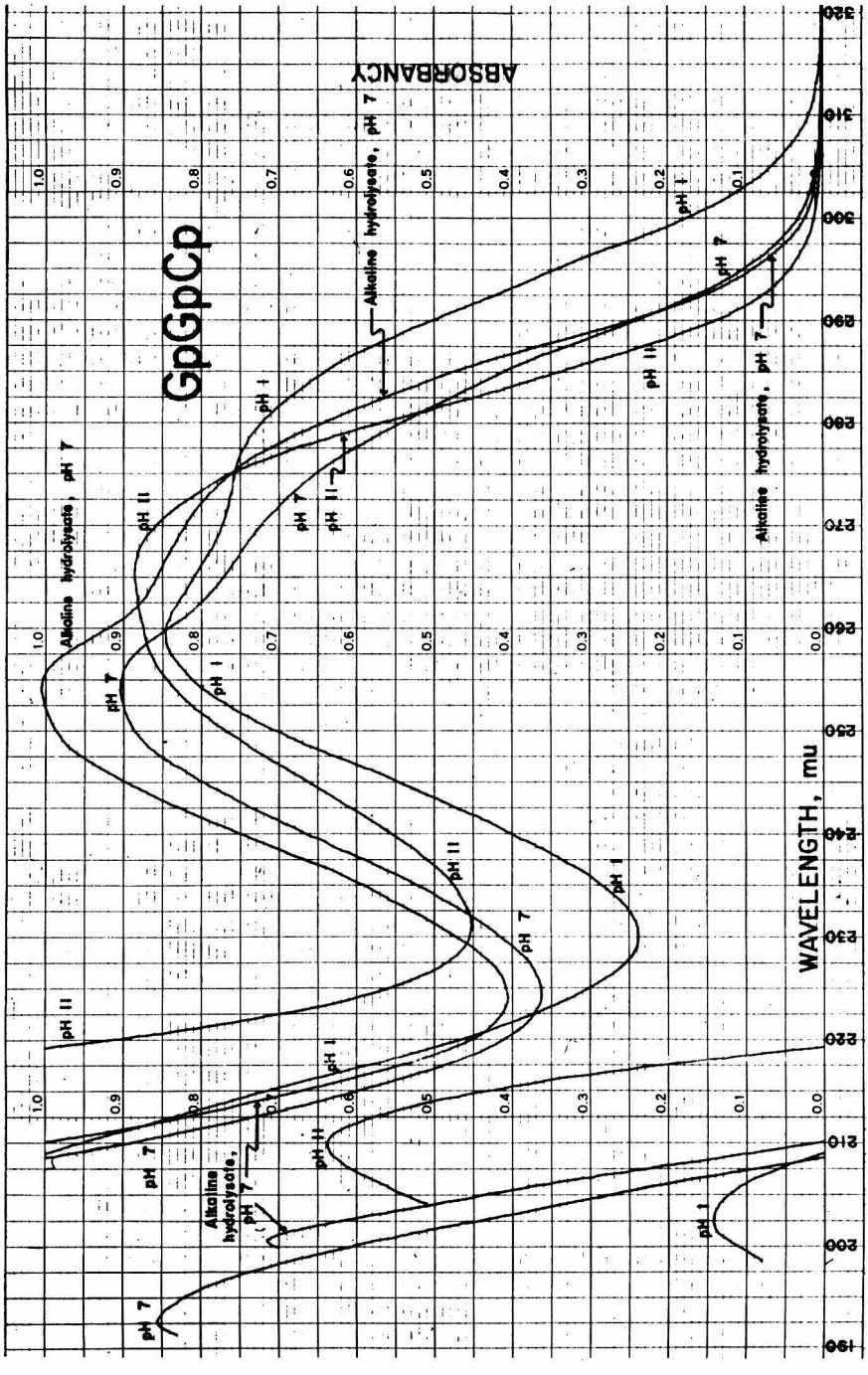
GpGpCp
ABSORBANCY
WAVELENGTH, mu
Alkaline hydrolysate, pH 7
pH 1
pH 7
pH 11
320
310
300
290
280
270
260
250
240
230
220
210
200
190

FIG. 3(11).

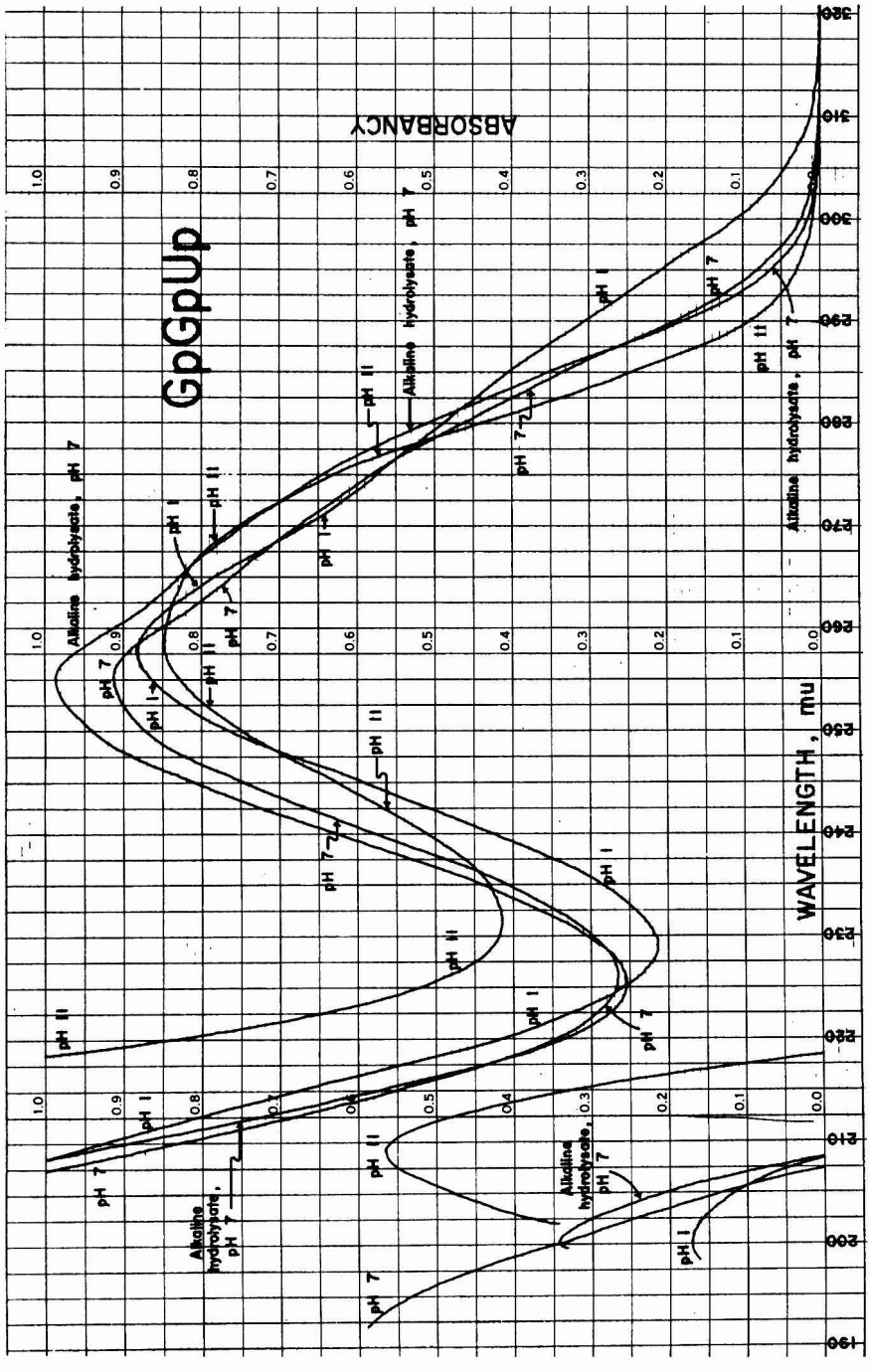
GpGpUp
ABSORBANCY
WAVELENGTH, mu
Alkaline hydrolysate, pH 7
pH 7
pH 1
pH 11
Alkaline hydrolysate, pH 7
Alkaline hydrolysate, pH 7
Alkaline hydrolysate, pH 7
Alkaline hydrolysate, pH 7

FIG. 3(12).

TABLE 4

SPECTROPHOTOMETRIC CONSTANTS OF PANCREATIC RIBONUCLEASE OLIGONUCLEOTIDES AT 20°[a]

Compound	λ max. and $a_m \times 10^{-3}$ at λ max.					
	pH 1		pH 7		pH 11	
	λ_{max}, mμ	$a_m \times 10^{-3}$	λ_{max}, mμ	$a_m \times 10^{-3}$	λ_{max}, mμ	$a_m \times 10^{-3}$
ApCp	265	21.3	261	21.2	261	21.2
ApUp	258	23.9	259	23.7	259	22.6
GpCp	277	19.6	256	18.8	267	19.1
GpUp	258	20.5	256	21.7	260	18.9
ApApCp	259	34.8	259	31.2	259	31.2
ApApUp	257	37.8	258	34.7	258	34.5
ApGpCp	259	32.2	257	31.5	260	32.2
GpApCp	259	31.6	258	30.9	260	31.4
ApGpUp	258	35.9	257	35.3	259	33.9
GpApUp	258	35.2	257	34.0	259	33.1
GpGpCp	259	28.2	254	30.1	265	29.6
GpGpUp	258	32.2	255	33.4	258	31.0

Compound	λ min. and $a_m \times 10^{-3}$ at λ min.					
	pH 1		pH 7		pH 11	
	$\lambda_{min.}$ mμ	$a_m \times 10^{-3}$	$\lambda_{min.}$ mμ	$a_m \times 10^{-3}$	$\lambda_{min.}$ mμ	$a_m \times 10^{-3}$
ApCp	233	6.5	227	9.2	228	10.0
ApUp	230	5.6	229	4.7	231	9.6
GpCp	233	5.8	224	9.6	230	11.3
GpUp	228	4.3	226	5.5	233	10.5
ApApCp	233	10.7	228	11.9	231	13.5
ApApUp	230	9.2	229	7.9	230	11.4
ApGpCp	232	10.2	227	12.5	229	14.1
GpApCp	232	10.5	227	13.2	230	14.6
ApGpUp	229	8.5	227	8.3	230	13.2
GpApUp	229	8.5	227	8.2	231	14.3
GpGpCp	230	8.0	225	12.1	231	15.1
GpGpUp	229	7.8	226	9.7	232	15.1

Compound	$a_m \times 10^{-3}$ at 260 mμ			Ratios at 250, 260, 280 mμ					
				As 250/260			As 280/260		
	pH 1	pH 7	pH 11	pH 1	pH 7	pH 11	pH 1	pH 7	pH 11
ApCp	20.8	21.2	21.2	0.74	0.81	0.81	0.72	0.41	0.41
ApUp	23.6	23.7	22.6	0.82	0.78	0.81	0.28	0.25	0.21
GpCp	17.5	18.1	18.3	0.76	1.01	0.89	1.09	0.69	0.70
GpUp	20.3	20.6	18.9	0.86	0.97	0.88	0.51	0.52	0.49
ApApCp	34.8	31.0	31.0	0.78	0.83	0.83	0.54	0.39	0.39
ApApUp	37.2	34.4	34.0	0.83	0.81	0.83	0.28	0.28	0.25
ApGpCp	32.2	31.0	32.2	0.81	0.94	0.87	0.69	0.47	0.48
GpApCp	31.6	30.6	31.4	0.81	0.93	0.86	0.69	0.50	0.51
ApGpUp	35.3	34.4	33.8	0.86	0.92	0.85	0.42	0.38	0.36
GpApUp	34.6	33.4	33.0	0.86	0.90	0.85	0.40	0.40	0.37
GpGpCp	28.2	28.1	29.2	0.83	1.05	0.88	0.85	0.64	0.65
GpGpUp	31.7	31.1	30.8	0.88	1.02	0.89	0.61	0.62	0.65

TABLE 4 (*Continued*)

Compound	Increase in a_m at 260 mμ upon Hydrolysis,[b] %	Compound	Increase in a_m at 260 mμ upon Hydrolysis,[b] %
ApCp	7.1	ApGpCp	10.9
ApUp	6.7	GpApCp	12.7
GpCp	5.0	ApGpUp	7.7
GpUp	4.8	GpApUp	11.0
ApApCp	22.7	GpGpCp	8.8
ApApUp	18.4	GpGpUp	6.9

[a] Spectrophotometric constants were calculated from the spectra reproduced in Fig. 3, utilizing the mononucleotide molar extinction coefficients listed in Table 1. Buffers employed were 0.10 *M* HCl, 0.02 *M* sodium phosphate, and 0.002 *M* NaOH for pH 1, 7, and 11, respectively.

[b] Per cent increases in molar extinction coefficients of the oligonucleotides upon alkaline hydrolysis were determined by recording the spectra at 20° of the compounds and their neutralized hydrolyzates in 0.02 *M* sodium phosphate, pH 7 (see Fig. 3).

has been recognized in short polynucleotides by some investigators for some time (19–21). However, since the presence of hypochromicity in long-chain polynucleotides usually has been interpreted in terms of hydrogen bonds between purine and pyrimidine bases, the occurrence of this effect in di- and trinucleotides (which have little or no opportunity to form hydrogen-bonded base pairs) has not been generally appreciated.

More recently, hypochromicity has been considered in terms of dipole interactions and hydrophobic forces between "stacked" or neighboring

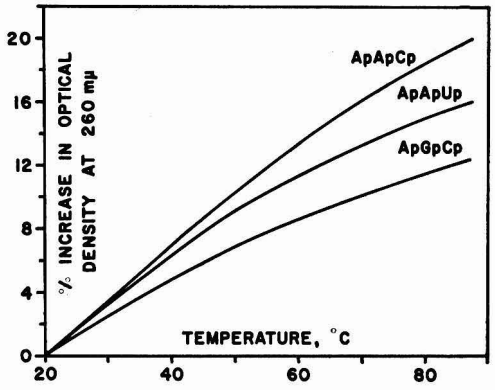

FIG. 4. Extinctions of ApApCp, ApApUp, and ApGpCp at 260 mμ as a function of temperature in 0.05 *M* NaH_2PO_4, 0.05 *M* Na_2HPO_4. Experimental details are given under "Materials and Methods." Optical density readings were recorded at temperature intervals of 2.5°; experimental points are not shown since scatter of the data was less than the width of the curves.

bases (21–25). This interpretation received strong support by the finding (26) that the helical form of polycytidylic acid at neutral pH is not destabilized by the complete reaction of the pyrimidine amino groups with formaldehyde. In addition, the breadth of the temperature transitions of the hypochromicity and the residual hypochromicity at extremes of pH observed in this work are both indicative of the presence of interactions other than hydrogen bonds between the bases. Consequently, the formation of hydrogen bonding between bases in short oligonucleotides is seen not to be a prerequisite for the hypochromic effect.

The marked influence of temperature upon the extinction in the ultraviolet of di- and trinucleotides has apparently not been investigated before. Unlike the larger natural polynucleotides, which in media of moderate ionic strength usually exhibit little temperature dependence of hypochromicity at temperatures near room temperature, the errors in the quantitative estimation of oligonucleotides due to this effect are most important under the usual conditions of measurement. For this reason, the spectrophotometric constants have been reported here at a controlled temperature of 20°.

Inspection of the spectra in Fig. 3 shows that the identification of di- and trinucleotides from pancreatic ribonuclease digests of RNA is possible by critical comparison of complete spectra taken under the experimental conditions chosen here. Comparison of ratios of absorbancy at selected wavelengths and pH's lends itself to convenient computer fitting (27, 28) of the spectra. The accumulation of spectral data such as that presented here will be essential for the eventual computerized analysis of base sequence and composition by spectral data alone.

SUMMARY

The di- and trinucleotides arising from the digestion of RNA with pancreatic ribonuclease were isolated by a combination of column and paper chromatography. The spectra of the oligonucleotides at pH 1, 7, and 11 were determined and from these data the spectrophotometric constants at 20° were calculated. These values were found to be a function of temperature and representative dependencies are given.

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ANALYTICAL BIOCHEMISTRY 13, 66–70 (1965)

Detection of Nanogram Quantities of Chloroquine by Gas-Liquid Chromatography

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Chloroquine [7-chloro-4-(4-diethylamino-1-methylbutylamino)quinoline] is a highly effective antimalarial which has gained wide acceptance not only in the treatment of malaria, but also in the palliation of several of the collagen diseases. Because of the tenacious binding of this drug to tissues, serum levels as low as 10 nanograms per milliliter are observed with suppressive dosages. The most sensitive method available at the present time for the determination of chloroquine is the spectrofluorophotometric method of Brodie *et al.* (1). We have found in our laboratory that the original sample must contain a minimum of 50 nanograms of the drug in order to observe a significant fluorescence. Even if the method were modified so that a larger fraction of the sample were used for the final determination, a minimum of 25 nanograms would be required. Because of this low sensitivity, the routine determination of chloroquine requires 5 ml of serum. A further difficulty with the method is that it is necessary to separate the parent drug from its de-ethylated metabolites by repeated washings and extractions into aqueous buffers.

Kazyak and Knoblock (2) demonstrated that microgram quantities of chloroquine could be separated from biological materials by solvent extraction, followed by gas-liquid chromatography. This suggested that, with the recent development of highly sensitive detection systems, a chromatographic method could be developed which would be more sensitive and rapid than the spectrofluorophotometric method. In the following work I have examined the conditions for the determination of pure chloroquine standards by gas-liquid chromatography and present a technique of potential value in the determination of this drug in biological samples.

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MATERIALS AND METHODS

The chloroquine base (m.p. 90°–92°C) was prepared by the extraction of chloroquine phosphate (Sterling-Winthrop, New York) from alkaline solution with diethyl ether. All other reagents were reagent grade and were used as obtained. All solutions of chloroquine standards were prepared daily.

The gas chromatograph used was a Chromalab model 110 (Glowall Corp., Willow Grove, Pa.) equipped with a 10-mc Sr-90 ionization detector. The column oven was maintained at 220°C, while the flash and detector ovens were both maintained at 250°. The detector voltage was set to 10 volts so that the chloroquine was detected by electron capture. The amplifier gave a steady baseline with a full scale sensitivity of 10^{-11} amps. The current was recorded on a 50-mv Brown Recorder (Minneapolis-Honeywell Regulator Co., Minneapolis, Minn.). Argon was used as the carrier gas.

The columns were 12 ft $\times$ 8 mm glass coils, except as noted. Most packings were prepared from a single batch of Gas-Chrom P (mesh 100/120) which had been acid-washed and siliconized (3). The liquid phases were applied by the method of Horning *et al.* (3). The support and liquid phases were obtained from Applied Science Inc. (College Park, Pa.).

All samples were dissolved in ethyl acetate or toluene. Injections of 1 to 5 μl were made with a Hamilton 10-μl syringe (Hamilton Co., Whittier, Calif.). Peak areas were determined with a coordinating polar planimeter (Keuffel & Esser Co., New York, N. Y.).

RESULTS AND COMMENTS

Several of the common liquid phases, which were examined for their applicability to this problem, are listed in Table 1. Since chloroquine is somewhat polar, adsorption becomes the limiting factor for the sensitivity of the system. This leads to severe tailing, especially when small amounts are placed on the column, and reduces the peak height. One method of circumventing this problem is to use either the trimethylsilyl or the amide derivative. Unfortunately the secondary amino group of chloroquine does not form either of these derivatives, so it was necessary to examine other liquid phases and support modifiers to find a system which would show little adsorption with the free base.

I have examined the three most commonly used support modifiers, i.e., dichlorodimethylsilane, polypropylene glycol 9000, and polyvinylpyrrolidone, for their effect on adsorption. It would appear that only polyvinylpyrrolidone coats the support sufficiently well to inhibit this ad-

TABLE 1

SENSITIVITY OF GAS-LIQUID CHROMATOGRAPHY WITH VARIOUS COLUMN PACKING MATERIALS

Liquid phase[a]	%	Modifier[b]	%	Sensitivity
S.E. 30	3.0	DMDCS[c]	1.0	100 ng
S.E. 30	1.0	DMDCS	1.0	50 ng
S.E. 30	4.0	DMDCS	1.0	50 ng
S.E. 30	1.0	Polyethylene glycol 9000	0.1	50 ng
QF-1	1.0	DMDCS	1.0	500 ng
DC 200*	1.0	—		10 mg
Neopentylglycol succinate	1.0	PVP[d]	1.0	50 ng
Neopentylglycol succinate†	1.0	PVP	1.0	5.0 ng

[a] All liquid phases were on Gas-Chrom P (mesh 100/120), except (*) which was on carbon ST-2700 (Cabot Corp., Boston, Mass.) (4). All columns were 12 ft long except (†) which was 2 ft in length.

[b] The support was slurried with 3 times v/w of a solution containing the percentages listed of the modifier in an appropriate solvent (3).

[c] Dimethyldichlorosilane.

[d] Polyvinylpyrrolidone.

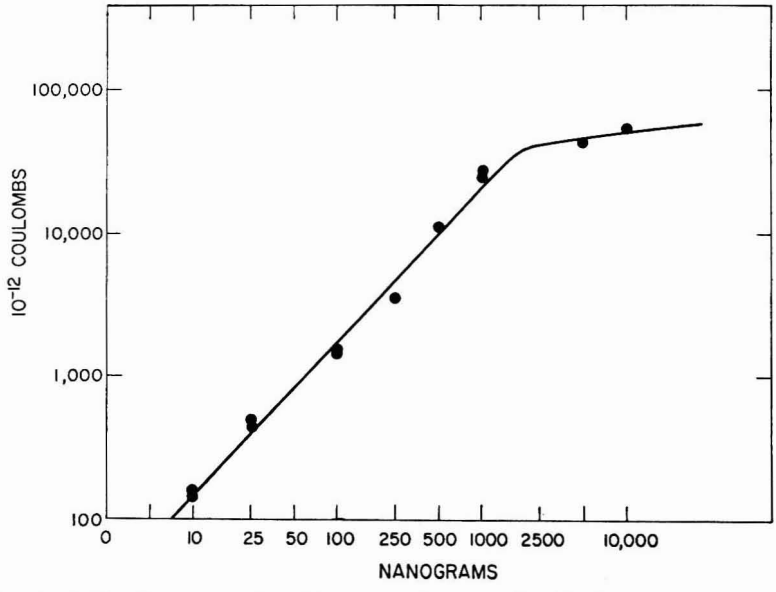

FIG. 1. Calibration curve for chloroquine in a gas-liquid chromatograph using a NGS 1%–PVP 1% liquid phase on Gas-Chrom P (mesh 100/120) support. Argon at 15 psi and 30 ml/min was the carrier gas. Column oven was at 220°C and flash and detector ovens at 250°. The detector was a 10-mc Sr-90 ionization unit with a voltage of 10 volts.

sorption. This support modifier has been reported to be effective only with polar liquid phases, and with these phases chloroquine gives long relative retention times. With a 12-ft column of 1% polyvinylpyrrolidone (PVP) and 1% neopentylglycol succinate (NGS) (5) the drug came off the column after 157 min with 4800 theoretical plates. By reducing the column length to 2 ft with only 20 in. of packing, I have been able to reduce the retention time to 8 min with 360 theoretical plates, increasing the sensitivity so that the minimum detectable amount is 5 nanograms. This is a fivefold improvement over the spectrofluorophotometric method. A calibration curve and typical chromatogram are shown in Figs. 1 and 2, respectively. The reduced column length probably leads to an increase in sensitivity by reducing the number of ad-

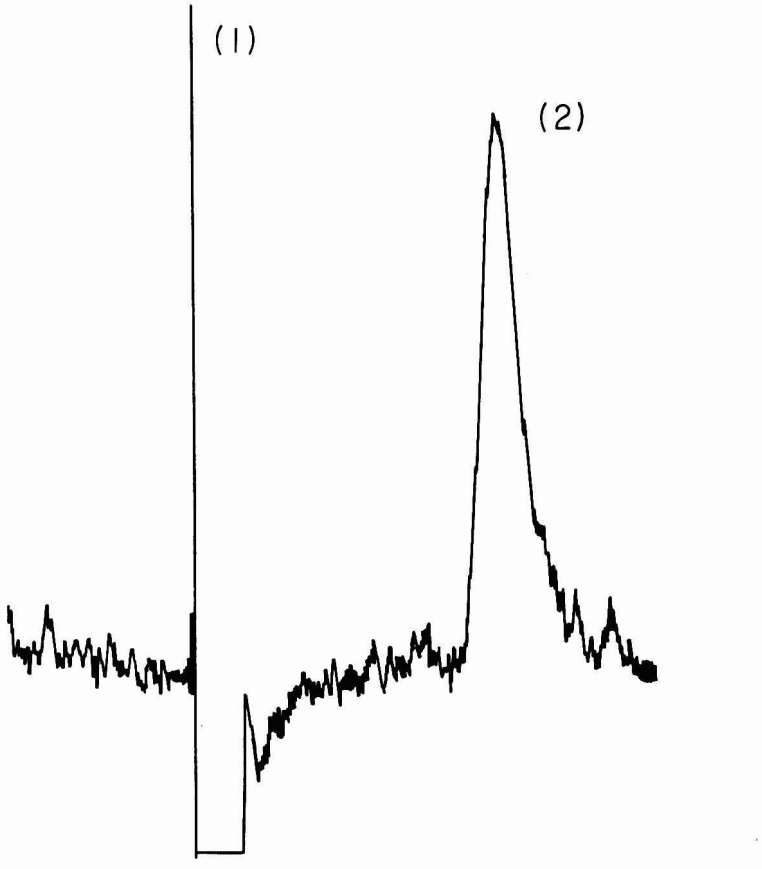

FIG. 2. Typical chromatograph of chloroquine. Conditions are as in Fig. 1. Peak (1) air; peak (2) 25 ng chloroquine. Retention time 8 min.

sorption sites and by allowing for an increased gas flow at reduced column pressures.

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I wish to thank Dr. Henry M. Fales of the Laboratory of Metabolism, National Institutes of Health, Bethesda, Maryland for his interest in this problem.

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ANALYTICAL BIOCHEMISTRY 13, 71–73 (1965)

Enzymic Assay of Adenosine 3′,5′-Monophosphate

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Received March 25, 1965

Adenyl cyclase and 3′,5′-AMP[1] have been implicated in the hormonal control mechanism of several biochemical reactions (1) and therefore the measurement of 3′,5′-AMP in biological material is of interest. In the method of assay used by Rall and Sutherland (2, 3) and later modified by Brown, Clarke, Roux, and Sherman (4), a dog liver extract was used as the source of the relatively inactive liver dephosphophosphorylase and its kinase and of the active liver phosphorylase and its phosphatase. This preparation was shown to carry out the following reaction:

$$\text{liver dephosphophosphorylase (inactive)} \xrightleftharpoons[\text{phosphatase}]{\text{ATP, } Mg^{++}\text{, kinase}} \text{liver phosphorylase (active)}$$

The addition of 3′,5′-AMP to this preparation results in increased formation of active liver phosphorylase. Rall and Sutherland (3) measured the formation of the active liver phosphorylase by following glycogen production from glucose 1-phosphate. Brown *et al.* (4) measured glucose 1-phosphate formed from glycogen by enzymically coupling it to NADP[1] reduction through phosphoglucomutase and glucose 6-phosphate dehydrogenase. Levels of 3′,5′-AMP as low as 10^{-6} *M* could be measured by this procedure.

Extracts of dog liver prepared by the method of Brown *et al.* (4) were found to contain only 10–12 mμmoles of pyridine nucleotides/ml. Since 200 mμmoles NADP/ml was required for their enzyme assay, NADP was added to the dog liver extract, but it was both utilized and destroyed by other systems present. In the present investigation these difficulties were overcome by (*a*) dialysis of the preparation to remove endogenous substrates of NADP oxidoreductases and (*b*) conducting the enzyme assay

[1] Abbreviation: 3′,5′-AMP = adenosine 3′,5′-monophosphate. NADP = Nicotinamide Adenine Dinucleotide Phosphate. ATP = Adenosine Triphosphate. $NADPH_2$ = Reduced NADP.

in two parts: after the dog liver extract was incubated with 3′,5′-AMP to activate phosphorylase, the incubation mixture was boiled at pH 7.4, an aliquot taken and, its glucose 1-phosphate content determined by following reduction of NADP at 340 mμ in the presence of phosphoglucomutase and glucose 6-phosphate dehydrogenase. Levels of 3′,5′-AMP as low as 10^{-8} *M* were determined by this method.

A dog liver extract (11,000 × *g* supernatant fraction of a dog liver homogenate) was prepared essentially as described by Rall and Sutherland (3). It was incubated at 37°C for 20 min to achieve maximal inactivation of phosphorylase and then quickly frozen in small tubes in a dry ice/ethanol mixture. The preparation was stored in dry ice, because it rapidly lost its ability to respond to 3′,5′-AMP at 0° or −15°C. Before use, the preparation was thawed and dialyzed against two changes of 50 vol of 0.002 *M* potassium phosphate buffer, pH 7.4, for about 3 hr at 2°.

Samples to be assayed for 3′,5′-AMP content were incubated at 30°C in 1 ml solution containing 0.4 ml dog liver extract, 0.125 *M* potassium phosphate buffer, pH 7.4, 0.02 *M* NaF, 0.001 *M* AMP, 0.003 *M* ATP, 0.0067 *M* caffeine, 0.005 *M* $MgCl_2$, and 2% glycogen. After 40 min the incubation mixtures were transferred to a boiling water bath for 3 min, then chilled and centrifuged. Aliquots (0.025 ml) of the supernatant fractions were added to 2 ml of a solution containing 0.05 *M* tris/HCl (pH 7.4), 0.01 *M* $MgCl_2$, 0.005 ml phosphoglucomutase (250 units/ml) and 0.005 ml glucose 6-phosphate dehydrogenase (250 units/ml), in a 1-cm cuvet at room temperature. The reaction was followed to completion at 340 mμ in a spectrophotometer, after addition of 300 mμmoles NADP. Thus the phosphorylase activity can be expressed in terms of mμmoles $NADPH_2$, and a typical curve is shown in Fig. 1, where phosphorylase activity is plotted against 3′,5′-AMP concentration in the initial incuba-

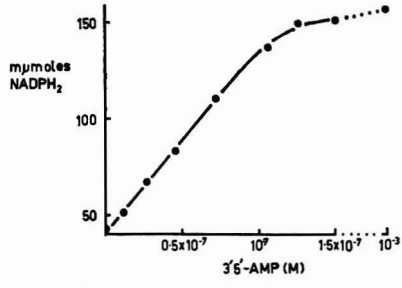

FIG. 1. Activation of phosphorylase in an 11,000 × *g* supernatant of a dog liver homogenate, in the presence of 3′,5′-AMP. Phosphorylase activity is expressed in terms of mμmoles $NADPH_2$ in a 1-cm cuvet and compared with the original concentration of 3′,5′-AMP as described in the text.

tion mixture. Liver extracts from several young healthy dogs have been used in this system, and have all had essentially the same properties.

This system could be used to measure 3′,5′-AMP produced by biological materials, after suitable dilution of samples to fit the assay curve. Experiments by other groups have shown the possibility of artifacts in the assay of 3′,5′-AMP (2, 5, 6). In our method, control samples were preincubated with 3′,5′-AMP phosphodiesterase to convert 3′,5′-AMP to AMP (7), to ensure that the activation of phosphorylase was due to 3′,5′-AMP.

Several samples of analogs of 3′,5′-AMP were generously supplied by Dr. G. I. Drummond of the University of British Columbia, and inosine 3′,5′-monophosphate was prepared from 3′,5′-AMP (8). These were tested in the assay system described above. In all cases in which a response was observed, it resembled that shown in Fig. 1. The molarities at which the various analogs caused maximal stimulation of the system were: 3′,5′-AMP, $1.5 \times 10^{-7} M$; deoxy-3′,5′-AMP, $10^{-4} M$; inosine 3′,5′-monophosphate, $10^{-6} M$; cytidine 3′,5′-monophosphate, $10^{-4} M$; uridine 3′,5′-monophosphate, $10^{-5} M$; deoxycytidine 3′,5′-monophosphate and thymidine 3′,5′-monophosphate had no effect at $10^{-2} M$.

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An Improved Gradient-Making Device for Density Gradient Centrifugation

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Several difficulties are encountered with the simple gradient-making devices of Britten and Roberts (1) and Martin and Ames (2). One is that a small amount of the light solution will occasionally pass through the channel connecting the two chambers and rise to the top of the heavy solution when the closure between the chambers is opened, even if the device is perfectly level. Another is that the centrifuge tube has to be lowered continuously as the gradient enters the tube if the heavy solution is in the mixing chamber. This operation requires constant attention, and alone requires about 10 min (2) per gradient. An obvious way to overcome this difficulty is to put the light solution in the mixing chamber and deliver the gradient to the bottom of the centrifuge tube. As the tube fills, the lighter solution continually floats above the entering gradient without further attention. With the latter method and the devices mentioned, a third difficulty arises as the centrifuge tube is lowered at the end of the filling operation: a heavy drop falls off the end of the delivery tip when the surface is broken and disturbs the gradient. Finally, the taper of the delivery tip causes disturbances as the centrifuge tube is lowered.

This paper describes an easily constructed convenient device for making reproducible gradients in the Spinco 5050 tube, or with modification in a Spinco 2236 tube. A choice of two alternative modes of operation is presented, depending on the quality of construction of the device.

DESCRIPTION OF DEVICE

Figure 1 shows the details of the device that is used to make a gradient in the Spinco 5050 tube. The volume of the two chambers can be increased by using 22-mm test tubes so that gradients for the Spinco 2236 tube can be made. The delivery tip is siliconized so that its outer surface near the tip is not wettable. The nonwettable surface prevents a drop from forming at the tip when it is withdrawn from the surface of the gradient

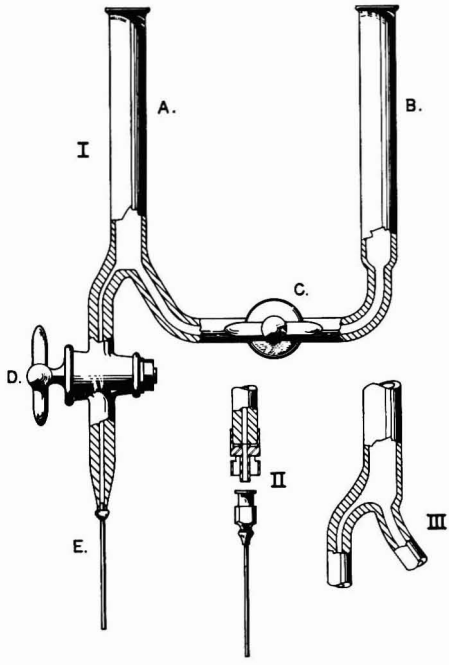

FIG. 1. Gradient-making device. (I) Overall view: (A and B) mixing and reservoir chambers, respectively, Pyrex or Kimax test tubes, 13 × 100 mm; (C) stopcock, Pyrex or Kimax, 2-mm bore with Teflon plug; (D) stopcock, Pyrex or Kimax, 1-mm bore, with Teflon plug; (E) delivery tip, 20 gage, stainless steel, fashioned from a 2- or 3-in. cannula, attached with Duco cement, siliconized. (II) Alternate method of delivery tip attachment via Luer-lock fitting cemented to capillary outlet of stopcock D. (III) An example of poorly executed construction.

at the end of the operation. A clean break is necessary to avoid disturbing the top layer of the gradient.

PROCEDURES

Preferred Method (Well-Constructed Device). To make a gradient in the Spinco 5050 tube the device is mounted on a ring stand with the mixing (A) and reservoir (B) chambers as nearly plumb as possible. With stopcock C open, the tip of a pipet containing the heavy solution is introduced into chamber B near the capillary that joins the two chambers and the capillary is filled with the solution. (Alternatively, excess heavy solution can be removed from chamber B by suction after closing stopcock C.) Stopcock C is closed, and a predetermined amount, 2.3 ml usually, of the heavy solution is pipetted into chamber B. With stopcock D closed, about 1 ml of the light solution is placed in the mixing chamber

A. Stopcock D is opened, and then closed when the solution has drained to the meniscus at the capillary opening. A predetermined amount, 2.3 ml $+ V_d$ ml, of the light solution is introduced into the mixing chamber (V_d is explained later).

A stirring blade attached to a mechanical stirrer[1] is placed in the mixing chamber. The blade is made from a stainless-steel spatula[2] by removing the blade completely at one end and giving a slight twist to the blade that remains attached to the cylindrical handle. The twist must be in the proper direction so that, as the blade rotates, the incoming heavy solution is directed upward. Thus, if the stirring motor turns counterclockwise (as viewed from above), the twist should be counterclockwise. The stirring must not produce cavitation or a deep vortex. It may be necessary to make the blade narrower (by filing) for free rotation in the reservoir.

The delivery tip, stopcock bore, and capillary of the delivery side contains the light solution, so that its volume (designated V_d) must be displaced by the initial portion of the density gradient. (V_d is easily determined by counting the number of drops it takes to empty the capillary—a little air pressure is needed to displace the last drop). Hence, with the device properly filled, and with the stirrer operating, stopcock C is opened. Stopcock D is opened, and then closed[3] after the number of drops representing V_d has been removed. Next, a Spinco 5050 tube is placed in a holder (a piece of wood drilled to hold the tube snugly and plumb) on a labjack, a small hydraulic jack, or similar raising and lowering device. The tube is raised until the delivery tip is centered less than 1 mm above the bottom of the tube. Stopcock D is now opened,[3] and as the gradient flows into the tube, it displaces the less dense solution that entered immediately before it so that the less dense solution rises continually as described previously for the syringe-type gradient-making device of Bock as used by Sinclair *et al.* (3). The remaining operation (10 min or so) to the point (see below) where stopcock D is closed, requires no further attention. Chambers A and B empty essentially at the same rate, so that at the end of the gradient-making operation the more dense solution in chamber B ceases flowing as its meniscus reaches the capillary. In a well-constructed apparatus, the meniscus of the gradient in the mixing chamber reaches the outflow capillary at the same time. The gradient is allowed to drain until flow stops. Stopcock D is

[1] Scientific Products Co., Catalog No. S-8005.

[2] Scientific Products Co., Catalog No. S-1565. The narrow end is discarded.

[3] It is necessary to close stopcock D in a direction opposite to that by which it was opened so that the gradient within the bore of stopcock D is not inverted. An oblique bore stopcock avoids all difficulty, but we have not found one with a Teflon plug.

closed at a convenient time, and the tube is lowered in a slow steady manner so as not to disturb the gradient. The prepared gradient is now ready for use. Curve B of Fig. 2 illustrates a gradient prepared in this manner.

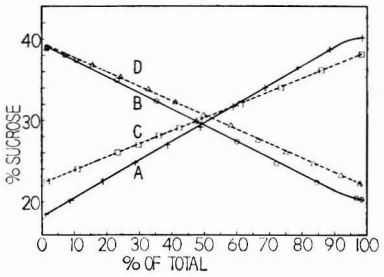

FIG. 2. Examples of gradients. (I) Well-constructed device (——): mixing chamber, 2.3 ml 17.0% (w/w) sucrose; reservoir, 2.3 ml 42.5% (w/w) sucrose; *curve A,* gradient directly from device, 400 drops; *curve B,* gradient from Spinco 5050 tube, 325 drops. (II) Alternative method, poorly constructed device (III of Fig. 1) (- - - -): *curve C,* gradient directly from device—chamber A, 3.3 ml 17.0% (w/w) sucrose, chamber B, 2.9 ml 42.5% (w/w) sucrose, V_d 20 drops, then 100 drops discarded, 320 drops collected, last 72 drops discarded; *curve D,* above gradient collected from Spinco 5050 tube, 312 drops.

The device is readily prepared for the next gradient-making operation by closing stopcock C, adding the required amount of heavy solution to the reservoir chamber, and refilling the delivery capillary and mixing chamber in accord with the initial filling procedure.

Alternative Method (Poorly Constructed Device). A device with imperfectly constructed chambers (often a result of unskilled efforts) still produces a gradient that is linear except at the extremes. Hence, a gradient of known linearity can be introduced into the Spinco 5050 tube if slightly excess volumes are introduced into the chambers and if the extremes of the gradient are discarded. Curve D of Fig. 2 illustrates such a gradient. Once the pertinent concentrations and volumes have been determined, the gradient can be reproduced at will.

RESULTS

Figure 2 shows curves A and C for gradients obtained directly from the device, and curves B and D for gradients obtained from the Spinco 5050 tube by the piercing technique described previously (4). In each case, 10-drop fractions were collected serially (the last fraction usually contains less than 10 drops).

The refractive index was determined on an aliquot of each thoroughly

mixed fraction, and was converted to per cent sucrose from the tables accompanying the refractometer.[4] Each 10-drop fraction is linear in sucrose concentration from the first to the last drop (a valid assumption), and the refractive index as determined is that of the average drop (No. 5½) of each fraction. Hence, the middle drop of each fraction is used to calculate per cent of total collected, and is plotted (abscissa) against per cent sucrose (ordinate). For example, in Fig. 2, for a well-constructed device, curve B, a total of 325 drops was collected;[5] fraction number 4 contained drop numbers 31 to 40 (37.7% sucrose) and is therefore 10.9% of the total:

$$\frac{35\frac{1}{2}}{325} \times 100 = 10.9$$

The devices described here have already been used successfully to prepare gradients which resolved the three hydroxylamine reductases of *Neurospora crassa* (5).

DISCUSSION

Each newly made device is tested initially for linearity by collecting fractions directly from the device. This tells the operator whether to use the preferred or the alternate mode of operation for that particular device.

For effective use in making a linear gradient, the design includes the following features. The capillary that connects the two chambers is at a lower elevation than the bottoms of the two chambers, and is always filled with the denser of the two solutions. This prevents the lighter of the two solutions from passing through the capillary and rising to the top of the denser solution. The rate of entry of heavy solution into the mixing chamber must be equal to the rate at which the gradient leaves the mixing chamber. To achieve this, the capillary that connects the two chambers has a larger bore than that of the delivery tip. To obtain a linear gradient with two solutions of *equal* density but differing in the concentration of one component, the shapes and volumes of the two chambers must be as nearly equivalent as possible (6) and the capillary inlets and outlets of the chambers must be at the same elevations. This allows the hydrostatic heads and the volumes of the two chambers to be equivalent throughout the making of the gradient, a condition that is necessary for a perfectly linear gradient to be formed.

[4] Bausch & Lomb Abbe-3L refractometer.

[5] The total numbers of drops varied because the hypodermic needles had different sized constrictions at the orifices due to differences in breaking-off procedures.

If the two solutions in the chambers are of *unequal* density, then the design features given above will not be expected to produce a linear gradient. It might be anticipated that the denser solution in the reservoir chamber will flow into the mixing chamber, when stopcock D is opened, at an initial rate somewhat greater than outflow from the mixing chamber, resulting in perturbation of the linearity of the gradient. However, our data do not reveal such a phenomenon probably because of a combination of factors such as (*a*) limitations in the accuracy of the measurement of the refractive index and (*b*) the slightly increased height of the meniscus in the mixing chamber due to V_d and the volume of the stirring blade. The initially greater solution height in the mixing chamber (because of the stirring blade) decreases at a rate roughly proportional to the changing difference in densities of the solutions in the two chambers as the gradient making progresses. Hence, these circumstances dictate that close adherence be paid to the details of construction as shown in Fig. 1. The capillary joints must be at the same elevations, the transitions from the chambers to the capillaries must be abrupt, and they must be executed in such a way as not to change the shapes of the bottoms of the chambers.

Even with devices in which seals of the capillaries to the test tubes are poorly executed, i.e., resulting in shape alterations near the bottoms of the chambers or in poor placement of seals, the gradients in general are linear except at the extremes, depending on the particular alteration of shape or placement of seals. This is generally not of great consequence for most separation problems, but does become significant for molecular weight calculations (2) if the component is found at one of the nonlinear extremes of the gradient.

SUMMARY

An improved gradient-making device for sucrose density gradient centrifugation is described. The device can be easily constructed with a minimum of glassblowing skill. An alternate mode of operation is presented which allows the production of a linear sucrose density gradient even from a poorly constructed device.

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ANALYTICAL BIOCHEMISTRY 13, 80–84 (1965)

Radioactive Assay for Serine Transhydroxymethylase

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Serine transhydroxymethylase, which is present in a variety of sources (1), catalyzes the reversible reaction:

L-serine + tetrahydrofolate $\rightleftarrows$ N^5,N^{10}-methylene tetrahydrofolate + glycine

The methods used to assay the enzyme have involved: (*1*) estimation of serine by periodate oxidation followed by chromotropic acid treatment (2, 3); (*2*) determination of serine by manometric measurement of CO_2 release upon periodate oxidation (4); (*3*) manometric determination of O_2 uptake during oxidation of the C-1 unit to CO_2 (5); or (*4*) colorimetric estimation of the disappearance of formaldehyde bound in N^5,N^{10}-methylene tetrahydrofolate with acetylacetone reagent (6). Recently, Schirch and Mason measured N^5,N^{10}-methylene tetrahydrofolate spectrophotometrically with N^5,N^{10}-methylene tetrahydrofolate dehydrogenase plus TPN (triphosphopyridine nucleotide) in a two-step assay procedure (7).

To study the metabolism of N^5,N^{10}-methylene tetrahydrofolate in *Escherichia coli* K_{12}, strain 2276, we have developed a simple radioactive assay for serine transhydroxymethylase. 3-^{14}C-Serine yields a radioactive C-1 unit in N^5,N^{10}-methylene tetrahydrofolate. Since this C-1 unit readily equilibrates with carrier formaldehyde (8) it can be trapped with dimedon (5,5-dimethyl-1,3-cyclohexadione) (9) and measured. Although our assay is not suitable for detailed kinetic studies, it is applicable for studies of enzyme levels in crude systems. The sensitivity is an order of magnitude greater than the above methods and the assay is specific for ^{14}C derived from the β-position of serine. The method also obviates the use of purified N^5,N^{10}-methylene tetrahydrofolate dehydrogenase as an indicator enzyme. Twenty assays can be performed conveniently in 1.5 hr.

Hatch *et al.* also have reported the use of a tracer assay for serine transhydroxymethylase based on the formation of a reaction product between dimedon and the 1-carbon unit of N^5,N^{10}-methylene tetrahydrofolate; however, no details are presented (10).

[1] Postdoctoral Fellow, Public Health Service.

METHODS AND MATERIALS

All compounds were purchased from commercial sources. 3-^{14}C-L-Serine (6.56 μc/μmole) and pyridoxal-5-phosphate were obtained from California Corporation for Biochemical Research. U-^{14}C-L-Serine (17.0 μc/μmole) and ^{14}C-formaldehyde (2 μc/μmole) were obtained from New England Nuclear Corporation. Tetrahydrofolate (80% purity) was purchased from Sigma Chemical Co. Radioactivity was measured with a Packard Tri-Carb liquid scintillation counter using a naphthalene-dioxane counting fluid (11). Protein was determined by the method of Lowry *et al.* (12).

Complete assay systems contained 0.1 μmole 3-^{14}C-L-serine (1.05×10^6 cpm/μmole), 0.1 μmole pyridoxal phosphate, 0.8 μmole *dl*,-L-tetrahydrofolate, 4.0 μmoles β-mercaptoethanol, 30 μmoles potassium phosphate, and enzyme in a total volume of 0.4 ml, pH 7.4. All components except serine were first incubated 5 min at 37°. Reactions were then initiated by addition of the substrate and were terminated 15 min later with 0.3 ml of 1.0 *M* sodium acetate, pH 4.5; 0.2 ml 0.1 *M* formaldehyde and 0.3 ml 0.4 *M* dimedon (in 50% ethanol) were added in succession and the vessels were heated 5 min in a boiling water bath to accelerate formation of the HCHO dimedon derivative. The tubes were then cooled 5 min in an ice bath before the dimedon compound was extracted by vigorous shaking with 5.0 ml toluene at room temperature. Two minutes of centrifugation separated the phases and 3.0 ml of upper phase were removed for counting. It was convenient to carry out the entire assay in 12-ml conical centrifuge tubes using marbles to cover the tubes during the boiling step.

RESULTS AND DISCUSSION

Figure 1 illustrates the dependence of the assay on time and enzyme concentration. The amount of HCHO recovered is proportional to time for at least 25 min and to the level of enzyme until 15 mμmoles of serine are consumed. The source of the enzyme here was a $10{,}000 \times g$ supernatant fraction obtained after a sonication of *E. coli* K_{12}, strain 2276 (13). The protein concentration was 2.8 mg/ml.

Table 1 defines the requirements for the assay system. There is an absolute dependency on enzyme, tetrahydrofolate, pyridoxal phosphate, and the ^{14}C-β-carbon of serine. When 3-^{14}C-L-serine was replaced by U-^{14}C-L-serine (1.05×10^6 cpm/μmole), the percentage appearing as dimedon product was decreased about two-thirds, indicating that only the β-carbon of serine was being released as formaldehyde. Substrate blanks are quite low ($<0.5\%$ of the 1.05×10^5 cpm added) and repro-

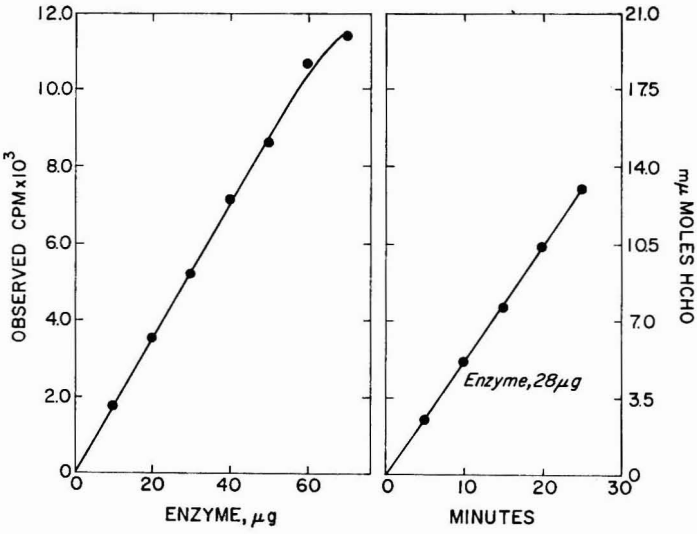

FIG. 1. Dependence of assay on time and enzyme concentration.

TABLE 1

REQUIREMENTS FOR SERINE TRANSHYDROXYMETHYLASE ASSAY

Omission from complete[a] system	Observed cpm	Formaldehyde formed/15 min, mμmoles
None	8570	15.0
Enzyme[b]	500	0
Tetrahydrofolate	465	0
Pyridoxal phosphate	515	0
3-^{14}C-L-Serine replaced by U-^{14}C-L-serine	3250	5.7

[a] Defined in the text.

[b] 50 μg protein from *E. coli* K_{12}, strain 2276.

ducible. Consequently, observed activities as low as 1×10^3 cpm are meaningful and correspond to the formation of 1.8 mμmoles of formaldehyde under the conditions outlined.

Table 2 shows that recovery of formaldehyde from substrate blanks prepared at 0° is approximately 90% for levels of formaldehyde over the range in which the assay is first order with respect to enzyme concentration (Fig. 1). The per cent recovery was not affected significantly by addition of bacterial protein and subsequent incubation at 37°.

Due to its sensitivity and specificity, the assay can be used to measure serine transhydroxymethylase in extracts of animal tissues having lower apparent specific activities than *E. coli* (Table 3). Animal tissues

TABLE 2
RECOVERY OF ADDED ^{14}C-FORMALDEHYDE FROM THE ASSAY SYSTEM

Conditions prior to recovery	Formaldehyde[a] added/tube, mμmoles	Per cent formaldehyde as dimedon derivative
Complete system[b] minus enzyme at 0°C	5	95
	10	92
	15	92
Complete system (+55 μg *E. coli* enzyme) at 0 min	5	95
	10	92
	15	91
Complete system after 15 min at 37°	5	93
	10	90
	15	91

[a] Observed radioactivity was 1400 cpm/mμmole.
[b] Complete system contained unlabeled L-serine (0.1 μmole) in place of 3-^{14}C-L-serine.

were homogenized in 3 vol of 0.1 *M* Tris chloride buffer, pH 7.4, and then centrifuged at 25,000 × *g* to yield supernatant fractions containing 14–48 mg protein/ml. Assays in Table 3 were performed with several levels of enzyme (0.1–0.6 mg of protein); however, it was noticed that, when higher levels of mouse spleen supernatant (1.5 mg of protein) were assayed, the observed cpm were not proportional to enzyme concentration. This difficulty can, however, be avoided by assaying with less protein and carrying out appropriate controls for recovery of formaldehyde.

In order to conserve radioactivity, the enzyme reactions were not saturated with respect to 3-^{14}C-L-serine, although saturating levels of *dl*,-L-tetrahydrofolate and other components of the system were used. A maximal rate in the *E. coli* system, obtained with 2.5×10^{-3} *M* 3-^{14}C-L-serine, gave specific activities 3 times that seen in Table 3. It should be pointed out that, if one wanted to obtain maximal specific activities for

TABLE 3
APPLICATION OF THE ASSAY WITH CRUDE TISSUE EXTRACTS

Enzyme source	Specific activity[c]
E. coli K_{12}, strain 2276	1.2
E. coli K_{12} S[a]	2.0
Mouse tumor[b]	0.08
Mouse liver	0.14
Mouse spleen	0.02

[a] Strain was kindly provided by Dr. A. Weissbach.
[b] Provided by Dr. M. A. Chirigos from Walker L-1210 tumor bearing animals.
[c] μmoles formaldehyde formed/hr/mg protein.

an individual tissue, it would be necessary to determine the reaction rate at saturating levels of serine.

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ANALYTICAL BIOCHEMISTRY 13, 85-90 (1965)

Absorption Spectra of Δ^5-3β-Hydroxy Steroids in Two Reagents[1]

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In the course of studies of Δ^5-3β-hydroxy steroids in human urine, the absorption spectra of these compounds in two sulfuric acid reagents proved of value in arriving at their identification (1). Wilson (2) has previously reported spectra of 11 steroids in sulfuric acid reagents. Smith et al. (3) have compiled spectra for a large number of steroids in sulfuric acid. This report extends these observations to 10 additional compounds and includes 5 previously described. These methods are helpful for the partial characterization of steroids in small quantity and as noted below one pair of isomers may be distinguished by these techniques.

MATERIALS AND METHODS

Reagents and Equipment

Concentrated sulfuric acid: Mallinckrodt, analytical grade.

Ethanol, absolute: Commercial Solvents Corporation.

Oertel reagent (4): 2 vol concentrated sulfuric acid was added to 1 vol of 95% absolute ethanol with cooling.

Cuvets: Pyrocell Corporation, 1.5-ml capacity, 10-mm light path.

Spectrophotometer: Beckman model DB with Photovolt model 43 varicord electronic recorder, set with a scanning speed of 40 mμ/min covering the region from 220 to 700 mμ.

Steroids: Some pure steroids employed were obtained from urine and fully characterized as described (1). Others were purchased from Mann Research Laboratories, 136 Liberty St., New York, N. Y., and Steraloids, Pawling, N. Y. Generous gifts of certain compounds were received from Dr. J. J. Schneider and Dr. D. K. Fukushima.

[1] These studies have been supported by grants from the U. S. P. H. S. National Institutes of Health #HD 00371-11 and T1-AM-5197.

TABLE 1

Steroid	Oertel reagent			Sulfuric acid		
	Time (mins.)	Maxima mμ (K)*	Minima mμ (K)	Time (mins.)	Maxima mμ (K)	Minima mμ (K)
3β-hydroxy-Δ⁵-androstene-17-one	210	305(412), 405(2765)	340(318)	210	305(1338), 400(2425)	340(453), 445(110)
Δ⁵-androstene-3β,17β-diol	30	325(460), 405(2157), 485(60)I	315(374), 450(58)			
	90	295(765), 405(1670), 485(1738)I, 525(260)I, 600(313)	335(365), 445(148), 545(226)	240	310(875), 408(1300), 440(845)I, 545(226)I	345(423), 430(906)
3β,17β-dihydroxy-Δ⁵-androstene-16-one	30	330(219)I, 405(2225)	325(223)	30	385(1070)I, 405(1250), 480(2130), 595(200)I, 655(267)	450(173)510(133)
	240	330(246)I, 405(2105), 605(165)	330(270), 445(547)	240	375(1040), 405(775)I, 430(510)I, 595(640), 665(587)I	330(561),420(614), 460(374)
3β,16α-dihydroxy-Δ⁵-androstene-17-one	30	375(1672), 400(1775), 610(617), 655(587)I	385(1600), 440(220)			
	90	255(587), 375(1380), 400(572)I, 610(1715), 655(1334)I	315(470), 395(572), 440(250)	240	270(102), 370(152), 405(144), 555(134), 600(121)I, 660(87)I	320(84),385(139), 450(84)
3β,7α-dihydroxy-Δ⁵-androstene-17-one	30	260(220), 293(165), 570(1000)	280(155), 325(91)	30	231(457)I, 296(293), 381(604)	250(220),324(145)
	180	255(229), 295(201), 570(940)		180	230(650)I, 295(730), 393(192)	249(302),330(110)
Δ⁵-pregnene-3β,20α-diol	30	335(651), 407(700), 530(615), 610(318), 690(149)I	255(248), 370(445), 470(382), 575(290), 650(141)			
	240	335(486), 410(658), 495(580)I, 526(608), 608(438)	370(354), 470(495), 505(574), 565(365)	240	310(334), 410(186), 450(139)I	370(151),400(177)
3β,17α-dihydroxy-Δ⁵-pregnene-20-one	15	265(544), 405(2380), 485(765)I	325(281)			
	90	265(553), 405(2345), 490(247), 525(213)I	325(306), 455(204), 505(212)	240	305(791), 340(712)I, 410(1000), 445(802)I, 490(836), 540(452)I	360(587), 460(769)
Δ⁵ pregnene-3β,17α,20β-triol	30	310(366)I, 345(459), 425(1090), 525(899), 615(1118)	320(365), 375(446), 465(473), 550(585)	30	335(820), 405(1190), 445(597)I, 495(657), 600(131)I	360(676), 465(560)

	90	310(372)I, 345(459), 421(645), 522(1562), 615(1110)	375(366), 455(619), 555(772)	210	310(774), 335(675), 405(933), 445(760)I, 495(657), 590(336)	325(662), 360(630), 430(765), 475(630), 560(308)
Δ^5-pregnene-3β,17α,20α-triol	30	310(382), 415(1235), 520(830), 575(387)I, 615(420)	255(270), 325(326), 465(490), 560(350), 595(387)	30	310(505), 335(505), 405(965), 445(393)I, 495(439)	320(495), 360(393), 435(419), 465(360)
	90	310(386), 405(933), 421(802)I, 515(1304), 580(424)I, 615(466)	335(240), 455(690), 575(419), 595(429)			
3β,16α-dihydroxy-Δ^5-pregnene-20-one	30	290(988), 405(2380), 485(443), 605(101)	325(517), 440(277), 545(92)	30	295(684), 340(633)I, 385(831), 405(735), 485(470), 535(294), 640(40)I	320(595), 400(727), 435(346), 520(286)
	180	290(1190), 405(2118), 485(535), 605(277)	325(535), 440(312), 545(203)	180	295(724), 370(882), 405(629)I, 475(544), 535(298)I, 650(55)I	320(610), 430(426), 580(81)
Δ^5-pregnene-3β,16α,20β-triol	30	315(1120), 405(2415), 475(745), 575(860)I, 615(985)	345(904), 435(530), 510(436), 590(857)			
	210	310(1308), 400(997), 475(1060), 575(2010)I, 615(2428)	355(701), 430(639), 510(874)	180	308(1110), 380(909), 470(985), 500(736)I	355(840), 425(675)
3β,17α,21-trihydroxy-Δ^5-pregnene-20-one	180	320(534), 405(1475), 485(296), 530(250)I	350(409), 440(247), 505(249)	180	320(775)I, 335(779), 405(820), 485(421), 660(106)I	365(520), 465(414), 570(152)
Δ^5-pregnene-3β,17α,20,21-tetrol	30	305(106), 405(2535), 480(674)	335(885), 440(594)			
	240	305(1240), 405(2220), 480(871)	345(924), 440(739)			
3β,11β,17α,21-tetrahydroxy-Δ^5-pregnene-20-one	30	310(753), 350(767), 410(1220), 510(1182)I, 550(1250)	335(715), 360(747), 445(845), 531(1200)			
	210	305(900), 470(1170)I, 510(1250), 555(1183)I	360(590)	180	265(675)I, 315(665), 400(1465), 495(772)	290(647), 345(531), 450(705)
3β,21-dihydroxy-Δ^5-pregnene-20-one	30	280(410)I, 401(2310), 480(157), 520(126)I	440(126)	30	285(966)I, 334(1530), 354(1470)I, 370(1385)I, 405(650), 480(346)I	360(1410), 390(630)
	180	280(505), 370(1240)I, 400(1765), 520(651), 600(157)I	320(440), 435(242)	180	285(1200), 371(1250), 405(975), 484(630)I	320(1060), 386(890)

The Table indicates wavelength and in parentheses absorbance of 0.1 M concentration (K)*.
I = inflection.

Procedure

Aliquots of steroids in ethanolic solution were transferred to dry 12×75 mm test tubes, to contain 5–20 μg, and dried *in vacuo;* 1 ml of concentrated sulfuric acid or the Oertel reagent was added to duplicate specimens, which were agitated briefly and transferred to the spectrophotometer cuvet. The spectra were determined at several time intervals for each compound.

RESULTS

The results are described in Table 1 after the manner of Bernstein and Lenhard (5) and are expressed as 0.1 molecular extinction coefficients. The time intervals selected for this presentation were those found most convenient and characteristic for each compound. In instances when two time intervals revealed typical changes, both sets of readings are given. Five of the fifteen steroids described here were previously reported by Wilson (2) and are included for purposes of comparison, in one instance to demonstrate the difference between isomers. Figure 1 portrays

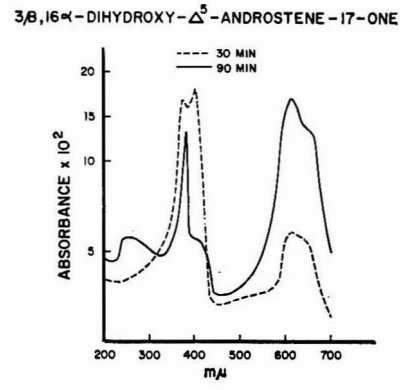

FIG. 1. Spectra in the Oertel reagent, 0.1 *M* concentration at two time intervals.

the characteristic alteration in the spectrum of 3β,16α-dihydroxy-Δ^5-androstene-17-one between 30 to 90 min in the Oertel reagent. Figure 2 demonstrates the readily detectable difference in the Oertel reagent between the 20-isomers of pregnenetriol.

DISCUSSION

Particular attention has been focused on the Δ^5-3β-hydroxy steroids in biological material in recent years in view of their importance in intermediary steroid metabolism. It is helpful to have available relatively

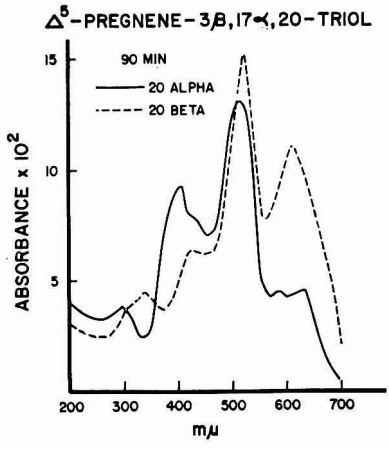

FIG. 2. Differing spectra of isomers of pregnenetriol in the Oertel reagent, 0.1 *M* concentration, at 90 mins.

simple means for the partial identification of these compounds after their isolation from mixtures. Special features of their absorption spectra in reagents of several types containing sulfuric acid have been informative. Characteristics of staining on paper chromatograms have also been helpful (6, 7).

As noted earlier, most but not all steroids with this grouping demonstrate high peaks of absorption in the region of 400–415 mμ in the Oertel reagent. It would appear that an oxygen at C-7 whether ketonic, as reported by Wilson (2), or a hydroxyl as noted herein deletes this typical peak. In addition, among the two isomers of pregnenetriol the 20α demonstrates a notable peak near 410 mμ although not the major one, whereas the 20β has a very weak and barely detectable maximum in this region (Fig. 2). This characteristic is somewhat dependent on time and in some instances is better observed earlier, since it fades thereafter while other peaks are augmented (Fig. 1). The spectra in concentrated sulfuric acid are considerably different from those in the Oertel reagent and the combination of both tests reinforces the ability to identify these compounds. In sulfuric acid there is often a maximum in the region of 400–415 mμ but it is frequently surpassed by other peaks.

SUMMARY

Absorption spectra of several Δ^5-3β-hydroxy steroids in concentrated sulfuric acid and the Oertel reagent have been reported. These spectra, when determined in two different reagents and at two time intervals, may aid in identification and yet require little material. The charac-

teristic alterations in the spectra with time are often sufficient to render characterization more exact. It has been demonstrated that one pair of isomers may be distinguished by these methods.

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ANALYTICAL BIOCHEMISTRY 13, 91–99 (1965)

A Simple and Specific Assay for Cyclodextrin Transglucosidase[1]

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Because of its unusual ability to produce cyclic dextrins and catalyze transglucosidation reactions, the amylase from *Bacillus macerans* has fascinated biochemists for many years (for reviews, see 1, 2). Although this enzyme has been the subject of many qualitative investigations (1–3), the lack of a suitable assay has impeded the rigorous quantitative study of the enzyme kinetics. The principal difficulty in designing an assay arises because the cycloamyloses, the products of starch digestion, are not amenable to routine reducing sugar analysis. The first semi-quantitative assay method for cyclodextrin transglucosidase, CT, developed by Tilden and Hudson (4) was very time consuming and imprecise. The end point was chosen as the time required for the onset of the formation of the characteristic cyclohexaamylose-iodine crystals.

Later, Hale and Rawlines (5) developed an alternate assay designed to measure CT activity which was based upon the time required to produce a starch-iodine solution having 50% transmission. While this new procedure significantly reduced the time required for assay, it was not readily adaptable to quantitative kinetic study of the transglucosidase. Another disadvantage of the new assay was its inability to distinguish between transglucosidase activity and hydrolytic activity. Because CT in a very pure state can now be obtained with relative ease (6, 7), the need for a direct quantitative assay is all the more imperative.

In this article we report a specific, rapid, and precise method for estimation of CT activity. The system employs a coupled enzyme assay which takes advantage of the ability of the cyclodextrin transglucosidase to transfer a cyclodextrin to a nonreducing acceptor (1, 2) and generate a linear substrate susceptible to hydrolytic cleavage by amylases. The

[1] Contribution 1324 from the Chemical Laboratories of Indiana University.

liberation of the reducing sugars measured by conventional methods gives the procedure the ease and convenience of routine sugar analysis. In addition, the production of several reducing ends for each mole of cyclodextrin coupled enhances the sensitivity of the procedure. The utility of the method is demonstrated by determining the apparent Michaelis constants and standard error for the donor, cycloamylose, and the acceptor, methyl-α-D-glucoside.

EXPERIMENTAL

Materials. Cyclohexaamylose was prepared according to the directions of French and co-workers (8) and then exhaustively digested with porcine α-amylase to hydrolyze small amounts of contaminating maltodextrins. Eastman reagent-grade methyl-α-D-glucoside was used as acceptor. α-Amylase of hog pancreas and β-amylase from sweet potato were purchased from Worthington Biochemical Research Corporation and the *Bacillus subtilis* amylase was purchased from Enzyme Development Corporation. All other chemicals were of reagent grade. The CT used in these experiments was prepared according to the directions of Schwimmer and Garibaldi (9) and the crude preparation used for all studies.

Solution A for the standard assay procedure contained 0.018 *M* cyclohexaamylose, 0.016 *M* methyl-α-D-glucoside, 0.25 mg/ml of porcine α-amylase, 0.02 *M* sodium chloride, and sodium acetate buffer at pH 5.4 at ionic strength 0.2. The stock solution A was prepared fresh daily by adding an appropriate amount of the crystalline α-amylase to a stock solution containing the other reagents stored in the cold (0–3°C). It has been observed that, upon standing even at 0°, the activity of the α-amylase is slowly lost.

Standard Assay Conditions. The assay is initiated by adding 0.5 ml of the CT solution to 0.5 ml of solution A, after thermostating at 35°C. The mixture is allowed to incubate at this temperature for 10 min and the reaction then terminated by the addition of copper solution used for the conventional reducing tests. For our experiments we have found it convenient to use a modified neocuproine assay which has recently been described by Dygert *et al.* (10).

Activity vs. Enzyme Concentration. To examine the system for endogenous activators and inhibitors, the relative activity as a function of CT concentration was determined under standard assay conditions at both 25° and 35°C.

Apparent Activation or Inhibition. A number of products are produced during the assay which could act as potential substrates to compete with the original acceptor, methyl-α-D-glucoside, and cause activation or inhibition of the reaction. Hence the time course of the reaction was

investigated under standard conditions at a low level of enzyme as a function of time.

Substrate Levels and Enzyme Activity. The effect on enzyme activity of various concentrations of acceptor and donor were investigated by systematically varying the concentration of either methyl-α-D-glucoside or cyclohexaamylose under otherwise standard assay conditions.

Effect of Amylase. Three commercially available enzymes, crystalline β-amylase from sweet potato, pig pancreatic α-amylase, and the amylase from *Bacillus subtilis*, were tested for their ability to hydrolyze the heptaose produced by coupling cyclohexaamylose to methyl-α-D-glucoside. The assays were conducted under the standard conditions except that the concentration of the enzymes was allowed to vary over a suitable range.

RESULTS

Activity as a Function of Substrate Concentrations. The effect of the methyl-α-D-glucoside and cyclohexaamylose concentration on the activity of the crude enzyme preparation are displayed in Figs. 1 and 2. On the

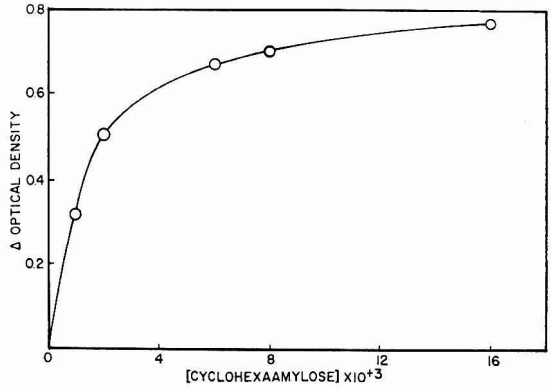

FIG. 1. Effect of cyclohexaamylose concentration at constant methyl-α-D-glucoside (1.5×10^{-3}) and α-amylase levels on CT activity. Samples were assayed under standard conditions except that cyclohexaamylose concentration was systematically varied.

basis of these experiments, the concentration of 9.0×10^{-3} *M* cyclohexaamylose and 8.0×10^{-3} *M* methyl-α-D-glucoside were selected as the appropriate substrate concentrations for the standard assay procedure for CT. It is interesting to note that there is apparently no cross-substrate inhibition apparent in this system under the conditions employed. This is somewhat surprising, since both donor and acceptor sites can apparently bind glucopyranoside residues.

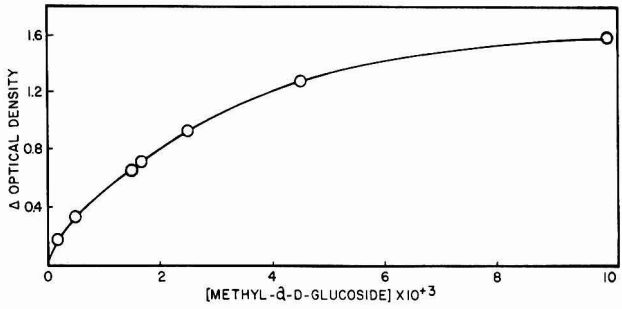

FIG. 2. Effect of methyl-α-D-glucoside concentration at constant α-dextrin concentration ($8 \times 10^{-3} M$) and α-amylase levels on CT activity. Samples were assayed under standard conditions except that methyl-α-D-glucoside concentration was systematically varied.

Amylases. A suitable amylase for this assay should: (*a*) give a large number of cleavage points in the linear substrate which was formed to enhance the sensitivity, (*b*) be available commercially, (*c*) not hydrolyze either of the substrates, and (*d*) yield a reducing value independent of enzyme concentration. The effects of three commercial enzymes, pig pancreatic α-amylase, sweet potato β-amylase, and *Bacillus subtilis* α-amylase, on the color yield are displayed in Fig. 3. While the *Bacillus*

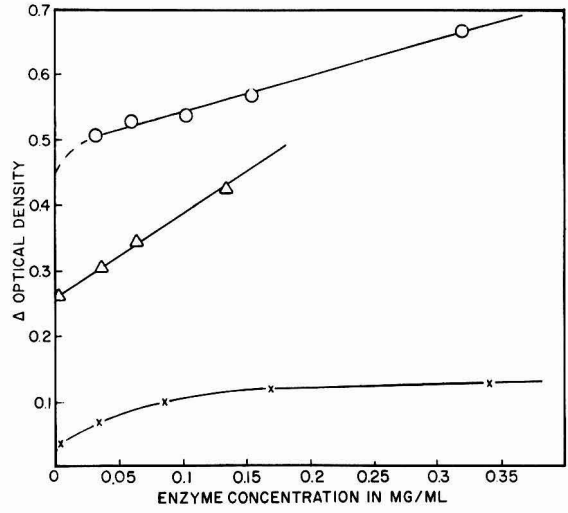

FIG. 3. Effect of concentration of different hydrolyzing enzymes on color yield. (○) α-amylase of hog pancreas, (△) β-amylase of sweet potato, (×) α-amylase of *Bacillus subtilis*. Standard assay conditions except that concentration of enzyme was varied.

subtilis enzyme has attributes *b*, *c*, and *d* listed above, it produces a significantly smaller amount of reducing sugar than the other enzymes and hence limits the sensitivity of the method. Because the sensitivity of reducing sugar assays is frequently the limiting factor in assessing initial steady-state velocities, we suggest the use of pig pancreatic α-amylase for enzyme kinetics at a level of 0.125 mg/ml as the hydrolytic enzyme (cf. Fig. 3) even though the extent of hydrolysis is somewhat dependent upon the enzyme concentration. Thus for precise analysis it will be imperative that the amylase concentration be carefully controlled for each run, but this is a small price to pay for high sensitivity. Obviously a glucosidase would give the highest reducing value, but unfortunately none of sufficient purity and activity is commercially available. The increase in the color yield at high α- and β-amylase concentration may result from the slow cleavage of the small oligosaccharides resulting from the degradation of the maltoheptaose (11, 12). Under the conditions of the assay it was found that none of these enzymes produces a significant amount of hydrolysis of either the cyclic dextrin or the acceptor, methyl-α-D-glucoside.

Endogenous Activators or Inhibitors. To test for the presence of endogenous activators or inhibitors in the crude enzyme, the activity was studied as a function of CT concentration at 25° and 35°C. The linearity of the two curves in Fig. 4 indicates the absence of observable endogenous activators or inhibitors in the CT preparation.

Time Course of Reaction. The time course of the production of reducing sugar in the CT digest is depicted in Fig. 5. The data were obtained

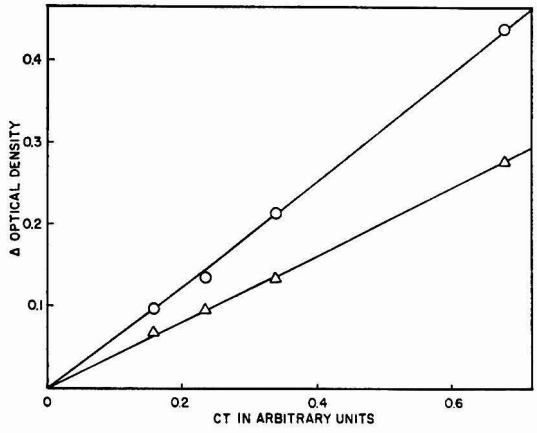

FIG. 4. Activity vs. CT concentration at 25° (△) and 35°C (○). Except for change in temperature, standard assay conditions were employed.

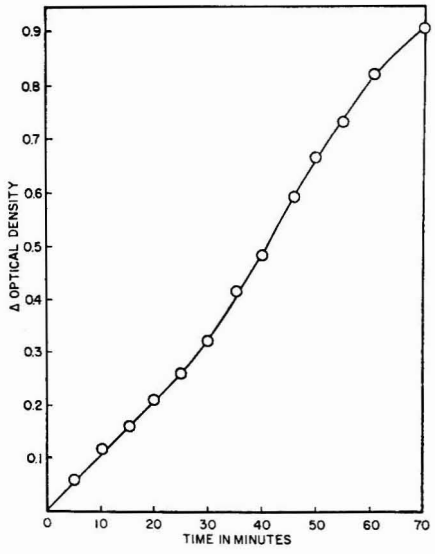

FIG. 5. Time course of reaction of CT under standard assay conditions. Samples of 0.1 ml were withdrawn from a standard assay mixture for determination of reducing power.

under standard assay conditions except that 0.1-ml samples were withdrawn for reducing sugar determinations, hence the optical density value under the standard conditions would be tenfold larger than those depicted in the figure. After about 30 min of incubation, an apparent activation of the enzyme can be observed. This phenomenon probably results from the ability of the hydrolytic products, the oligosaccharides, to act as better acceptors for the transglucosidation reaction than methyl-α-D-glucoside. Ultimately, as the cyclodextrin is consumed the velocity diminishes. In view of these opposing effects on enzyme velocity when studying enzyme kinetics, it will be necessary to ascertain the conditions under which the velocity does not change with time if extrapolation techniques are not employed to obtain initial velocities. Thus it will be necessary to show that product vs. time curves are linear at *both* the highest and the lowest substrate concentrations.

Use and Precision. As a demonstration of the use and precision of the assay method, apparent Michaelis constants for cyclohexaamylose and for methyl-α-D-glucoside have been ascertained. The data were fit by Cleland's (13) least-squares program to a hyperbola. The apparent Michaelis constant determined for cyclohexaamylose at a constant concentration of methyl-α-D-glucoside of $1.5 \times 10^{-4}\ M$ was $2.43 \times 10^{-3}\ M$ with a standard error of 6.3%. The apparent Michaelis constant for the

methyl-α-D-glucoside at a constant cyclohexaamylose concentration of 0.008 M in two different experiments was determined to be 3.06×10^{-3} M and 3.04×10^{-3} M with standard errors of 9.3 and 6.7%, respectively. The data are graphically displayed in Fig. 6 and 7. Since these assays were conducted with crude enzyme preparations which had a high blank value, it can be anticipated that the precision of the method will even

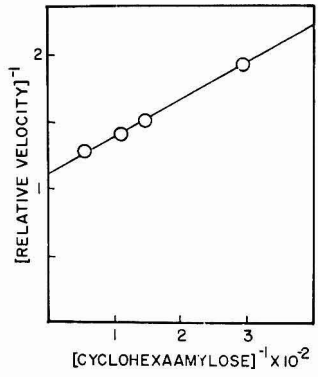

FIG. 6. Double reciprocal plot with cyclohexaamylose as variable substrate. *Conditions:* Standard assay, except that the concentration of cyclohexaamylose varied. Curve is constructed with Michaelis constants determined by unweighted least-square fit of data to a hyperbola (13).

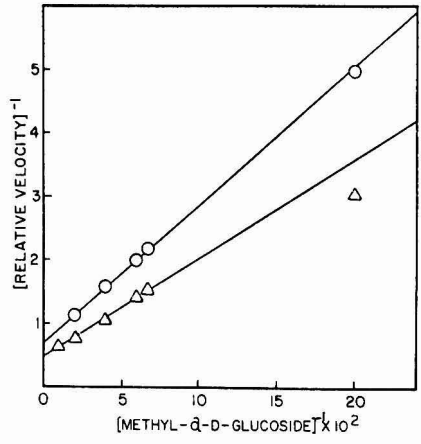

FIG. 7. Double reciprocal plots with methyl-α-D-glucoside as variable substrate. Standard assay conditions at two different CT concentrations except that concentration of methyl-α-D-glucoside were varied. Curves are constructed with Michaelis constants determined by unweighted least-square fit of data to a hyperbola (13).

further increase as the purity of the enzyme is enhanced and the background value of the blank is further reduced.

If an arbitrary unit of activity is defined as the amount of enzyme required to produce 100 μg of glucose equivalents in 10 min under standard assay conditions, then one unit of activity is equivalent to 0.05 unit of activity as defined by Tilden and Hudson (4) and to 0.002 unit of activity defined by Hale and Rawlins (5).

DISCUSSION

The method described in this paper is easy and precise, yet it has the advantage of specifically measuring coupling activity in the presence of hydrolytic enzymes. The large amount of α-amylase added to the system should effectively mask the activity of contaminating amylases. The presence of other amylases would only increase the sensitivity of the method and give a slightly higher apparent activity. If cyclodextrinases (6, 7) are present in the system their activity can be corrected for by running a control in the absence of the acceptor. This control is suggested for general usage because trace quantities of acceptors may contaminate crude preparations and lead to spurious activity values. The pig pancreatic α-amylase under the conditions of our assay is incapable of hydrolyzing the substrates; hence the reducing value of the blank remains at a low level for purified enzyme preparations, assuring good precision for enzyme kinetics.

The application of this procedure for the determination of initial steady-state kinetics of the coupling reaction of the transglucosidase is too obvious to require further elaboration. The precautions mentioned under "Results" should, however, be observed.

SUMMARY

A new assay for the cyclodextrin transglucosidase of *Bacillus macerans* is presented. It is based upon the ability of the enzyme to couple the cyclic dextrins to substrates to produce linear nonreducing oligosaccharides. These saccharides are then hydrolyzed with α-amylase and the reducing sugars released are measured by conventional tests. The utility and precision of the method are demonstrated by determining enzyme kinetic parameters.

ACKNOWLEDGMENT

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Colorimetric Determination of ε-*N*-Methyl-L-lysine

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ε-*N*-Methyl-L-lysine has been shown to occur in flagellae of *Salmonella typhymurium* (1) and in mammalian histones (2), but its presence in the flagellae of *Spirillum serpens* has yet to be confirmed (3). ε-*N*-Methyl-L-lysine has been characterized by paper chromatography (1, 2), ion-exchange chromatography and mass spectrometry (2), and ε-alkyllysinase (4). Evidence is present here that, when ε-*N*-methyl-L-lysine is reacted with ninhydrin by the method previously described (5), the visible spectrum of the colored product has a very characteristic maximum absorption peak at 464 mμ. Of the compounds so far tested only ε-*N*-monoalkyl derivatives of lysine gives this pattern. The following investigation deals with the specificity and application of the method to a mixture of α-*N*-, and ε-*N*-methyl-L-lysine.

MATERIALS AND METHODS

N-Alkyl derivatives of L-lysine and L-ornithine were prepared according to the method described earlier (6) and the rest of the compounds described in Table 1 were purchased from Sigma Biochemical Company. Ninhydrin, ethylene glycol monoethyl ether (ethyl Cellosolve), and ethylene glycol monomethyl ether (methyl Cellosolve) were obtained from Fisher Scientific Company. ε-*N*-(^{14}C-Methyl)-L-lysine was prepared as described (6).

The ninhydrin method described previously (5) was slightly modified. Coleman colorimetric tubes (1.8 × 10.2 cm) are charged with 0.2 ml of an aqueous solution of the compound and 0.9 ml of 4% ninhydrin dissolved in methylCellosolve. The content of the tube is mixed and each tube is capped with a rubber stopper equipped with a capillary tube. The tube is heated in a boiling water bath for 10 min. After cooling the tube for a few minutes, 6.9 ml of water is added. As our results from this paper describe, the color so formed is rather stable, so that the precaution taken in the case of ε-*N*-acetyl-L-lysine is not necessary. The optical density is measured either with a Coleman spectrophotometer or with a

Bausch & Lomb Spectronics 505 recording spectrophotometer. The ratio of optical density measured by Coleman colorimeter (light path of 1.8 cm) and that measured by Bausch & Lomb spectronics (light path of 1.0 cm) was found to be 1.281.

RESULTS AND DISCUSSION

Characteristics of Absorption Spectrum of Ninhydrin Color of ε-N-Methyl-L-lysine. Figure 1 illustrates the characteristics of visible spectra

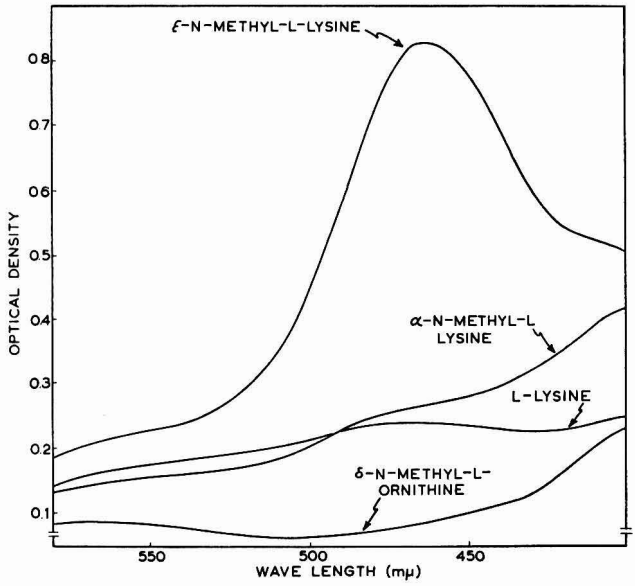

FIG. 1. Characteristic absorption spectrum of ninhydrin color of ε-*N*-methyl-L-lysine: 1.0 μmole of an aqueous solution of the compounds was treated according to the procedure described in "Methods."

of ninhydrin colors produced with L-lysine, α-*N*-methyl-L-lysine, ε-*N*-methyl-L-lysine, and δ-*N*-methyl-L-ornithine. It can be seen in the figure that, even though four compounds tested in the figure very closely resemble each other, ε-*N*-methyl-L-lysine is the only compound to have a maximum absorption peak at 464 mμ. Table 1 shows that among the compounds tested so far only ε-*N*-methyl-L-lysine, ε-*N*-ethyl-L-lysine, and ε-*N*-benzyl-L-lysine give this spectrum. Even compounds such as δ-*N*-methyl-L-ornithine (one-carbon-less analog), α-*N*-methyl-L-lysine (isomer), ε-*N*, ε-*N*-dimethyl-L-lysine or secondary amines (dimethylamine and L-adrenaline) do not give this spectrum. It is noteworthy here that, when both hydrogens of the ε-amino group are substituted as in

TABLE 1
NINHYDRIN COLOR SPECTRA OF VARIOUS COMPOUNDS

Compound	Maximum absorption at[a]	
	570 mμ	464 mμ
1. ε-*N*-Methyl-L-lysine		0.832
2. ε-*N*-Ethyl-L-lysine		0.818
3. ε-*N*-Benzyl-L-lysine		0.697
4. δ-*N*-Methyl-L-ornithine	(0.043)[b]	(0.077)
5. α-*N*-Methyl-L-lysine	(0.124)	(0.264)
6. Sarcosine	(0.010)	(0.004)
7. *N*-Methyl-DL-valine	0.281	
8. ε-*N*,ε-*N*-Dimethyl-L-lysine	0.744	
9. ε-*N*-Tosyl-ε-*N*-methyl-L-lysine	0.875	
10. Asparagine	0.583	
11. L-Lysine	(0.194)	(0.264)
12. Cadaverine	(0.034)	(0.018)
13. L-Arginine	0.756	
14. L-Isoleucine	0.810	
15. L-Methionine	1.010	
16. L-Proline	(0.057)	(0.127)
17. *N'*-Methylnicotinamide	0	0
18. Monomethylamine	0.142	
19. Dimethylamine	0	0
20. Trimethylamine	0	0
21. Noradrenalin	(0.020)	(0.047)
22. L-Adrenalin	0	0
23. *N'*-Methyladrenalin	0	0
24. Choline	0	0
25. Hydroxylamine	0	0

[a] The spectrum was measured by Bausch & Lomb Spectronics 505. 1.0 μmole of the compound was reacted with ninhydrin. For detailed procedure, see "Methods."

[b] Parentheses indicate that the spectrum does not have a maximum absorption peak.

the case of ε-*N*,ε-*N*-dimethyl-L-lysine and ε-*N*-tosyl-ε-*N*-methyl-L-lysine, the compounds give an absorption peak at 570 mμ. When methyl Cellosolve is replaced with ethyl Cellosolve, the distinctive characteristics of the spectrum of ε-*N*-methyl-L-lysine are less predominant; when water is used instead of methyl Cellosolve, they disappear completely.

Stability. Figure 2 illustrates the stability of ninhydrin color of ε-*N*-methyl-L-lysine developed by the present method and by Moore and Stein's method (7). After color was developed, the Coleman tubes were left at room temperature in light. It can be seen that, when ninhydrin color was developed with the present method, the color is far more stable than that developed by the other method. Heating beyond 10 min did not have an adverse effect for the color yield.

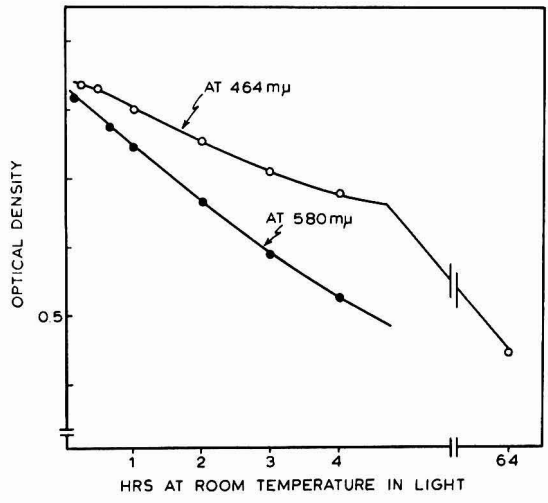

FIG. 2. Stability of ninhydrin color of ε-*N*-methyl-L-lysine: 0.8 and 0.4 μmole of ε-*N*-methyl-L-lysine were treated by the present and by Moore and Stein's method (7). The final volume of both cases is 8.0 ml. The OD of the color developed by the present method was determined at 464 mμ and by the latter method at 580 mμ with a Coleman spectrophotometer.

Effect of Buffer during Color Development. The pH of the final mixture (after adding 6.9 ml of water) was found to be between 3.5 and 3.8. However, when 0.1 *M* acetate or formate buffer (final concentration) at pH 3.8 was used in order to fix the pH during color development, this resulted invariably in the loss of the characteristic spectrum. In the presence of buffers, the ninhydrin color produced with ε-*N*-methyl-L-lysine gave a spectrum with a maximum absorption peak at 570 mμ. Providing the compound is dissolved in water, the result is very reproducible.

Sensitivity. The sensitivity of the present method in comparison with that of Moore and Stein is illustrated in Fig. 3. It can be seen in the figure that, when Moore and Stein's method was used for both L-leucine and ε-*N*-methyl-L-lysine, the color intensity does not differ significantly and the OD curve is completely linear. However, when ε-*N*-methyl-L-lysine was treated by the present method, the curve is not linear, indicating a disproportionality at the lower concentrations of the compound. In the straight region, the present method is 2.60 times less sensitive than the method of Moore and Stein. However, 0.05 μmole of ε-*N*-methyl-L-lysine can still be detected.

When different proportions of the two isomers α-*N*-methyl-L-lysine and ε-*N*-methyl-L-lysine are mixed and treated by the present method,

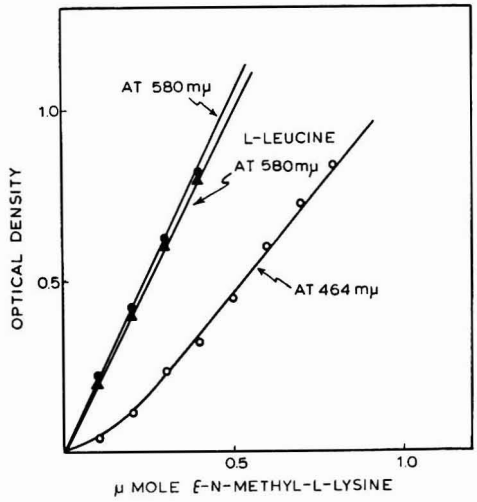

FIG. 3. Comparison of sensitivity of present method and that of Moore and Stein: The conditions are the same as in Fig. 2. L-Leucine was determined by Moore and Stein's method only.

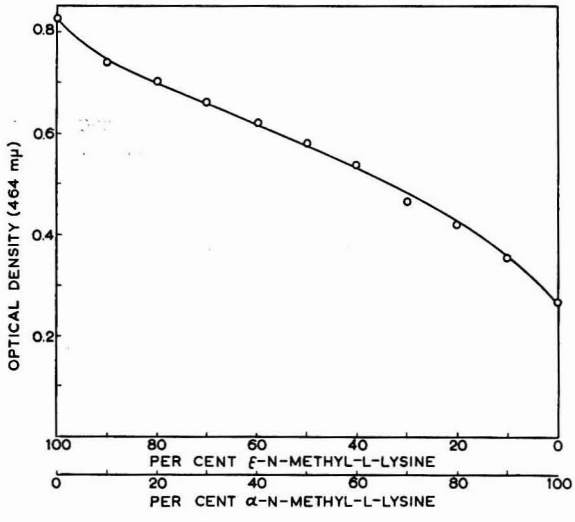

FIG. 4. Plot of $OD_{464\ m\mu}$ versus varying proportions of a mixture of α-*N*- and ϵ-*N*-methyl-L-lysine: The total amount of compounds in varying proportions of mixture was 1.0 μmole. The OD was read by Bausch & Lomb Spectronics 505. For experimental detail, see "Methods."

the curve illustrated in Fig. 4 is obtained. It can be seen that 1.0 μmole of pure α-*N*-methyl-L-lysine gives an OD of 0.26, and 1.0 μmole of ε-*N*-methyl-L-lysine, an OD of 0.83. Even though the curve in the figure is not straight between these two values, it is still obvious that it is quite possible to find a minimum of 5% contamination of the compound with

TABLE 2

PAPER CHROMATOGRAPHY OF NINHYDRIN-COLORED SUBSTANCE OF ε-*N*-METHYL-L-LYSINE PREPARED BY DIFFERENT METHODS

Method of ninhydrin reaction	Solvent system[a]	Ninhydrin spot			Remark	R_f of	
		R_f	Color	cpm		L-Lysine	ε-*N*-Methyl-L-lysine
Present method	Pyridine	0.78	Y	193			
	Ethyl acetate	0.96	Y	179			
	n-Butanol	0.88	Y	225			
	Propanol	0.87	Y	169			
	Formix	0.80	Y	188			
	Phenol	0.81	R	159			
Moore and Stein	Pyridine	0.54	V	35		0.22	0.31
		0.72	Y	132			
	Ethyl acetate	0.48	V	11	V spot appears	0.09	0.08
		0.90	Y	160	after spraying[b]		
	n-Butanol	0.65	V	48		0.34	0.36
		0.83	Y	196			
	Propanol	0.75	V	90		0.18	0.21
		0.90	Y	92			
	Formix	0.43	V	26	V spot appears	0.18	0.25
		0.84	Y	233	after spraying[b]		
	Phenol	0.64	V	98		0.44	0.67
		0.82	Y	184			

[a] Y = yellow. R = red. V = violet.

Solvent system	Composition
Pyridine	Pyridine:water:absolute alcohol (4:20:80)
Ethyl acetate	Ethyl acetate:formic acid:water (70:20:10)
n-Butanol	*n*-Butyl alcohol:acetic acid:water (50:25:25)
Propanol	70% propan-1-ol
Formix	*t*-Butyl alcohol:formic acid:water (70:15:15)
Phenol	Lower phase from mixture of phenol and water

[b] Sprayed with 0.1% ninhydrin in *n*-butyl alcohol.

In the Moore and Stein method, 0.18 ml ε-*N*-methyl-L-lysine (10 μmoles/ml), 0.02 ml radioactive amino acid [ε-*N*-(^{14}C-methyl)-L-lysine, 2 μmole/ml, and 29,480 cpm/0.01 ml], and 0.8 ml of water were boiled for 3 min with 1.0 ml of the reagent (7). In the present method, half the amount used in the previous case was treated by the method described. Thus, the final concentration of the ratioactive ε-*N*-methyl-L-lysine is the same. Instead of diluting with water, 0.010 ml of the sample was applied on Whatman No. 1 paper and the paper chromatogram developed at room temperature.

the other isomer; that is, 0.05 μmole of contamination of isomer can be detected as long as only two isomers exist in the mixture, and the total concentration is known.

Study of the Mechanism. According to the postulated mechanism of the ninhydrin reaction, the colored substance results from the combination of two molecules of reduced ninhydrin with ammonia arising from the amino acid. While liberating carbon dioxide, the α-amino acid becomes the aldehyde analog deprived of one carbon atom. Thus, except for proline and hydroxyproline, the color will be the same regardless of the nature of the rest of the molecule. However, published data (5) and the present results demonstrate that the ninhydrin color developed under specified conditions is very much dependent on the nature of the whole molecule. Thus, the colored compound produced from ϵ-*N*-(^{14}C-methyl)-L-lysine when treated according to the method described here or by the method of Moore and Stein, was subjected to paper chromatographic analysis. As shown in Table 2, when treated by the present method the yellow ninhydrin color produced moved together with the radioactivity, whereas, by Moore and Stein's method, the violet color (absorption peak at 580 mμ) did not coincide with that of the radioactivity. By the latter method, it is noted that a highly radioactive yellow spot was also separated on the chromatogram. The fact that treatment of ϵ-*N*-methyl-L-lysine with Moore and Stein's method resulted in two ninhydrin colors was also demonstrated from the spectrum. As shown in Fig. 5, even

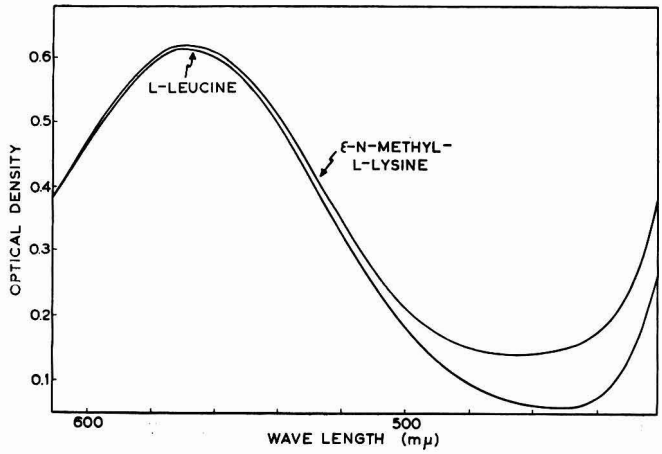

FIG. 5. Comparison of ninhydrin color of L-leucine and ϵ-*N*-methyl-L-lysine developed by the Moore and Stein method: 0.4 μmole each of L-leucine and ϵ-*N*-methyl-L-lysine was treated by the Moore and Stein method (7). Thus, the reaction was carried out in 0.05 *M* acetate buffer at pH 5.0. The final volume was 8.0 ml.

though the optical density at 570 mμ of L-leucine and ε-*N*-methyl-L-lysine is same, the optical density at 464 mμ is different with these two amino acids: the OD of ε-*N*-methyl-L-lysine is much higher than that of L-leucine. Thus, it can be said that some part of the amino acid molecule besides ammonia participates in the formation of this yellow color. Since even the one-carbon-less analog, δ-*N*-methyl-L-ornithine, does not give rise to this spectrum, it is possible that ε-*N*-methyl-L-lysine somehow forms an intermediate cyclic compound which then results in the formation of the characteristic color. It is a well-known fact that cyclic amino acids such as proline and hydroxyproline yield yellow colors, but no ammonia, when treated with ninhydrin by the method of Moore and Stein.

SUMMARY

When ε-*N*-methyl-L-lysine is treated with ninhydrin under specific conditions, the spectrum is very characteristic, having a maximum absorption peak at 464 mμ. Among the compounds tested so far, this amino acid is unique in this respect. Even though the method is 2.60 times less sensitive than that of Moore and Stein, the stability of the color is better. A minimum 0.05 μmole of this amino acid in a total of 1.0 μmole of mixture with α-*N*-methyl-L-lysine can be easily detected.

ACKNOWLEDGMENT

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A Micromethod for the Differentiation of Amino Acid-Specific sRNA Molecules

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Several chromatographic and extraction methods have been used to fractionate soluble RNA's[1] from various sources (1–4) and to demonstrate the species differences in amino acid-specific sRNA molecules (2, 5–7). We have been interested in comparing sRNA's from tumor and normal mammalian sources. Since the supply of material is often limited, it was necessary to devise micromethods for the aminoacylation reactions, the chromatography, and the detection of the specific aminoacyl sRNA's presented in this paper. The chromatography of aminoacyl sRNA's by these new thin-layer chromatographic techniques is fast and requires only microgram quantities of sRNA. Autoradiography is used to locate the C^{14}-labeled aminoacyl sRNA's after chromatography. Rat liver and P388 lymphocytic leukemia cells (8) were the sources of mammalian sRNA used. The method is not generally suitable for the separation and isolation of individual aminoacyl sRNA's, but should be useful in studies of the differences in aminoacyl sRNA's from one source, in specific aminoacyl sRNA's from different sources, in the effects of drug treatment, and in the use of heterologous activating enzymes.

MATERIALS AND METHODS

Preparation of sRNA and pH 5 Activating Enzymes

P388 Leukemia Cells. Seven days after an intraperitoneal inoculation of 10^6 P388 lymphocytic leukemia cells in BDF_1 mice, the P388 cells were collected and the sRNA and enzymes were extracted according to a modification of the method of Littlefield and Keller (9). The modifications were removal of the red blood cells by washing with Medium A minus sucrose and the carefully controlled homogenization of the cells in a Dounce homogenizer. The sRNA was phenol extracted from the pH

[1] Abbreviations: sRNA, soluble ribonucleic acid; CTP, cytidine triphosphate; ATP, adenosine triphosphate; PEP, phosphoenol pyruvate; CCD, countercurrent distribution.

5 precipitate of the 105,000 × *g* supernatant solution, while the pH 5 supernatant fraction, which contained the activating enzymes, was dialyzed against Medium A overnight.

Rat Liver sRNA and Enzymes. The livers from 150-gm Sprague-Dawley rats were excised, homogenized, and fractionated according to the procedure of Hoagland (10). Either a 25–85% or a 35–95% ammonium sulfate fraction of the pH 5 supernatant fraction was used as the activating enzyme. Centrifugation of several sRNA preparations in a sucrose density gradient revealed only a 4 *s* peak, indicating that the preparations contained no ribosomal RNA.

Preparation of C^{14}-Aminoacyl sRNA

Batches of sRNA were labeled with various amino acids by a micro version of the method of Stephenson and Zamecnik (1). Incubation mixtures contained 0.1 ml of lyophilized enzyme, 200 μg of sRNA, 20 μmoles tris-HCl buffer at pH 7.8, 28 μmoles KCl, 7.5 μmoles $MgCl_2$, 0.25 *M* sucrose, 0.25 μmole CTP, 5 μmoles ATP, 5 μmoles PEP, 25 μg pyruvate kinase, and about 0.44 μg of a uniformly labeled C^{14}-amino acid in a total volume of 0.5 ml. After a 10-min incubation at 30°C, the sRNA was extracted for 20 min with 1.5 ml of a solution containing 60% phenol and 40% 0.08 *M* tris-HCl at pH 7.8. The aqueous and phenol layers were separated by centrifugation at 700 × *g* for 30 min; then the sRNA was precipitated from the aqueous solution by addition of 0.1 vol of 2 *M* potassium acetate at pH 5 plus 2.5 vol of cold ethanol. Following a second alcohol precipitation, the sRNA was dialyzed. Recoveries were nearly quantitative when the incubation, phenol extraction, and centrifugation steps were all carried out in the same test tube. The sRNA consistently contained some adenine nucleotides as suggested by the absorbancy ratio of 260:280 greater than 2.0.

CTP, ATP, PEP, and pyruvate kinase were obtained from Calbiochem, uniformly labeled C^{14}-amino acids were obtained from New England Nuclear Corp., and other chemicals were the best grades obtainable.

Preparation of Plates for Thin-Layer Chromatography

The Desaga/Brinkmann spreading apparatus was used to apply a 500-μ coat of chromatographic medium on 200 × 200 mm carrier plates. Several adsorbents and methods of preparation of the adsorbents were used. For instance, carboxymethylcellulose TLC powder (Macherey-Nagel) and polyethyleniminecellulose TLC powder (Gallard-Schlesinger) were homogenized in a specifically loosened Potter-Elvejhem homogenizer to obtain an even suspension before spreading, while the substituted cross-linked dextrans, DEAE Sephadex A-50 fine and CM Sephadex A-50

fine (Pharmacia), were washed according to the supplier's directions before a water-saturated suspension was applied to the plates. Micronization of these types of Sephadex resulted in smoother looking plates and longer developing time, but no change in the R_f values of the sRNA molecules. All the plates were air-dried overnight because activation in the oven at various temperatures and times resulted in binding of aminoacyl sRNA at the origin. Since the addition of any buffer to the absorbent caused cracking after drying the plates and caused binding of the sRNA to the origin, the chromatographic media were spread in distilled water (pH 6).

Thin-Layer Chromatography

As many as eight aliquots containing 5–200 μg of C^{14}-aminoacyl sRNA (600–850 dpm) were spotted 3 cm from the bottom of a plate with cool air drying. Either the upper phase of Holley's (11) biphasic isopropanol/formamide/phosphate buffer at pH 6.2 (the phosphate solvent) or the lower phase of Zachau's (12) solvent system, *n*-butanol/water/tri-*n*-butylamine/acetic acid/di-*n*-butyl ether at pH 6.2 (the organic solvent) was used to separate the various sRNA's. Prior to chromatography, 140 ml of solvent was put into a rectangular developing tank (Desaga) which was tilted on its side and kept at room temperature. Equilibration was improved by putting a 15-cm filter paper saturated in solvent on the upper side of the tank. After the plate was inserted and propped up on a glass rod (1 cm in diameter), the tank was raised until the solvent came 1.5 cm up the plate. In this way, the plate was nearly horizontal and the chromatography time was minimized. The separation of sRNA took 2 hr on the Sephadex plates and 30 min on the cellulose plates. After chromatography the plates were air-dried overnight, then exposed to Kodak Medical X-ray film, No-Screen, for ten days (13). After the films were developed according to the directions from the supplier, the R_f's of the various sRNA's were calculated. In all systems used, free amino acids migrated in distinctive spots near the solvent front. There was no evidence that amino acids were hydrolyzed from the charged sRNA during chromatographic development.

Reproducibility. From two to seven preparations of each of the aminoacyl sRNA's from both sources were made, and samples from each of the preparations were chromatographed from two to six times. Reproducibility of the R_f's was very good in spite of the tailing. Neither the use of different preparations of amino acid activating enzymes nor variations in the amount of sRNA applied to the plates had any effect on the separations.

RESULTS AND DISCUSSION

Different combinations of solvents and chromatographic media have resulted in different degrees of separation of specific aminoacyl sRNA's and differentiation of apparent species of these amino acid-specific sRNA's. The number, pattern, and relative concentration[2] of these spots varied according to the chromatographic medium used. Most spots (apparent species) for various amino acid-specific sRNA's were obtained with the organic solvent with polyethyleniminecellulose, carboxymethylcellulose, or CM Sephadex plates.

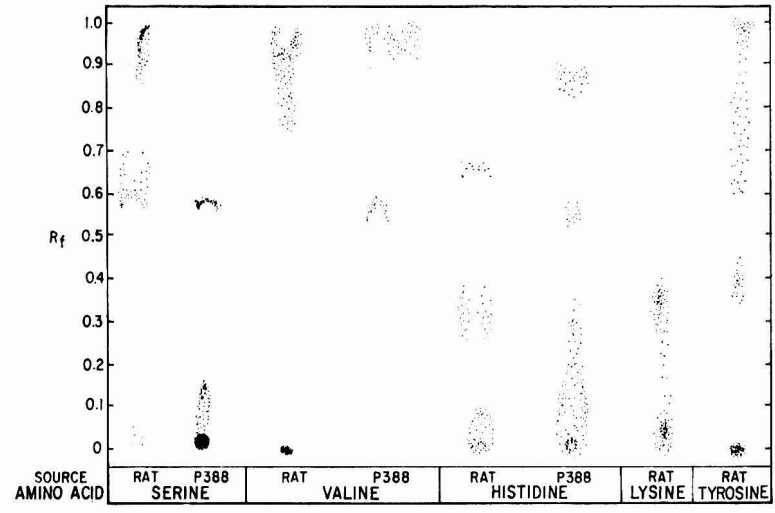


FIG. 1. Tracing of chromatogram of C^{14}-aminoacyl sRNA's on polyethylenimine-cellulose with organic solvent. Estimated from the general darkness of the spots on the autoradiogram, not from grain counts. See "Methods" for experimental details.

Figure 1 shows that the patterns of the R_f's of rat liver seryl, valyl, histidyl, lysyl, and tyrosyl sRNA's on polyethyleniminecellulose were significantly different from each other, although individual species of certain amino acid-specific sRNA's may have the same R_f. The precaution was taken in these studies to expose the x-ray films long enough for all possible components of the sRNA to be detected when 700–850 dpm was spotted. In addition to finding several apparent species for four of the five amino acid-specific sRNA's from rat liver shown here, the data of

[2] Estimated from the general darkness of the spots on the autoradiogram, not from grain counts.

Fig. 1 show distinct differences between the R_f's of the species of seryl sRNA from rat liver and P388 leukemia cells and differences in the relative concentrations of species of histidyl sRNA from rat liver and P388 leukemia cells.

The reproducible variations in concentration between the species of histidyl sRNA's from the two sources were also evident using carboxymethylcellulose and the organic solvent system (Fig. 2). The major spot

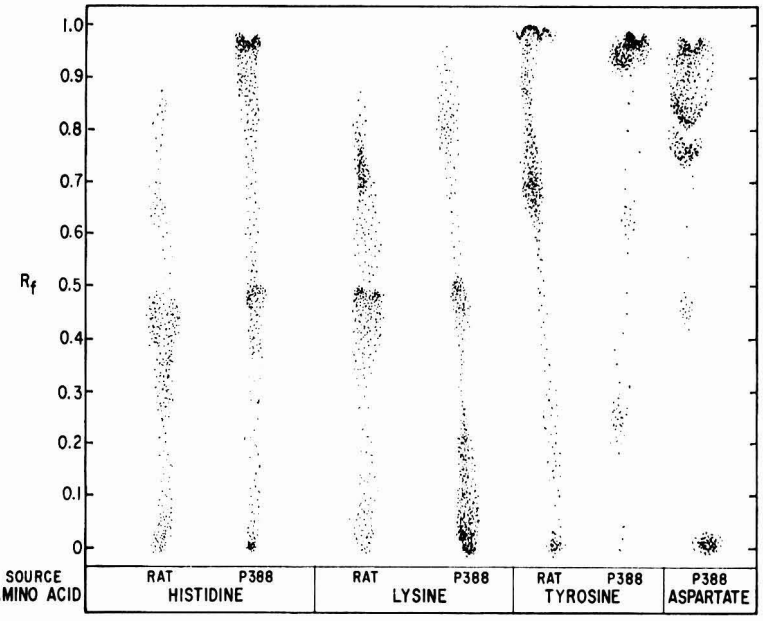
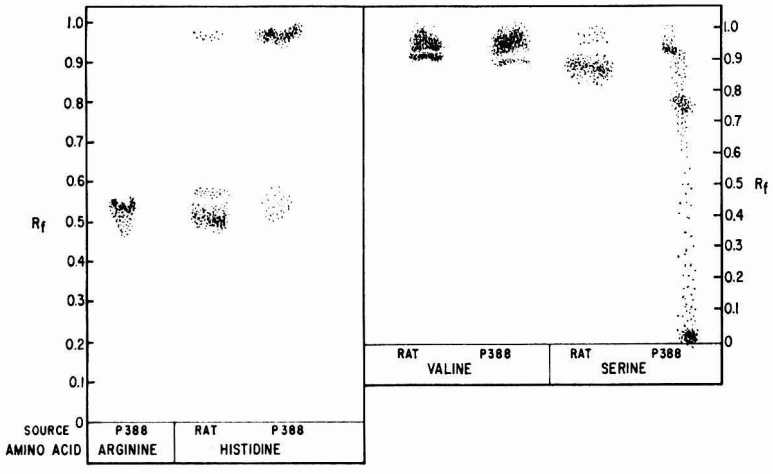


FIG. 2. Tracing of chromatogram of C^{14}-aminoacyl sRNA's on carboxymethylcellulose with organic solvent. Estimated from the general darkness of the spots on the autoradiogram, not from grain counts. See "Methods" for experimental details.

of the P388 histidyl sRNA was just behind the solvent front, while the major spot of the rat liver histidyl sRNA was near the middle of the plate. In this chromatographic system the lysyl sRNA's migrated as three spots with streaking in between, whereas, with polyethyleniminecellulose (Fig. 1), only two spots were seen. The organic solvent on carboxymethylcellulose was the only system studied thus far which revealed small differences between the two valyl (not shown) and tyrosyl sRNA's of rat liver and P388 cells.

The use of the phosphate solvent resulted in better separation of amino acid-specific sRNA's but less differentiation of species of particular sRNA's. Single, compact spots were observed on polyethylenimine-

cellulose of arginyl sRNA from P388 cells (Fig. 3) and lysyl sRNA's from both sources (not shown). Although the C^{14}-histidyl sRNA's from rat liver and P388 cells ran as two similar spots, the relative intensities



FIGS. 3 and 4. Tracings of chromatograms of C^{14}-aminoacyl sRNA's on polyethyleniminecellulose with phosphate solvent. Estimated from the general darkness of the spots on the autoradiogram, not from grain counts. See "Methods" for experimental details.

of the spots differed. Again, there was no apparent difference in migration of spots from the valyl sRNA's from the two sources, but there was a difference between the two seryl sRNA's (Fig. 4).

When the phosphate solvent was used with DEAE Sephadex plates, separation of certain specific sRNA's resulted despite the diffuse nature of the spots. In the composite chromatogram (Fig. 5), it is seen that of the ten aminoacyl sRNA's tested complete separation of the P388 seryl, lysyl, and either tyrosyl, alanyl, or aspartyl sRNA's was obtained. Complete separation of rat liver alanyl and leucyl or lysyl sRNA's was also obtained. Modifications of the phosphate solvent according to the more recent method of Holley might improve specific separations (14).

P388 arginyl and aspartyl sRNA as well as histidyl, lysyl, seryl, and tyrosyl sRNA from rat liver and P388 cells have been fractionated into two, three, or even four apparent species by using the organic solvent on polyethyleniminecellulose, carboxymethylcellulose, or CM Sephadex plates. We found the same number of species of rat liver lysyl and tyrosyl sRNA by using the organic solvent on polyethyleniminecellulose plates as Apgar did by using Holley's CCD method (3). Although there is a

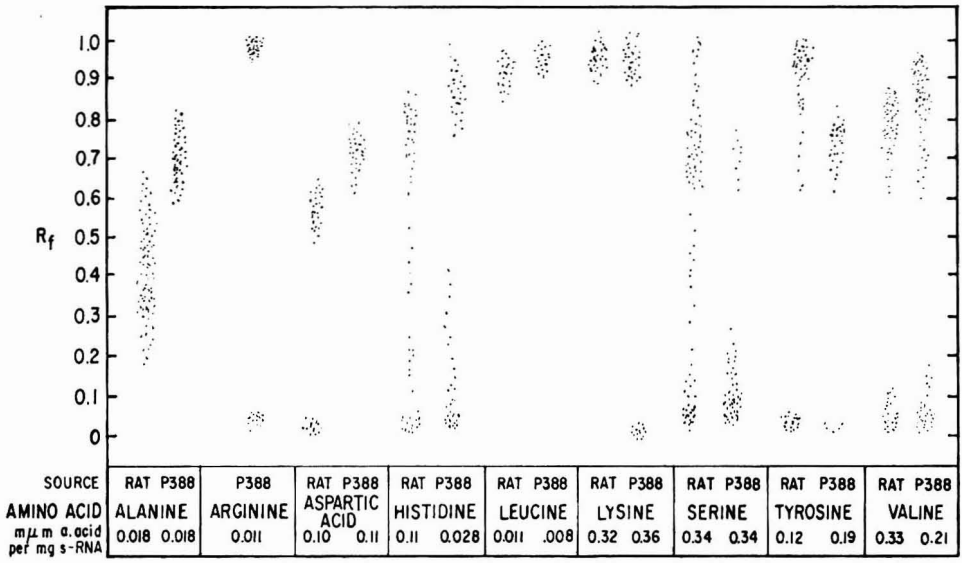


FIG. 5. Tracing of composite chromatogram of C^{14}-aminoacyl sRNA's on DEAE Sephadex with phosphate solvent. Estimated from the general darkness of the spots on the autoradiogram, not from grain counts. See "Methods" for experimental details.

growing number of reported species of sRNA's from various sources (6, 15–18), we have yet to prove that our results are due to species variations. This could be done by eluting the spots and testing for aminoacylation, coding properties, and ability to transfer the amino acids to ribosomes. Preliminary results indicate that it is feasible to use thicker layers for the chromatography of larger quantities of sRNA which will be required for these tests.

SUMMARY

A micromethod is described for the differentiation of amino acid-specific sRNA's by thin-layer chromatography. Specific rat liver and mouse P388 lymphocytic leukemia aminoacyl sRNA's were detected by autoradiography. Several spots were found for almost every aminoacyl sRNA chromatographed, and reproducible differences in chromatographic behavior were found between several amino acid-specific sRNA's from liver and leukemia cells.

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Identification and Estimation of Indole Analogs by Gas-Liquid Chromatography

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Gas-liquid chromatography (GLC) has not been employed extensively for the analysis of indole analogs. Lloyd *et al.* (2) examined the behavior of certain indole alkaloids on a GLC column, but the alkaloids are generally of more complex structure than those indole analogs which would be useful in studies of tryptophan biochemistry. Both Janak and Hrivnac (1) and Mossini and Vitali (3) employed somewhat higher temperatures, 200–225°C, and carrier gas-flow rates, 70 ml/min, the combination of which would not separate satisfactorily the simpler indole analogs we wanted to study.

As will be described elsewhere, some of the indole analogs employed are acceptable as substrates for the tryptophan synthetase of *Escherichia coli* and the product is the corresponding tryptophan analog. In most paper, column, and thin-layer chromatographic procedures tested, the tryptophan analogs and the indole analogs often behaved indistinguishably from the parent compound, and appreciably greater analysis time was required. GLC treatment of the indole analogs, as described below, provided sufficient separation to permit identification and quantitative estimations of the indole analogs. Consequently, tryptophan analogs to be analyzed may be converted to indole analogs by enzymic degradation with tryptophanase from *E. coli*, and subjected to GLC.

MATERIALS AND METHODS

Indole analogs were obtained commercially (Aldrich Chemical Co., Regis Chemical Co.).

Aqueous solutions were prepared by prolonged (when necessary) shaking of a suspension of the analog at 37°C. Usually, overnight shaking was sufficient to dissolve the suspended material. To avoid, insofar as possible, artifacts due to microbial contamination, sterile flasks were employed for the preparation of solutions of analogues.

GLC was performed in an Aerograph model 665 instrument fitted with

a hydrogen-flame ionization detector. A column, 5 ft × ⅛ in., of 5% silicone oil DC-550 on Chromosorb W, hexamethyldisilazane treated, 80/100 mesh (Wilkens Instrument and Research, Inc.) was maintained at a constant temperature as indicated, or, for identification purposes, was programed for a linear increase in temperature. The injector, with Pyrex liner, and detector temperatures were maintained at 215° and 156°C, respectively. The nitrogen carrier gas-flow rate was 15 ml/min, and the hydrogen flow rate was approximately 15 ml/min.

RESULTS AND DISCUSSION

Table 1 lists the retention times, relative to indole, of the indole analogs studied. Two columns, packed by different operators, were employed. Relative retention times were nearly identical in the two columns. A clear identification of the analog is possible under the conditions employed. The identification may be made more emphatic by preliminary extraction procedures to concentrate the indole solution. The toluene extract of an aqueous solution may be reduced to a small volume, or dried at low temperature (about 60°C), the residue dissolved in water, and the aqueous solution or the concentrated toluene solution subjected to GLC. The extraction procedure is not useful for quantitative estimation, owing to the appreciable volatility of most of the indole analogs. The toluene extract is not employed at lower temperatures owing to interference, by tailing of the toluene, in identification of the early emergent peaks.

TABLE 1
RELATIVE RETENTION TIMES FOR INDOLE ANALOGS

Analog	Soln.[a]	Column temp., °C	Relative retention time,[b] min
Indole	A	160	1.00
5-Fluoroindole	A	162	1.17
1,2-Dimethylindole	A	163	1.73
2,7-Dimethylindole	A	163	2.02
2,5-Dimethylindole	A	163	2.36
5-Chloroindole	A	162	3.24
5-Methoxyindole	A	164	3.54
5-Bromoindole	A	163	5.24
5-Methoxyindole	A	200	0.86
5-Methylindole	T	200	0.56
5-Aminoindole	T	200	1.47
5-Nitroindole	T	200	2.41
5-Cyanoindole	T	200	2.87

[a] Either aqueous (A) or toluene (T) solutions were employed.
[b] Indole retention time was 5.5 min.

A programed column temperature increase permits a more convenient identification routine. Figure 1 shows a typical analysis of a mixture of

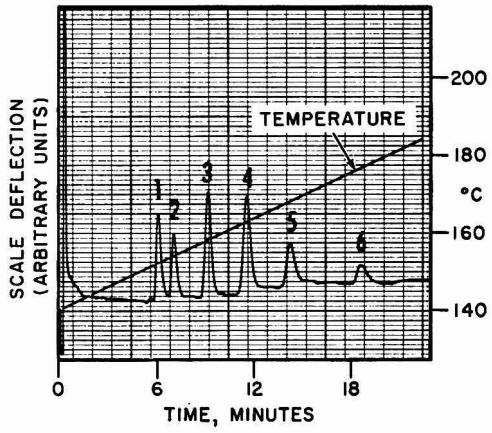

FIG. 1. Temperature programed gas-liquid chromatogram of indole analogs. A mixture of analogs was prepared in aqueous solution. The sample volume was 1.0 μl. Initial column temperature was 140°C. Immediately upon injection of the sample, the column temperature was increased linearly at the rate of 2°/min as shown. Peak identities, with retention times (min) are: (1) indole, 6.1; (2) 5-fluoroindole, 7.0; (3) 1,2-dimethylindole, 9.0; (4) 2,5-dimethylindole, 11.3; (5) 5-chloroindole, 13.9; (6) 5-bromoindole, 18.1.

analogs: they are well separated and form reasonably sharp peaks. The procedure is highly reproducible.

Figure 2 relates peak height to amount of indole in 1.0-μl samples. Similar relationships were obtained with 5-fluoroindole or 5-chloroindole. The GLC method provides a useful relationship for quantitative estimation of indole and indole analogs, providing uniform sample volume is employed and providing column temperatures are chosen such that the peak to be estimated emerges with less than 6-min retention time. The peak height measurement method is less reliable if the retention time is greater than about 6 min.

It is necessary to inject a constant sample volume to obtain reproducible results. The use of varying sample volume of a standard indole solution led to variable peak heights.

The relationship of retention time as a function of column temperature for the halogenated indole analogs is illustrated in Fig. 3.

The method described here provides a rapid and accurate estimation for indole and some of its analogs. The method has been employed in

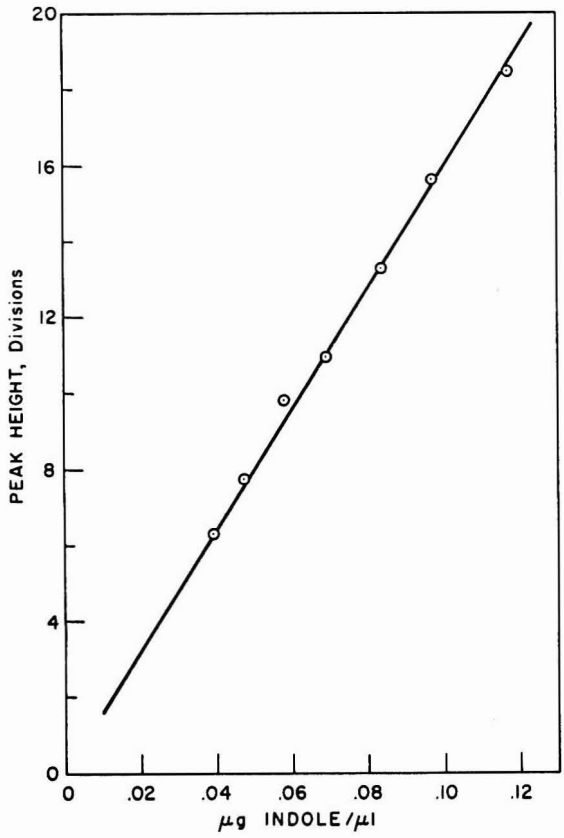

FIG. 2. Peak height as a function of concentration. Appropriate dilutions of indole in water were prepared such that a constant sample volume, 1.0 μl, could be injected. Column temperature was 160C.

this laboratory for following enzymic reactions involving either indole utilization or indole production. Samples of the entire reaction mixture may be injected directly, without treatment, with no apparent change in column characteristics after 1000 estimations, though it is, of course, advisable to replace the injector septum and the Pyrex injector liner periodically.

SUMMARY

The qualitative and quantitative estimation of indole and a series of simple indole analogs is described. The method may be employed for the direct estimation of indole analogs in enzyme reaction mixtures.

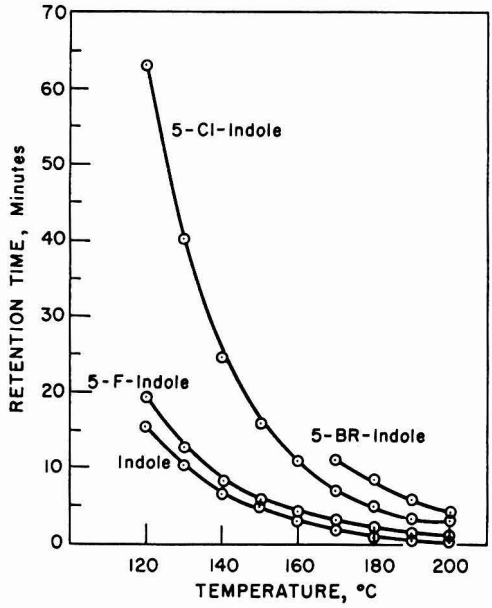

FIG. 3. Retention time as a function of column temperature. Aqueous 1.0-μl samples of a solution containing three analogs for temperature range 120°–160°C, or four analogs for temperature range 170°–200°C were injected. Retention time was calculated as interval between time of injection and time at which analog in question began to enter the detector and is the mean value for at least six trials at each temperature.

ACKNOWLEDGMENT

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ANALYTICAL BIOCHEMISTRY 13, 121–132

The Interpretation of Gel Electrophoresis

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There are two types of zone electrophoresis—those where the supporting medium is designed to interact as little as possible with the materials being separated, which approximate to electrophoresis in free solution, and those where an interaction is deliberately aimed at as an aid to resolution.

Starch gel electrophoresis was the first example of the latter type, and was shown by Smithies (1) to depend for its effectiveness on a molecular filtration effect. Large protein molecules are retarded relative to small molecules of similar charge. This has been used in an empirical way, and Smithies (2) has given an empirical treatment relating mobility to gel concentration and also to molecular size.

Recently (3) polyacrylamide gels were introduced as support media for zone electrophoresis. They give results very similar to those obtained with starch gels, but are much easier technically, and in particular can be prepared in a wider range of gel concentrations.

The simple relationships found empirically relating mobility to gel concentration over a narrow range do not hold over a wide concentration range, and we have developed a new theory to cover all gel concentrations. In addition a relationship between molecular size and mobility is discussed.

EXPERIMENTAL

Formation of Gels. The following method has been found most convenient for general purposes. Cyanogum 41 (B. D. H. Ltd) is a mixture of about 5% *N,N'*-methylenebisacrylamide and 95% acrylamide; it is dissolved in water to the required concentration and then to each 100 ml of solution 0.5 ml of 2-dimethylaminoethyl cyanide followed by 1.0 ml of freshly made 10% ammonium persulfate is added.

This mixture consistently gelled within 10 min in a sealed trough. Oxygen has a markedly inhibiting effect and must be excluded during gel formation. The initial solution of Cyanogum is stable for 24 hr but a fresh one is recommended. The gel was formed in a trough 8 × 14 cm and 2 mm deep, with a glass plate as lid. Four sample slots were formed at the same time by Perspex spacers 12 × 0.5 × 1.5 mm deep. It is important that the slots do not pass completely through the gel.

After gelation the gel slabs were removed from the mold and washed in a bath of the appropriate buffer for electrophoresis. Equilibration was fairly rapid and was complete in about 2 hr, though 24 hr with buffer changes was usually given. Formation of the gels in water has the advantage of standardization of the reaction, and the gel will not form in some electrophoresis media, particularly acidic ones. In addition, unpolymerized acrylamide should be removed by washing before electrophoresis.

After washing and equilibration, the gel was placed in a trough 8 × 14 cm and 2 mm deep and where necessary trimmed to size. The weaker gels swell during washing, introducing small errors into the nominal concentration. Swelling was less than 5% for a 5% gel and had almost disappeared at 7.5% gel strength. Other errors are introduced because acrylamide monomer is never fully converted to polymer and the extent of conversion varies with concentration.

Electrophoresis. Insertion of the sample is usually the most critical operation in zone electrophoresis. Generally, zones moving from a region of high mobility to a region of low mobility will automatically sharpen at the junction. The starting slot must therefore be made very cleanly and as straight as possible to utilize this effect as much as possible.

It was found that, if the starting slot passed through the gel, the protein gave very bad "bottom effects," and for this reason the slots were restricted in depth. No top effects have so far been observed, and slicing of the gel is not necessary (cf. starch gel).

Samples (0.01 ml) were applied without supporting medium and the gels run horizontally in a Shandon Universal tank. So far, no electrodecantation effects have been seen, and a vertical position apparently has no advantage. Filter paper wicks were used and the gel was open on top. Currents up to 25 mamp per gel had no ill effects.

Staining and Storage of the Gels. The dye solution used to find protein zones after electrophoresis has two functions. First the protein must be precipitated in the gel, preferably irreversibly. Most dye solutions use acid-alcohol for this, though it is not always effective. In this case alcohol caused marked shrinkage, opacity, and twisting of the gel. It was found, however, that glycerol controlled the effect of alcohol, and the following dye mixture was used successfully: 50 parts methanol, 50 parts water, 20 parts glycerol, 1 part glacial acetic acid, saturated (~1%) with naphthalene black (= amido schwartz 10B).

After staining for 30–60 min the gels were washed with dilute acetic acid until the background was clear. Overwashing is possible, and in one case the gel was successfully restained, proving the stability of the zones. Electrophoretic destaining was possible but not usually employed.

By using glycerol in the dye mixture it was possible to avoid changes

in the dimensions of the gels during staining and washing and the gels over the whole range of concentrations showed very little size variation. They were photographed at a standard magnification and the distance from the center of the zone to the starting slots measured on the prints with an estimated accuracy of ±3%.

It was found impossible to dry the gels satisfactorily—the method described by Wieme (4) was erratic. In the end the gels were stored wet in Metathene bags and have remained stable for 2 years. Some gels turned pink and the zones disappeared—they could, however, be restored by washing in dilute acetic acid.

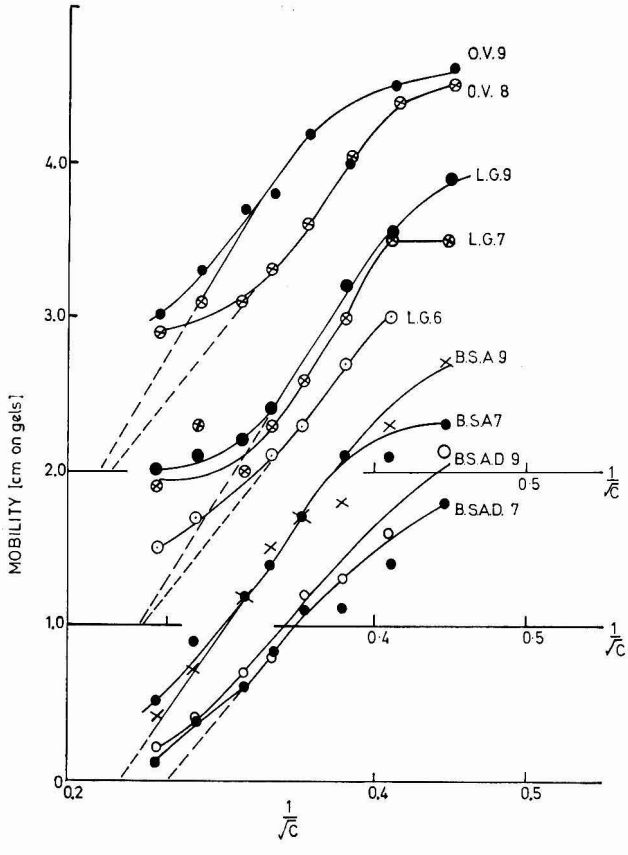

FIG. 1. Plots of mobility vs. $1/\sqrt{c}$ where c is gel concentration. The $1/\sqrt{c}$ axis is shifted for clarity. (O.V. 9) Ovalbumin at pH 9. (O.V. 8) Ovalbumin at pH 8. (L.G. 9, L.G. 7, L.G. 6) β-Lactoglobulin at pH 9, 7, and 6. (B.S.A 9, B.S.A 7) Bovine serum albumin at pH 9 and 7. (B.S.A.D 9, B.S.A.D 7) Bovine albumin dimer at pH 9 and 7. For further details see text.

RESULTS AND DISCUSSION

Polyacrylamide gels, unlike starch, can easily be prepared in a wide range of concentrations. It thus becomes feasible to study the way in which mobility varies with concentration when pH and all other variables are kept constant.

Some experiments with ovalbumin, bovine serum albumin, and β-lactoglobulin are shown in Fig. 1. These proteins were run in phosphate buffers in gels varying from 5 to 15% acrylamide. In Fig. 1 the mobility M, is plotted against $1/\sqrt{c}$, and in Fig. 2 some results are shown for M plotted against $1/c$. Clearly an inverse function of c is required. $1/\sqrt{c}$ was chosen because, according to Raymond and Nakamichi (5):

$$\bar{p} = kd/\sqrt{c} \tag{1}$$

where $\bar{p}$ is the mean pore size, c is the gel concentration, d is the diameter of the strands making up the gel structure, and k is a constant depending

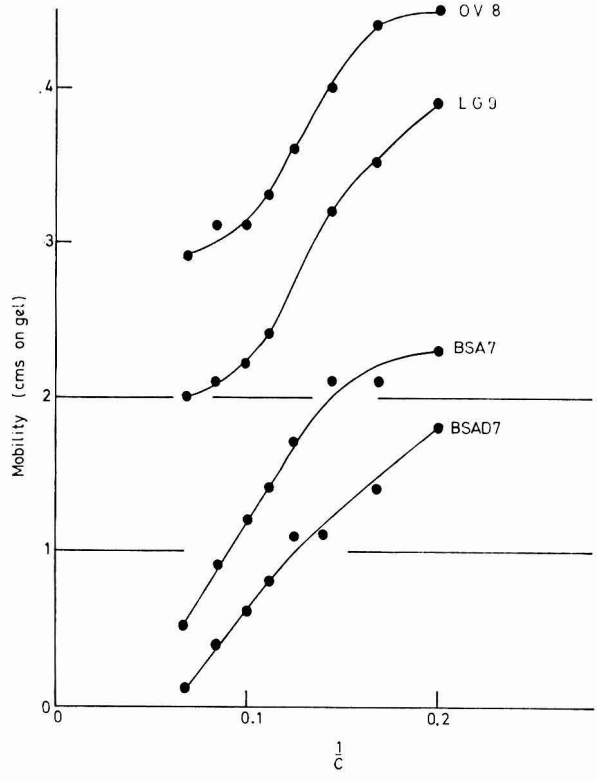

FIG. 2. As for Fig. 1, except that mobility is now plotted against $1/c$.

on the way they cross. On the other hand, empirically $1/c$ has been used (2) and one approach suggests that:

$$\bar{p} = (k'd/c) + k'' \tag{2}$$

where k' is a constant, and d and c are the same as above.

Whichever relationship is chosen, it is clear that the mobility is not proportional to the mean pore size of the gel over the whole concentration range. There is curvature at both high and low gel strengths. It is easy to see that in, for example, a very weak gel where the molecule is effectively unretarded this must be so. Equally, even in very strong gels there will always be some pores that can pass the protein molecule and the mobility will never drop completely to zero.

Suppose, rather, that the mobility is proportional to the proportion of pores larger than a certain limiting size. It is easy to see that, while a large molecule might migrate the same total distance as a small one, its progress through the gel must be retarded because, in order to find sufficiently large pores, it will be forced to zigzag much more than a small molecule.

By making the assumption that the distribution of pore sizes in the gel is Gaussian, and that the standard deviation of the distribution does not vary significantly with gel strength, it can be shown that the proportion of pores greater than a certain limiting size P_L is given by the integral:

$$\int_{(P_L-\bar{p})/\sigma}^{\infty} \frac{1}{\sqrt{2\pi}} e^{-(1/2)p^2} dp = Q\left(\frac{P_L - \bar{P}}{\sigma}\right) \tag{3}$$

where Q is a standard normal probability integral (6). Putting the mobility proportional to this:

$$\frac{M}{D} = Q\left(\frac{P_L - \bar{p}}{\sigma}\right) \tag{4}$$

where D is the mobility at zero gel concentration and can be estimated from the mobility at very low gel concentrations. Since we take σ as constant we can either use Eq. (1) for $\bar{p}$ to obtain:

$$\frac{M}{D} = Q\left(\alpha + \frac{K}{\sqrt{c}}\right) \tag{5}$$

or equation (2) for $\bar{p}$ when:

$$\frac{M}{D} = Q\left(\alpha + \frac{K}{c}\right) \tag{6}$$

where α and K are constants.

These equations can be tested by plotting the appropriate function of c against M/D on arithmetical probability paper, which is linear in Q. The

results of such plots for arachin monomer and dimer and a conarachin component (7), together with some of the data from Figs. 1 and 2, are shown in Figs. 3 and 4. In both cases the points fall on or near straight lines and

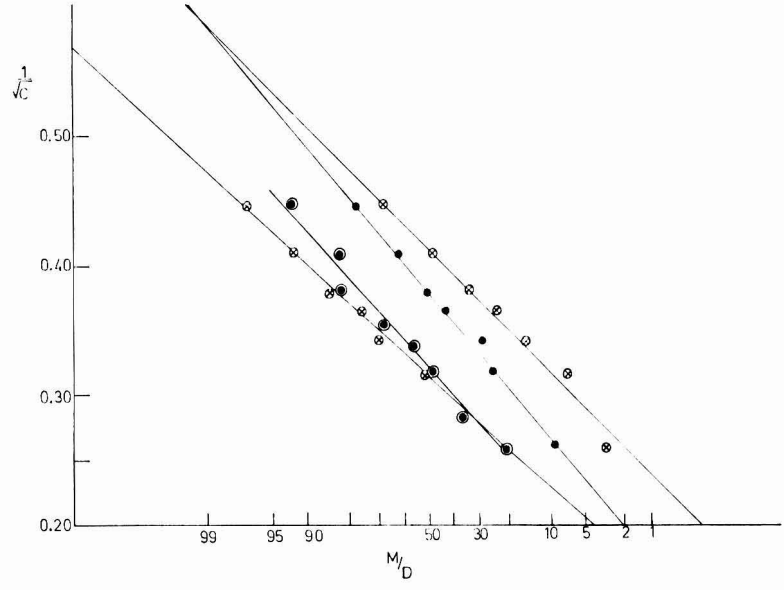

FIG. 3. Plots of M/D against $1/\sqrt{c}$ on arithmetical probability paper for: (⊗) arachin dimer, (●) arachin monomer, (⊙) ovalbumin, (⊗ to the left) β-conarachin.

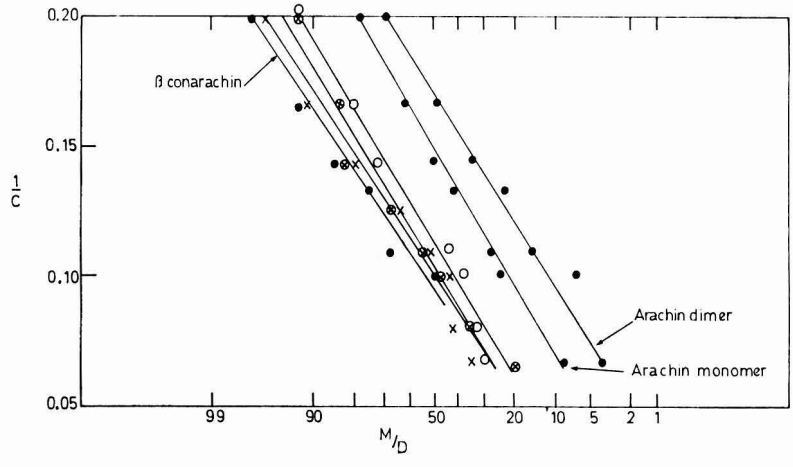

FIG. 4. As for Fig. 3, except that M/D is now plotted against $1/c$: (×) ovalbumin, (⊗) albumin, (○) β-lactoglobulin.

it appears that this sort of plot cannot distinguish between the two possible relationships for mean pore size. The linearity of the plot was much more sensitive to the value of D selected than to the function of concentration chosen. However the results are consistent with either Eq. (5) or (6), both of which correctly predict the sigmoid relationship seen in Figs. 1 and 2. It is emphasized that this result shows no more than consistency and does not prove that the model is correct.

Limiting Pore Size. The concept of a limiting pore size, which the protein molecule cannot quite enter, was introduced in obtaining Eq. (4). Now when $M/D = 0.5$, the limiting pore size must be equal to the average pore size, i.e.:

$$(\bar{p})_{M/D=0.5} = P_L \tag{7}$$

If the protein molecules are taken as roughly spherical, then the volume of the molecule:

$$V = \frac{4}{3}\pi\left(\frac{P_L}{2}\right)^3 \tag{8}$$

and the molecular weight would be expected to be approximately proportional to $(P_L)^3$.

Now the gel concentration at $M/D = 0.5$ can be found, and using Eq. (1) or (2), to relate this to the average pore size, the molecular weight (M.W.) should be approximately proportional either to:

$$\text{M.W.} = K\left(\frac{1}{\sqrt{c}}\right)^3_{M/D=0.5} \tag{9}$$

from Eq. (1) or to:

$$\text{M.W.} = K\left(\frac{1}{c}\right)^3_{M/D=0.5} \tag{10}$$

from Eq. (2).

Figures 5 and 6 show the data from Figs. 1 and 2, together with data for arachin and glycinin components obtained in exactly the same way. It is clear that the results fit Eq. (10) much better than (9), suggesting that Eq. (2) and hence $1/c$ are the appropriate functions of concentration.

It is not easy to calculate the true relationship between gel concentration and pore size. Raymond and Nakamichi (5) assumed a model in which the gel strands crossed at right angles, but appear to assume at one stage in their calculation that the pore size is very large compared with the gel strand diameter. If this assumption is not made, a different relation is obtained. Thus, consider a cube of gel, of side l, and volume l^3. Suppose

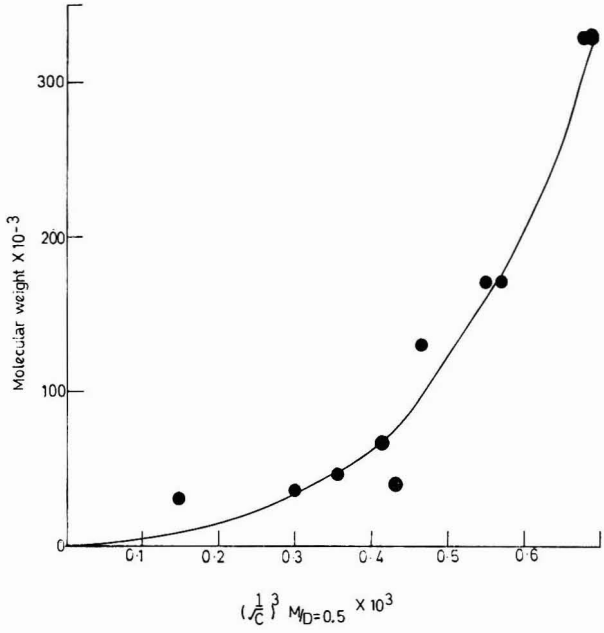

FIG. 5. Molecular weights vs. $(1/\sqrt{c})^3$ at $M/D = 0.5$. Note marked curvature.

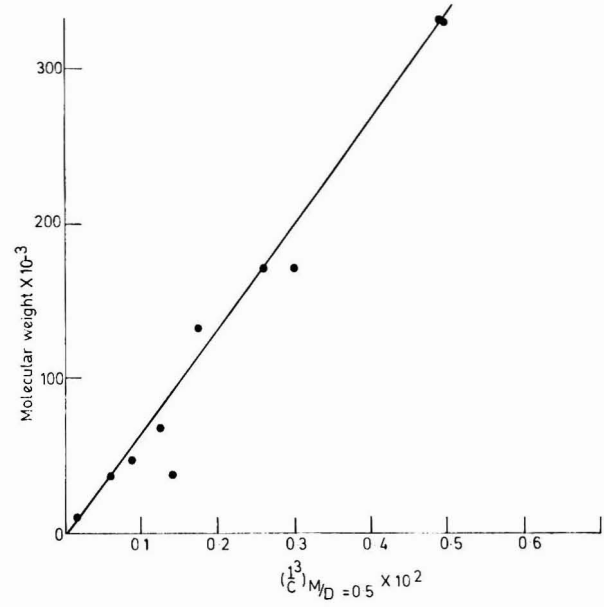

FIG. 6. Molecular weights vs. $(1/c)^3$ at $M/D = 0.5$. Note linearity of the plot and its passage through the origin.

there are n strands in each direction, so that there are $3n^2$ strands making up the mesh:

$$l = nd + (n - 1)p$$

and since n is very large, $n - 1 \doteq n$, and:

$$l = nd + np$$

The total volume of the gel strands is:

$$3n^2 l\pi(0.5d)^2$$

assuming them to be cylindrical, and the volume concentration is:

$$c = 3n^2 \frac{(nd + np)\pi(0.5d)^2}{(nd + np)^3}$$

This simplifies to:

$$c = \frac{3\pi(0.5d)^2}{(d + p)^2}$$

and:

$$p + d = kd/\sqrt{c} \tag{11}$$

which is not the same as Eq. (1) from a similar model, though if d is assumed to be very small compared with p, it approximates to it.

Ornstein recently published a similar derivation (8) and Ogston has obtained an analogous result from a random mesh (9).

Other models give rather different results—consider, for example, a sheet of gel made up of n parallel strands crossing another set of n parallel strands at right angles. It can be shown that, for such a sheet:

$$p + d = kd/c \tag{12}$$

and if the gel were made up of such sheets:

$$\bar{p} = (k'/c) - k''$$

which is the relationship between pore size and concentration suggested by the experimental data, although it is very unlikely that this is the actual structure of the gel.

Ornstein (8) has described a complex gel structure which could be employed to explain our results. This involves, however, the assumption that the gel structure changes as its concentration changes.

It is difficult to explain our results unless some structure such as Ornstein's is used, but it would appear that no useful calculations of the relationship between average pore size in the gel and volume concentration of the gel are possible at present. Since the pore size appears to be the major

factor determining mobility, a much clearer knowledge of the gel structure at all concentrations remains the key to further understanding.

Measurement of P_L. It is possible to make some calculations of the value of P_L from the molecular weights of the proteins, though these of necessity are very approximate. If the molecule is assumed spherical, and the density of protein is taken as 1.3, then

$$\text{M.W.} = \rho N \left(\frac{4}{3}\right) \pi \left(\frac{P_L}{2}\right)^3 \times 10^{-24}$$

where N is Avogadro's number and P_L is in Å, and:

$$P_L = 2\left(\frac{\text{M.W.}}{3.28}\right)^{1/3} \tag{13}$$

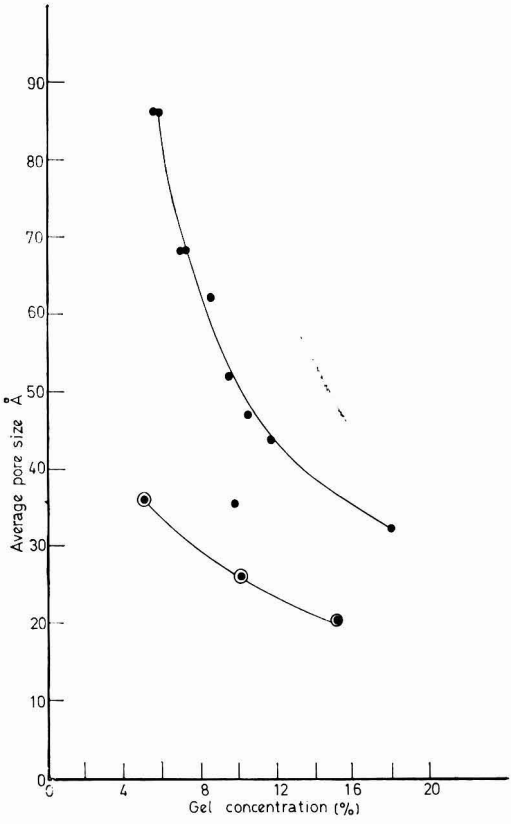

FIG. 7. Estimates of average pore size, calculated from molecular diameters (●). Data of White (10) (⊙). For further explanation see text.

By equating P_L with the average pore size at a gel concentration where $M/D = 0.5$, the curve shown in Fig. 7 is obtained.

White (10) has calculated pore sizes from the water permeability of these gels, making a number of assumptions. Some of his data are shown in Fig. 7. It is clear that his values are much lower than those found in this work, and Raymond and Nakamichi (5) have already pointed out that they cannot account for the permeability toward large protein molecules. However, White (10) suggests that errors may make his results too low, and that the error is likely to exceed 25%. It will be seen that one point in Fig. 7, that due to β-lactoglobulin, also lies well away from the curve and since our calculations also involve considerable approximations the discrepancy is not surprising. They are in better agreement with a calculation of Ornstein suggesting 50 Å pores at 7.5% (8).

We may conclude that the way in which mobility in gels varies with gel concentration is consistent with the hypothesis that mobility is proportional to the proportion of pores larger than a limiting size, and can be accounted for solely on the basis of a pore-filtration mechanism. The limiting pore size is determined by the size of the molecule and a simple relationship (Eq. 10) relating molecular weight, gel concentration, and mobility has been found.

SUMMARY

1. A method of polyacrylamide gel electrophoresis is described and the way in which mobility varies with gel concentration examined.

2. A relationship between mobility and the distribution of pore sizes in the gel is derived and fitted to results obtained with various globulins.

3. A constant, the limiting pore size, is derived and related to the molecular weight of the species. Some estimates of pore sizes in the gels are also calculated.

ACKNOWLEDGMENT

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ANALYTICAL BIOCHEMISTRY 13, 133–142

Effect of Acetaldehyde, Acetic Acid and Ethanol on the Resorcinol Test for Fructose[1]

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The color reaction of ketoses with resorcinol in the presence of hydrochloric acid is commonly known as the Seliwanoff test for ketoses (1). This test is the basis for numerous quantitative procedures for determining fructose (2). In the course of using the resorcinol test to determine 3,6-anhydrogalactose (3), we observed that the results obtained within some procedures varied greatly. Preliminary investigations employing fructose indicated this to be due to the use of ethanol and acetic acid in these procedures. Other workers have also attributed variations in their results to the use of these solvents (4–6). Although these solvents are used in almost all procedures, their effects on the color reaction have never been thoroughly examined. Roe (7, 8) recommended their use, merely stating that they had a hyperchromic effect.

In a preliminary communication (2) we described a procedure designed to study the effect of impurities in commercially available acetic acid and ethanol on the absorption spectrum of the product formed in the resorcinol test for fructose. When commercial acetic acid or ethanol was used in this procedure the wavelength of maximum absorption (λ_{max}) of the colored product was at 555 mμ. However, when purified acetic acid or ethanol was used, as well as when both of these compounds were excluded, λ_{max} was at 480 mμ. Purified acetic acid or ethanol to which a trace of acetaldehyde had been added behaved in the same manner as the commercial solvents. At the time we postulated that acetaldehyde was responsible for the spectral shift observed and in this paper we show that in the sample of acetic acid examined acetaldehyde alone was responsible for this spectral shift. The main purpose of this paper is to describe the effect on several resorcinol test procedures of concentration changes in acetaldehyde, purified acetic acid, and purified ethanol.

[1] Issued as NRC No. 8689.

EXPERIMENTAL

Materials and Reagents

The chemicals used are described elsewhere (9).

The 2,4-dinitrophenylhydrazine reagent was a saturated solution in 2 *N* HCl (10).

Standards. A stock solution of *fructose* was prepared by dissolving 27 mg (0.15 mmole) in 50 ml of a saturated aqueous solution of benzoic acid; standard solutions were prepared by diluting the stock solution with distilled water. Stock solutions of *1,1*-diethoxyethane were prepared by weighing 0.822 gm (6.96 mmoles; about 1 ml) in a 100-ml volumetric flask and diluting immediately to volume with distilled water, with ethanol, or with acetic acid. This was the only practical method found to prepare stock solutions of 1,1-diethoxyethane which gave reproducible results. Dilutions were made with the appropriate solvents.

Determination of Fructose

The spectrophotometric determinations of fructose were performed using either procedure A, which we described earlier (2), or procedure B, which is a modification of the method of Roe and Papadopoulos (11). Unless it is specified otherwise, all determinations were carried out using 0.18 μmole of fructose per test. When 1,1-diethoxyethane was included in a test mixture, 0.21 μmole per test was used unless otherwise mentioned.

Absorbance measurements were made with a Beckman DK-2 spectrophotometer using a 10-mm light path, or, where specifically noted, with a Coleman Junior spectrophotometer using 19-mm round cuvets.

Purification of Acetic Acid and Ethanol

Criterion of Purity. Procedure A was used to establish a criterion of purity. Acetic acid or ethanol was considered pure when the absorbance at 555 mμ of test mixtures containing 8% acetic acid or 8% ethanol was less than 10% larger than the absorbance of test mixtures without these solvents.[2] In such a case λ_{max} was always at 480 mμ.

Purification of Acetic Acid. The specimens of glacial acetic acid used were Eastman Organic Chemicals #763 (99.7+% acetic acid) and British Drug Houses Laboratory Reagent (lot 20363, 99.5+% acetic acid). Each was purified by fractional distillation. The statement by Hers *et al.* (5) that the impurity responsible for the absorption band at 555 mμ could

[2] See reference 2, figure 1, for absorption spectra of test mixtures containing 0 and 8% purified acetic acid.

not be removed from acetic acid by fractional distillation conflicts with our results, and can only be attributed to a less efficient distillation.

Purification of Ethanol. Absolute ethanol (Consolidated Alcohols Limited, Toronto, Canada) was purified by refluxing with 2,4-dinitrophenylhydrazine and 85% orthophosphoric acid.

Concentration and Identification of Acetic Acid Impurity

Method of Concentration. A 500-ml three-necked round-bottom flask was fitted with a gas dispersion tube (coarse fritted disk), a Graham condenser (30-cm jacket length), and a stopper. Water at 40° was circulated through the jacket of the condenser. The outlet of the condenser was connected to a U-shaped trap maintained at —20°. The outlet of the —20° trap was connected to a spiral trap which was packed near the outlet with a 5-cm plug of glass wool. The spiral trap, including the portion packed with glass wool, was bathed in liquid air. A micro gas washing bottle containing 3 ml of 2,4-dinitrophenylhydrazine reagent solution was connected to the outlet of the liquid air trap. Acetic acid (250 ml of E.O.C. #763) was placed in the round-bottom flask and refluxed for 4 hr while dry, purified nitrogen was passed through at a rate of 120 ml/min. Most of the material removed (about 4 ml) consisted of acetic acid and was in the —20° trap. The volume of condensate in the liquid air trap was about 50 μl. The 2,4-dinitrophenylhydrazine reagent solution remained clear. The condensate in each trap was used in carrying out the resorcinol test (procedure A). These tests showed that the 4-hr purge was sufficient to ensure complete removal from the commercial acetic acid of the impurity responsible for the spectral shift. The bulk was in the liquid air trap with a minute amount in the —20° trap. There was no loss of this impurity from the apparatus during the concentration process since the amount found in the combined —20° and liquid air traps was the same as in the original sample of commercial acetic acid.

Gas-Liquid Partition Chromatography of Liquid Air Trap Condensate. An F & M model 500 gas chromatograph was used to separate the liquid air trap condensate into 5 fractions on a column packed with 5% (w/w) Carbowax 1540 on Chromosorb W (60-80 mesh). Only fraction 1 (the fraction with the shortest retention time) shifted λ_{max} to 555 mμ when each of the 5 fractions from 2.9 μl of condensate was used separately in procedure A and afforded a precipitate when each of the 5 fractions from 4.3 μl of condensate was tested with 2,4-dinitrophenylhydrazine reagent. Fraction 1 was indistinguishable from acetaldehyde by gas chromatography.

Preparation of 2,4-Dinitrophenylhydrazine Derivative of Impurity.

A yield of 6.7 mg of acetaldehyde 2,4-dinitrophenylhydrazone was obtained from the liquid air trap condensate from 500 ml of acetic acid (E.O.C. #763). From a plot of absorbance at 555 mμ vs. concentration of acetaldehyde in purified acetic acid similar to the plot shown in Fig. 1, the amount of acetaldehyde in the commercial acetic acid used was estimated to be 63 mμmole/ml on the assumption that acetaldehyde was the only impurity present in that sample of commercial acetic acid responsible for the shift to 555 mμ. The theoretical yield of acetaldehyde 2,4-dinitrophenylhydrazone from 500 ml would thus be 7.06 mg and the yield obtained was 95% of theoretical.

RESULTS AND DISCUSSION

The absorption spectra shown in Fig. 2 illustrate the shift in λ_{max} from 480 to 555 mμ when acetaldehyde is added to test mixtures. In the single brand of acetic acid examined we found acetaldehyde to be the only impurity responsible for this spectral shift. Since acetaldehyde is an impurity in commercial acetic acid and ethanol prepared by present methods (12), and since these solvents are widely used in the resorcinol test, we undertook to study the effect of acetaldehyde, ethanol, and acetic acid on this color reaction.

1,1-Diethoxyethane was used, rather than acetaldehyde, because it is less volatile and more stable at room temperature and is readily hydrolyzed to acetaldehyde by an acid catalyst. With procedure A we found that equimolar amounts of 1,1-diethoxyethane and acetaldehyde afforded identical results.

The change in absorbance with increasing concentration of 1,1-di-

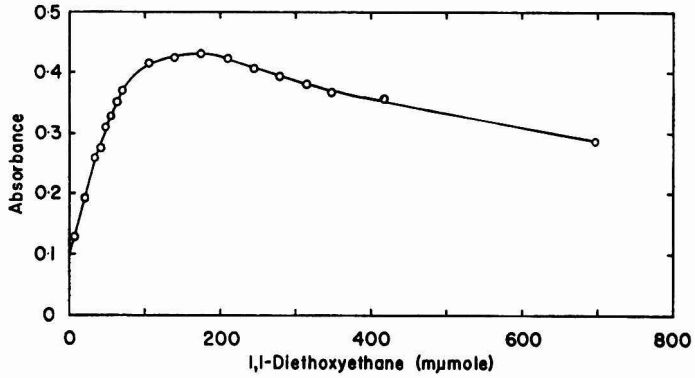

FIG. 1. Change of absorbance at 555 mμ with concentration of 1,1-diethoxyethane (mμmole/test) in resorcinol test for fructose carried out according to procedure A, in the absence of organic solvent. Absorbance measured with Coleman Junior spectrophotometer.

ethoxyethane is shown in Fig. 1. Maximum absorbance was reached when equimolar amounts of fructose and 1,1-diethoxyethane were present. From a study of the relationship between absorbance and fructose concentration for several concentrations of 1,1-diethoxyethane we concluded that Beer's law holds for the determination of fructose at 555 mμ so long as the 1,1-diethoxyethane concentration equals or exceeds that of fructose.[3]

The effect of purified ethanol or purified acetic acid on the resorcinol test for fructose carried out according to procedure A is negligible when compared with the effect of acetaldehyde. This is due to the high hydrochloric acid concentration used. For example, the incorporation of 8% ethanol or 8% acetic acid in test mixtures increased the absorbance at 480 mμ in the absence of 1,1-diethoyxethane by 8.5% or 10%,[2] respectively. On the other hand, the incorporation of 1,1-diethoxyethane in test mixtures increased the absorbance at 555 mμ in the absence of ethanol and acetic acid by 690% (Fig. 2). There was a further increase at

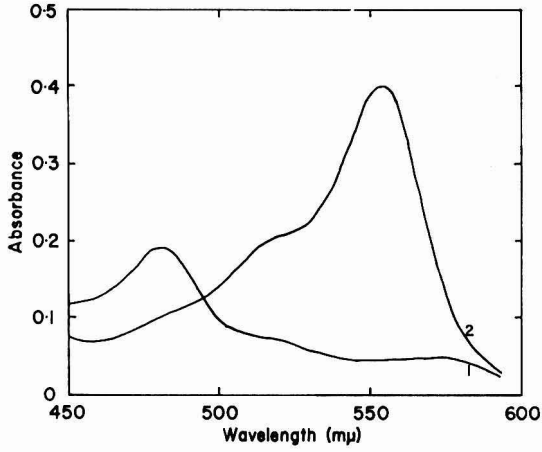

FIG. 2. Absorption spectra of colored product formed in resorcinol test for fructose carried out according to procedure A, in the absence of organic solvent: (1) without acetaldehyde; (2) with 0.21 mμmole acetaldehyde in test mixture.

555 mμ of 13 or 17%, respectively, when the ethanol or acetic acid concentration in test mixtures containing 1,1-diethoxyethane was 8%.

When the concentration of hydrochloric acid in the resorcinol test is decreased from 9 to 7 *N*, as in going from procedure A to procedure B, there is an increase in the effect of ethanol and acetic acid on the

[3] An example is given in reference 9.

TABLE 1
PROCEDURE B—COMPOSITION OF TEST MIXTURES[a]

Concentration in test mixture		Volume of reagents[b] (ml)					
Purified acetic acid[c] (%)	1,1-Diethoxy-ethane (μmole)	Resorcinol reagent[d] in purified acetic acid[c]	1,1-Diethoxy-ethane in purified acetic acid[c]	1,1-Diethoxy-ethane in water	Resorcinol reagent[d] in water	Purified acetic acid[c]	Glass distilled water
30	—	1	—	—	—	2	—
30	0.21	1	1	—	—	1	—
20	—	1	—	—	—	1	1
20	0.21	1	1	—	—	—	1
10	—	1	—	—	—	—	2
10	0.21	1	—	1	—	—	1
0	—	—	—	—	1	—	2
0	0.21	—	—	1	1	—	1

[a] Time of heating at 80° was 10 min. Absorption spectra are shown in Figs. 3 and 4.
[b] Test mixtures contained 6 ml of concentrated hydrochloric acid and 1 ml of a solution of fructose. The total volume of each test mixture was 10 ml.
[c] Purified ethanol was substituted for acetic acid when required.
[d] Preparation of the resorcinol reagent was described by Roe and Papadopoulos (11).

test while the effect of acetaldehyde decreases. However the most conspicuous effect encountered in procedure B is neither the effect of acetaldehyde nor the effect of ethanol or acetic acid but the *combined effect of acetaldehyde plus ethanol or acetic acid.* The composition of test mixtures used in procedure B are shown in Table 1. Figures 3 and 4

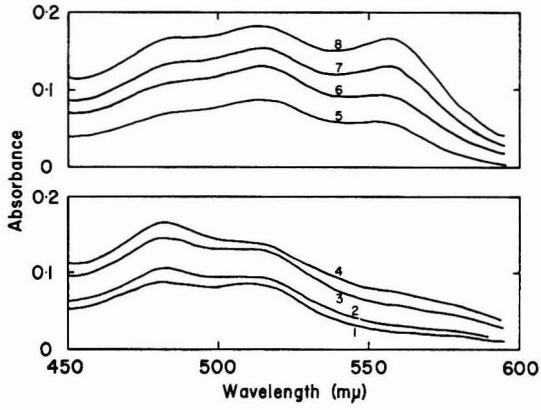

FIG. 3. Study of effect of increasing the concentration of purified ethanol in procedure B. Absorption spectra in lower half were obtained from test mixtures in which 1,1-diethoxyethane was excluded: (1) 0% ethanol; (2) 10%; (3) 20%; (4) 30%. Absorption spectra in upper half were obtained from test mixtures in which 1,1-diethoxyethane was included: (5) 0% ethanol; (6) 10%; (7) 20%; (8) 30%.

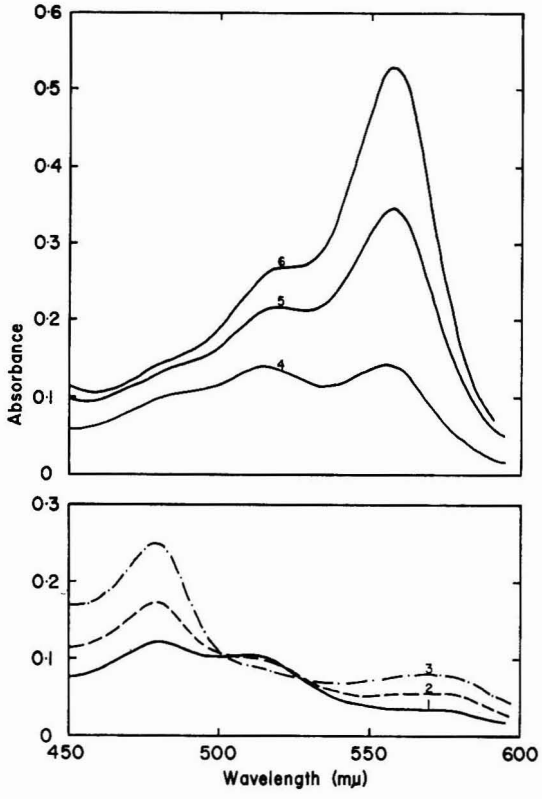

FIG. 4. Study of the effect of increasing the concentration of purified acetic acid in procedure B. Absorption spectra in lower half were obtained from test mixtures in which 1,1-diethoxyethane was excluded: (1) 10% acetic acid; (2) 20%; (3) 30%. Absorption spectra in upper half were obtained from test mixtures in which 1,1-diethoxyethane was included: (4) 10% acetic acid; (5) 20%; (6) 30%.

show the absorption spectra of the colored products obtained using a 0 to 30% range of ethanol and acetic acid concentration, with or without added 1,1-diethoxyethane. In the absence of 1,1-diethoxyethane, the absorbance at 480 mμ was increased by 85% as the ethanol concentration increased from 0 to 30% (Fig. 3, curves 1 to 4). The effect of acetic acid was somewhat more pronounced (Fig. 3, curve 1, and Fig. 4, curves 1 to 3). The increase in absorbance at 480 mμ amounted to 184% when the acetic acid concentration was increased from 0 to 30%. In the absence of ethanol and acetic acid, incorporation of 1,1-diethoxyethane in test mixtures increased the absorbance at 555 mμ by 118% (Fig. 3, curves 1 and 5). This increase is much smaller than that obtained with procedure A (Fig. 2). The absorbance at 555 mμ of test mixtures containing

1,1-diethoxyethane increased by 64, 116, and 180%, respectively, when 10, 20, and 30% ethanol were used (Fig. 3, curves 5 to 8). Acetic acid is far more effective in increasing the absorbance at 555 mμ of test mixtures containing 1,1-diethoxyethane. This increase amounted to 144, 470, and 770%, respectively, for 10, 20, and 30% acetic acid (Fig. 3, curve 5, and Fig. 4, curves 4 to 6).

As the hydrochloric acid concentration in the resorcinol test was decreased further, the trends established using procedures A and B continued. Thus in the procedure of Roe (7) in which the hydrochloric acid concentration is 6 N and the ethanol concentration is 19%, ethanol was responsible for a 184% increase in absorbance at 480 mμ but acetaldehyde *had no effect whatsoever on the absorbance at 555 mμ.*[4]

The use of commercial ethanol and acetic acid in resorcinol test procedures in which the hydrochloric acid concentration is above 6 N (8, 11) would undoubtedly lead to conflicting results being obtained by workers using the same procedure in different laboratories since the acetaldehyde content of the commercial solvent used would determine the position of λ_{max}, the adherence to Beer's law, and the sensitivity of the procedure. The acetaldehyde content of commercial ethanol and acetic acid varies a great deal. Thus we found that the acetaldehyde content of 8 brands of acetic acid varied from 10 to 500 mμmoles/ml and a single sample of ethanol contained 195 mμmoles/ml. Hence these commercial solvents should be used with caution, and with proper understanding of their effects on the color reaction.

On the basis of the data presented in this paper we have developed another resorcinol procedure for determining fructose. Organic solvents have been excluded from this procedure which relies on the inclusion of 1,1-diethoxyethane in the test mixture for its high sensitivity. This method is reported in the following paper (9).

The chemical reactions responsible for color formation in the resorcinol test for fructose are undoubtedly complex. Resorcinol probably reacts with 5-(chloromethyl)-2-furaldehyde or a degradation product thereof to form a condensation product (13) with maximum absorption at 480 mμ (procedure A). Acetaldehyde may react with this condensation product to form another one with increased conjugation (λ_{max} at 555 mμ). This is supported by the shift of λ_{max} to 555 mμ observed when

[4] The procedure of Kulka (6) in which the ethanol and hydrochloric acid concentrations are 38% and 4 N, respectively, is not quite comparable to other procedures discussed in this paper because of the longer heating time (40 min) and the use of Fe^{+++}. Nevertheless it is yet another procedure in which acetaldehyde has no effect on the absorbance at 555 mμ. There was, however, a 15% increase in absorbance at 480 mμ (measured with a Coleman Junior spectrophotometer) when 2 μmoles of 1,1-diethoxyethane was used in a test mixture.

acetaldehyde was added to the colored solution (λ_{max} at 480 mμ) and the solution allowed to stand overnight at 0° in the dark.

Whenever purified ethanol or purified acetic acid was used in a given procedure, the absorbance measured at 480 and at 555 mμ was always higher than when these solvents were excluded from test mixtures. This increased absorbance was not due to an interaction of ethanol or acetic acid with the condensation products since the addition of these solvents to the reaction mixture after completion of the test procedure resulted in a decrease in absorbance accounted for by dilution. It is suggested that the presence of these solvents effectively increases the amount of condensation products available for reaction with resorcinol. The mechanism by which this is accomplished may not be the same in both cases. Thus fructose is known to be degraded by acetic acid in the absence of mineral acid (14), and acetic acid may simply increase the formation of degradation products suitable for reaction with resorcinol. On the other hand, ethanol may stabilize fructose degradation products suitable for reaction with resorcinol since it is known that the rates of acid-catalyzed conversion of 5-(hydroxymethyl)-2-furaldehyde to levulinic and formic acids are less in aqueous ethanol than in water (15).

SUMMARY

Acetaldehyde was shown to be the impurity in a sample of commercial acetic acid responsible for shifting λ_{max} in the resorcinol test for fructose from 480 to 555 mμ. The effect of acetaldehyde, purified acetic acid, and purified ethanol on several resorcinol procedures was determined. It was shown that maximum absorbance at 555 mμ was obtained when equimolar amounts of fructose and acetaldehyde were present in the test mixture and that Beer's law held for the determination of fructose at 555 mμ as long as the acetaldehyde concentration was equal to or exceeded that of fructose. The effect of acetaldehyde on resorcinol procedures decreased while the effect of purified ethanol and purified acetic acid increased as the hydrochloric acid concentration in the test was lowered from 9 to 6 *N*. Acetic acid was always more effective than ethanol in increasing the sensitivity of the test.

ACKNOWLEDGMENT

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ANALYTICAL BIOCHEMISTRY 13, 143–148

Improved Resorcinol Reagent for the Determination of Fructose, and of 3,6-Anhydrogalactose in Polysaccharides[1]

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Several resorcinol reagents are in use for the quantitative determination of fructose (1) and other sugars which yield a positive Seliwanoff test (2, 3). In the preceding paper (4) we described the effect of concentration changes in acetaldehyde, purified acetic acid, and purified ethanol on several resorcinol procedures for determining fructose. This study was necessary since acetaldehyde was shown previously to have a marked effect on a resorcinol procedure (5). It is likely that in other procedures acetaldehyde was inadvertently included in the reaction mixture, being present as an impurity in the acetic acid or ethanol used as a solvent for resorcinol (4).

In this laboratory we required a method for the determination of sugars that give a positive Seliwanoff test and that are present in polysaccharides. On the basis of our previous findings (3–5) we developed an improved resorcinol method in which 1,1-diethoxyethane was deliberately included as a source of acetaldehyde. The improved resorcinol-1,1-diethoxyethane reagent is herein proposed as a highly sensitive reagent for the quantitative determination of fructose, and 3,6-anhydrogalactose in agar, carrageenan, and other algal polysaccharides.

EXPERIMENTAL

Materials and Reagents

The sugars used were: fructose (Anachemia Chemicals Ltd.), m.p. 100.5–102°, $[\alpha]_D^{22}$ $-90 \pm 1°$ (equil.; *c* 1.0, water); sucrose (British Drug Houses Ltd.); raffinose (Nutritional Biochemicals Corp.); and carrageenan (Marine Colloids Inc.). The washed agar (Difco Bacto-agar),

[1] Issued as NRC No. 8688.

kappa-carrageenan, and 3,6-anhydro-L-galactose diethyl mercaptal were prepared as previously described (3). The porphyran, from *Porphyra umbilicalis,* was a gift from Dr. J. R. Turvey.

Standards. The 3,6-anhydro-L-galactose diethyl mercaptal, 25.4 mg (94.6 μmoles), was dissolved in 50 ml of distilled water. Aliquots (3 ml) of this stock solution were placed in 50-ml volumetric flasks and stored at −20°. The free sugar was prepared (6) by adding 1.5 ml of a 1% solution of $HgCl_2$ (55 μmoles) and an excess of $CdCO_3$ to 3 ml (5.7 μmoles) of the sugar mercaptal stock solution. The mixture was heated for 1 hr at 50°, made up to 50 ml with distilled water, and filtered through a fine sintered-glass filter. This working standard contained 0.114 μmole of 3,6-anhydrogalactose per milliliter.

A stock solution of fructose was prepared as described previously (4). The working standard, 0.09 μmole of fructose per milliliter, was made by diluting the stock solution with distilled water.

A stock solution of 1,1-diethoxyethane (acetal, Eastman Organic Chemicals) in distilled water was prepared as described previously (4). The working standard, 2.78 μmoles of 1,1-diethoxyethane per milliliter, was made by dilution with distilled water. This standard was stable for at least one month.

A stock solution of resorcinol (Anachemia Chemicals Ltd., m.p. 109–111°) was prepared by dissolving 150 mg (1.36 mmoles) in 100 ml of distilled water. This solution was prepared weekly and stored in a brown bottle.

Resorcinol Reagent. The reagent was prepared by adding 100 ml of concentrated hydrochloric acid (sp. gr. 1.188–1.192) to 9 ml of resorcinol stock solution and adding to this mixture 1-ml of the aqueous solution of acetal (2.78 μmoles). The reagent developed a color on standing, but was stable for at least 3 hr.

Procedure for Color Development

Two milliliters of a solution containing up to 0.25 μmole of fructose or 3,6-anhydrogalactose, in the free or combined state, was transferred to a boiling tube, 25 × 150 mm, and covered with a glass marble. The tube was placed in an ice bath, and 10 ml of the resorcinol reagent was added. The contents were mixed in the ice bath and cooled for at least 3 min but not longer than 30 min. The tube was placed in a 20° water bath for 4 min and then heated for 10 min at 80°. It was then cooled for 1.5 min in an ice bath, and the absorbance was measured within 15 min at 555 mμ in a Coleman Junior spectrophotometer. The reaction was carried out in diffuse light since the color fades on exposure to sunlight. Cold acid degrades 3,6-anhydrogalactose and reproducible results are

obtained only with careful attention to the details of the procedure. The reaction mixture contained 11.1 μmoles of resorcinol and 0.25 μmole of 1,1-diethoxyethane and was 9 *N* with respect to hydrochloric acid.

RESULTS AND DISCUSSION

The improved reagent described here stems from our earlier work with several resorcinol procedures (3–5). The color development curves with fructose or 3,6-anhydrogalactose were identical to that previously reported for the latter sugar (3), the absorbance reaching a maximum value after 9 min at 80°. The absorption spectrum between 450 and 600 mμ had maximum absorbance at 555 mμ and minimum absorbance at 457 mμ (3–5). The absorbance at 555 mμ was proportional to concentration in the range of 0 to 0.25 μmole of fructose or 3,6-anhydrogalactose (Fig. 1). If the concentration of 1,1-diethoxyethane in the test mixture

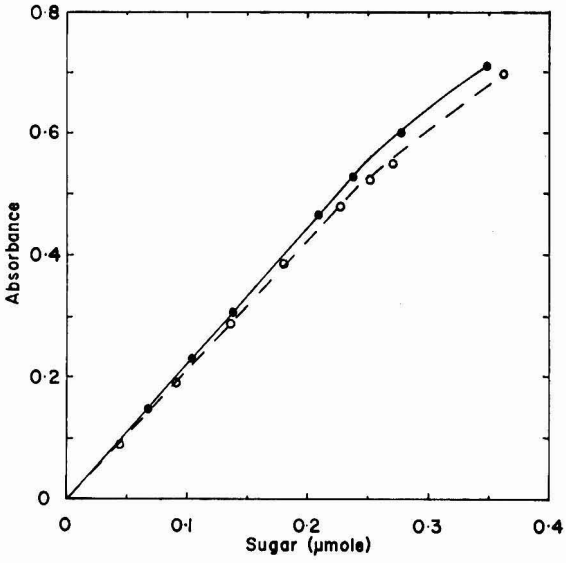

FIG. 1. Absorbance vs. concentration curves: (●), fructose; (○), 3,6-anhydrogalactose.

were increased, adherence to Beer's law could be extended to a wider range of concentration of fructose and 3,6-anhydrogalactose (4).

The sensitivity of this method is greater than that obtained with resorcinol tests described previously (Table 1). This is only partially due to the high concentration of acid in the reaction mixture. The increase in sensitivity is mainly associated with the use of an optimum concentra-

TABLE 1
COMPARISON OF SENSITIVITY OF DIFFERENT RESORCINOL PROCEDURES

Method[a]	λ[b] (mμ)	Absorbance[c]	Sensitivity (%)
Improved reagent	555	0.407	100
Kulka (2)	480	0.355	87
Roe (1)			
Pure ethanol[d]	520	0.096	24
Pure ethanol + 1,1-diethoxyethane[e]	520	0.099	24
Roe, Epstein and Goldstein (8)			
Pure acetic acid[d]	520	0.151	37
Pure acetic acid + 1,1-diethoxyethane[e]	520	0.190	47
Roe and Papadopoulos (7)			
Pure acetic acid[d]	520	0.166	41
Pure acetic acid + 1,1-diethoxyethane[e]	520	0.306	75
McRary and Slattery (9)			
Pure ethanol[d]	540	0.054	13

[a] Tests were performed as described, except with the procedure of Roe and Papadopoulos (7), when solutions were heated for 10 min at 80°.

[b] Wavelength at which absorbance was measured.

[c] Measured for 0.18 μmole fructose in a Coleman Junior spectrophotometer with 19-mm round cuvets.

[d] Solvent for resorcinol.

[e] 1,1-Diethoxyethane (0.209 μmole) added to test mixture in the solvent shown. This amount of 1,1-diethoxyethane was in slight excess of the minimum amount for optimum absorbance. The amount of acetaldehyde used in the original work is unknown.

tion of 1,1-diethoxyethane, which causes a significantly higher absorbance at 555 mμ (4, 5). Of the methods compared in Table 1, only those of Roe and Papadopoulos (7) and Kulka (2) are at least half as sensitive as the improved method. It should be noted that, prior to the addition of 1,1-diethoxyethane, the method of Roe and Papadopoulos (7) was less than half as sensitive as our method. The effect of 1,1-diethoxyethane on the method of Kulka (2) has been described (4). The present method is the only highly sensitive resorcinol procedure available today in which the concentration of all the reactants is clearly defined.

It is important to note that the specificity of the test has not been significantly altered. The ratio of the absorbance obtained with various sugars to that obtained with fructose (Table 2) shows that, in mixtures, equimolar amounts of these sugars would not significantly interfere with the determination of either fructose or 3,6-anhydrogalactose. The 2% error produced by glucose compares favorably with the 0.05 to 2% values reported for other colorimetric tests (2). The absorbance with 0.18 μmole of 3,6-anhydrogalactose was 92% of that of an equimolar amount of fructose. Thus with a correction factor, fructose may be used as a

TABLE 2
SPECIFICITY OF IMPROVED RESORCINOL REAGENT

Sugar	Color ratio (%)[a]
Fructose	100
3,6-Anhydro-L-galactose	92
Glucose	2
Galactose	1
Mannose	2.5
Fucose	<1
Rhamnose	1
Xylose	4
Arabinose	1
Ribose	5
Sorbose	69

[a] $\frac{\text{Absorbance 0.18 } \mu\text{mole sugar}}{\text{Absorbance 0.18 } \mu\text{mole fructose}} \times 100.$

reference sugar to determine the concentration of 3,6-anhydrogalactose in agar, carrageenan, and other algal polysaccharides.

This procedure was designed to determine the concentration of sugars in oligosaccharides and polysaccharides. The high concentration of acid in the reaction mixture ensures complete hydrolysis of these compounds. An analysis of sucrose and raffinose yielded 1 mole of fructose per mole of these sugars. The values obtained for 3,6-anhydrogalactose in agar, carrageenan, and porphyran may be considered to be valid since similar results have been obtained with other methods (Table 3).

TABLE 3
CONCENTRATION OF 3,6-ANHYDROGALACTOSE IN ALGAL POLYSACCHARIDES

Polysaccharide	3,6-Anhydrogalactose (%)[a]			
	Present method	Resorcinol (3) method	Anthrone (3) method	O'Neill (10) method
Washed agar (Difco)	42.5	42.9	43.6	
Carrageenan	18			
Kappa-carrageenan	24.8	23.2	23.8	24–28.1 (11)
Porphyran	12			11.7 (12)

[a] As reported in reference cited.

The usefulness of the modified reagent is undoubtedly not limited to the applications we have discussed. It should also be useful in determining fructose in fructosans, and its high sensitivity could well make it a method of choice in purely chemical problems in which fructose must be determined.

SUMMARY

An improved, highly sensitive resorcinol reagent is described for the colorimetric determination of fructose, and of 3,6-anhydrogalactose in agar, carrageenan, and other algal polysaccharides.

ACKNOWLEDGMENT

The technical assistance of J. Berrigan is greatly appreciated.

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ANALYTICAL BIOCHEMISTRY 13, 149–161

Automated Determination of NAD-Coupled Enzymes. Determination of Lactic Dehydrogenase[1]

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The analytical complexity and increased clinical demand for NAD-coupled enzyme[3] determinations has pointed up the need for a simple, accurate, and reproducible enzymic assay which will also be applicable to the analysis of many specimens.

Several automated procedures for serum lactic dehydrogenase have been described for use on the Technicon AutoAnalyzer. The earliest, reported by Schwartz, Kessler and Bodansky (1) assayed enzyme preparations and tissue homogenates. In 1964, Strandjord (2) described an automated spectrophotometric procedure for serum assays differing in various flow diagram characteristics as well as other details. Both procedures measured the decline in the 340-mμ absorbance of $NADH_2$ based on the "backward reaction": pyruvate + $NADH_2$ $\rightleftarrows$ lactate + NAD, to assay enzyme activity. Later, Brooks (3) using the "forward reaction," presented an automated procedure which measured NAD fluorometrically.

This report presents an automated assay procedure which appears generally applicable to NAD-$NADH_2$ coupled enzyme systems. In the present example, the determination of serum lactic dehydrogenase activity is illustrated. The procedure has been adapted to the Technicon AutoAnalyzer, which will permit a net analysis of 20 specimens an hour. Further, two automated flow systems have been developed that will

[1] Portions of this work are taken from a thesis submitted by Stanley Morgenstern in partial fulfillment of the requirements for the M.A. degree, Brooklyn College, 1965. Presented at the 17th National Meeting of the American Association of Clinical Chemists, Chicago, Ill., August, 1965.

[2] Present address: Bio-Science Laboratories, Los Angeles, Calif.

[3] Abbreviations used: NAD, nicotinamide adenine dinucleotide; $NADH_2$, reduced form; LDH, lactic dehydrogenase.

permit the analyst, depending on the equipment available, to choose either a colorimetric or a spectrophotometric determination of enzyme activity.

The colorimetric assay is based on the coupled reactions:

$$\text{lactate} + \text{NAD} \rightleftarrows \text{pyruvate} + \text{NADH}_2 \quad (1)$$

$$\text{Cu}^{++} + \text{NADH}_2 \rightleftarrows \text{Cu}^{+} + \text{NADH} \quad (2)$$

$$\text{Cu}^{+} + \text{neocuproine} \rightarrow \text{Cu}^{+}\text{-neocuproine complex} \quad (3)$$

The spectrophotometric assay, based on the increase in the 340-mμ absorbance of $NADH_2$, measures reaction (1).

METHODS

I. Reagents

1. *Sulfuric acid, 0.433 N:* Dilute 12 ml of reagent-grade concentrated sulfuric acid to 1000 ml with distilled water.

2. *2-Amino-2-methyl-1-propanol* (Eastman Organic Chemicals, #P4780), 0.75 *M*: Dilute 285 ml of 50% by weight 2-amino-2-methyl-1-propanol to 1000 ml with distilled water.

3. *Cupric sulfate, 1%:* Dissolve 10 gm of cupric sulfate pentahydrate in 1000 ml of 0.433 *N* sulfuric acid.

4. *Buffered lactate control solution, 0.67 M:* Adjust a solution of 35.80 ml of lactic acid, 85%, in 896 ml of 0.75 *M* 2-amino-2-methyl-1-propanol (No. 2 above) to pH 9.0 with 5 *N* sodium hydroxide and diluted to 1 liter.

5. *Buffered lactate assay solution:* Nicotinamide adenine dinucleotide (NAD) (P-L Biochemicals, Inc., Milwaukee, Wis.) in 2-amino-2-methyl-1-propanol/lactic acid buffer. Dissolve 1.21 gm NAD in 250 ml of control buffer, (No. 4 above).

6. *Copper-neocuproine reagent:* Cupric sulfate, 3×10^{-3} *M* and 8.0×10^{-3} *M* neocuproine (G. Frederick Smith Chemical Co., Columbus, Ohio) in 0.433 *N* sulfuric acid. Dilute 75 ml of the 1% stock cupric sulfate solution to approximately 950 ml with 0.433 *N* sulfuric acid, add 1.74 gm of neocuproine, and when dissolved dilute to 1 liter with 0.433 *N* sulfuric acid.

7. *Standard:* Reduced nicotinamide adenine dinucleotide ($NADH_2$)[4] (Sigma Chemical Co., St. Louis, Mo.). Prepare a stock standard solution containing 4.13 μmoles/ml by dissolving 81.25 mg of $NADH_2$ in 25 ml of 0.67 *M* 2-amino-2-methyl-1-propanol buffer, pH 9.0.[5] In the 3.5 ml

[4] $NADH_2$ was used as the disodium salt with a F.W. of 763 and 97% purity.

[5] The $NADH_2$ standards are prepared in 0.67 *M* buffer to provide identical final molarity of both the standard solutions and the reaction mixture.

reaction mixture[6] containing 0.1 ml stock standard, the $NADH_2$ concentration is 0.118 μmole/ml. Additional standards are prepared by diluting 0.5, 1.0, and 1.5 ml stock solution with 1.5, 1.0, and 0.5 ml 0.67 *M* 2-amino-2-methyl-1-propanol, pH 9.0. These solutions will give 0.0295, 0.0590, and 0.0885 μmole $NADH_2$/ml reaction mixture.

8. *Enzyme control:* A vial of Versatol E (General Diagnostics Division, Warner Chilcott, Morris Plains, N. J.) is reconstituted with 3 ml of distilled water according to the manufacturer's specifications. Further dilutions with saline are made to give solutions containing 25, 50, 75, and 100% of the label value activity.

II. Flow Diagram

See Figures 1 and 2.

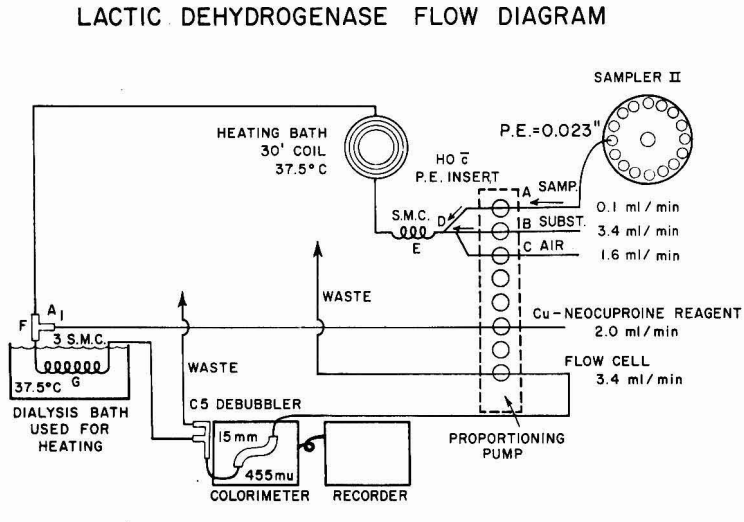

FIG. 1. Flow diagram: automated colorimetric determination of lactic dehydrogenase activity. D is an HO cactus fitting with an 0.011" polyethylene tubing insert. E is a glass single mixing coil. F is an A-1 glass fitting. G indicates three glass single mixing coils connected in series submerged in the 37° Technicon dialyzer bath. (The recently developed H-3 fitting can be substituted for the HO fitting and eliminates the need for the insert.)

Chart Paper. Chart paper ruled in optical density (absorbance) is used. Chart speed is 18"/hr.

Filters. Filters absorbing maximally at 455 mμ are used in the colori-

[6] The 3.5 ml reaction mixture is the cumulative volume when 0.1 ml sample and 3.4 ml buffered substrate are mixed at D in Fig. 1.

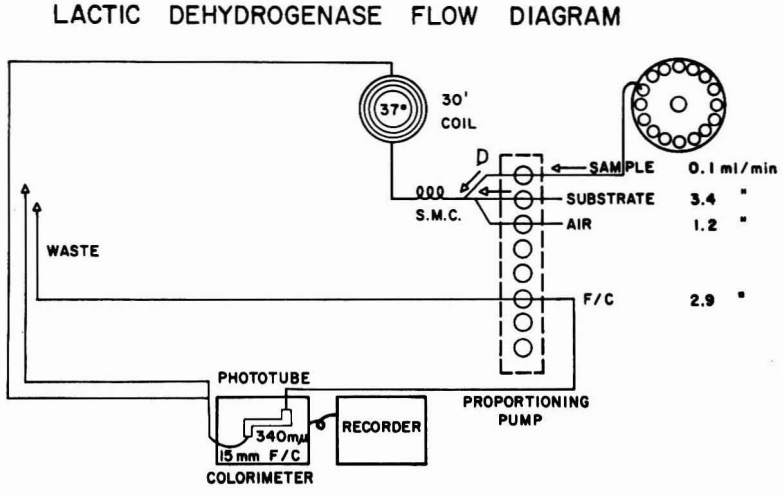

FIG. 2. Flow diagram: Automated spectrophotometric determination of lactic dehydrogenase activity. D is an HO cactus fitting with an 0.011″ polyethylene tubing insert. (The recently developed H-3 fitting can be substituted for the HO fitting and eliminates the need for the insert.)

metric procedure; 340 mμ filters are used in the spectrophotometric procedure.

Incubation Time. The incubation time is determined daily to accommodate changes in the manifold, and for activity calculation. The incubation time, measured with a stopwatch, is the transit time of a colored solution (e.g., 1% potassium dichromate) taken between points D and F. The distances between the end of the single mixing coil E and the heating bath and the A-1 glass fitting at F are kept as short as possible.

III. Operating Procedure

In the colorimetric procedure, buffered lactate control solution (No. 4) is pumped for 5 min, then the acidified copper-neocuproine reagent is aspirated, and when the combined reagent stream passes through the flow cell the baseline is set at 0.01 absorbance. Standards and specimens are aspirated at 40 samples/hr. When this control assay is complete, the buffer line is transferred to the buffered lactate assay solution (No. 5), the baseline is reset to 0.01 absorbance, and the enzyme assay is begun.

In the spectrophotometric method, the procedure is similar and differs only in the elimination of the colorimetric reagent line. Thus, lactate control buffer is pumped as described above, the baseline is adjusted to 0.01 and both standard solutions and specimens are aspirated. When

the control analysis is complete, the buffer line is transferred to buffered lactate assay solution and the process is repeated.

IV. Calculation

The *lactic dehydrogenase activity* is expressed as μmoles of NAD converted to μmoles of $NADH_2$ per milliliter of reaction mixture per minute (1). This value is obtained by relating net absorbance (total assay absorbance — control absorbance) to μmoles $NADH_2$/ml reaction mixture from the $NADH_2$ calibration curve, and dividing the result by incubation time.

Example: Net absorbance: $0.385 = 0.0545$ μmoles/ml (from the $NADH_2$ calibration curve). With an incubation time of 5 min, the activity is:

$$\frac{0.0545\ \mu\text{mole NADH}_2\text{/ml}}{5\ \text{min}} = 0.0109\ \mu\text{mole/ml/min}$$

This value may be expressed also as μmoles/liter/min, and would then be 10.9.

To express enzyme activity in clinically useful units, pooled high activity serum is analyzed, in replicate, by the method of Amador, Dorfman, and Wacker (5). The serum is carefully diluted with saline and the dilutions are reanalyzed by the present procedure and a calibration curve relating absorbance to "Wacker units" (5) is constructed. One such calibration curve is illustrated in Fig. 5. The range of normal human serum is 70 ± 14 units (5).

RESULTS

Figure 3 presents a typical calibration curve and the strip chart recording from which it was constructed, using $NADH_2$ standards. The comparative continuous sampling ("steady-state") record is also shown. At the 40 specimen/hr sampling rate, the maximum absorbance is 93% of this "steady-state" value, indicating very good correlation.

The absorbance of the standard $NADH_2$ solutions was also measured in a phototube colorimeter at 340 mμ. This is similarly shown in Fig. 4. It will be particularly noted that no sample interaction occurred when the sample containing 0.109 μmole $NADH_2$/ml reaction mixture was immediately followed by a sample containing 0.0273 μmole $NADH_2$/ml reaction mixture.

In addition, an enzyme activity curve based on dilutions of Versatol E and the chart recording used in its preparation are given in Fig. 5. Also shown for comparison are the continuous sampling patterns given by the 12.5 and 50% dilutions, which again demonstrate a close correla-

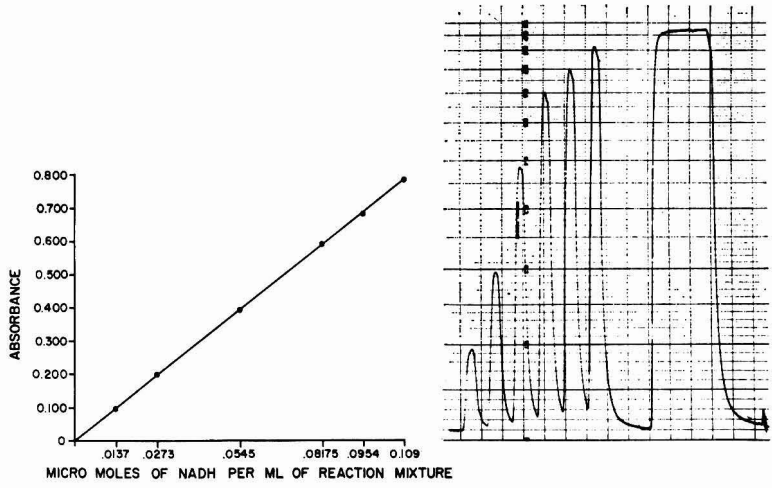

FIG. 3. Calibration curve: automated colorimetric determination of $NADH_2$ standards at 455 mμ. $NADH_2$ concentrations can be converted to units by dividing by incubation time.

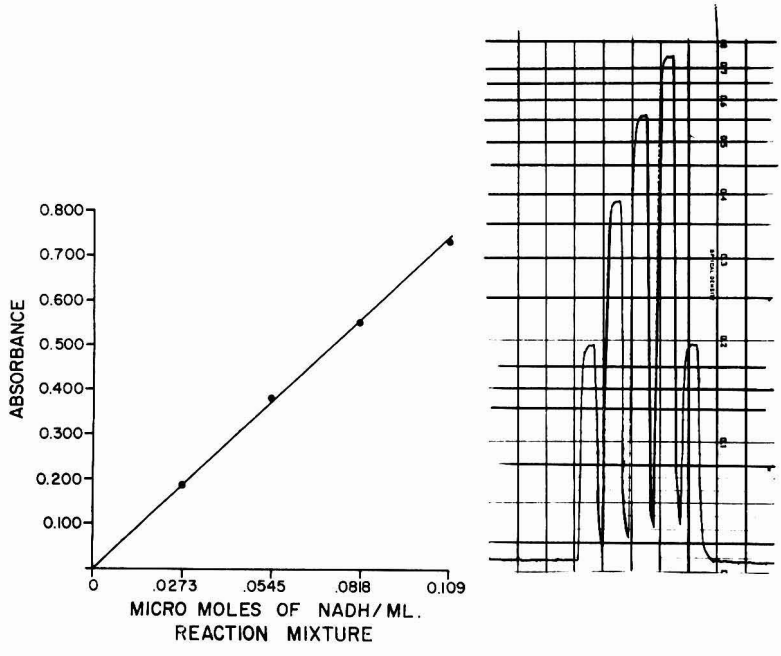

FIG. 4. Calibration curve: automated spectrophotometric determination of $NADH_2$ standards at 340 mμ. $NADH_2$ concentrations can be converted to units by dividing by incubation time.

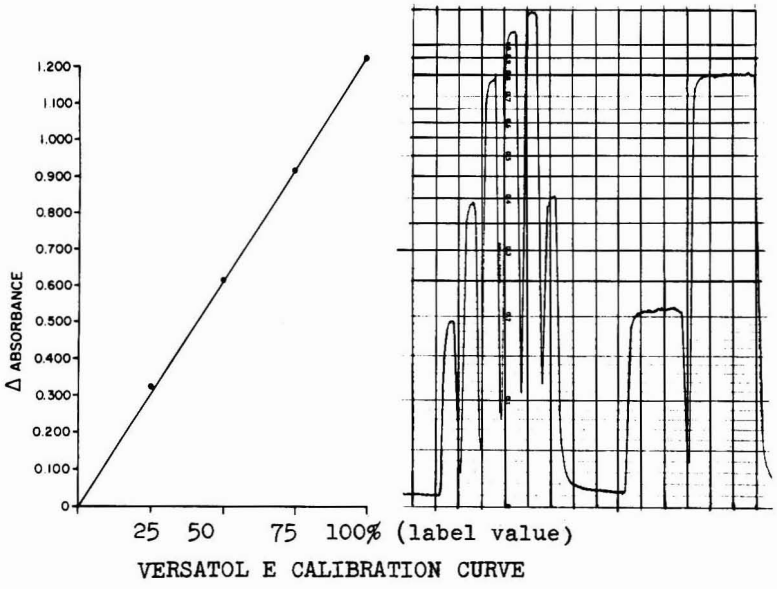

FIG. 5. Calibration curve: automated colorimetric assay of Versatol E dilutions for lactic dehydrogenase activity. Enzyme activity is expressed as percentage of reconstituted material. In this lot 100% assayed as 0.0193 μmole $NADH_2$/ml reaction mixture/min. The control absorbance recordings which were subtracted from these recordings are not shown.

tion between enzyme activity at the sampling rate and with continuous sampling.

The enzyme activity of identical samples was determined by the present colorimetric method and by direct spectrophotometric meas-

TABLE 1

COMPARISON OF SPECTROPHOTOMETRIC (340 mμ) AND COLORIMETRIC ASSAYS ON HUMAN SERUM BY AUTOANALYZER PROCEDURES

Specimen	340 mμ	455 mμ	Ratio 340/455
1	588	525	1.06
2	995	1007	0.99
3	384	364	1.06
4	1062	986	1.08
5	531	546	0.97
6	404	396	1.02
7	464	450	1.03
8	434	431	1.01
9	581	557	1.04
10	649	650	1.00

Values are expressed as μmoles $NADH_2$/ml reaction mixture/min and have been multiplied by 10^5 to obtain whole numbers.

urement of $NADH_2$ at 340 mμ in a phototube colorimeter. The concentrations and volumes of reagents used for the enzyme portion of the assay were identical. Identical $NADH_2$ standards (see "Methods") and serums were used for both procedures. With identical incubation the activities were calculated from standard curves and expressed as μmoles $NADH_2$/ml reaction mixture per minute. Excellent agreement is seen (Table 1).

DISCUSSION

The present procedures also use the "forward reaction" studied by Wacker and associates (4). This approach was chosen because it obeys zero-order reaction kinetics over a wide range of lactate concentrations as demonstrated by Amador *et al.* (5), who followed the reaction for 5 min. In the present study to determine whether this approach would be feasible for automated analysis the reaction was followed for 10 min and repeatedly was found to be consistently linear at many levels of activity (Fig. 6). The merits and disadvantages of both approaches have been thoroughly reviewed by Amador *et al.* (5) and Thiers and Vallee (6) and the reader is referred to these discussions.

Existing manual colorimetric methods for assay of NAD-$NADH_2$ coupled enzymes present various difficulties. Of the two reactions which

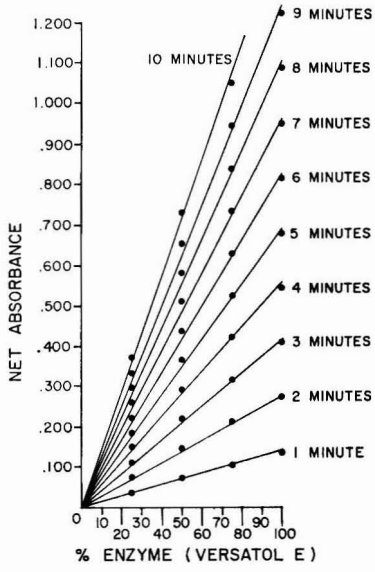

FIG. 6. Serum LDH activity: reaction rates at varying activity levels. Measurements taken on a Beckman DU spectrophotometer at 340 mμ; method of Amador, Dorfman, and Wacker (5).

have been generally used, one involves color changes in tetrazolium dyes (7–12). The principal defect in this method is the slow reduction of the dye by $NADH_2$. Intermediate electron carriers ("soluble diaphorase" and the structurally similar phenazine methosulfate) have been used to accelerate the reaction. "Soluble diaphorase," a pig heart preparation, has variable reproducibility and its solubility is limited, especially at the concentrations required for the assay. Phenazine methosulfate, somewhat superior to diaphorase, is photosensitive and comparatively unstable. As an additional complication, the formazans formed by reduction of the tetrazolium salts are water insoluble and are usually extracted into organic solvents, or are colloidally suspended with gelatin, for ultimate measurement. The disadvantages of these methods when applied to automated analyses are quite evident.

The other method commonly used is based on the photometric measurement of pyruvic dinitrophenylhydrazone (13, 14). Aside from the experimental difficulties connected with the measurement of alkaline 2,4-dinitrophenylhydrazone solutions, this assay procedure requires specially purified reagents (14) and is often erratic, and poor correlation with the spectrophotometric method has been claimed (5).

The present procedure couples the linear reaction kinetics of the "forward reaction" with a sensitive direct-reacting hydrogen acceptor reagent. The reagent contains acidified cupric sulfate and neocuproine (2,9-dimethyl-1,10-phenanthroline). The latter specifically chelates cuprous ion, producing a bright orange solution with a molar absorbance of 7950 at 454 mμ (15). Enzymically produced $NADH_2$ directly reduces $Cu^{++} \rightarrow Cu^{+}$ (Eq. 2), eliminating intermediate electron carriers. Color development occurs over a wide pH range, but by developing the color at pH 3.5 further enzymic oxidation of lactate is also halted.

During the experimental development of the present procedures, three segments of the over-all determination were studied in detail; first, factors pertaining to the enzyme reaction, e.g., buffer, substrate, and NAD concentration, pH and incubation time; second, factors pertaining to the colorimetric reaction, e.g., copper and neocuproine concentration, pH and color development; third, factors related to the automated flow system. Based on these studies the manifolds and flow diagrams presented in Section II of "Methods" were evolved (Figs. 1, 2).

Published studies (16) indicate that excess lactate altered the rate of LDH-catalyzed reactions and substrate inhibition has also been observed in other dehydrogenase reactions. In the present study, in contrast to these reports, lactic acid inhibition was not observed up to a 500 mM concentration (Fig. 7). A continuous increase in enzyme activity paralleled increases in substrate concentration, reaching a plateau at

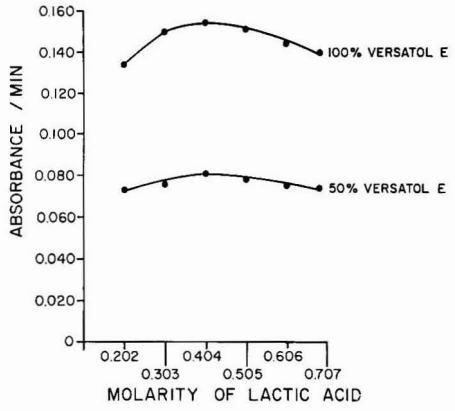

FIG. 7. Determination of optimum substrate concentration in 0.67 *M* 2-amino-2-methyl-1-propanol buffer, pH 9.0. Reactions measured at 340 mμ (Beckman DU spectrophotometer).

300 m*M*. This was true at both low (normal) and elevated (abnormal) activity levels. This observation suggested that both the buffer and its concentration influenced the reaction. This view is supported by Robins *et al.* (17), who demonstrated that with 0.1 *M* 2-amino-2-methyl-1-propanol, lactate did not cause inhibition even at 200 m*M*, a level well beyond the optimum previously determined. As shown in this laboratory, at a buffer concentration of 0.67 *M*, even higher substrate levels were permitted, an obvious advantage. The utility and advantages of 2-amino-2-methyl-1-propanol were commented on in an earlier paper (18) from this laboratory.

Wacker and Snodgrass (19) reported an initial nonlinear period during the "forward reaction" which they ascribed to the superposition of endogenous α-hydroxy acid oxidation. This could influence 340-mμ spectrophotometry at low lactate substrate concentration, especially in some disease states. At substrate concentrations above 2 m*M* (normal endogenous lactate levels) such influence is nullified, and the waiting period required in the "backward reaction" is no longer required.

Since linear kinetics were demonstrated manually over a 10-min interval (Fig. 6), considerable flexibility was permitted in the selection of the analytical procedure. A 5-min incubation on the AutoAnalyzer gave adequate range and sensitivity and this time interval was well within the limits of this procedure. The incubation time could be further increased with no loss of reaction characteristics, if only a flow cell with shorter light path (e.g., the gravity cell) was available, and thereby required increased $NADH_2$ production for adequate sensitivity.

Winer and Schwert (20) noted a 5% decrease in NAD stability in pH 10.0 tris buffer after 6 hr. Earlier, Lowry *et al.* (21) had shown that $NADH_2$ had a stability in 0.1 *M* 2-amino-2-methyl-1-propanol buffer, pH 9.9, about equal to 0.1 *M* tris at pH 8.7. This was corroborated during the present study, where the absorbance at 340 mμ of a solution of NAD in 0.67 *M* 2-amino-2-methyl-1-propanol buffer, pH 10, increased 82% after 3 hr at 25°, indicating nonenzymic reduction of NAD. By contrast in 2-amino-2-methyl-1-propanol at pH 9.0, the optimum for the present procedure, no change in absorbance at 340 mμ was observed over 3 hr either at 25° or 37°. Thus, sufficient NAD can be incorporated into the buffered substrate solution for the day's analysis without appreciable loss.

Another advantage of the present procedures is that errors and uncertainties are obviated in the pyruvate → lactate assay resulting from the development of an inhibitor in the $NADH_2$ solutions (22). In the colorimetric procedure, the $NADH_2$ formed is reacted immediately with the color reagent. In the spectrophotometric procedure, the $NADH_2$ formed is measured immediately.

Thiers and Oglesby (23) demonstrated that one inherent deficiency of the continuous-flow technique upon which the AutoAnalyzer system is based is specimen interaction, which contributes to inaccuracy and loss of precision. When the Technicon Sampler I was used, at 40 specimen per hour sampling, a specimen producing 0.0024 μmole $NADH_2$/ml mixture/min following one producing 0.0193 μmole $NADH_2$/ml mixture/min exhibited 12% interaction. With the Sampler II, and at a 40 specimen 2:1 sampling to wash ratio, this was reduced to about 3% (Figs. 4 and 5).

There are conflicting reports concerning lactic dehydrogenase stability in stored serum samples. One recent evaluation was made by Kreutzer and Fennis (24), who found that sera stored at room temperature showed little change in total activity or the isoenzymes over a 10-day period. Refrigeration below 0° caused losses among the isoenzyme fractions in the order 4, 5, 3, 2, and 1.

Although the purpose of the present investigation was primarily developmental, a short-term (24-hr) enzyme stability study was included. The result is shown in Table 2, which indicates no real loss of activity over a 24-hr period whether refrigerated (4°) or stored at room temperature (21°). It is suspected that such reported losses of total enzyme activity on refrigeration may reflect varying decline of the individual isoenzyme fractions.

The procedure can also be used advantageously to process in successive samplings duplicate specimens, one untreated, and one which has

TABLE 2
STABILITY OF SERUM LACTIC DEHYDROGENASE ACTIVITY

Specimen	Freshly drawn	Refrigerated sera stored (capped) 24 hr	Room-temperature stored sera (capped) 24 hr
1	0.160	0.170	0.166
2	0.095	0.118	0.097
3	0.145	0.164	0.150
4	0.293	0.310	0.290
5	0.165	0.164	0.170
6	0.213	0.203	0.208
7	0.136	0.131	0.123
8	0.345	0.359	0.343
9	0.154	0.147	0.161
10	0.227	0.212	0.242

Values are given as net absorbance at 455 mμ (AutoAnalyzer).

been inactivated at 65°. This procedure is claimed to differentiate the heat-stable cardiac isoenzyme from the heat-labile liver isoenzyme (25).

Investigations on the application of this reaction to the determination of activity of other enzyme systems are in progress.

SUMMARY

Procedures have been developed for the automated colorimetric and spectrophotometric determination of serum lactic dehydrogenase activity. The former is based on the measurement of a cuprous-neocuproine complex formed by coupled reaction with enzymically generated reduced nicotinamide adenine dinucleotide ($NADH_2$). The latter measures the 340-mμ absorption of the $NADH_2$ similarly produced. Excellent agreement is obtained between the two methods.

ACKNOWLEDGMENTS

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SHORT COMMUNICATIONS

Fast Determination of Aromatic Aldehydes[1]

The current micromethod employed for the separation and quantitative estimation of the aldehydes produced upon the alkaline nitrobenzene oxidation of lignin samples involves a rather lengthy paper chromatographic procedure (1). In a recent investigation in this laboratory of the lignin from *Metasequoia* wood, the techniques of thin-layer chromatography and multiple unidimensional development (2) were applied. Employing these modifications, the aromatic aldehydes can be determined as accurately as, and more rapidly than, by the earlier method.

Chromatographic Technique. The reaction mixture from an alkaline nitrobenzene oxidation (3) of a lignin was pipetted into a 2-dram vial, and the bomb washed with 1.0 ml water and with 2.0 ml methylene chloride. The washings were added to the vial, which was then capped, and shaken in a Vortex mixer for 1 min. When two layers had separated, 1.0 ml of the aqueous phase was pipetted into another vial and 1.0 ml 6 *N* HCl was added. This was extracted as before with 2.0 ml methylene chloride. After standing for a few minutes, the lower layer was transferred carefully with a Pasteur pipet to a small separatory funnel, and the organic phase removed.

A 1.0-ml aliquot of this solution was spotted on an 8″ × 8″ glass plate coated with silica gel (Adsorbosil-2, Applied Science Laboratories, Inc.) to a thickness of 50 μ. As a control, a mixture of *p*-hydroxybenzaldehyde, vanillin, and syringaldehyde was spotted on each end of the plate, which was then developed with methylene chloride-acetone (99:1 by volume). The solvent front was allowed to ascend 15 cm from the origin, and the plate was air-dried. It was redeveloped in this manner two additional times.

The locations of the aldehyde-containing zones were observed and marked under ultraviolet light (3660 A). Each of these zones as well as a blank were scraped from the plate and the resulting powders were placed in separate centrifuge tubes. The powders were washed with three

[1] Communication No. 400.

TABLE 1

STANDARDIZATION OF CHROMATOGRAPHIC SEPARATION

Sample	Mg spotted	Mg recovered		% recovery
p-Hydroxybenzaldehyde	0.094	0.095		101.1
	0.094	0.094		100.0
	0.094	0.097		103.2
			Average	101.4
	0.188	0.190		101.1
	0.188	0.187		99.5
	0.188	0.188		100.0
			Average	100.2
	0.282	0.278		98.6
	0.282	0.280		99.3
	0.282	0.277		98.2
			Average	98.7
	0.376	0.374		99.5
	0.376	0.363		96.5
	0.385	0.375		97.4
			Average	97.8
Vanillin	0.094	0.093		98.9
	0.094	0.093		98.9
	0.094	0.093		98.9
			Average	98.9
	0.188	0.185		98.4
	0.188	0.183		97.3
	0.188	0.184		97.9
			Average	97.9
	0.283	0.276		97.5
	0.283	0.273		96.5
	0.283	0.275		97.2
			Average	97.1
	0.377	0.367		97.3
	0.377	0.361		95.8
	0.377	0.370		98.1
			Average	97.1
Syringaldehyde	0.093	0.088		94.6
	0.093	0.089		95.7
	0.093	0.086		92.4
			Average	94.2
	0.187	0.177		94.7
	0.187	0.178		95.2
	0.201	0.192		95.5
			Average	95.1
	0.280	0.272		97.1
	0.280	0.265		94.6
	0.300	0.281		93.7
			Average	95.1
	0.373	0.360		96.5
	0.373	0.345		92.5
	0.399	0.387		97.0
			Average	95.3

5-ml portions of ethanol. For quantitative estimation of the aldehydes, the ethanol extracts were transferred to volumetric flasks; 4 ml 0.2% KOH in ethanol was added, and the solutions were diluted to 50.0 ml. The optical densities were read on a Zeiss PMQ-II spectrophotometer at 335 mμ for *p*-hydroxybenzaldehyde, at 352 mμ for vanillin, at 368 mμ for syringaldehyde, and at each of these wavelengths for the blank. After correcting for the blank, the observed optical densities were compared with those of standard curves relating optical density and concentration (in mg aldehyde/50.0 ml solution). Standardization corrections were applied (Table 1) to the weights of aldehydes, and these values were multiplied by an aliquot factor of 40, to obtain the total amounts of aldehydes produced.

The chromatographic separation was standardized by spotting previously known amounts of the aldehydes on thin-layer plates, developing, and isolating as described above. The results are recorded in Table 1.

To establish the efficiency of the aldehyde-extraction procedure, samples of vanillin were weighed into small vials and dissolved in 1.0 ml 2 *N* NaOH. The extraction was then performed as described above. The results, shown in Table 2, indicate that the extraction is quantitative.

TABLE 2
EFFICIENCY OF VANILLIN EXTRACTION

Wt. vanillin, mg	Aliquot wt. (calculated), mg	Aliquot wt. (experimental), mg	Recovery %	Deviation
2.28	0.570	0.545	95.6	3.8
1.56	0.390	0.402	103.1	3.7
1.47	0.368	0.363	98.6	0.8
1.75	0.438	0.430	98.2	1.2
2.04	0.510	0.498	97.6	1.8
1.77	0.443	0.420	94.8	4.6
1.32	0.330	0.322	97.6	1.8
1.55	0.387	0.401	103.6	4.2
1.59	0.397	0.398	100.3	0.9
1.40	0.350	0.356	101.7	2.3
2.11	0.527	0.527	100.0	0.6
1.70	0.425	0.430	101.2	1.8
1.74	0.435	0.437	100.5	1.1
		Average	99.4 ± 2.7%	

To ensure that no reactions were occurring which would change the compositions (and hence the concentrations) of the aldehyde solutions, solutions of varied concentration were prepared and their optical densities measured immediately, after 2 days, and after 3 days. The results showed only statistical scatter, as seen from the data listed in Table 3.

TABLE 3
VARIATION OF OPTICAL DENSITY WITH TIME

Sample	Optical density		
	On first day	After 2 days	After 3 days
p-Hydroxybenzaldehyde	0.980	0.981	0.982
	0.782	0.769	0.773
Vanillin	0.665	0.660	0.664
	0.488	0.484	0.490
Syringaldehyde	0.586	0.572	0.580
	0.453	0.449	0.453

Thus this procedure forms an accurate and rapid technique for the separation and quantitative estimation of the aromatic aldehydes produced on the alkaline nitrobenzene oxidation of lignin.

ACKNOWLEDGMENT

This study was supported in part by a grant of the National Science Foundation to Dr. F. F. Nord. Thanks are extended to Dr. D. D. Clarke of this department for discussions and assistance in the course of these experiments.

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Determination of Ferric and Other Metal Chelates of Ethylenediamine Di-(o-hydroxyphenylacetic Acid) in Plant Tissues[1, 2]

The increasing uses of the ferric chelate of ethylenediamine di-(*o*-hydroxyphenylacetic acid) (EDDHA) in the nutritional and medical

[1] This work was supported in part by the Geigy Chemical Corporation, Ardsley, New York.
[2] University of Arizona Technical Paper No. 959.

fields, for the correction of lime-induced iron chlorosis in plants (6) and the differentiation of anemia (3) and possible control of carcinogenesis in man (2) have created the need for a method for the direct determination of EDDHA metal chelates in biological tissues. A previous report from this laboratory by Batra and Maier (1) describes a combination solvent extraction and spectrophotometric method for the determination of FeEDDHA in plant tissues. The method of Batra and Maier is time consuming and not suitable for large-scale analysis. It is the purpose of this note to present a modification of the existing method for FeEDDHA determination in plant tissues that reduces the analysis time but does not sacrifice accuracy or sensitivity.

Quick frozen and lyophilized plant samples, which had been ground through an 80-mesh stainless-steel screen in a Wiley mill, were homogenized with a Vir Tis "45" homogenizer for 20 min at 45,000 rpm. The mixture for homogenization consisted of 1.0 gm lyophilized plant tissue, 20 ml 0.2 *M* sodium acetate buffer (pH 4.6), 15 ml ethanol stabilized tetrachloroethylene, and 0 to 7000 μg aqueous FeEDDHA. The homogenate was quantitatively transferred with deionized water to 50 ml centrifuge tubes and centrifuged at 1500 $\times$ *g* at 4°C for 10 min. The aqueous phase was removed and retained and the pellet and tetrachloroethylene layers were resuspended with 5 ml of the acetate buffer and again centrifuged at 1500 $\times$ *g*. The suspension was repeated twice and the aqueous supernatants combined. The pellets and tetrachloroethylene layers were discarded.

EDDHA and EDDHA metal chelates are insoluble in tetrachloroethylene and highly soluble in the acetate buffer. The tetrachloroethylene extracted the plant chlorophylls, carotinoids, and other slightly polar and nonpolar compounds, eliminating those compounds which have absorption spectra that interfere with absorption maximum of FeEDDHA at 480 mμ.

The combined aqueous extract were filtered through a 0.22-μ Millipore filter and vacuum desiccated at 35°C until the volume was less than 20 ml. This solution was then brought to a volume of 50 ml, the spectra obtained from 350 to 700 mμ on a Perkin-Elmer 202 recording spectrophotometer, and the absorbance at 480 mμ recorded. Comparison of this absorbance data and the spectra obtained with a standard curve and spectra of FeEDDHA yielded the FeEDDHA concentration in the plant samples and confirmed that there was no absorption interference from other plant compounds. The molar absorptivity of FeEDDHA at 480 mμ was 4086.

The recovery data of FeEDDHA, from a pure solution and from tomato leaf tissue to which various amounts of FeEDDHA had been

added (Table 1), show an accuracy and sensitivity that is comparable to the Batra and Maier method (1).

TABLE 1
RECOVERY OF ADDED FeEDDHA FROM SOLUTION AND TOMATO TISSUE

FeEEDHA added, μg/ml	FeEDDHA solution recovery, μg/ml[a]	S.D.	FeEDDHA tissue recovery, μg/ml[a]	S.O.
10	9.9	±0.1	10.0	±0.1
20	19.9	±0.1	19.7	±0.2
30	30.0	±0.1	29.9	±0.2
40	39.8	±0.1	40.0	±0.3
60	59.9	±0.2	59.5	±0.4
100	99.8	±0.3	99.7	±0.2

[a] All data are the results of triplicate samples.

The FeEDDHA microgram value obtained by this method cannot be considered as only the residual FeEDDHA. A segment of this value is part of the residual ligand and other metal chelates of EDDHA which have combined with the sodium acetate soluble plant iron during the homogenization and extraction steps.

For the determination of the remaining EDDHA and other EDDHA metal chelates (total EDDHA metal chelates and ligand other than FeEDDHA are designated as M-EDDHA), an aliquot of the combined aqueous extracts was diluted 1:1 with a 0.02 *M* $FeSO_4$-0.1% $NH_2OH \cdot HCl$ solution and the spectra again read from 350 to 700 mμ and the absorbance at 480 mμ recorded. This last step converts the remaining sodium acetate soluble EDDHA and EDDHA metal chelates to the ferric EDDHA form. This is accomplished either by oxidative chelation of the ferrous ion by EDDHA or by mass action replacement of other metals from EDDHA by ferric ion due to the higher stability of FeEDDHA than the other metal chelates of EDDHA.

Addition of the $FeSO_4$-NH_2OH solution did not show an interference absorption at 480 mμ in the pure solution tests and has been used as a method of detection for the EDDHA ligand and metal chelates (4).

This method has been used satisfactorily in this laboratory for the determination of residual EDDHA metal chelates in tomato leaf, stem, and root tissue (5).

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A Simplified Preparation of the Unusual Disaccharides, 3-Ketosucrose and Allosucrose

We would like to report a rapid and convenient method for preparing the two unusual disaccharides, α-D-ribohexopyranosyl-3-ulose β-D-fructofuranoside ("3-ketosucrose") and D-allosyl-β-D-fructofuranoside ("allosucrose"), first isolated by Fukui *et al.* (1), and a method for preparing isotopically labeled sucrose and allosucrose. These sugars are currently being used to study properties of invertase and sugar uptake by *Neurospora conidia* in this laboratory.

A strain of *Agrobacterium tumefaciens*, which accumulates 3-ketosucrose in its medium when grown on sucrose, was kindly provided by Dr. R. M. Hochster. The bacteria were grown and polysaccharides and salts were precipitated with ethanol from the growth medium containing the 3-ketosucrose by the methods of Fukui *et al.* (1). 3-Ketosucrose was identified by its mobility on paper chromatograms and its characteristic staining reactions (1). For quantitative estimation of 3-ketosucrose, the extent of reduction of triphenyltetrazolium chloride by 3-ketosucrose was measured by dissolving the red reduction product in 7 ml of ethyl acetate and determining the optical density at 490 mμ. A standard curve was prepared by determining the absorbancy of completely reduced equivalents of triphenyltetrazolium.

Approximately 2 gm of crude 3-ketosucrose was applied to a Dowex

1 column (2×75 cm) in the borate form (2) and eluted with a linear gradient of boric acid from 0.0 to 0.125 *M*. Sucrose emerged first and was completely separated from the later eluting 3-ketosucrose. An alternative method used was to elute with 0.05 *M* boric acid until no more sucrose emerged and then to elute the 3-ketosucrose with 0.100 *M* boric acid. Fractions containing 3-ketosucrose were combined and taken to dryness at 30° with a rotary evaporator, and boric acid was removed by evaporation with methanol (2). The resulting residue of 3-ketosucrose was completely free of other sugars. The recovery of the 3-ketosucrose from the column was approximately 40% of that applied, indicating a substantial loss on the column, perhaps due to oxidation and/or polymerization.

3-Ketosucrose was reduced with sodium borohydride at 4° (1). A 3- to 4-fold excess of $NaBH_4$ in 0.2 *M* phosphate buffer, pH 6.5, was added to 3-ketosucrose dissolved in the same buffer, the pH being held at 6.5 with 0.5 *M* oxalic acid. The mixture was stirred in the cold for several hours and allowed to stand overnight. The reduction mixture was treated with mixed-bed resin to remove salts, reduced in volume, and treated with methanol to remove boric acid. The mixture, which contained sucrose and allosucrose, was then applied to a Dowex 1-borate column (2×75 cm) equilibrated with water and eluted with a linear gradient of 0.0–0.1 *M* boric acid. Sucrose emerged first and was completely separated from the major component, allosucrose, that appeared at approximately 0.08 *M* boric acid. Following removal of boric acid from the combined fractions containing allosucrose, the product was observed to be completely free of other sugars. It was found possible to carry out the borohydride reduction on the polysaccharide-free crude medium and then to isolate the allosucrose by the methods given above. This procedure eliminates the need for prior separation of 3-ketosucrose and the approximate 60% loss of the latter that occurs during ion-exchange chromatography.

Sucrose and allosucrose specifically labeled with tritium in the 3 position of the afructone (glucose and allose, respectively) were prepared by reduction of 3-ketosucrose with sodium borotritiide. Sodium borotritiide (10 μmoles, 200 mc/mmole) was added directly to 25 μmoles of 3-ketosucrose in 2.5 ml of 0.04 *M* phosphate buffer, pH 6.5, and stirred for 1 hr; then, 100 μmoles of sodium borohydride was added to ensure complete reduction of the 3-ketosucrose. The reduction mixture was treated with mixed-bed resin and applied to a Dowex 1-borate column (4×50 mm). A linear gradient of boric acid, 0.0–0.1 *M*, was used for elution. Fractions (12 ml) were collected and examined for tritium by counting aliquots in a Packard scintillation counter—using 10 ml of Bray's solvent (3) +1 ml water. Two sharp, completely separated peaks

of radioactivity were apparent. The first compound, which contained approximately 10% of the total radioactivity, emerged at the correct position for sucrose and gave a positive chemical test for sucrose (positive glucose oxidase test following cleavage with invertase). The second compound, at the correct position for allosucrose, was a disaccharide which contained fructose, and was concluded to be allosucrose. Samples (10 μl) of the H^3-labeled sucrose and allosucrose were spotted onto Whatman No. 1 paper along with much larger chemical amounts of nonradioactive sucrose and allosucrose, respectively; all of the radioactivity moved with the disaccharides in the solvent system *n*-propanol/ethyl acetate/water (7/1/2, v/v). Upon fermentation of the labeled allosucrose with washed yeast cells, an unfermentable labeled monosaccharide was obtained which was chromatographically identical with D-allose. In similar fashion, D-glucose-3-H^3 might be prepared by splitting the labeled sucrose with invertase or by acid hydrolysis.

ACKNOWLEDGMENTS

We thank Dr. Nelson K. Richtmyer for furnishing an authentic sample of D-allose. This work was supported in part by a grant from the U. S. Public Health Service (GM-08995-04) and by a National Science Foundation Postdoctoral Fellowship to one of us (G. A. M.).

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A New Method for the Determination of Deoxyribonucleic Acid[1]

Recently Seydel and Garrett (1) described an ultraviolet chromophore with a maximum absorption at 261 mμ, which is unique to solu-

[1] This Research was supported by NIH Grants CA 18402 and CY 6506.

tions of 2′-deoxy-D-ribose heated at 80°C in 1 *N* HCl for at least 5 hr. Under these conditions the amount of the chromophore produced is directly proportional to the deoxyribose concentration. The concentration of the chromophore can be measured either by calculating the difference in absorbance at 261 mμ before and after destruction of the chromophore with alkali, or by extracting the chromophore with chloroform and measuring the absorbance of the extract at 261 mμ. In view of the simplicity as well as the specificity of the reaction it seemed desirable to investigate its applicability to the determination of deoxyribonucleic acid (DNA). As nucleic acids and their breakdown products produce high extinctions in the region of 261 mμ, it was decided to use the latter method because, as Seydel and Garrett pointed out, nucleosides or bases are not extracted into the chloroform phase. The procedure was carried out as follows: 3 ml 1 *N* HCl containing the sample was heated for at least 5 hr at 80° and extracted with 2 vol (1.5 ml each) of chloroform.

As is shown in Fig. 1, a straight line was obtained with deoxyribose as well as with a commercial preparation of sperm DNA (Nutritional

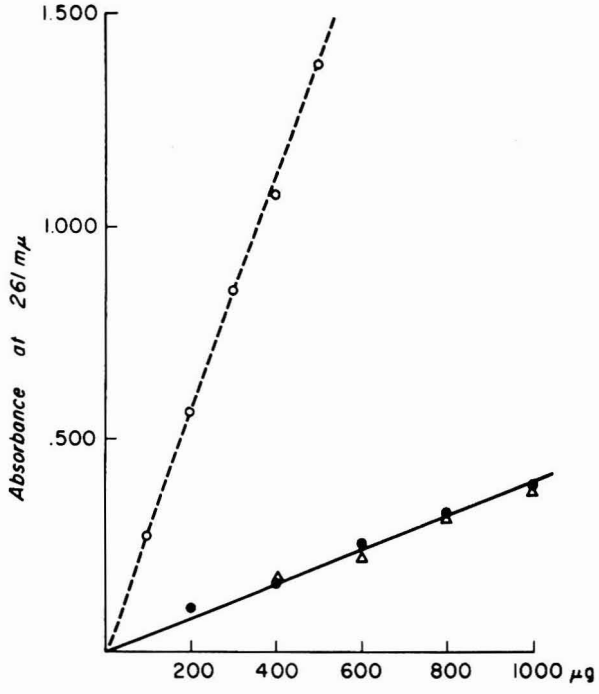

FIG. 1. Absorbances of various concentrations of deoxyribose (○) and of DNA, untreated (●) and hydrolyzed with 1 *N* perchloric acid (△+) prior to incubation in 1 *N* HCl.

Biochemicals Corp.). Hydrolysis of the DNA in 1 *N* perchloric acid for 30 min at 90° prior to the incubation in 1 *N* HCl did not change the results. Identical samples containing 900 μg DNA were incubated in 0.25, 1, and 2 *N* HCl for varying periods of time at 80°. The optical densities of the chloroform extracts plotted against time are presented in Fig. 2.

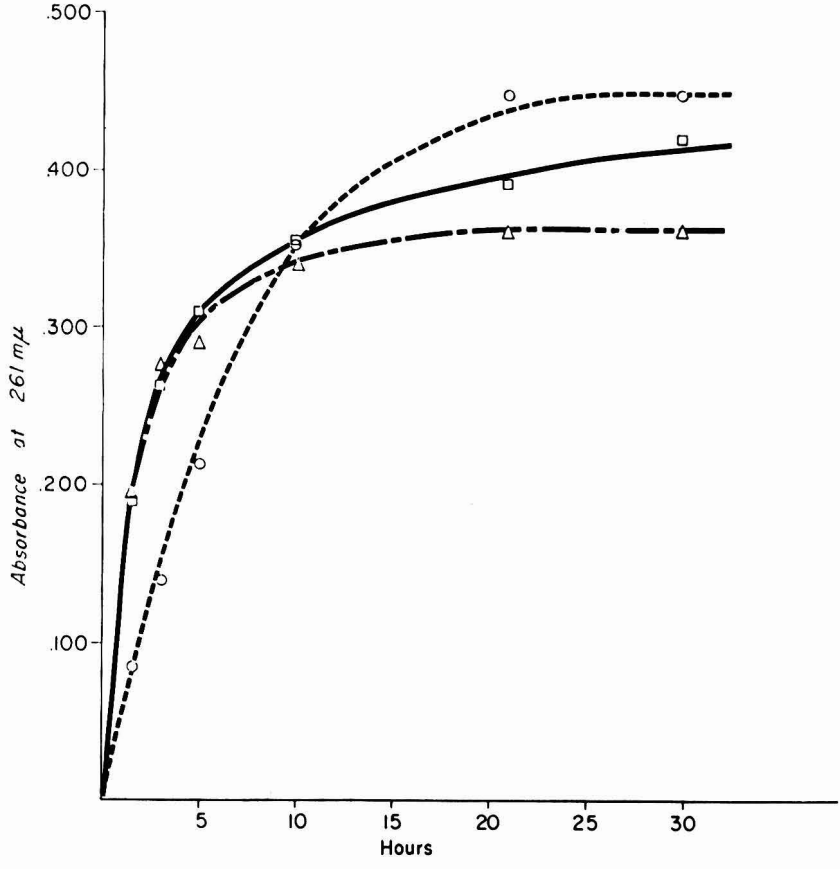

FIG. 2. Development of the 261 mμ chromophore with time in 0.25 *N* HCl (○---○), 1 *N* HCl (△—△), and 2 *N* HCl (□—□).

It appears that the formation of the chromophore in 1 and 2 *N* HCl is approximately 80% after 5 hr and approaches the maximal concentration after 10–30 hr. With 0.25 *N* HCl only 50% of the maximal concentration is obtained after 5 hr incubation. The maximal yield is slightly higher than with 1 or 2 *N* HCl and is approached after 20 to 30 hr, so that this procedure could be used for overnight incubations.

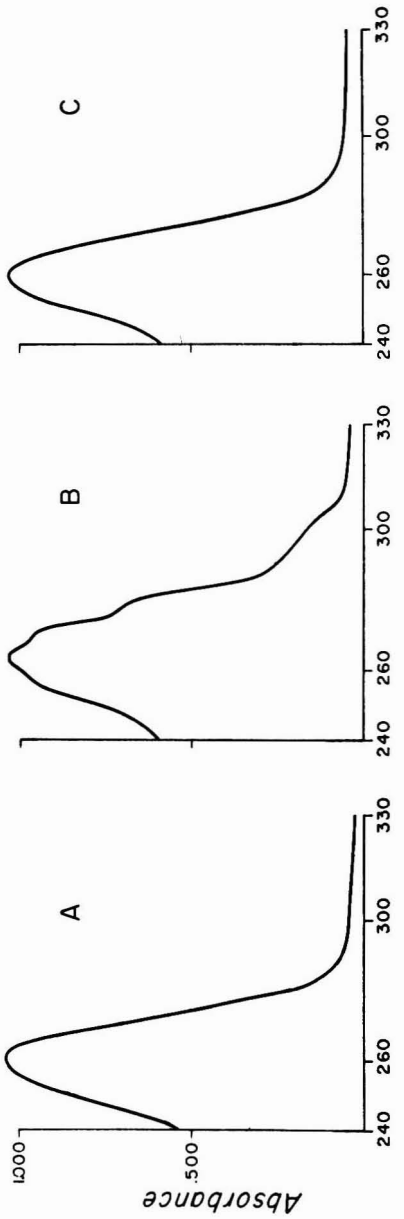

FIG. 3. Spectra of chloroform extracts of 1 *N* HCl "hydrolyzates" of DNA after incubation at 80°C for 18 hr: (A) sperm-DNA, (B) sheep spleen DNA, (C) sheep spleen DNA treated with perchloric acid for 20 min at 90°C followed by ether extraction prior to incubation in 1 *N* HCl.

In order to test the method on other DNA preparations, DNA was extracted from rat liver and sheep spleen with phenol as described by Colter *et al.* (2), and then treated according to Schmidt and Thannhauser (3). The final precipitates were washed with ethanol and ether and air-dried. Determination of the DNA concentration of solutions of these samples, according to the method presently described, yielded values which were too high. Apparently substances are released from the DNA during the incubation, which also absorb in the ultraviolet region around 261 mμ. As is shown in Fig. 3A and B, spectra of the chloroform extracts of, respectively, sperm DNA (Nutritional Biochemicals Corp.) and sheep spleen DNA indicate that, in contrast to the sperm DNA, the sheep spleen DNA gave rise to chloroform-soluble materials with absorption maxima at 265, 270, 280, and 295 mμ, in addition to the chromophore with $\lambda_{max} = 261$ mμ. The same sample of sheep spleen DNA was therefore extracted with 0.5 *N* perchloric acid at 90° for 20 min, and the resulting solution extracted 3 times with ether. The ether was removed by bubbling through nitrogen. The solution was then made up to 3 ml and 1 *N* HCl and extracted 18 hr at 80°. The spectrum of the chloroform extract, shown in Fig. 3C, indicates that the extraneous absorbing materials had been removed by this procedure. DNA preparations from rat liver and sheep spleen were therefore both pretreated with perchloric acid and ether. As can be seen in Table 1, good agreement was obtained between the DNA concentrations determined according to the method presently described and the diphenylamine reaction (4).

TABLE 1

COMPARISON OF VALUES OBTAINED BY THE PRESENT METHOD AND THE DIPHENYLAMINE REACTION

DNA preparation	DNA concn. in mg/ml as determined by	
	Diphenylamine reaction[a]	HCl-chloroform
Rat liver (1 mg/ml)	0.94[b]	1.01
Sheep spleen (1 mg/ml)	0.90	0.97

[a] The diphenylamine reaction was carried out and the difference of the absorbance at 595 and 650 mμ was measured according to Dische (4).

[b] The values are averages of four determinations.

In summary, it appears that the reaction described by Seydel and Garrett can be applied to isolated and purified DNA. The DNA is heated in 0.5 *N* perchloric acid, and the supernatant of this extract is washed three times with ether. After removal of the ether by bubbling through nitrogen, aliquots are made up to 3 ml and 1 *N* HCl and incubated for

18 hr at 80°C. The solutions are extracted twice with 1.5 ml chloroform after cooling to room temperature and the extract is read at 261 mμ.

ACKNOWLEDGMENTS

The author wishes to thank Dr. E. R. Garrett for his interest and encouragement and Mrs. Mary Jo Heeb for expert technical assistance.

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Errata

Vol. **11,** No. 1, 1965, (pages 1–5), in the article entitled "The Fluorometric Analysis of Ultramicro Quantities of Hydrogen Peroxide" by Albert S. Keston and Richard Brandt:

page 2, under the heading "Procedure," the sentence "II. LADCF was prepared as in I and contained 0.001 mg/ml peroxidase" should read: "II. LDADCF (alkali activated) was prepared as in I and contained 0.001 mg/ml peroxidase."

Vol. **11,** No. 1, 1965, (pages 6–9), in the article entitled "Synthesis of Diacetyldichlorofluorescin: A Stable Reagent for Fluorometric Analysis" by Richard Brandt and Albert S. Keston:

page 6, under the heading, "Synthesis Procedure," the sub-heading "Preparation of Dichlorofluorescein (LDCF)" should read: "Preparation of Dichlorofluorescin (LDCF)."

page 7, the sub-heading "Preparation of Diacetyldichlorofluorescein (LDADCF)" should read: "Preparation of Diacetyldichlorofluorescin (LDADCF)."

Vol. **11,** No. 2, (pages 335–341), in the article entitled "A Simple Procedure for the Extraction and Esterification of Some Organic Acids" by Nancy W. Alcock:

page 338, lines 1–3 following the legend to Fig. 1 should read as follows:

Hydrogen flow rate 2.5 cm on rotameter with 12 lb/sq in. pressure
Air flow rate 3.0 cm on Rotameter with 12 lb/sq in. pressure
Helium flow rate 3.0 cm on rotameter with 30 lb/sq in. pressure

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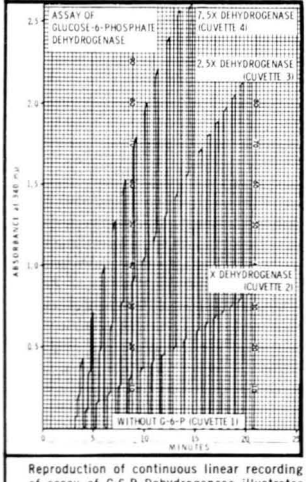

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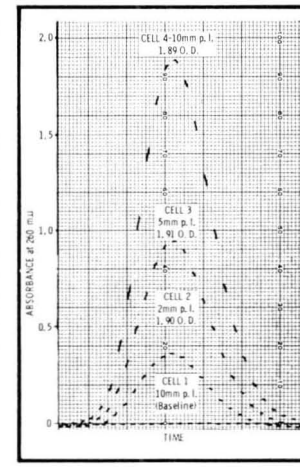
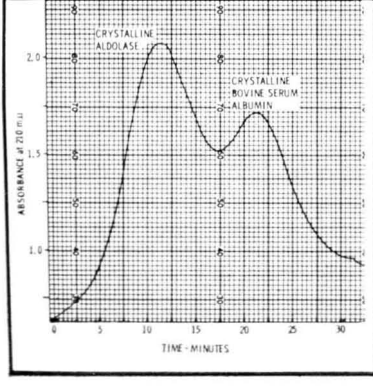


Chromatogram of Dowex-1 column eluted with Formic Acid shows section with AMP peaks only. Record made with Gilford Model 2000 equipped with Gilford Flow-Through Cuvettes and Automatic Blank Compensator.

Salmon Sperm DNA profile illustrates linear registration of both temperature and absorbance changes on the same scale. Gilford Model 2000 equipped with Gilford Linear Thermosensor which measures temperature changes in cuvette chamber.



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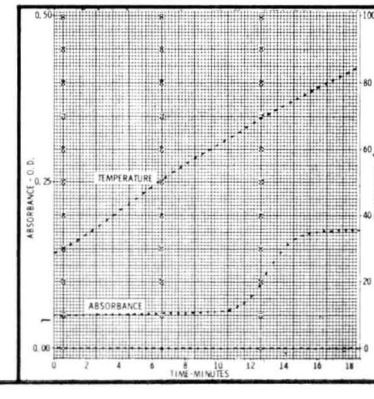

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Contents:

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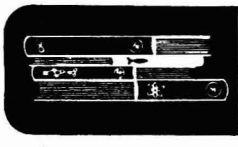

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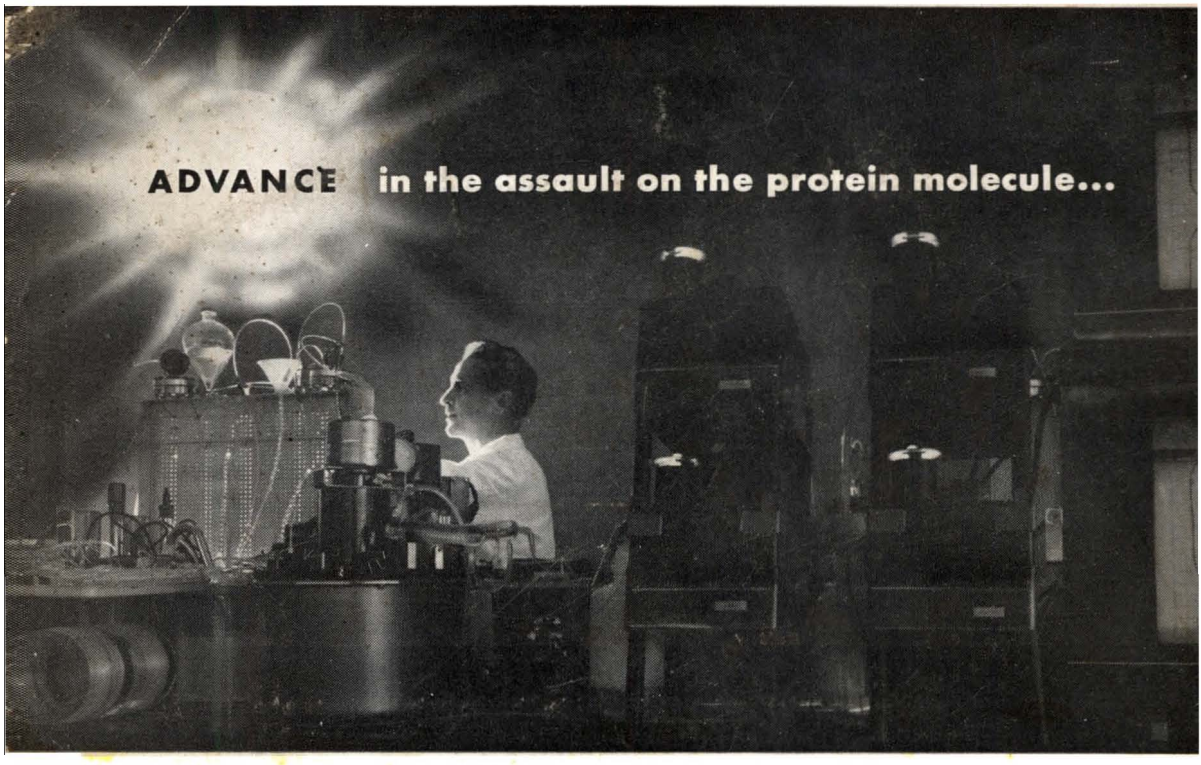

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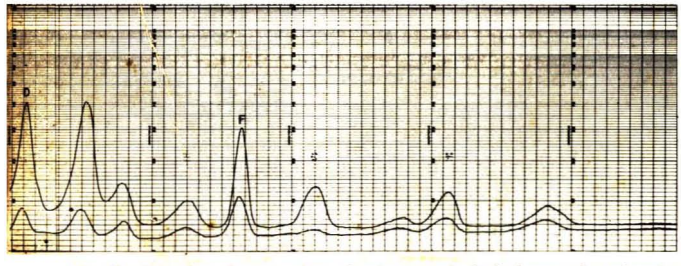

Section of a chromatogram showing tryptic hydrolyzate of performic acid oxidized bovine pancreatic Ribonuclease before and after alkaline hydrolysis.

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