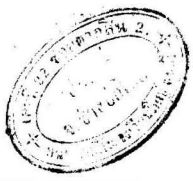

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SHORT COMMUNICATION

ANALYTICAL BIOCHEMISTRY 4, 1–9 (1962)

pH Gradient Elution in Column Chromatography[1]

JOHN M. REINER AND BUENA REINER

From the Department of Microbiology and Department of Pharmacology, Division of Basic Health Sciences, Emory University, Georgia

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INTRODUCTION

The popular and useful chromatographic technique of elution by means of concentration gradients is susceptible to a simple mathematical analysis, so that an experimenter may arrange to get gradients of given shape and amount. Some of the formulas have been collected by Svensson (1). No comparable theory has been developed for use in connection with gradients of pH. Since the latter have proved especially helpful in the chromatography of macromolecules (2), it seemed worthwhile to investigate the theory. Inasmuch as a combination of pH gradient and ionic strength gradient proved most useful, we attempted to extend the theory by correcting for ionic strength effects. Comparison with experimental data revealed the usual deficiencies in the classical corrections, as well as some interesting individual idiosyncrasies of certain ions. A satisfactory empirical correction, however, was developed.

THEORY

a. Gradients of Concentration. We shall confine ourselves for simplicity to systems in which a single mixing chamber is employed. If the reservoir containing the strong solution is hermetically connected to the mixer, the incompressibility of the solvent in the case of aqueous systems insures constancy of volume of fluid in the mixing chamber. Under these conditions, the concentration of the eluting solute in the efflux from mixer into column is given (1) by:

$$C = C_r - (C_r - C_0)e^{-rt} \tag{1}$$

In this equation, C_r is the concentration in the reservoir, C_0 the initial concentration in the mixer, t the time, e the basis of the natural logarithms, and r the ratio of the volume flow rate through the reservoir-mixer system to the volume V of fluid in the mixer (e.g., if the flow rate is

[1] This investigation was supported in part by grants from the United States Public Health Service and the National Science Foundation.

regulated at 15 ml/hr, and the liquid volume in the mixing chamber is 300 ml, $r = 15/300 = 1/20$). Thus rt is also equal to total volume flow that has occurred at time t, divided by mixer volume.

Suppose a dilute buffer at given pH is added to the mixer, while the reservoir, in addition to the neutral electrolyte, contains the same buffer at a different pH and concentration (as an extreme case, the free base of the buffer system). As the buffer enters the mixer from the reservoir, the pH will shift as the ratio of base to acid in the buffer system is raised. The pH at any time, aside from corrections for ionic strength, will depend on the time variation of base and acid in the mixer.

Let the initial concentrations of base and acid in the buffer system of the mixer be B_0 and A_0. Suppose buffer is added to the reservoir with base at concentration B_r, and acid at concentration A_r. The changes in concentration of the components in the mixer are governed by the obvious differential equations:

$$dB/dt = r(B_r - B), \qquad dA/dt = r(A_r - A) \tag{2}$$

where B and A are the concentrations of base and acid in the mixer at time t. The terms for the rate of interconversion of base and acid in the mixer vanish on the customary assumption that the dissociation equilibria of electrolytes are established almost instantly.

The solution of the differential equations is:

$$B = B_r - (B_r - B_0)e^{-rt}, \qquad A = A_r - (A_r - A_0)e^{-rt} \tag{3}$$

If pure base rather than buffer is placed in the reservoir, the parameters B_r and A_r are calculated from the concentration B_t of added base in the conventional fashion, A_r being approximately equal to the concentration of OH^-. If pure acid A_t is added, a comparable calculation results.

The extension to cases in which mixing occurs in several stages, or in which mixer volumes are not constant, is obvious, and will not be treated in detail. No new principles are involved beyond those already familiar for solutions of nonelectrolytes or neutral electrolytes.

The calculation of the pH at time t, when a volume Vrt has flowed through the system, involves the ratio of B and A as given by eq. (3), with corrections to be considered in the next section.

b. pH and Ionic Strength. Consider a buffer system of the kind just described; for instance, let the buffer be tris(hydroxymethyl)aminomethane (commonly referred to as Tris). The stoichiometric equation for the buffer equilibrium may be written:

$$B + H^+ \leftrightharpoons A^+ \tag{4}$$

where A^+ is the acid (cation) corresponding to the base B. At any ionic strength, the equilibrium equation is:

$$a_b a_h / a_a = K \tag{5}$$

where a_b is the *activity* of the base, etc., and K is the true thermodynamic acid dissociation constant. Since activities are related to concentrations by the activity coefficients (e.g., $a_b = y_b B$), while by definition pH = $-\log a_h$, we can take the logarithm of both sides of Eq. (5) and rearrange to get:

$$\text{pH} = \text{p}K + \log (B/A) + \log y_b - \log y_a \tag{6}$$

Corrections for effects of environment (such as ionic strength in the solvent) on the buffer equilibrium are embodied in the expression $\log y_b - \log y_a$. Since this expression is added to the simpler and more familiar expression $pK + log(B/A)$, the correction may be applied to the buffer system independently of the value of B/A. The correction is sometimes considered to add to the term pK to give a term pK', K' being called the *apparent* dissociation constant.

There is a large body of attempts at theoretical calculation of activity coefficients in systems like this (3–8). The best-known expression, applicable to simple ions at not too great values of the ionic strength, is (3) of the form $FI^{1/2}/(1 + fI^{1/2})$, where I is the ional concentration (twice the ionic strength) and F and f are constants depending on the temperature and the dielectric constant of the solvent. An improved expression by Fuoss and Onsager (7) has the same functional form. Various terms that are linear in the ional concentrations have been proposed, either as empirical corrections to take care of the anomalous behavior of activity coefficients at high ionic strengths, or to account for the effects of electrolytes on the solubilities of nonelectrolytes ("salting-in" or "salting-out" terms), or (7) as the result of more rigorous solutions of the equation for the electrical potential due to an ion. The linear terms may be positive or negative in sign, depending on their origin.

Both kinds of terms apply to the charged form of the buffer (A^+), only the linear form to the uncharged base B. We may therefore write:

$$\log y_b = k_b C, \qquad \log y_a = -M\kappa a/(1 + \kappa a) + k_a C \tag{7}$$

In these equations, C is the concentration of the electrolyte, a is the mean distance of closest approach to the ion under consideration (in this case A^+) by all other ions, and:

$$M = z\epsilon^2/2DkTa, \qquad \kappa = (4\pi\epsilon^2 NI/1000DkT)^{1/2}, \qquad I = \Sigma C_i z_i^2 \tag{8}$$

where ϵ is the charge on the electron in esu, D is the dielectric constant of the solvent, k is Boltzmann's constant, T is the absolute temperature, N is Avogadro's number, and I is the ional concentration, given by the summation (Σ) of concentration times square of valence for all ions in the solution, while z is the valence of the ion under consideration (A^+).

In view of Eq. (7), we can express the correction terms in Eq. (6) as:

$$\log y_b - \log y_a = M\kappa a/(1 + \kappa a) - k_s C \qquad (9)$$

where $k_s = k_a - k_b$. It is frequently assumed that electrostatic effects have been segregated, as it were, in the first term of Eq. (9), and that the "salting-out" effect is the same for the neutral base and the cationic acid (4). This implies $k_a = k_b$, or $k_s = 0$; if it were correct, the second term of Eq. (9) would disappear. Since the remaining term is positive, it would be predicted that the pH of such a buffer system would rise with increasing ionic strength.

EXPERIMENTAL METHODS

Measurements of pH were made with a Beckman pH meter, Model G, using external glass and calomel electrodes with shielded leads. The Beckman Type E-2 glass electrode (which is made of special glass to minimize salt effects at high pH) was used in some experiments to check results obtained with the ordinary (General Purpose Glass) electrode.

For pH measurements requiring some precision, electrodes must be flushed heavily with distilled water between samples, until the pH returns to neutrality. The usual rinse from a wash bottle is wholly inadequate when dealing with concentrated salt solutions at elevated pH values.

Readings were taken within a few seconds after immersion of the electrodes in the test solutions, to avoid subsequent drift due to the establishment of a liquid junction potential. This source of artifact is not negligible in concentrated solutions of LiCl in particular, since the mobility of Li^+ is only half that of Cl^- (5). Application of the Henderson equation (6) indicates that the liquid junction potential may raise the apparent pH by as much as 0.1 unit in 2 M LiCl; and our observations are in agreement with this expectation. Readings in simple aqueous solutions or dilute salt showed no such drift (if electrodes had been adequately rinsed).

RESULTS

The most casual observation demonstrated that the assumption $k_s = 0$ must be wrong. Thus, if 2-amino-2-methyl-1,3-propanediol (Diol) was dissolved in 5 M LiCl, we did not observe a higher pH than the 10.9 of the simple aqueous solution of the base; instead, the measured pH was about 9.8. The pH of Tris in strong LiCl solutions was erratic, occasionally dropping below 7.

If the shape of the buffer molecule or its degree of hydration were to be affected by the presence or absence of the proton, one would expect that k_s would not vanish. In order to get at the mechanism of the observed effect (and also in order to get estimates of k_s values), a series of measurements was undertaken, employing various substituted amines dissolved

in a series of salt concentrations. In addition to LiCl, we tried NaCl, $NaNO_3$, and KCl. In addition to Tris and Diol, we used isopropylamine, 2-amino-2-methylpropanol, and *tert*-butylamine.

In Fig. 6, theoretical and experimental gradient curves are compared. The conditions are those usually employed (2) for elution of the high molecular weight fraction of nuclear ribonucleic acid from Ecteola cellulose. The mixer contained 300 ml of 0.005 M Diol in 2 M LiCl, adjusted to pH 9.0 with HCl. The reservoir contained 0.1 M Diol (free base) in 6 M LiCl. Duplicate 5-ml fractions of the effluent from the mixer were collected at a total flow rate of about 30 ml/hr.

By using Eqs. (1) and (3) to calculate C and B/A, the correction terms were calculated from Fig. 2. The pK of Diol at 25°C is 8.8. The values of the other parameters used were: A_0, $1.2 \times 10^{-3} M$; B_0, $3.8 \times 10^{-3} M$; A_r, $8 \times 10^{-4} M$; B_r, $10^{-1} M$.

The pH values of Ecteola column effluents are slightly higher than either the theoretical or the experimental values of our Fig. 6. This is not altogether surprising, since the exchanger is a buffer, and also because it removes some of the electrolyte.

DISCUSSION

First of all, it may be observed that the calculated and measured pH values of Fig. 6 are in fair agreement. Our method for empirical correction of pH gradient curves would seem to be a practical one.

In the second place, we note that the theory of Eqs. (6) to (9) fails us at low at least as badly as at high salt concentrations. In Figs. 1–4, many

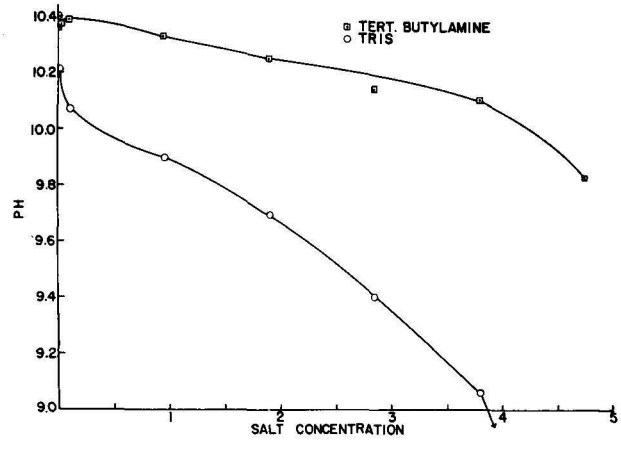

FIG. 1. Variation of pH with salt concentration in moles per liter for 0.1 M solutions of *tert*-butylamine and Tris in LiCl.

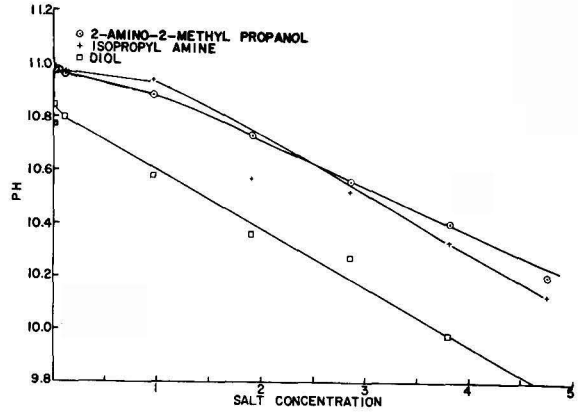

FIG. 2. Variation of pH with salt concentration in moles per liter for 0.1 M solutions of 2-amino-2-methylpropanol, isopropylamine, and Diol in LiCl.

of the curves either show an initial sharp drop in the region below 0.1 M salt, or, after a very slight initial rise, drop with a marked inflection rather than in simple linear fashion.

The analysis of the linear portions of the curves at high salt concentration is, as usual, problematical. Scatchard (4) has derived an equation formally identical with our Eq. (9), by taking into account the variation of dielectric constant with concentration. Scatchard's Eq. (183) gives a linear term of the right sign and right order of magnitude if we approximate the dielectric constant of the solution by a linear function of salt concentration in the usual fashion ($D = D_0 - gC$, so that the factor

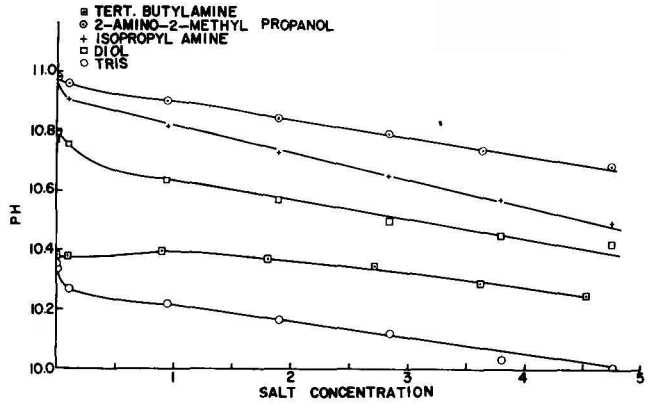

FIG. 3. Variation of pH with salt concentration in moles per liter with all five bases at 0.1 M in NaCl.

$1 - D/D_0$ becomes gC/D_0). (The constant R should of course be replaced by k.) Using the data of Harris and O'Konski (9) (and ignoring the fact that the concentration curves for the dielectric constant are not strictly linear), we obtain not only the right order of magnitude, but the correct ratio for the constants in LiCl and NaCl solutions, respectively. (The case of KCl will be considered shortly.)

If alternatively we attempt to account for the differences between k_a and k_b by applying Kirkwood's treatment of ion-dipole interactions (4), the terms come out, for any reasonable values of the dipole moments, to account for but a small fraction of the observed k_s values.

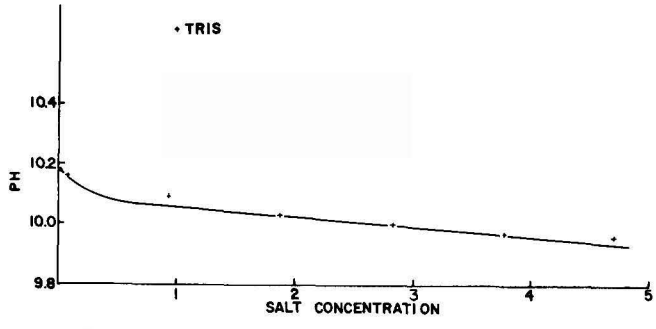

FIG. 4. Variation of pH with salt concentration in moles per liter with Tris at 0.1 M in $NaNO_3$.

The difficulty with accepting the relatively pleasing results obtained using Scatchard's equation is the nature of the higher terms in the expansion of Fuoss and Onsager (7). The second term (in G_3) is small and presents no problem. The third (in H(b,2)), however, is of the order of 1 or 2, and has the wrong sign, so that it would easily cancel the term from Scatchard's equation. Since the activity coefficients of electrolytes generally pass through a minimum with increasing concentration, the problem is not confined to our particular experimental situation, as Fuoss and Onsager (7) among others have pointed out.

In view of the first four figures, the results obtained with KCl as the electrolyte (Fig. 5) are remarkable. Since the curves asymptotically approach a linear course at high salt concentrations, it does not seem likely that $k_s = 0$ in this case. Instead, it would appear that k_s is negative—in other words, that k_a is less than k_b, or that k_a itself is negative. This would amount to a salting-in effect. Such a result would fit Scatchard's equation if potassium salts were to *increase* the dielectric constants of their aqueous solutions. Such a situation is suggested by experiments which have been interpreted to mean (10) that potassium ions show negative hydration—

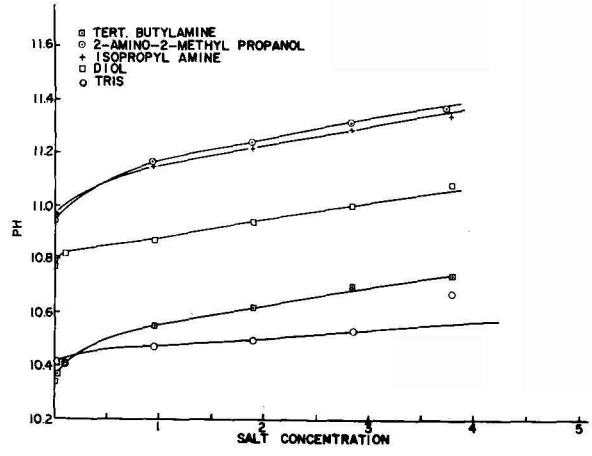

FIG. 5. Variation of pH with salt concentration in moles per liter with 0.1 *M* solutions of all five bases in KCl.

that is, increase rather than decrease the mobility of water molecules in their neighborhood. Unfortunately for the interpretation, the data of Harris and O'Konski (9) show a decrease of dielectric constant with concentration for potassium salts, albeit a much weaker decrease than for other electrolytes. The suggestion is also contradicted by the results of Brady (11), which indicate that potassium fits into the structure of water without disturbance, while it is lithium that behaves abnormally.

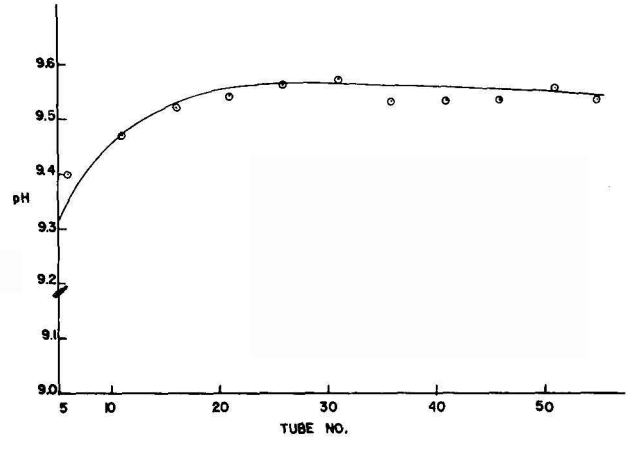

FIG. 6. Comparison of theoretical and experimentally observed pH gradients in presence of a salt concentration gradient. The buffer system was Diol, the salt LiCl; conditions specified in the text. The solid line is the calculated curve; the open circles are the experimental points.

The choice of buffer substances was based in part on the desire to see whether the hydroxyl groups in Tris and Diol are important for the salt effects. It is evident that the initial anomaly is most marked for the buffers with two or three hydroxyl groups, while those with only one hydroxyl or none show a relatively weaker dependence on salt concentration at low salt levels. Moreover, the contrast is more marked with LiCl as the electrolyte than with NaCl. The nature of the mechanism is not clear; but the numerical insignificance of the dipole moments (mentioned above) suggests by elimination that hydration of the buffer ion may be involved. The fact that 2-amino-2-methylpropanol groups itself with isopropylamine rather than with Diol hints at internal hydrogen bonding among the hydroxyl groups as one of the factors (rather than hydrogen bonding of the N and OH groups).

SUMMARY

A theory of the development of pH gradients in chromatographic eluents is presented, including an attempt at theoretical corrections for ionic strength. Experimental data on the variation of pH with salt concentration present features not fully accounted for by any theory. In seeking agreement between theory and experiment, it is necessary to postulate that salting-out effects are not the same for a neutral molecule and its charged form. Among the uniunivalent electrolytes tested, KCl differs radically from the others in showing a salting-in effect. The significance of the results is discussed.

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Quantitation of Keratan Sulfate in the Presence of Other Corneal Glycosaminoglycans[1]

BERNARD WORTMAN[2]

From the Department of Ophthalmology, Washington University, School of Medicine, St. Louis, Missouri

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At least half of the glycosaminoglycans of connective tissues such as cornea and nucleus pulposus is composed of keratan sulfate. The amount of keratan sulfate present in cartilage is found to increase with age of the animal (1) and is closely associated with the chondroitin sulfate-protein complex (2, 3). Keratan sulfate has not been found to exist in the absence of other glycosaminoglycans in any single tissue. Therefore, studies of keratan sulfate can be complicated by the presence of other glycosaminoglycans and by its interaction with other polysaccharide-protein complexes. The study of keratan sulfate metabolism (4) could be facilitated if there were available a simple colorimetric method for its detection in the presence of corneal glycosaminoglycans. The availability of ion exchangers (5, 6) and methods for determining hexoses (7) suggested that it would be feasible to devise simple colorimetric techniques which would permit the quantitation of uronic acid-deficient glycosaminoglycans in the presence of other forms. The cornea has been selected to demonstrate the feasibility of these techniques.

MATERIALS AND METHODS

Chemicals. Glucuronic acid, hyaluronic acid (from umbilical cord), Ecteola, chondroitin sulfate, *N*-acetylglucosamine, and galactosamine were obtained from Sigma Chemical Company, St. Louis, Missouri. Glucosamine and *N*-acetylgalactosamine were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Hyaluronic acid (from vitreous), keratan sulfate,[3] and corneal glycosaminoglycans were isolated from beef eyes by previously described methods (8, 9).

[1] Supported in part by research grant B-1911 from the National Institutes of Health, U. S. Public Health Service.

[2] This work was done during tenure as an Established Investigator of the American Heart Association.

[3] Authentic keratan sulfate was obtained from Dr. Torvard C. Laurent, Medical Chemistry Institute, University of Uppsala, Sweden.

Anion-Exchange Chromatography. Ecteola used in this study had an exchange capacity of 0.3 meq/gm. Ecteola was washed in 1 *M* NaOH, water, ethanol, and ether, and air dried. Six grams of Ecteola were suspended in 0.05 *M* HCl, and columns packed under 6 psi pressure and equilibrated overnight with 0.05 *M* HCl. Corneal glycosaminoglycans were dissolved in distilled water and applied to the Ecteola columns, which were then eluted with 0.05 *M* HCl and with a linear gradient to 1 *M* NaCl in 0.05 *M* HCl. The eluent was assayed for hexuronic acid by a modification of the carbazole method and hexose by the cysteine-H_2SO_4 method and compared with glucuronic acid and galactose standards, respectively (7).

RESULTS

Absorption Spectrum. In the presence of cysteine, keratan sulfate and chondroitin sulfate showed absorbancy maxima at 410 mμ and at 400 mμ, respectively (Fig. 1). In the absence of cysteine, absorbancy was very

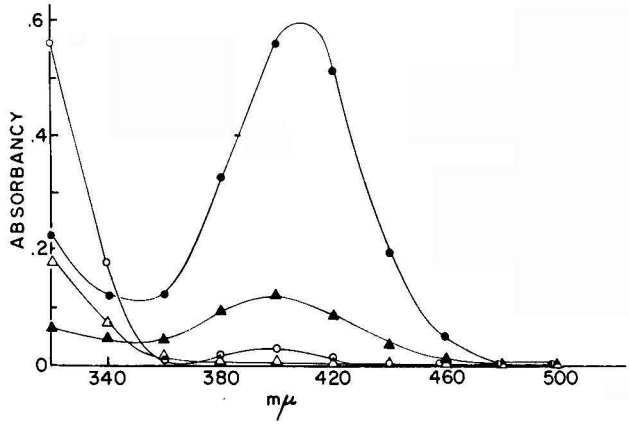

FIG. 1. Absorption spectra of products formed from keratan and chondroitin sulfates. Method: 200-μl volumes containing 68 μg of keratan sulfate or 62 μg of chondroitin sulfate were mixed with 1 ml of an 85% H_2SO_4 solution and heated for 3 min at 100°; the absorption spectra were measured in the presence and absence of 20 μl of a 3% cysteine HCl solution. The symbols have the following designation: keratan sulfate, with (●) and without (○) cysteine; chondroitin sulfate, with (▲) and without (△) cysteine.

low but increased at lower wavelengths. At 410 mμ, the absorbancy due to keratan sulfate is 5.5 times as great as that due to chondroitin sulfate when corrected for absorbancy measurements made in the absence of cysteine (Fig. 1). Similarly, the absorbancy ratio at 405 mμ for equivalent weights of keratan sulfate and chondroitin sulfate is 5.6 (Table 1).

TABLE 1

EFFECT OF VARIOUS COMPOUNDS ON COLOR DEVELOPMENT

Compound[a]	Amt. (μg)	Absorbancy (405 mμ) with cysteine	
		Present[b]	Absent
N-Acetylglucosamine	24.7	0.026	0.003
N-Acetylgalactosamine	21.0	0.020	0.000
D-(+)-Glucosamine	24.7	0.336	0.005
D-(+)-Galactosamine	24.5	0.241	0.004
D-(+)-Galactose	31.5	0.584	0.028
Glucuronic acid	26.6	0.134	0.005
Hyaluronic acid (vitreous)	96.0	0.108	0.006
Hyaluronic acid (umbilical cord)	83.0	0.168	0.011
Chondroitin sulfate	87.7	0.128	0.012
Keratan sulfate	93.4	0.710	0.022
Corneal glycosaminoglycans	97.1	0.356	0.019
Water blank		0.023	0.000

[a] 200 μl containing the listed amount of compound to be tested was mixed with 1 ml of 85% H_2SO_4, placed in a water bath at 100° for 3 min, and then cooled in an ice bath.

[b] 20 μl of a 3% solution of cysteine HCl was added and the absorbancy measured at 405 mμ after standing 30 min at room temperature.

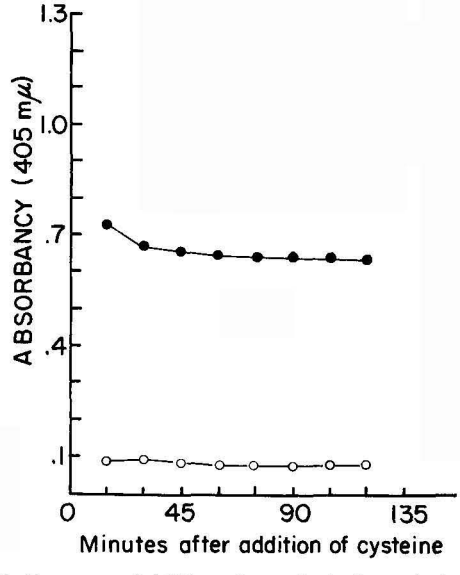

FIG. 2. Effect of time on stability of product formed from keratan sulfate. Method and symbol designations are the same as in Fig. 1. Measurements were made with 72.3 μg of keratan sulfate.

Effect of Time on Color Development. In the presence of cysteine, the color intensity due to keratan sulfate reached a maximum during the first 15 min at room temperature (Fig. 2). The color is stabilized after 30 min and is followed by a slight reduction of color intensity during the remaining minutes tested at room temperature.

Effect of Keratan Sulfate and Chondroitin Sulfate Concentration. Absorbancy due to keratan sulfate and to chondroitin sulfate is linear over the concentration range tested (Fig. 3).

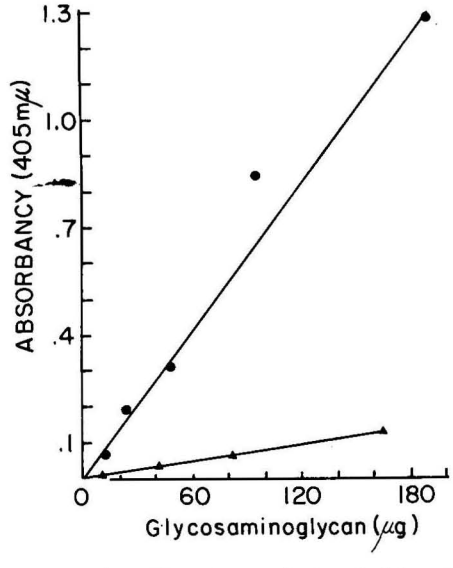

FIG. 3. Effect of concentration of keratan and chondroitin sulfates on absorbancy. Method and symbol designations are the same as in Fig. 1. Absorbancy values have been corrected for reagent blanks.

Measurement of Glycosaminoglycans from Beef Cornea after Resolution on Ecteola. Corneal glycosaminoglycans were detected in the column effluent by use of carbazole and cysteine methods (forms I, II, and III in Fig. 4). No cysteine-positive material was found associated with form I. The heterogeneity of forms II and III is suggested by their complex shape. Authentic chondroitin and keratan sulfates eluted from Ecteola columns in approximately the same NaCl concentrations as did forms II and III, respectively. Measurements of hexuronic acid and hexose after resolution of corneal glycosaminoglycans showed that a total of 3.87 mg of glucuronic acid and 3.85 mg of galactose was recovered. These values indicate that half of the corneal glycosaminoglycans used in this study is composed of keratan sulfate and recoveries are complete after passage through the anion exchanger.

Effect of Various Compounds on Color Development. Chondroitin sulfate and hyaluronic acid produce color in the cysteine method (Table 1). The amount of color from chondroitin sulfate is much less than that produced by keratan sulfate and can be accounted for on the basis of its glucuronic acid content. Keratan sulfate does not contain glucuronic

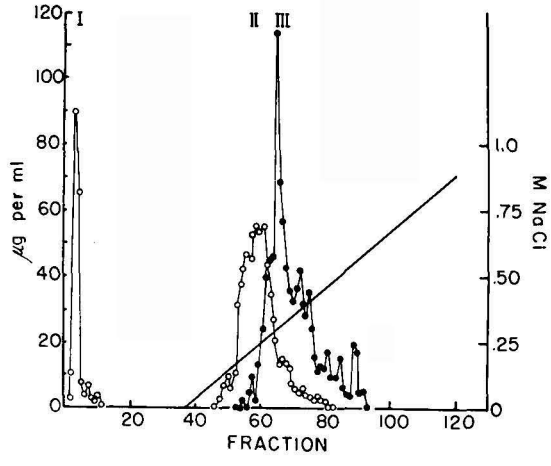

FIG. 4. Ecteola column (0.9 × 23 cm) was prepared as described in text. Corneal glycosaminoglycans (20 mg) were applied to the column and 4-ml volume fractions were collected at the rate of 24 ml/hr. Forms I and II contained 973 and 2892 μg of hexuronic acid, respectively; form III contained 3850 μg of hexose. The symbols have the following designations: uronic acid-containing glycosaminoglycans (○); uronic acid-deficient glycosaminoglycans (●).

acid but does contain galactose. The acetyl derivatives of glucosamine and galactosamine do not contribute to color development to the same extent as the simple hexosamines. It is calculated that 93.4 μg of keratan sulfate contains 36.4 μg of galactose and that 97.1 μg of corneal glycosaminoglycans contains 18.8 μg of galactose (Table 2). Approximately 50% of corneal glycosaminoglycans is composed of keratan sulfate of

TABLE 2
MEASUREMENT OF HEXOSE IN GLYCOSAMINOGLYCANS[a]

Substrate	Hexose[b] (μg)		Error (%)
	Calculated	Observed	
Keratan sulfate, 93.4 μg	36.4	39.3	+8
Corneal glycosaminoglycans, 97.1 μg	18.8	18.6	−1

[a] Based on values in Table 1.
[b] Measurements are based on galactose standard.

which approximately 39% is galactose. This means that the observed amount of galactose in keratan sulfate and in corneal glycosaminoglycans was within approximately 8 and 1% of theoretical values, respectively.

DISCUSSION

Glycosaminoglycans have been extracted from tissues by several techniques (10). All methods are based on separation of the polysaccharides from their protein complex and subsequent precipitation of the polysaccharides in alcohol or as an aliphatic ammonium salt complex. Tissues usually contain mixtures of glycosaminoglycans and further resolution of the mixture has been attempted by use of ion exchangers, by alcohol solubility, and by aliphatic ammonium salt-complex dissociation, with varying degrees of success. Glycosaminoglycans which contain hexuronic acid can be detected by rapid colorimetric tests which involve the use of carbazole or orcinol. Glycosaminoglycans which do not contain hexuronic acid present a different problem and use of the cysteine-H_2SO_4 method as employed in this study offers a simple, rapid, sensitive, and reproducible method for their detection. Application of these techniques will facilitate further study of keratan sulfate metabolism in cornea.

At pH 1.3 (0.05 *M* HCl) the anion exchanger is functioning at a pH below that of the p*K* values reported for carboxyl groups of glycosaminoglycans (11). In 0.05 *M* HCl, sulfate groups are dissociated while carboxyl groups are not, and the former are in part responsible for the observed binding of sulfated glycosaminoglycans to the anion exchanger. Chondroitin is the low sulfated form of chondroitin 4-sulfate, which comprises approximately half of the total hexuronic acid containing glycosaminoglycans in cornea (8). The high resolving power of the Ecteola column demonstrates that approximately 12.6% of the total corneal glycosaminoglycans are not bound to the anion exchanger and are assumed to be sulfate-free; the remaining 87.4% probably vary in sulfate content (9). Keratan sulfate shows a complexity in its elution pattern which is probably due to the degree of sulfation. Therefore, keratan sulfate as well as chondroitin sulfate may occur in tissues in various stages of sulfation.

The present report confirms earlier investigations which demonstrated that corneal glycosaminoglycans are composed of 50% keratan sulfate and the remaining 50% is composed of chondroitin and chondroitin 4-sulfate (8). These data are not in full accord with the recent report that cornea contains over 66% keratan sulfate and no sulfate-free chondroitin (9). This discrepancy may be explained by the knowledge that isolation methods in the recent study (9) are not identical with those used in the present and in the earlier investigation (8).

SUMMARY

Glycosaminoglycans were isolated from beef cornea and resolved by anion-exchange chromatography on Ecteola columns. Keratan sulfate was measured in the presence of other glycosaminoglycans by a cysteine-H_2SO_4 method. The interference due to other saccharides and their derivatives was investigated.

ACKNOWLEDGMENT

The author thanks Mrs. Franklin Black for technical assistance.

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ANALYTICAL BIOCHEMISTRY 4, 17–23 (1962)

A Method for the Colorimetric Estimation of Glycogen with Iodine[1]

CLARA R. KRISMAN[2]

From the Instituto de Investigaciones Bioquímicas "Fundación Campomar" and Facultad de Ciencias Exactas y Naturales, Obligado 2490, Buenos Aires, Argentina

Received October 10, 1961

The estimation of glycogen is usually carried out by precipitation with ethanol followed by hydrolysis and measurement of free sugars (1–4). Analytical procedures based on the color that glycogen gives with iodine are not much used because they lack sensitivity and are affected by temperature (5, 6). Following the observation of Sumner and Somers (7) that ammonium sulfate deepens the color produced by iodine, a study has been carried out with different salts. It has thus been possible to develop a procedure which is simpler than the classical methods and has about the same accuracy.

EXPERIMENTAL

Reagents

All the reagents used were analytical grade.

Glycogen was prepared as described by Somogyi (8) or was a commercial preparation (Amend Drug & Chemical Co., New York).

Amylopectin and amylose prepared according to Cowie and Greenwood (9) were a gift from Dr. C. E. Cardini.

β-Limit dextrins were prepared by the action of β-amylase on amylopectin or glycogen (10).

Phosphorylase-limit dextrins were obtained by the action of muscle phosphorylase (10).

Effect of Different Salts

The action of several salts on the color reaction was tested. As shown in Table 1 ammonium sulfate, calcium chloride, and sodium citrate are

[1] This investigation was supported in part by a research grant (No. G-3442) from the National Institutes of Health, U. S. Public Health Service, by the Rockefeller Foundation, and by Consejo Nacional de Investigaciones Científicas y Técnicas. The data in this paper are part of a thesis for the degree of Doctor of Chemistry at the Buenos Aires University.

[2] Investigator, Instituto Nacional de Microbiología.

TABLE 1

EFFECT OF SALTS ON GLYCOGEN-IODINE COMPLEX

The reaction mixture contained: glycogen, 0.06 mg; I_2, 0.52 mg; KI, 5.2 mg; salt[a]; in a total volume of 1.52 ml. A blank was made for each salt. Though addition of different salts displaced the wavelength of maximum absorption, absorbancy was measured at 500 mμ, which was the maximum corresponding to the glycogen-iodine complex when ammonium sulfate was added.

Added salt	Amount (gm)	Absorbancy
$Ba(CH_3COO)_2$	0.72	0.270
$(NH_4)_2HPO_4$	0.65	0.295
$(NH_4)H_2PO_4$	0.43	0.315
Sodium citrate	0.81	0.350
NaCl	0.42	0.270
NH_4Cl	0.40	0.190
$(NH_4)_2SO_4$	0.57	0.370
$CaCl_2$	0.87	0.340
—	—	0.050

[a] The amounts of salts indicated in the table correspond to 1.3 ml of saturated solution, except for ammonium sulfate.

the most effective in increasing the color. Ammonium sulfate and sodium citrate led to the formation of a precipitate at the higher concentrations of glycogen; therefore calcium chloride was selected for further study.

Absorption Curve

In the presence of $CaCl_2$ the maximum absorption is shifted to 460 mμ (Fig. 1) and the color is increased tenfold. The effect of $CaCl_2$ on

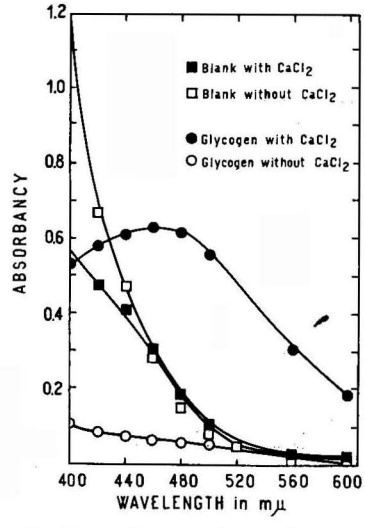

FIG. 1. Absorption spectrum of glycogen.

the reagent blank is very small in the range 460–600 mμ; at lower wavelengths the absorbancy of the blank is about 0.6 with $CaCl_2$ and much higher without it (greater than 1.2). The influence due to each of the reagents, when the others were kept constant, was studied. Figure 2

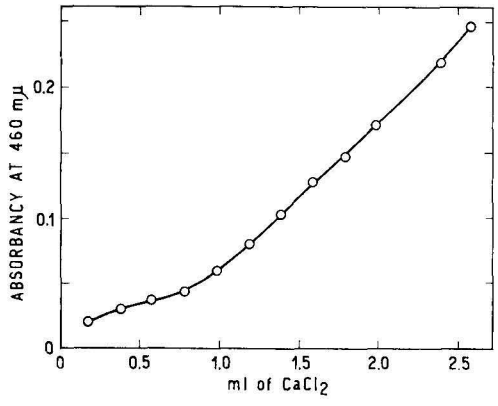

FIG. 2. Effect of $CaCl_2$ concentration. The reaction mixture consisted of: 0.1 mg glycogen, 0.26 mg I_2, 2.6 mg KI, and the indicated amounts of $CaCl_2$ (saturated solution), in a total volume of 3.0 ml. A blank was made for each $CaCl_2$ concentration.

shows the effect of different concentrations of $CaCl_2$. Since the absorbancy was not much increased by adding more than 0.26 mg of I_2 (see Fig. 3), this amount was selected in order to avoid higher blanks.

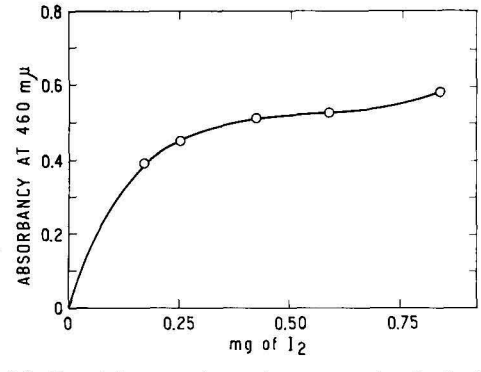

FIG. 3. Effect of iodine. The reaction mixture consisted of: 0.2 mg glycogen, 1.7 gm $CaCl_2$, 9.38 mg KI, and the indicated amounts of I_2, in a total volume of 3.0 ml. A blank was included for each I_2 concentration.

A similar study showed that, in order to obtain a color intensity independent from the concentration of KI, this salt must be present in a tenfold excess (w/v) with respect to iodine.

RECOMMENDED PROCEDURE

Reagents

Iodine-iodide solution: 0.26 gm of I_2 and 2.6 gm of KI are dissolved in 10 ml of water.

Calcium chloride: saturated solution at room temperature.

Iodine reagent: 130 ml of $CaCl_2$ solution is mixed with 0.5 ml of iodine-iodide solution. This solution may be stored for a week in a brown glass-stoppered bottle in the ice box.

Ammonium chloride: saturated solution at room temperature.

Procedure

Iodine reagent (2.6 ml) is added to 0.4 ml of a solution containing from 0.06 to 0.75 mg of glycogen per milliliter. After mixing, a clear solution is obtained and the yellowish brown color is measured at 460 mμ. The absorbancy is proportional to glycogen concentration up to 1 mg/ml.

Tissue Glycogen

Glycogen can be extracted by the usual KOH method (11) as follows: 0.9 ml of 33% KOH solution is added to 0.1 ml of tissue homogenate or to approximately 100 mg of freshly excised tissue. The mixture is heated at 100° for 20 min and cooled. Cell debris, if present, is removed by centrifugation. After adding 1.3 ml of 96% ethanol and mixing, the solution is heated to boiling, and immediately cooled in an ice bath to aid the precipitation of glycogen. The supernatant fluid obtained by centrifugation at 3000 rpm for 15 min is poured off and the tube drained onto a filter paper. At this stage it is unnecessary to reprecipitate several times, as in the usual procedures. However, it is important to neutralize the excess alkali. This can be accomplished as follows: 0.2 ml of saturated NH_4Cl is added to the tube and carefully mixed with a glass rod. It is important that the NH_4Cl solution should come into contact with the walls of the tube. The tubes are then heated during 5 min at 100°. After cooling, 0.2 ml of distilled water and 2.6 ml of iodine reagent are added and the absorbancy is measured at 460 mμ. A blank and a glycogen standard are treated in the same way.

Glycogen from different species does not always give the same color; if desired, a standard from the same source may be included.

RESULTS

Recovery

The method was tested by adding known quantities of glycogen to tissue extracts, in which case 100 $\pm$ 5% recoveries were obtained.

Effect of Temperature, pH, and Color Stability

In the absence of salts an increase in temperature produces a decrease in color (6), which amounts to 37% for a 10° interval. In the presence of $CaCl_2$ the corresponding decrease was only 11%.

The absorbancy is the same at neutral or acid pH, but decreases rapidly at a pH higher than 7, due to the formation of hypoiodite.

TABLE 2
COLOR STABILITY
Reaction mixture as described under "Recommended Procedure."

Time (min)	Absorbancy	Time (min)	Absorbancy
0	0.525	140	0.490
10	0.520	215	0.490
30	0.520	After 24 hr	0.400
50	0.515		

As can be seen in Table 2, the absorbancy of the solution does not change appreciably within 1 hr.

Specificity

Glycogen of different origins, and from animals in different conditions, was assayed by the iodine and phenol-sulfuric acid (1) methods using rat liver glycogen as standard. The ratio of the amounts obtained (R = iodine/phenol-sulfuric) was taken as 1, for the standard. The following values for R were obtained: rabbit liver 1.02, normal rat liver 0.94, female serpent liver 0.86, fasted rat liver 0.84, fasted rat muscle 0.85, normal rat diaphragm 1.1, insulin-treated rat diaphragm 1.1, and a lower value for diabetic rat muscle 0.57.

Glycogen degraded with muscle phosphorylase (phosphorylase-limit dextrin) gave a value of R 0.53, while glycogen degraded with β-amylase (β-amylase-limit dextrin) gave a value of R 0.45; in both cases the λ_{max} was displaced to values lower than 400. For amylase-limit dextrin R was 0.20.

The results with commercial soluble starch were as follows: a violet-brown color with λ_{max} at 520 mμ was obtained. In the absence of $CaCl_2$ the color varied from green to blue with increasing concentration. Absorbancy was proportional to concentration between 0.05 and 0.2 mg. Amylopectin gave a λ_{max} at 520–540 mμ (Fig. 4), which shifted to 560 mμ in the absence of $CaCl_2$. Absorbancy was approximately doubled by addition of $CaCl_2$. With amylopectin degraded with β-amylase (β-amylase-limit dextrin) absorbancy was 30% lower and the λ_{max} remained unchanged. Experiments performed with amylose showed that it gives

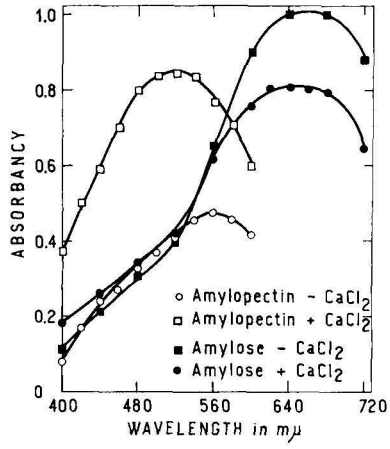

FIG. 4. Absorption spectrum of amylopectin and amylose.

the same blue color and a λ_{max} at 690 mμ with or without $CaCl_2$ (Fig. 4). Addition of the latter somewhat diminished absorbancy. Dextran, inulin, and mannan produce no color.

DISCUSSION

The method described is rapid and simple. Furthermore there is no interference by polysaccharides, which give no color with iodine but which release reducing sugars by hydrolysis. The color is stable and does not vary appreciably with temperature. By reducing the volume of the reagents to one-fourth of the values given above, quantities as small as 0.02 mg may be estimated.

Experiments carried out with mixtures of glycogen and amylopectin show that the wavelength of maximum absorption shifts toward higher values on passing from glycogen (λ_{max}, 460 mμ) to amylopectin (λ_{max}, 520 mμ), therefore this displacement of the λ_{max} is related to the degree of branching of the polysaccharide. This agrees with data recently published by Archibald *et al.* (12).

The exterior chains of glycogen play a considerable role in the color reaction. Thus, phosphorylase, which leaves only about 4 glucose residues (13) in the exterior branches, reduces the absorbancy 25%, and β-amylase, which leaves 2–3 glucose residues in the outside branches (14), diminishes absorbancy 43%.

No strict correlation is to be expected between the results of the iodine and the classical methods, since the latter measure hexose residues, while the color developed with iodine depends on chain length (15–17). Therefore the iodine method is more specific for glycogen and, with the addi-

tional information provided by the value of λ_{max}, it should prove useful in the study of glucogenoses, when combined with other methods.

SUMMARY

A rapid, simple, and specific method for the colorimetric estimation of glycogen in concentrations varying from 0.15 to 1 mg/ml with an iodine-iodide reagent in the presence of salts has been studied. It eliminates interference by polysaccharides, which do not develop color in the presence of iodine. This colorimetric procedure can be applied to the estimation of tissue glycogen.

ACKNOWLEDGMENTS

I wish to express my gratitude to Dr. Luis F. Leloir for his inspiring guidance and support, and to the other members of the Instituto de Investigaciones Bioquímicas "Fundación Campomar" for many helpful discussions and criticisms.

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ANALYTICAL BIOCHEMISTRY 4, 24–27 (1962)

An Improved Method for Determination of Uric Acid in Serum[1]

MARIE H. CARR[2] AND BERTON C. PRESSMAN[3]

From the Research and Radioisotope Laboratories, Kansas City Veterans Administration Hospital, Kansas City, Missouri

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The large numbers of papers dealing with the determination of uric acid which have appeared in the literature in recent years imply a dissatisfaction of analysts with current methods (1–7). Prevalent colorimetric procedures lack specificity and hence reliability, while the enzymic methods employing uricase are complicated by technical problems arising from the ultraviolet absorption of serum protein. This is particularly troublesome in the case of experimental animals having low serum uric acid levels. Since one of our studies involved the following of slight changes in serum uric acid levels in dogs, we were motivated to develop a reliable method giving good precision at low uric acid concentrations.

METHOD

Principle

Separation of uric acid from protein is accomplished by passage of serum or other biological fluid through a strong anion-exchange resin in the acetate form. It has been found that such a column removes the uric acid from the fluid with greater ease and consequently is more reliable than the chloride form previously recommended (4, 6). Following the removal of extraneous UV absorbing materials by washing, the uric acid is eluted from the column and determined by enzymic assay.

Materials and Reagents

Ion-Exchange Resin. Dowex 2-X8 (100–200 mesh) in the acetate form. This resin is purchased in the chloride form and converted to the acetate

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[2] Deceased, April 28, 1962.

[3] Present address: Johnson Foundation, University of Pennsylvania, Philadelphia 4, Pennsylvania.

form by washing on a column with 10% sodium acetate until tests for chloride in the effluent are not more than faintly positive.

Standards. Uric acid stock, 1 mg/ml, is prepared by dissolving uric acid[4] in *M*/15 glycine buffer. This solution is stable almost indefinitely if stored frozen in small vials. For a working standard, the stock solution is diluted 0.5 ml to 10 ml with water. For assay purposes, 0.5 ml of the working standard is diluted to 6 ml with *M*/15 glycine buffer to correspond to the dilution of the biological fluids analyzed.

Eluant. To 500 ml of 0.04 *M* HCl add 500 ml M NaCl.

Glycine Buffer, ⅔ *M*, pH 9.35.

Glycine Buffer, M/15. This solution is prepared by diluting 10 ml of the ⅔ *M* buffer to 100 ml with water.

Uricase,[5] purified, 125 units/ml. Dilute the amount required for a day's work 1:20 with M/15 glycine buffer.

Procedure

1. Prepare resin columns (2 cm in height × 0.4 cm in diameter).
2. Pass 0.5 ml biological fluid containing 2 to 100 μg uric acid through the column. If whole blood is to be analyzed, a volume of diluted hemolyzed blood equivalent to 0.5 ml whole blood is used.
3. Wash twice with 10 ml H_2O. Discard the washings.
4. Elute the uric acid with 5 ml of the HCl-NaCl eluant.
5. Add 1 ml of ⅔ *M* glycine buffer, pH 9.35, to which has been added 6.25 ml 10 *N* NaOH per liter to compensate for the HCl in the eluant.
6. Measure the absorption of the buffered eluate at 293 mμ.
7. To a 3.5-ml aliquot of buffered eluate, add 0.1 ml of diluted uricase described above, and incubate at 45°C for 2 hr.
8. Redetermine the 293 mμ absorption. The decrease following incubation is a measure of the uric acid present. A standard containing 5.00 mg % uric acid in *M*/15 glycine buffer gave an absorption change of 0.306 OD in the enzymic assay.

RESULTS AND DISCUSSION

The results obtained by this method in the analysis of human and canine subjects are given in Table 1. Reproducibility was 0.013 mg % in dogs at a level of 0.30 mg % and 0.063 mg % in humans at a level of 4.31 mg %.

Concentrations of HCl in 0.5 *M* NaCl of 0.050 to 0.005 *M* were tested

[4] The reagent-grade product purchased from Matheson, Coleman and Bell was found to have the same absorption as that purified by the method of Liddle and Associates (5).

[5] Sigma Chemical Company, St. Louis, Missouri.

as eluting agents. The level of 0.02 *M* HCl as described above was found optimal.

An incubation period of 1 hr at 45°C was found to be adequate for the oxidation of uric acid by the amount of uricase used. A 2-hr period is used as a safety factor.

TABLE 1
NORMAL VALUES

	Dog serum	Human serum
Number	64	38
Mean	0.30	4.19
S.D.	0.155	1.22

Assays for uric acid were linear with concentration over a wide range. Following passage of aqueous uric acid solutions through the columns the values obtained were somewhat low. This error was decreased when assaying uric acid solutions prepared in deionized serum and no longer appeared significant when uric acid was dissolved in untreated dog serum (see Table 2).

TABLE 2
RECOVERY OF URIC ACID FROM VARIOUS SOLVENTS

Added (mg %)	Recovery (%)		
	Water	Deionized serum	Dog serum
10.0	93	98	102
7.5	90	96	98
5.0	89	96	98
2.5	83	94	98

It is evident that the presence of other anions in plasma, as well as the protein, plays a significant role in facilitating subsequent elution of uric acid from the resin. Comparison of elution graphs of both aqueous uric acid and a serum solution in the same concentration range of uric acid shown in Fig. 1 further illustrates this point. Accordingly, the use of this method for additional types of biological fluids should be checked out by running recovery determinations in the presence of the fluid.

Dowex 2-X8 in the chloride form has been used by several investigators (4, 6) to separate uric acid from interfering components present in biological fluids. Since the resin is known to have a much stronger affinity for chloride ion than for organic ions, it follows that uric acid should displace acetate with greater ease than it can chloride. Accordingly, urate ions are bound with greater ease by the acetate column with

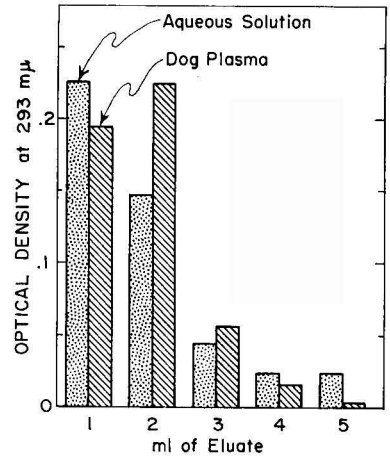

FIG. 1. Effect of extraneous ions on the elution of uric acid from resin.

no sacrifice of their susceptibility to subsequent elution by acidic chloride solutions. This reasoning has been corroborated by us experimentally; under otherwise identical conditions, a given specimen giving 95% recovery from acetate columns resulted in an 85% recovery of uric acid from equivalent chloride columns.

SUMMARY

A method is described for the determination of uric acid in serum which is sufficiently precise and reliable to follow small changes at low levels.

The advantage of the use in the acetate form of the anion exchange resin Dowex 2-X8 is pointed out.

The influence of protein and other anion components of serum on the absorbance of uric acid and on its elution from the resin is noted.

Normal values of serum uric acid in dogs as well as man, with sampling deviations, and reproducibility of the method at both levels is demonstrated.

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ANALYTICAL BIOCHEMISTRY 4, 28–38 (1962)

Improved Apparatus for Centrifugally Accelerated Electrochromatography and Its Application to Continuous Separation of Serum Proteins[1]

HUGH J. McDONALD AND PAUL A. LIBERTI

From the Department of Biochemistry, Graduate School, and Stritch School of Medicine, Loyola University, Chicago, Illinois

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INTRODUCTION

In earlier publications (1–5) the basic principles of centrifugally accelerated paper electrochromatography were described, and the technique was applied principally to the discontinuous separation of lower molecular weight materials such as indicator dyes, amino acids, etc. In this paper the authors develop the principles to a greater extent, contrast them with those of curtain electrophoresis, and apply the technique to the continuous separation of serum proteins.

The appartus has undergone extensive revision, including a more functional size, improved electrode assembly, continuous sample application through a wick arrangement, and an improved collecting system.

APPARATUS AND EXPERIMENTAL TECHNIQUE

A schematic diagram of the apparatus is presented in Fig. 1. The drive mechanism and auxiliary components, such as brake, tachometer, and automatic timer, are similar to those described earlier (5).

The rotatable head or rotor consists of a lower (Bakelite) and an upper (transparent Lucite) shallow tray (each section measuring 60 × 70 cm) which are clamped together to form a completely sealed chamber. Attached along the full length of the long sides of the bottom tray are platinum tube electrodes ($\frac{1}{16}$ in. outside diameter, 0.01 in. wall thickness, containing a copper core for strength) connected to the high voltage direct current source through two brass slip rings (one connected to the positive and one to the negative supply) and spring-loaded graphite brushes. The potential source has a variable DC output, 0–100 ma at

[1] Portions of this paper were presented at the 139th (St. Louis, Mo., April, 1961) and 140th (Chicago, Ill., September, 1961) meetings of the American Chemical Society.

0–1000 volts. It is line-operated from 115 volts, 60 cycles, and is designed to give a highly regulated DC voltage output.[2] Over the upper surface of the bottom tray are placed numerous shallow plastic pins which serve to prevent the paper sheet, when wetted during a run, from making area contact with the bottom tray. The rectangular paper sheet (Whatman No. 3MM, 53 × 55 cm) with 25 V-shaped points along each end is made up of 2 equal sections cut from larger sheets, by using a template. The half sections are arranged in such a way that the Whatman paper machine direction is at right angles to the direction of the electrodes. This arrangement is advantageous because the movement of substances through the paper is slower in a direction at right angles to the direction of the paper than when parallel to it (5) and, as a consequence of this fact, the resident time of a particular migrant on the paper is increased, thus allowing for a maximum effect of a given electrical field. The paper sheet, when positioned properly, rests lightly on the multiple plastic pins protruding upward from the surface of the lower tray and occupies a plane approximately midway between the upper and lower trays.

A series of 25 collecting vessels, made up of test tubes, (inside diameter, 8 mm; length, 6 cm) are positioned at each end of the rotor to correspond to the dual set of 25 drip points in the paper sheet. Each series of 25 collectors is held in a rack which can readily be replaced by a new set when necessary, and access to the separate tubes, to withdraw samples for analysis, is available through sealed portholes in the top tray or cover.

The solution to be fractionated is placed in the circular plastic (acrylic) cup illustrated in Fig. 2, which is designed as an annular trough (outside diameter, 10 cm; inside diameter, 6 cm; wall thickness, 4.5 mm; channel depth, 11 mm). The trough is attached to the drive shaft of the rotor by two slender spokes, thus maintaining a fixed position with respect to the rotating paper sheet. It is provided with two outlets (approximately 1.5 mm in diameter) for the wicks which feed the solution to be fractionated to the rotating paper sheets. A useful wick is made by cutting a triangular section, 1¼ in. on each edge, from standard facial tissue (e.g., Kleenex) and rolling it up, as a tight pointed cone, starting along one edge. The point of a cone is threaded through each of the two outlets in the annular cup and is pulled through until the tips just touch the surface of the paper sheet. The cup, rotating with the head, is positioned in such a way that a line passing through the wick outlets is parallel to the electrodes along the sides of the rotor while the spokes

[2] Electro-Chromatofuge Power Supply, Cat. No. 5090, Labline, Inc., Chicago 22, Illinois.

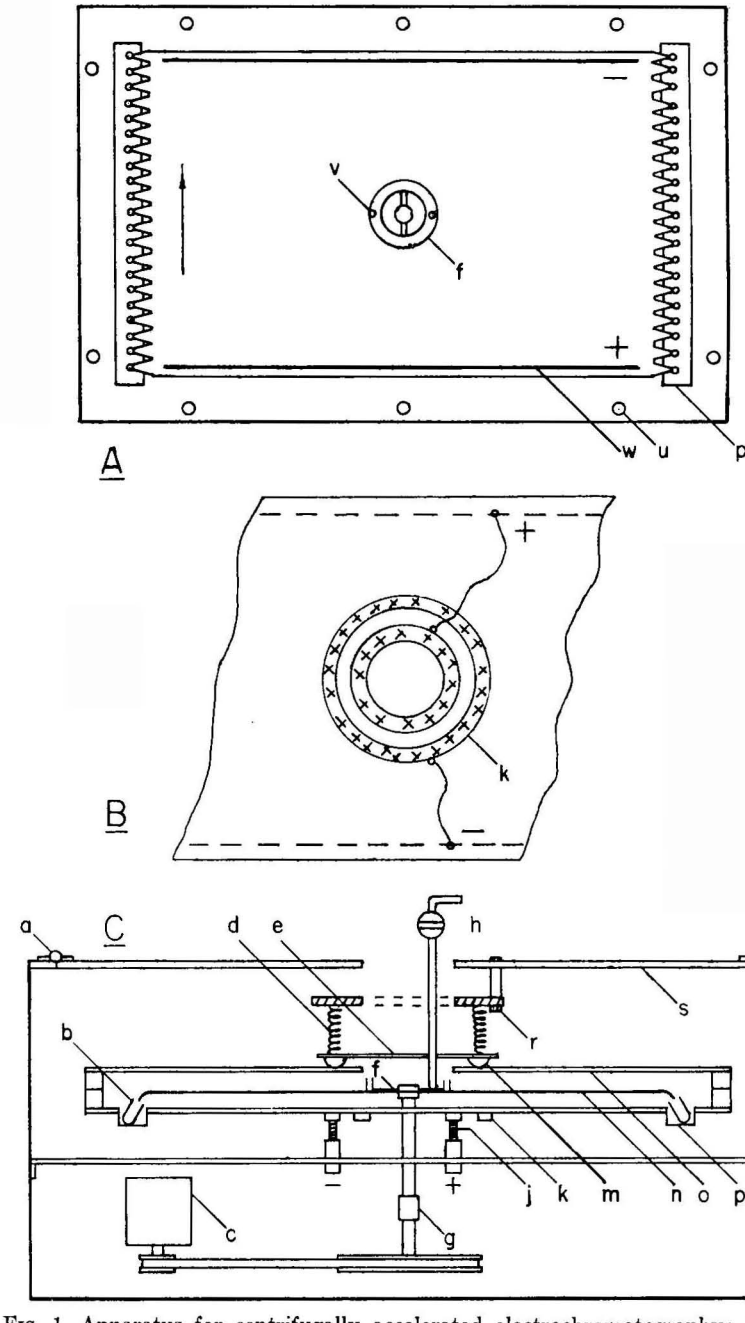

FIG. 1. Apparatus for centrifugally accelerated electrochromatography: (A) Top view of rotor, with upper tray or lid removed and paper sheet in place; unmarked

holding the cup to the shaft of the rotor are at right angles to the direction of the electrodes. The solution to be fractionated is directed by the

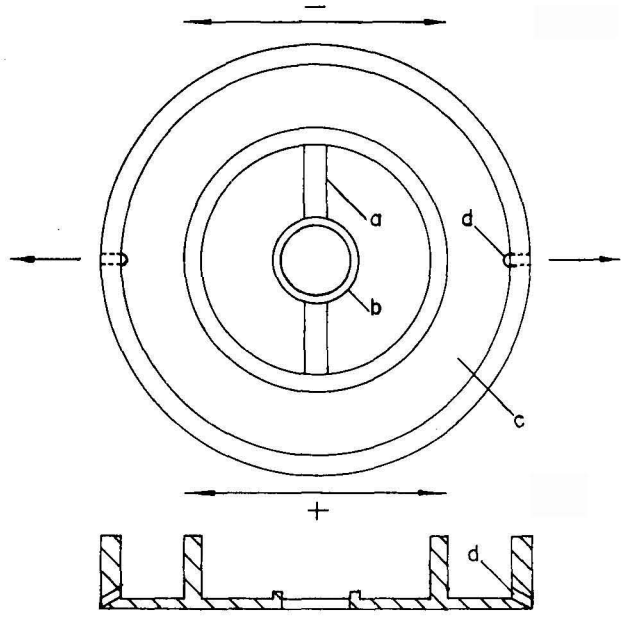

FIG. 2. Annular cup, for admittance of sample, attached to drive shaft of rotor by circular hub and two spokes. Two outlets provided for wicks to feed solution to paper sheet. Double pointed arrows indicate direction of electrodes; unmarked single pointed arrows, direction of flow of sample from cup to paper sheet. a, spokes attaching hub, b, to annular cup; b, hub of cup, attached to drive shaft; c, annular cup; d, holes through which wick is threaded to direct sample to paper sheet.

wicks into the paper sheet at two point sources, symmetrically positioned away (approximately 5 cm) from the central point of the rectangular sheet and along the median line parallel to the direction of the electrodes.

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arrow indicates machine direction of paper sheet; u, screw clamps to attach upper and lower trays of rotor; v, opening for wick through which sample is fed to paper; w, electrode. (B) View of rotor from below, showing slip rings which connect DC power to leads attached to positive and negative electrodes, respectively, along sides of rotor. (C) Side view of apparatus, with brake, tachometer, and automatic timer deleted; a, hinge for exterior cover; b, collecting cups; c, ¼ horsepower variable speed drive; d, coil springs; e, tempered-glass plate; f, feeder cup for admittance of sample; g, drive shaft; h, developing solvent pipet; j, brushes for DC power admittance; k, slip rings for DC power; m, ring seal bearing (Teflon); n, paper sheet; o, upper tray of rotor; p, removable rack with space for 25 collecting cups; r, spring-loaded closure device, attached to one-half of exterior cover; s, hinged exterior cover.

Additional amounts of the solution being fractionated may be added to the annular cup, while the rotor is in motion, by insertion of a pipet or feed tube through a sealable porthole in the glass floating closure shield (Fig. 1, e) over the center opening in the top tray of the rotor.

The developing liquid or background electrolyte is added to the rotating paper sheet in a thin, continuous jet-like stream from a capillary tip. The tip, inserted through an opening in the floating closure shield, directs the liquid into the area containing the spokes of the feed cup. As the sheet rotates, centrifugal force causes the initial circle of liquid to spread out uniformly underneath the cup in a steadily widening circular area so as to saturate the paper sheet thoroughly from the center on out to its perimeter. As the front of this circular wave reaches the sides of the sheet where the electrodes are attached, it divides and flows along the electrodes to the outside corners of the paper sheet, where it is flung off into the lower tray of the rotor. In this way interfering electrode products are continually washed away.

In practice, the paper sheets are prewetted and placed on the head, and the centrifugal field and the background electrolyte are turned on, but the sample is not added until a steady state of saturation is reached.

RESULTS

For the fractionation of serum proteins or lipoproteins a veronal buffer (pH, 8.6–8.9; ionic strength, 0.02) containing 20% glycerol is used as the developing liquid or background solvent. Other experimental conditions are: paper, Whatman No. 3MM; potential gradient, 13–17 volts/cm across the rectangular paper sheet, that is, a total impressed voltage of 700–900 volts; rotor speed, 200–230 rpm; temperature, 25–26°C. Prior to undergoing electrochromatographic fractionation, the serum is diluted with the glycerol-buffer solution in the ratio 1:1 by volume. Before beginning the actual separation of a serum sample, the rotor is run for about 0.5 hr with the voltage on, the background solvent being added at the same rate as is used in the actual separation, namely, about 0.8 ml/min. Once a steady state is reached, as determined by constancy of amperage and voltage, the serum preparation is added to the cup without stopping the unit and collection of fractions from the 25 drip points at each end of the paper sheet begins approximately 1 hr later. The serum preparation is run through the unit at the rate of 1.0–1.5 ml/hr.

In the specific case described here, the human serum was an aliquot from pooled samples drawn in early morning from approximately 50 unselected patients. It was used the same day it was drawn. The sample was prepared for fractionation by adding together equal volumes of serum and buffer. It was then prestained for albumins by adding 1 mg of

bromophenol blue per 5 ml of preparation. The experimental conditions were: buffer, veronal; pH, 8.6; ionic strength, 0.02; 20% glycerol; rotor speed, 229 rpm; potential across the sheet, 700 volts, i.e. 13.2 volts/cm; current, 10 ma; flow of background solvent, 1 ml/min. The liquid from the cups on each side was analyzed further, and its components identified by comparison with known serum proteins, using the technique of ionography (6). The experimental conditions were: horizontal strips, cellulose acetate, 20 × 2.5 cm; buffer, veronal; pH, 8.6; ionic strength, 0.02; temperature 25°C; potential gradient, 7.5 volts/cm. Figure 3a

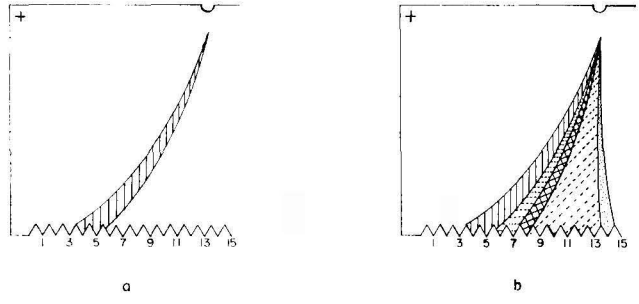

FIG. 3. (a) Electrochromatographic map of one-half of the paper sheet, showing path of albumin prestained with bromphenol blue; (b) showing path of other protein fractions following poststaining.

represents the significant portion of one-half of the paper sheet, showing the path of the prestained albumins, as it appeared at the close of the run. Figure 3b represents the other half of the paper sheet, which was oven-dried at 120°C for 1 hr, stained with bromphenol blue solution, and cleared with distilled water. Three distinct migration paths are visible, against a much lighter background. On ionographic analysis using cellulose acetate strips, of the material in the cups corresponding to the drip points at the end of the path the one on the left was found to correspond to albumin, and matches the path in Fig. 3a. The material in the cups corresponding to the middle migration path was found to be α_1- and α_2-globulin plus a trace of albumin. The material in the cups corresponding to the migration path on the right of Fig. 3b was almost pure α-globulin. In Fig. 4, the weight of protein, in gammas per 0.2 ml of collected sample, as determined by the method of Lowry (7), is plotted against the number of the receiving tubes. Also in Fig. 4, the ionographic analysis of the collected material is given. Albumins and gamma globulins of high purity, as indicated by subsequent ionographic analysis on cellulose acetate strips, have been isolated by the method, repeatedly. Mixtures of the α_1- and α_2-globulins and of the β_1- and β_2-globulin components are

collected by this method. The mixtures have been further resolved by "partial" lyophilization and a second run through the apparatus.

In another series of experiments, it was of interest to determine whether the α and β lipoproteins would migrate to regions corresponding to their location in the traditional ionographic separation. This was done by preparing two serum solutions, one prestained for albumins with bromophenol blue and the other prestained for lipoprotein with Sudan black B. The trough of the annular cup was divided into two compartments so that one wick delivered the prestained albumin solution and

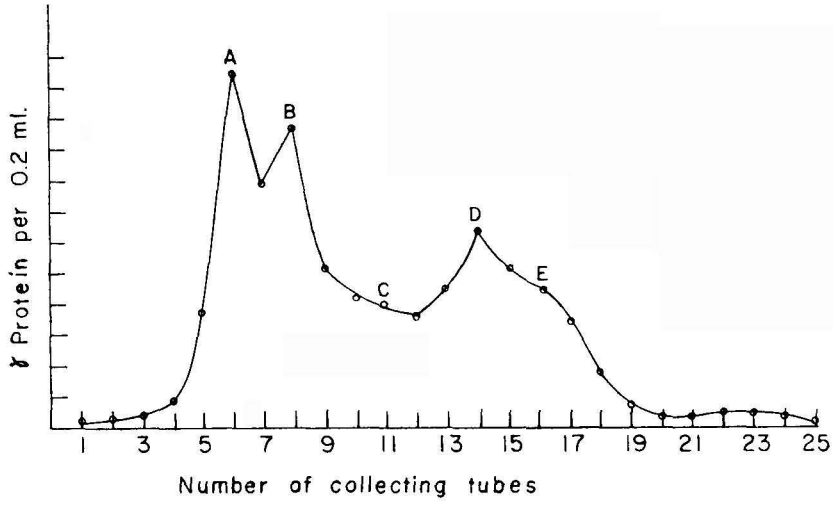

FIG. 4. Amount of protein, in gammas per 0.2 ml, plotted against the number of collecting tubes, starting with 1 nearest to positive electrode and 25 nearest to negative electrode: (A) albumin; (B) α_1- and α_2-globulin plus trace albumin; (C) α_2- and β-globulin; (D) γ-globulin and trace β-globulin; (E) γ-globulin.

the other prestained lipoprotein preparation. At the close of the runs, inspection of the portion of the paper sheet containing the migration path of the prestained lipoprotein showed two visible fractions. When it was superimposed upon the portion of the paper sheet containing the migration path of the prestained albumin it was found to correspond to the region of α- and β-globulins. Further identification by the cellulose acetate technique, as described above, gave conclusive evidence that the α- and β-lipoproteins had indeed migrated to the same regions as found in the traditional ionographic spectrum (9).

In the final experiments to be reported here, a sample of serum from a patient who had just suffered a myocardial infarction, was prepared for analysis. The serum glutamic-oxalacetic transaminase (SGOT) level

in the sample was determined, using the method of Reitman and Frankel (8), to be 125 units/mg. The serum was prepared for analysis and run through the apparatus as previously described.

The relative position of SGOT in the ionographic spectrum of serum proteins has been determined by Shepherd and McDonald (9) for normal serum and by Emmrich and Zimmermann (10) for pathological serum. The major portion of the transaminase activity was found to be associated with the α_2-globulin fraction of the serum proteins.

The transaminase activity of the liquid in each tube was determined and found to be highest (approximately 1000 units/mg protein) in tube 8 (see Fig. 5), i.e., nearer the positive electrode, and in the region of the

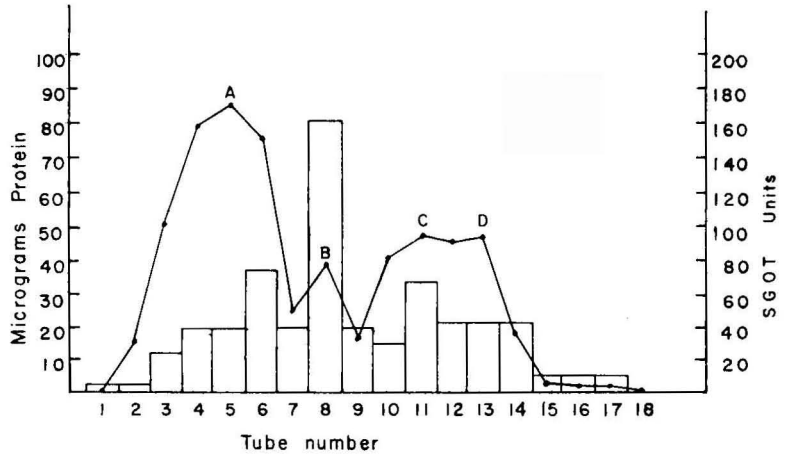

FIG. 5. Contour graph. Micrograms of protein per 0.1 ml of liquid collected in cups is plotted against the number of the collecting cups. (A) Albumin; (B) α_1- and α_2-globulins; (C) β_1- and β_2-globulins plus trace γ-globulin; (D) γ-globulin.

α_1 to α_2-globulins. The liquid samples (0.2 ml) from tube 8, at each end of the rotor were combined to give a total of 0.4 ml, which was, in turn, made up to a volume of 1.2 ml by the addition of distilled water. The sample was cooled to 4°C, and acetone (also at 4°C) was added to it in the ratio, acetone/solution of 2:1, by volume. The sample was allowed to stand for 0.5 hr, at 4°C, then centrifuged (International Clinical, Model CL, Rotor No. 808) for 10 min at 6400 rpm and the supernatant discarded. The slightly yellow precipitate was agitated, for 15 min, with sufficient water (twice distilled) to yield a volume of 0.2 ml. The globulins remained as a grey-white precipitate while the SGOT dissolved in the supernatant. On determining the SGOT activity, it was found to have been increased to approximately 2600 units/mg protein.

DISCUSSION

Consider a case (Fig. 6) in which the migrant mixture is made up of two components, both of which bear negative charges, e.g., a protein mixture at a pH above the isoelectric point of each component. Now suppose the electric field is turned on for a period of time just sufficiently long to cause a small ionographic separation of the two components and is then shut off. On rotating the head, the initial small separation

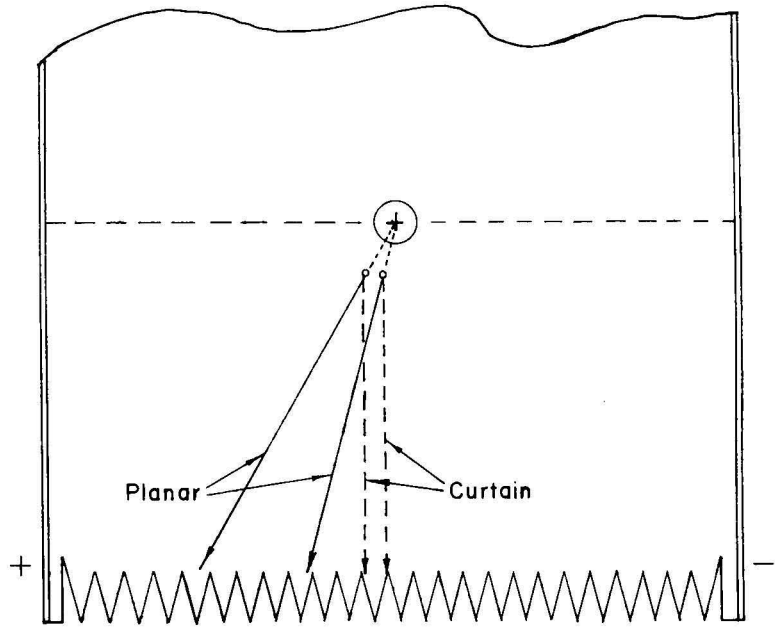

FIG. 6. Schematic idealized diagram illustrating the synergistic effect of the electric and centrifugal fields in planar electrophoresis as contrasted to the nonsynergistic effect of the electric and gravitational fields in curtain electrophoresis.

achieved by the electric field will continue to expand as the two spots move out along discrete radial lines which diverge farther and farther as the distance from the center increases. These lines, at any particular moment and time, will be such that they pass, ideally, through the center of rotation of the sheet and through the center of the spot. If the components are separated electrophoretically, even a short distance, the separation continues to increase under the influence of the centrifugal field alone, when the electric field is turned off. It is evident that in "planar electrophoresis" the electric and centrifugal forces act together to yield an enhanced separation of the components of the mixture. For

convenience of expression, the term "planar electrophoresis" (deriving from the fact that the paper sheet lies in a horizontal plane) is sometimes used, especially when the technique is being compared with, or contrasted to, so-called (vertical) curtain electrophoresis.

If the two lines in Fig. 6 represent the components of a multicomponent mixture with the fastest and slowest ionographic mobilities, all the other components will lie between these lines. It is evident, then, that a greater spread of the components has been achieved in the planar than in the curtain technique, i.e., the spread of the mixture in the former case corresponds to seven drip points while in the latter case, it corresponds to one point only. It is obvious, also, that because of the synergistic effect of the combined electric and centrifugal fields the length of the paper sheet would, in principle at least, not need to be as great for a given spread of the components of a mixture in planar as in curtain electrophoresis.

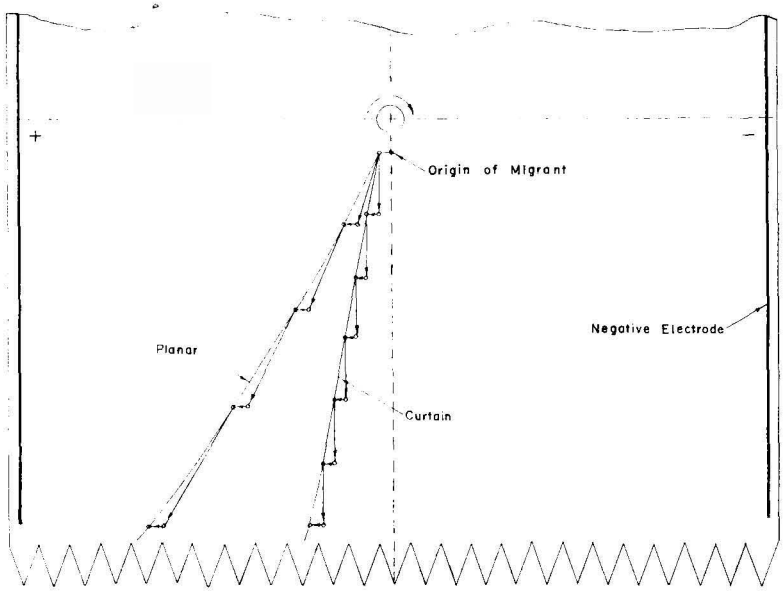

FIG. 7. Schematic representation of events, contrasting paths, for a single negatively charged moiety, in planar and curtain electrophoresis.

Figure 7 is a schematic representation of a single negatively charged migrant traversing the paper sheet under the conditions of curtain and of planar electrophoresis; in each case it is assumed, for purposes of elucidation, that the electrophoretic and centrifugal fields are acting in an interrupted, step-wise fashion. It is evident, again, that the path of

transit, under planar conditions, is in the form of a pronounced arc while, under curtain conditions, this characteristic is less prominent.

SUMMARY

The technique of centrifugally accelerated electrochromatography has been applied to the separation of serum proteins and lipoproteins. At a rotor speed of 200–230 rpm and a potential gradient of 15–17 volts/cm across the rectangular paper sheet (Whatman No. 3MM, 53 × 55 cm) and using a veronal buffer (pH, 8.6; ionic strength, 0.02) containing 20% glycerol, the serum preparation (serum and buffer, 1:1 ratio) has been fractionated at the rate of 1.0 ml/hr. The fractions are collected from 25 drip points at each end of the sheet.

ACKNOWLEDGMENTS

This project was supported in part by a grant-in-aid from the Chicago Heart Association. The pooled human serum and the sample of serum high in glutamic-oxalacetic transaminase were obtained from Cook County Hospital through the courtesy of Mr. James Q. Kissane of the Hektoen Institute for Medical Research. The authors are indebted to Mr. Alexander I. Newman and Mr. Robert J. Falk of Labline, Inc., Chicago, for their advice in the design of the apparatus and for building the prototype Electro-Chromatofuge used in this work.

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ANALYTICAL BIOCHEMISTRY 4, 39–45 (1962)

Simultaneous Multiple-Column Chromatography: Its Application to the Separation of the Adenine Nucleotides of Human Erythrocytes[1]

CHESTER DE LUCA, JOSEPH H. STEVENSON, JR., AND EUGENE KAPLAN

From the Departments of Pediatrics, Sinai Hospital of Baltimore, and The Johns Hopkins University School of Medicine, Baltimore, Maryland

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INTRODUCTION

In recent years the technique of column chromatography has become increasingly popular as an analytical tool for the experimentalist. When used for this purpose, it is the custom, usually, to process a single column many times employing a given set of conditions. By analogy to general laboratory practice, it would be desirable to employ a minimum of two columns simultaneously, one as a control for the other; a multiplicity of columns developed simultaneously would be more ideal. This is possible to a limited extent with column chromatography.

Directions for building an apparatus for developing at least six columns at once have been reported by Vestergaard (1). Described here is a method for easily handling three columns concurrently employing only readily available commercial equipment.

To illustrate the feasibility and facility of using such an apparatus, results are presented from an application to separating the adenine nucleotides and quantitating levels of ATP[2] in human erythrocytes.

MATERIALS AND METHODS

Chromatography Apparatus

The chromatographic apparatus, shown in Fig. 1, is a model V 15[2] fraction collector manufactured by Gilson Medical Electronic Co. This model has a three-section, square collecting stage which executes boustro-

[1] This work was supported by Grant No. 2A-5271 of the U. S. Public Health Service.

[2] Abbreviations used: AMP, ADP, and ATP; adenosine-5′-mono-, di-, and tri-phosphate, respectively; rbc, red blood cell(s).

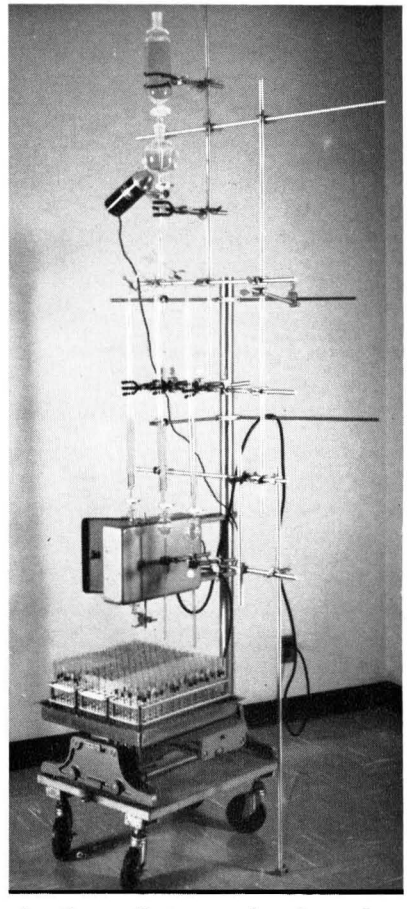

FIG. 1. The Gilson fraction collector equipped as described in the text for simultaneous multiple-column chromatography.

phedon movement. During the chromatography process a column is placed over the first tube position of each section of the stage.

The fraction collector may be equipped with any of the various devices available for activating the mechanism which changes the position of the collecting tubes. When either the drop-counting or fixed-volume type of tripping device is used, only one column need be aligned with it. The other two columns merely communicate with their respective collecting tubes through appropriately placed thistle tubes.

A common elution system is positioned above the columns. This communicates with all columns below through a *radially* symmetrical manifold. In the present study a nonlinear gradient elution is employed (2).

This is achieved by connecting, in series, two separatory funnels as shown in the figure. The uppermost funnel serves as a reservoir and the lower funnel acts as a mixing chamber. The latter is equipped with a Teflon-covered stirring bar and a magnetic stirrer of the type manufactured by Tri-R Instrument Co. The eluting mixture, adapted from the work of Hurlbert (2), was modified to give a one-stage, continuous gradient system capable of an uninterrupted elution of the three common nucleotides of adenine.

The chromatographic medium used is Dowex 1-X2 ion-exchange resin, 200–400 mesh, purchased from J. T. Baker Chemical Co. The resin, obtained in the chloride form, is first cycled through NaOH and HCl, then washed with acetone as recommended by Cohn (3). Finally, it is converted to the formate form and washed exhaustively with distilled water until no ultraviolet light absorbing material is eluted.

Preparation of Erythrocyte Extract

One to three milliliters of heparinized, fresh blood is centrifuged at 3000 rpm for 10 min in a refrigerated Lourdes centrifuge, using the high-speed angle rotor 9RA-V. The plasma and buffy coat are removed by suction. The red blood cells are then washed twice by resuspension in about two volumes of cold 0.9% NaCl and centrifuged as before. The cells are transferred to a graduated vessel with repeated washings of isotonic saline, and the suspension is finally adjusted to an exact volume equal to three to four times that originally occupied by the cells alone. A sample is removed at this point with a capillary tube, and a microhematocrit is determined using the Adams Micro-Hematocrit Centrifuge (Clay-Adams, Inc., New York). The remaining cell suspension is hemolyzed with cold distilled water during quantitative transfer to a 40-ml Lusteroid centrifuge tube and adjustment of the final volume to at least five times that of the saline suspension. Deproteinization is effected by adding 1.1 ml of cold 3 *M* $HClO_4$ for every 10 ml of the hemolyzate and blending for 1 min with a Lourdes Multi-Mixer at a rheostat setting of 50. The blade assembly is then washed down with 0.3 *M* $HClO_4$ from a polyethylene wash bottle. The washings are collected in the homogenate, and the entire coagulum is centrifuged at 10,000–12,000 rpm for 10 min. The supernatant fluid is decanted as quantitatively as possible and neutralized immediately with cold 2 *M* KOH. The resulting precipitate of potassium perchlorate is removed by a final centrifugation. The neutral extract is stored at —15°C. During the extraction procedure, all operations are carried out as rapidly as possible, and all materials are kept on ice.

Chromatographic Procedure[3]

Neutralized extracts of red blood cells estimated to contain 0.5–1.0 μmoles of ATP are placed on Dowex columns, measuring 1 $\times$ 13 cm, with the aid of slight positive pressure. The extracts are washed into the resin with three 3 ml water rinses, and the columns are closed at their bottoms. Next 1 *M* formic acid is layered over the resin bed to a height of 7 cm.

For the adapted gradient elution system, 1 *M* formic acid is adjusted in the mixing chamber to a precalibrated 400-ml mark after the flow lines to the manifold and columns are filled and all air is excluded. One liter of 0.5 *M* ammonium formate in 4 *M* formic acid is then placed in the reservoir, which is positioned above the mixing chamber. The connections from the manifold to each column are now secured.

To begin the elution all stopcocks are opened sequentially from the uppermost on the reservoir to those at the bottoms of the columns. A variety of steps may be designed for this procedure so that no air is trapped in the system.

A flow rate of 1 ml/min from each column is used routinely. Eluate volumes to be collected may be set at 1.5 or 2.0 ml per tube. Pressure systems may help to maintain a constant flow rate and to insure the collection of constant volumes when the tube changer operates on a time basis. Calibrated tubes may be placed at regular intervals on the collecting stage to serve to spot check the actual volumes obtained. Using a volumetric device routinely, we have found a gravity feed system to be adequate. The slight change in rate of flow observed is readily adjusted by the use of Kimble stopcocks equipped with Teflon plugs with metering valves. The total eluting time is 3–4 hr.

The eluates are read in the Beckman Model DU spectrophotometer at 260 mμ against water. For general chromatography the use of a multiple column system provides a ready opportunity for obtaining a true blank when the eluting agent contributes to the values obtained by the analytical method employed. We find it unnecessary to include a blank column each time as its contribution to the absorbance at 260 mμ equals the baseline values seen in the nucleotide profile. Correction for this background due to the formate ion is made only in the area where the ATP levels are determined. These levels are obtained by a summation of values of individual tubes comprising the nucleotide peak. For this quantitization, a molar absorbancy of 14.2×10^{-3} for ATP was used (5).

[3] For greater detail on the technique of chromatography of nucleotides, references (2), (3), and (4) are recommended to the reader.

RESULTS

Figure 2 shows a typical elution profile obtained during a single run with the system described above. It can be seen that the one-stage

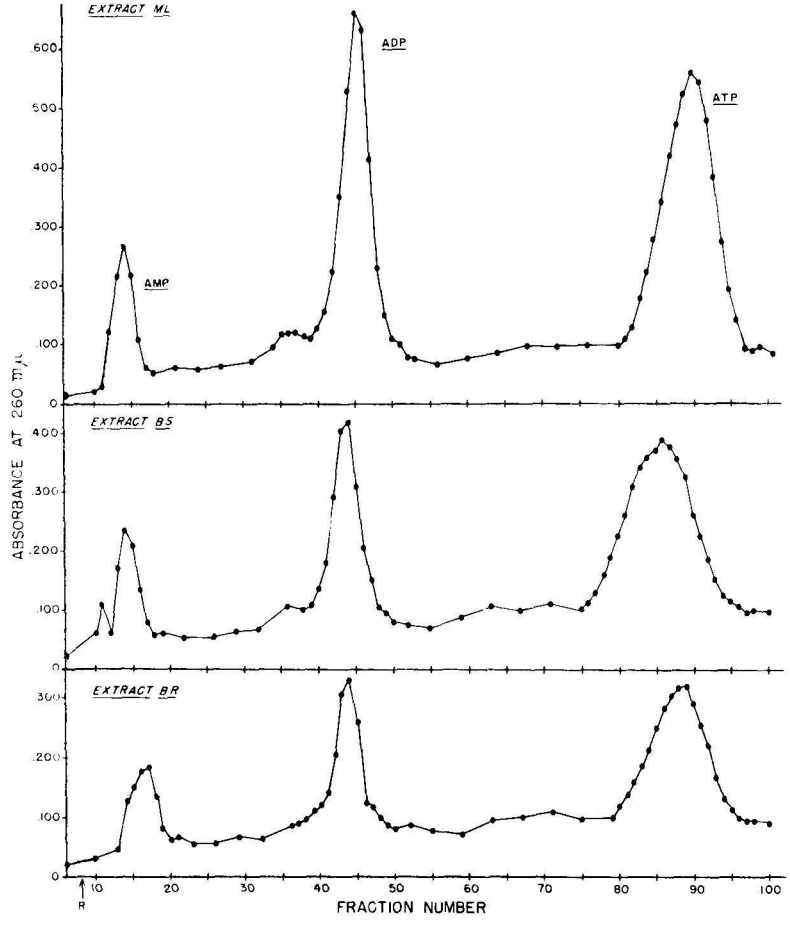

FIG. 2. Typical elution patterns observed during the simultaneous chromatography of three different extracts of human erythrocytes. This separation is obtained on 1 × 13 cm columns of Dowex 1-X2 ion exchange resin using a formic acid-ammonium formate gradient as described in the text. The arrow at the abscissa marks the initiation of the elution process. Two-milliliter fractions are collected at a rate of 1 ml/min.

eluting system adopted is capable of distinct separation of the major nucleotides contained by the human red blood cell. Also illustrated here

is the typical perfect correspondence of peak position that is repeatedly possible only with simultaneous multiple column development. Experiments with known mixtures of nucleotides average 95% recovery.

Table 1 gives a summary of mean values for ATP obtained in this study on red cells of normal human adults and newborn infants up to 36 hr of life. Statistical analysis of the data shows a significantly higher ATP content in the erythrocytes of the newborn infant. The p value for the difference in the ATP levels of these two groups is ca. 0.001 (6).

TABLE 1
COMPARATIVE LEVELS OF ATP IN HUMAN ERYTHROCYTES

	ATP (μmole/100 ml rbc)
Adult (14)[a]	69.2 ± 14.3
Newborn (14)	86.4 ± 10.4

[a] The figures in parentheses denote the number of samples.

While this report was in preparation, the work of Stave and Cara on whole blood appeared (7). Their results indicate a difference exists in the ATP levels of whole blood of infants and adults. Many others have published ATP values for adults only; for examples see references (8), (9), and (10).

DISCUSSION

Sequential chromatography of single columns, although remarkably reproducible when performed with care, leaves something to be desired as a strict analytical procedure. The packing of columns using a standard method and the same batch of prepared resin can be expected to yield relatively uniform columns and, therefore, need not be a factor limiting replicate development. More difficult to attain is the identity of elution which is the factor most important for precise reproducibility. The rigorous comparability needed for certain analyses can be claimed only for a set of columns processed as a unit. That the manner and rate of change of composition of the eluant be the same for every column can be insured only with a single eluting system common to all columns.

Described here is an approach to a minimum analytical unit of the type described above. The biological system for which it was adopted is relatively simple in that the nucleotides detectable, with the methods and amounts of blood employed, are limited. The technique of multiple chromatography is applied here not only to conserve time, but also to insure exact replication of analysis. The use of this method would be most important where the qualitative examination of peak position is of primary concern. Such would be the case, for example, with the current

work on the multiple molecular forms of enzymes separable by column chromatography (11).

SUMMARY

A readily available apparatus is described for the simultaneous development of three chromatographic columns. Also described is a single-stage, nonlinear gradient elution system capable of the uninterrupted separation of the adenine nucleotides of human erythrocytes.

The practicality of these methods is exemplified in their application to the determination of the ATP content of red cells of normal adults and full-term newborn infants.

The results show a significantly higher level of the triphosphate in the cells of the infant as compared to those of the adult.

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ANALYTICAL BIOCHEMISTRY 4, 46–51 (1962)

Photographic Detection of Zones after Centrifugation in Density-Gradient Columns of Particles Containing Nucleic Acid

A. D. THOMSON

From the Plant Diseases Division, Department of Scientific and Industrial Research, Christchurch, New Zealand

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INTRODUCTION

Density-gradient centrifugation is a useful fractionation procedure for separating and purifying plant and animal viruses (1) and ribosome particles (8). The position of particles in a gradient column may be observed by light scattering; otherwise fractions are withdrawn from the column and assayed for the presence of the particles. Identification of the position of particles by light scattering is often difficult and the method does not specifically identify particles containing nucleic acid. The present paper describes a convenient and sensitive photographic method for recording the position of particles containing nucleic acid after centrifugation in sucrose or potassium tartrate density-gradient columns.

MATERIALS AND METHODS

Preparation of Density-Gradient Columns

Gradient columns were prepared in quartz centrifuge tubes measuring 1×4.5 cm. Gradients were made by layering successively in the tubes 0.3 ml of sucrose solutions containing 400, 300, 200, 100, and 0 gm of sucrose per liter of distilled water or 0.05 M (pH 7.0) potassium phosphate buffer. Some experiments were also made using gradient columns prepared with potassium tartrate (7): 0.3 ml of solutions containing 600, 500, 400, 300, 200, and 100 gm of potassium tartrate per liter of distilled water were layered successively in quartz tubes. Columns were kept at room temperature for at least 6 hr before centrifugation; 0.1 ml of the preparation to be fractionated was floated on top of the gradient column and then centrifuged.

Centrifugation

Sucrose and potassium tartrate gradient columns were centrifuged at 25,000 rpm for 60 and 90 min, respectively, in a Spinco model L ultracentrifuge using the S.W. 39 rotor. Centrifugation runs were made at 4°C.

Ultraviolet Photography

After centrifugation the tubes were placed in a suitable holder and photographed at a distance of 2 ft from the source of ultraviolet light, which was a 15-watt low-pressure mercury vapor lamp manufactured by Hanovia Ltd. The manufacturers state that 80% of the total radiation is at 2537 A. Some improvement in resolution was obtained by attaching a mask to the tube holder so that the ultraviolet light passed mainly through the central region of the tube. Photographic paper was placed behind and in contact with the quartz tube. Best results were obtained with an extra hard contact paper such as Ilford grade 4. The time of exposure to ultraviolet light depended on the concentration of particles and nucleic acid, but satisfactory results were usually obtained with an exposure of 1–4 sec. The presence of virus in a zone in the gradient columns was confirmed by infectivity tests on fractions removed from the column.

Virus Preparations

Tobacco mosaic virus was grown in tobacco and purified by salt precipitation and differential centrifugation. Nucleic acid was prepared from the purified tobacco mosaic virus by the heat method of Lippincott (5). Turnip yellow mosaic virus and carnation mottle virus (3) were grown in *Brassica pekinensis* Rupr. and *Chenopodium amaranticolor* Coste and Reyn., respectively, and were purified by differential centrifugation. Cauliflower mosaic virus was purified by the method described by Pirone, Pound, and Shepherd (9). Virus particles were suspended in 0.05 *M* (pH 7.0) potassium phosphate buffer or in distilled water. Preparations of cauliflower mosaic virus and carnation mottle virus, made without a heat clarification step or when the particles were suspended in water instead of phosphate buffer, contained nucleoprotein particles which were presumably ribosomes. Similar particles were also obtained from uninfected cauliflower and *C. amaranticolor* plants by differential centrifugation.

RESULTS AND DISCUSSION

Lusteroid centrifuge tubes are opaque to ultraviolet light. However, quartz tubes are suitable for photography with ultraviolet light and

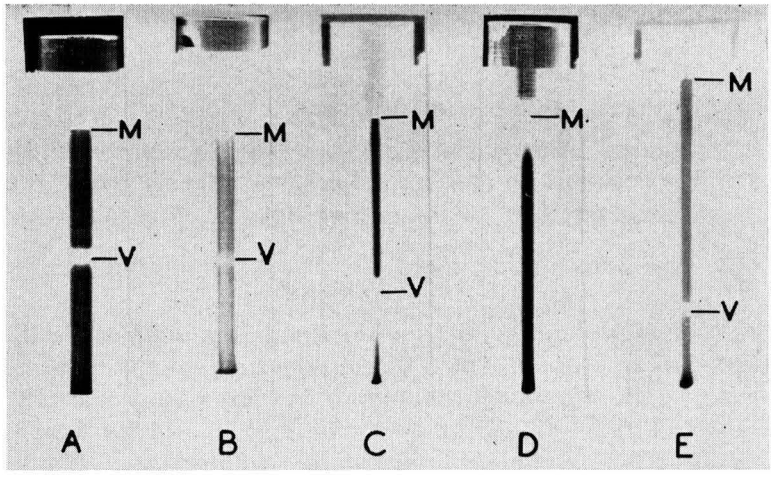

FIG. 1. Photographs taken in ultraviolet light and showing the position in sucrose (A, B, C, and D) and potassium tartrate (E) gradient columns after centrifugation of virus (V) particles. M indicates the position of the meniscus. A, 20 μg of turnip yellow mosaic virus. B, 2 μg of turnip yellow mosaic virus. C, 130 μg of tobacco mosaic virus. D, Nucleic acid prepared from same preparation of tobacco mosaic virus as in C. E, 130 μg of tobacco mosaic virus.

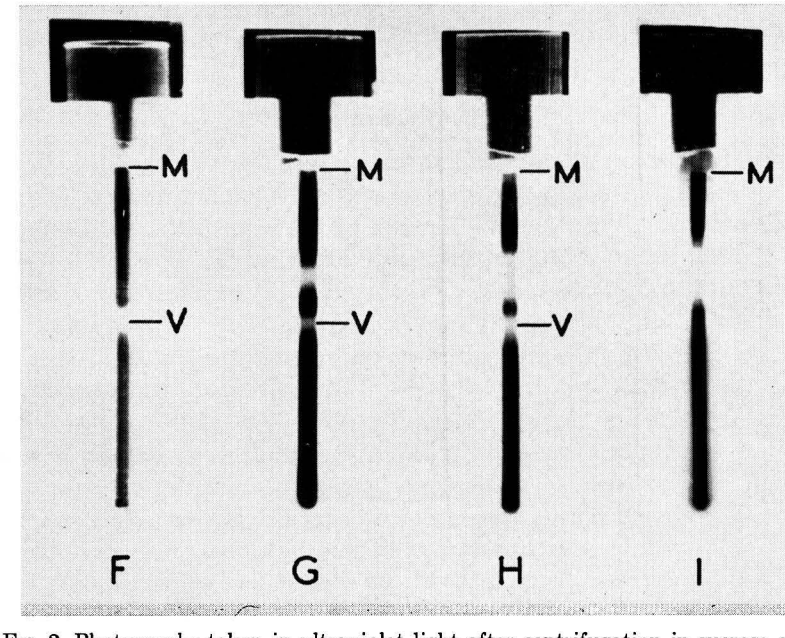

FIG. 2. Photographs taken in ultraviolet light after centrifugation in sucrose gradient columns of: F, Purified carnation mottle virus; G, partially purified carnation mottle virus heated at 55° for 5 min; H, unheated partially purified carnation mottle virus; I, ribosomes from uninfected *Chenopodium amaranticolor*.

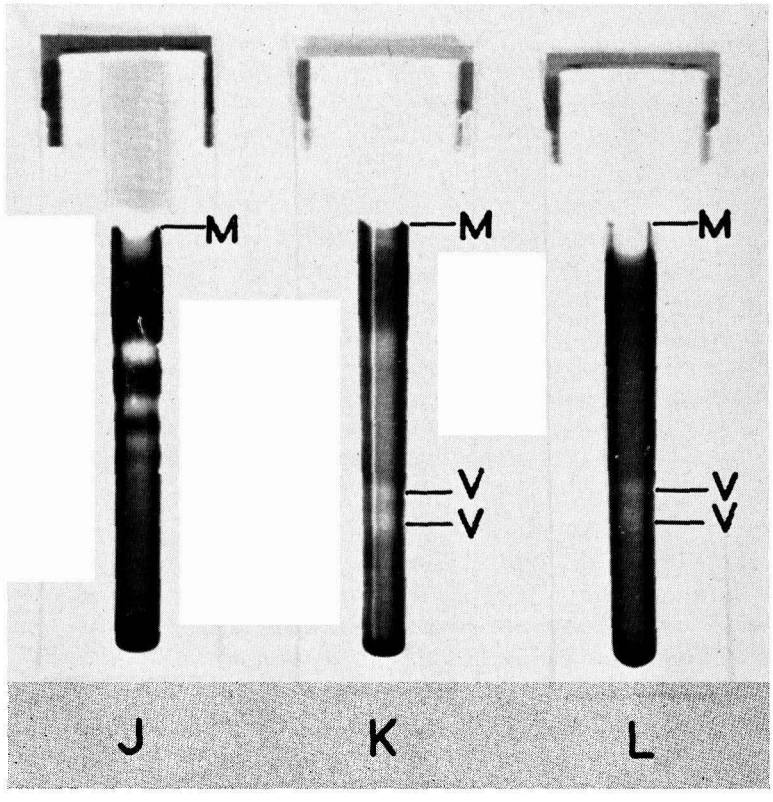

FIG. 3. Photographs taken in ultraviolet light after centrifugation in sucrose gradient columns of: J, ribosomes from cauliflower; K, unheated partially purified cauliflower mosaic virus; L, same preparation as in B, but heated at 55°C for 5 min and centrifuged at 5000 × *g* for 15 min before centrifugation in the gradient column.

particles containing nucleic acid, which are present in the gradient column, absorb ultraviolet light and appear on the photographic paper as white zones. The zones thus located can be removed from the tube with a fine pipet and the identity of the fraction can be confirmed by further tests. The method has been found useful for rapidly and accurately identifying the position of particles containing nucleic acid in sucrose and potassium tartrate gradient columns and provides a permanent record of the centrifugation run.

Figures 1, 2, and 3 show photographs taken in ultraviolet light of tubes in which purified preparations of turnip yellow mosaic virus (Fig. 1, A and B), tobacco mosaic virus (Fig. 1, C and E) and carnation mottle virus (Fig. 2, F) have been centrifuged. The sensitivity of the method depends on the concentration of particles and the percentage of

nucleic acid they contain. Tobacco mosaic virus contains 5% (4) and turnip yellow mosaic virus contains 37% (6) ribonucleic acid. With the present method it has been possible to identify approximately 12 μg of tobacco mosaic virus and 2 μg of turnip yellow mosaic virus (Fig. 1, B). Virus at these concentrations produced no light-scattering zones in the gradient columns. Although the protein and nucleoprotein components of turnip yellow mosaic virus sediment as two zones in sucrose gradients (2), only the nucleoprotein appears on the ultraviolet print (Fig. 1, A and B). Two zones frequently appear in columns after centrifugation of preparations of cauliflower mosaic virus (Fig. 3, L), but their significance is at present unknown. The present method clearly identifies the position of nucleic acid in gradient columns, and the tube shown in Fig. 1, D, was layered with nucleic acid prepared from tobacco mosaic virus. Under nonequilibrium conditions, isometric viruses such as turnip yellow mosaic virus (6), cauliflower mosaic virus (9), and carnation mottle virus (3) produce relatively narrow bands in the sucrose gradient columns, whereas tobacco mosaic virus which is anisometric (6) produces a broad zone (Fig. 1, C). Thus the type of zone appears to give a clue to the shape of the virus particle.

The presence of particles containing nucleic acid, which are presumably ribosomes, are also clearly visible in tubes in which partially purified virus preparations (Fig. 2, H; Fig. 3, K) and preparations from uninfected *C. amaranticolor* (Fig. 2, I) and cauliflower (Fig. 3, J) have been centrifuged. The four fractions shown in Fig. 3, J, may represent subunits from the ribosomes. Heating partially purified preparations of viruses at 55°C for 5 min removes much of this material (Fig. 2, G; Fig. 3, L). Heated preparations which are layered on gradient columns and then centrifuged show a conspicuous ultraviolet-absorbing fraction near the top of the column (Fig. 3, L). This fraction is nucleic acid released from ribosomes by the heat treatment.

The use of the present method for separating viruses from untreated infective extracts was also investigated. A large amount of ultraviolet-absorbing material in the column after centrifugation made identification of the virus zones very difficult. However, in the case of turnip yellow mosaic virus and carnation mottle virus, sap heated at 55°C for 5 min and then centrifuged at $5000 \times g$ for 15 min gave a preparation which, when centrifuged in a sucrose gradient column, produced an ultraviolet-absorbing zone containing the virus. In the case of turnip yellow mosaic virus, protein and nucleoprotein fractions were also visible by light scattering, although no light-scattering zones were visible in the tube layered with carnation mottle virus.

SUMMARY

A procedure is described for detecting particles containing nucleic acid following centrifugation of the particles in sucrose or potassium tartrate density-gradient columns. Quartz centrifuge tubes containing the gradient columns were photographed in ultraviolet light after centrifugation. The method has been used to identify 12 μg of tobacco mosaic virus and 2 μg of turnip yellow mosaic virus.

ACKNOWLEDGMENTS

I am grateful to Professor J. A. R. Miles of the University of Otago for the use of a Spinco model L ultracentrifuge and Dr. R. E. F. Matthews of Plant Diseases Division for the preparation of turnip yellow mosaic virus.

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ANALYTICAL BIOCHEMISTRY 4, 52–56 (1962)

A Quantitative Colorimetric Assay for Squalene[1]

GEORGE H. ROTHBLAT, DOLORES S. MARTAK, AND DAVID KRITCHEVSKY

From the Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania

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INTRODUCTION

The role of squalene as an obligatory intermediate in sterol biosynthesis is well established. The isolation and purification of this compound as the hexahydrochloride (1), or by paper (2), or column chromatography (3, 4) has been reported; but no rapid, sensitive method for squalene estimation is available. Sobel (5) described a rapid quantitative test based on the Liebermann-Burchard reaction which has a sensitivity of 300–1500 μg of squalene. This report describes a rapid colorimetric method which can be used for quantitative estimation of 10–150 μg of squalene. The procedure is a modification of a test used by Golodetz (6) for cholesterol determination and later adapted by Weigel (7) for fatty acid assay.

MATERIALS AND METHODS

The squalene (reagent grade) used was purchased from Eastman Organic Chemicals and California Corporation for Biochemical Research. All solvents were redistilled. Formaldehyde solution (36–38%) was reagent grade. Activated silicic acid (Unisil) was purchased from Clarkson Chemical Company, Williamsport, Pennsylvania.

Liver lipids were obtained by extraction of rat livers with ethyl ether-petroleum ether 2:1 using 60–75 ml solvent per liver. The extracts were dried over anhydrous sodium sulfate and reduced to dryness under nitrogen. The residues were taken up in hexane and chromatographed on Unisil columns (10 gm) according to the method of Horning *et al.* (8). Squalene was eluted with 6% benzene in hexane (150 ml).

Colorimetric Method

Solutions containing squalene were reduced to dryness under a stream of nitrogen. Thorough drying is necessary since even traces of solvent

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interfere with color development. One milliliter of concentrated sulfuric acid was added to the lipid residues and the tubes placed in a water bath maintained at 70°C for 5 min. A pale yellow color began to appear immediately after addition of the sulfuric acid. The intensity of the color is usually weak. After this initial warming period, 0.5 ml of formaldehyde solution was slowly added and the tubes shaken to ensure thorough mixing. The addition of formaldehyde intensifies and stabilizes the color. The tubes were then capped and placed in a boiling water bath for 10 min. The level of the water was adjusted so that each tube was ¾ immersed in the boiling water. Immediately after removal from the water bath, 2.5 ml of glacial acetic acid was added to bring the volume up to 4.0 ml, and the solutions were mixed thoroughly.

The purpose of adding the glacial acetic acid is primarily for dilution, in order to obtain a satisfactory volume for colorimetric assay. Other diluents were tested, but glacial acetic acid appeared to preserve maximum color intensity. The volume of diluent may be varied to suit the requirements of the assay procedure. The resulting yellow to brown color is stable for several hours. The optical density was read at 400 mμ using a Coleman Junior or a Bausch and Lomb Spectronic 20 spectrophotometer.

RESULTS AND DISCUSSION

The absorption spectrum of the squalene chromogen (Fig. 1) shows that no distinct absorption peak is attained; rather, a plateau is observed between 380–420 mμ. Readings taken at 400 mμ follow the Beer-Lambert law (Fig. 2).

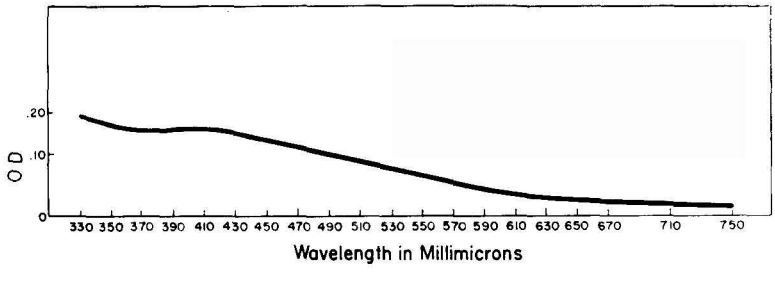

FIG. 1. Absorption spectrum of squalene-HCHO-H_2SO_4 chromogen.

Tests were conducted to determine if concentrated hydrochloric or glacial acetic acid could be substituted for sulfuric acid in the color reaction, but no color was obtained with either acid. Since other aldehydes can be used in conjunction with sulfuric acid to yield colored products with cholesterol (9–11), we tested furfural, *p*-anisaldehyde,

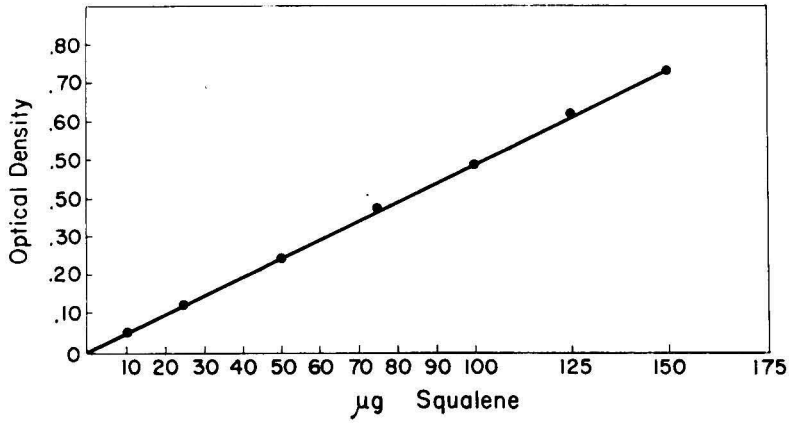

FIG. 2. Agreement of squalene-HCHO-H_2SO_4 chromogen with Beer-Lambert law at 400 mμ.

salicylaldehyde, and benzaldehyde. In no case was a satisfactory color reaction obtained.

The sulfuric acid-formaldehyde test was performed on a number of compounds other than squalene to determine the specificity of the color reaction. Oleic and linoleic acids, cholesterol, triolein, trilinolein, and tripalmitin were tested. All of the compounds containing one or more double bonds gave a positive color reaction, tripalmitin alone giving no significant color. The color could be deepened by prolonging the time of heating, confirming similar observations of Weigel (7). The chromogens produced with the other compounds all demonstrated spectra very similar to that of the squalene chromogen.

To establish the accuracy and applicability of the squalene assay procedure, the recovery of squalene added to natural rat liver lipid mixtures was investigated. The lipid extract from a pool of three rat livers was divided into several equal aliquots. The first fraction was used as a control and varying amounts (25–1000 μg) of squalene were added to each of the others. After silicic acid chromatography, the squalene content of the hydrocarbon fraction was assayed colorimetrically. It may be seen from Table 1 that close to quantitative recoveries were obtained. The failure to obtain 100% recovery reflects loss of squalene due to chromatographic procedures.

The recovery of squalene from rat liver indicates a normal level of about 40–50 μg/gm liver. This figure is somewhat higher than that Langdon and Bloch (12) adduced by other procedures.

In view of the fact that the color reagent will react with any unsaturated compound, we tested to see whether this slight discrepancy could

be due to small amounts of some carotenoid substance being eluted in the hydrocarbon fraction. Spectrophotometric analysis of the squalene containing eluate obtained upon column chromatography of several different samples of liver lipids failed to show any absorption maxima.

TABLE 1
RECOVERY OF SQUALENE ADDED TO RAT LIVER LIPIDS

Aliquot	Squalene (μg)			% Recovery
	Added	Found	Calculated	
1	0	250	—	—
2	25	272	275	88.9
3	75	320	325	93.1
4	250	491	500	96.3
5	500	738	750	97.5
6	1000	1217	1250	96.7

The presence of carotenoids in this fraction can be detected spectrophotometrically (and proper corrections made) or more easily by the intense blue color obtained when concentrated sulfuric acid is added to the dried lipid residue. The blue color is readily observable when small (5–10 γ) quantities of β-carotene are added to the squalene fraction prior to color development. Chromatography of a mixture of squalene and carotene was not attempted.

We have found the colorimetric method described above to be satisfactory for the assay of squalene in lipid extracts from cells grown in tissue culture.

SUMMARY

A rapid and sensitive quantitative colorimetric test for squalene has been devised. The color reaction involves the use of sulfuric acid and formaldehyde. The test will give a positive reaction with other compounds which contain double bonds and must consequently be used in conjunction with other methods which yield purified squalene. This method has been found very useful for rapid quantitation of the amounts of squalene eluted from silicic acid columns.

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ANALYTICAL BIOCHEMISTRY 4, 57–68 (1962)

Chromatography of Nitrofluoresceins, Aminofluoresceins, and Fluorescein Isothiocyanates

HOWARD S. COREY, JR.,[1] AND ROGER M. McKINNEY

From the U. S. Department of Health, Education, and Welfare, Public Health Service, Communicable Disease Center, Technology Branch, Technical Development Laboratories, Savannah, Georgia

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INTRODUCTION

Adaptations of the fluorescent antibody technique to biological research and medical diagnosis have been increasing markedly since its introduction by Coons and Kaplan (1).[2] The original labeling substances, fluorescein isocyanate I or II, were hazardous to prepare and tedious to use because of their instability. A major improvement resulted from the synthesis of the stable fluorescein isothiocyanate (2) and its demonstrated superiority as a labeling agent (2, 3).

Although the identification of aminofluorescein I and II by elemental analysis of their hydrochlorides has been claimed (1), there have been no reports of the preparation of the free amines or of the fluorescein isothiocyanate isomers I and II in an analytically pure state. Since 1958, aminofluorescein I and II and their isothiocyanates have become available from a number of commercial sources. The isothiocyanates have been prepared by treatment of the appropriate aminofluorescein, or derivative thereof, with thiophosgene. The amines are obtained by reduction of the corresponding nitrofluoresceins, which are the main condensation products from the reaction of 4-nitrophthalic acid with resorcinol.

The only satisfactory method that has been available for evaluating the fluorescein isothiocyanates has been to test their utility by conjugation with antibody serum, which is then treated with antigen; the resultant complex is examined for fluorescence microscopically. Since there is a

[1] Present address: Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

[2] "The Bibliography of Literature on Fluorescent Antibody and Fluorescence Microscopy" (compiled by Microbiological Section, Laboratory Branch, Communicable Disease Center, Public Health Service, U. S. Department of Health, Education and Welfare, Atlanta, Ga.) contains 39 references from the years 1905 to 1949 and 287 for the period 1950–1959.

wide variation in the relative staining ability of different lots of these products, the development of a simple rapid method of evaluating the currently available aminofluoresceins and fluorescein isothiocyanates would be a definite advance in the field. We report the use of paper chromatography combined with instrumental analysis to evaluate the chemical purity of aminofluorescein and fluorescein isothiocyanate products from commercial and other sources. The intermediate nitrofluorescein isomers were also studied and their R_f values determined in order to establish whether they are contaminants in any of the end products.

EXPERIMENTAL PROCEDURE

Materials. Aminofluorescein I and II and fluorescein isothiocyanates I and II were obtained from several commercial sources as well as from preparations made in this and other laboratories.[3] Nitrofluorescein I and II were prepared according to the procedure of Coons and Kaplan (1).

Chromatography was done with 100-cm strips of standard Whatman No. 1 paper. Reagent solvents were used to dissolve samples and to develop chromatographs.

The papers were dipped in a phosphate buffer and dried before use. The buffer was prepared by adjusting a 0.05 *M* solution of K_2HPO_4 to the desired pH (7.0–7.5) with concentrated phosphoric acid.

Chromatography. Solutions containing 2 mg/ml of aminofluorescein were prepared by weighing 1 ± 0.002 mg into vials, and then adding 0.5 ± 0.01 ml of *n*-butanol. Solutions of the fluorescein isothiocyanates were prepared similarly in *n*-butanol and methanol at a concentration of 1 mg/ml using 0.4 to 1.0 mg of each sample. The nitrofluoresceins were dissolved in 0.01 *N* HCl in methanol and stored at —4°.

Samples were spotted on the buffered sheets in increments of 2 μl using disposable 2-μl pipets (Drummond Scientific Co. Microcaps). When a 4-μl charge was chromatographed, the first spot was dried before adding the second aliquot. The micropipets were not rinsed. After equilibration with the atmosphere in standard Pyrex chromatography tanks containing water saturated with *n*-butanol for 45–60 min, the sheets were developed in the descending manner with *n*-butanol that had been equilibrated with three-fourths of its volume of buffer. The development at 24 to 26° required 13 to 16 hr.

[3] The commercially prepared products used in this study were purchased from the following companies: Baltimore Biological Laboratory, Baltimore, Maryland; Sylvana Chemical Company, Orange, New Jersey; Dajac Laboratories, Philadelphia, Pennsylvania; Nutritional Biochemicals Corporation, Cleveland, Ohio. Other products utilized in this study were prepared in this and other laboratories of the U. S. Public Health Service.

Analytical Procedure

Visual Inspection. The chromatograms were inspected under an ultraviolet lamp after being fumed with ammonia vapors. Fuming with ammonia intensified the yellow-green fluorescence exhibited by the amines and isothiocyanates. The nitrofluoresceins changed from yellow-orange to dark orange in the presence of ammonia under UV light. The R_f's were determined with a Chromatogrid (distributed by California Corporation for Biochemical Research) measuring from the origin to the centers of the spots.

Fluoroscanning Apparatus. The fluoroscanning apparatus consisted of an ultraviolet light source, a diffuse reflectance attachment with light-source slit, a paper-strip transport mechanism, a photomultiplier tube with an amplifier, and a strip chart recorder. The reflectance component was equipped with a Corning 4010 filter, which permitted maximum transmittance of the yellow fluorescent light from the spots on the strip, but excluded long-wavelength visible light and reflected ultraviolet light used to induce the fluorescence.

Fluoroscanning. All samples subjected to fluoroscanning were chromatographed at least six times. A representative chromatogram for each sample was selected, from which 1″-wide strips were prepared. The strips were joined together with cellophane tape to form a continuous ribbon, and a leader of blank, buffered Whatman No. 1 paper was attached to the solvent front end of the first strip to be analyzed.

The strips were drawn past the light-source slit in the reflectance component by means of the strip transport mechanism. A constant fraction of the fluorescent light from each spot was focused on the photomultiplier tube. The signal from the photomultiplier tube was transmitted, after amplification, to the strip chart recorder.

Chart paper graduated from 0 to 100 was used, and the baseline signal was set at 10 units while the leader strip was passing across the slit in the reflectance component. Then the center of the major spot in the reference sample (chosen arbitrarily for intense fluorescence during visual inspection of the strips) was scanned, while adjustments were made so that the signal deflected the pen 80 to 90 units. The chromatogram strips and recorder paper were synchronized to move at the same rate, so that the position of detectable spots on the chromatograms corresponded in position to deviations of the chart record from the baseline. The over-all chart record gave an approximate concentration profile curve (4)[4] in terms of fluorescence. The proportionality of concentration to fluorescence is good for similar substances. Since mixtures of substances with variations in

[4] See pp. 214 to 216 of reference (4).

intensity of fluorescence have been encountered in this study, the curves have been designated fluorescence profile curves. The R_f of any spot can be determined from the point of maximum deflection of the chart line.

RESULTS

Chromatography of Nitrofluoresceins. Samples of nitrofluorescein I and nitrofluorescein II, prepared by the procedure of Coons and Kaplan (1), were chromatographed in order to determine their R_f values and relative intensity of fluorescence. Four micrograms of each isomer were chromatographed on Whatman No. 1 paper buffered with pH 7.0 K_2HPO_4-H_3PO_4 and developed with *n*-butanol. Both isomers had their predominant spot at R_f 22.[5] The average area of this spot, as determined by the fluoroscanning apparatus, was 29 cm^2 for several isomer II samples and 16 cm^2 for isomer I samples.

TABLE 1

AREAS CALCULATED FOR COMPONENTS OF AMINOFLUORESCEIN I SAMPLES FROM FLUORESCENCE PROFILE CURVES[a]

Sample No.	Areas in cm^2 at R_f[b]					Total A (cm^2)
	0	32	56	82	Misc.	
NH_2-1[c]	6.4	91.5		2.8	@ 7[d]	100.7
-5[c]	2.4	95.8		2.0	1.8 @ 46	102.0
-7[c]	2.4	90.3	2.2	1.8		100.1
-10[c]	66.0	33.7		2.0		100.7
-12	1.5	103.2	2.7			107.4
-14	1.8	109.2		3.8		114.8
-16	1.4	94.3		2.8	2.9 @ 67	101.4
-17	2.0	103.5		4.0		109.5
-20	1.4	50.5		5.4	11.6 @ 22	68.9
-26	3.4	95.5	1.8	1.6		102.3

[a] Chromatography was done with the *n*-butanol/pH 7.0 K_2HPO_4-H_3PO_4 system on Whatman No. 1 paper at 24 to 26°. Each sample was 4 μg.

[b] The R_f values are the average of 6 to 10 determinations.

[c] Denotes commercial samples.

[d] Only a trace amount was present.

Chromatography and Instrumental Analysis of Aminofluoresceins. The R_f values and relative fluorescence of the spots for ten isomer I samples, as determined with the fluoroscanning apparatus, are listed in Table 1. Similar data for eleven isomer II samples are given in Table 2. Isomer I, with R_f 32 for the major spot, was less mobile than isomer II, which had an R_f value of 82 for the major spot. The *n*-butanol solutions of the aminofluoresceins were stable, as shown by the fact that samples several

[5] R_f values have been multiplied by 100 throughout this paper.

months old gave the same chromatographic pattern as fresh samples. The data obtained by fluoroscanning the amine chromatograms for isomer I samples and isomer II samples were taken in a single instrumental analysis, so that comparisons between Tables 1 and 2 can be made.

TABLE 2

AREAS CALCULATED FOR COMPONENTS OF AMINOFLUORESCEIN II SAMPLES FROM FLUORESCENCE PROFILE CURVES[a]

Sample No.	Areas in cm² at R_f[b]					Total A (cm²)
	07	32	56	82	Misc.	
NH_2-2[c]	2.2	1.2	3.2	64.8		71.4
-3[c]	[d]		1.4	74.6		76.0
-4[c]	1.2	1.2	1.7	60.3	1.5 @ 14	65.9
-6[c]		5.7	15.2	41.3		62.2
-8[c]	[e]	[e]	[e]	[e]		—
-9[c]		11.5	4.2	49.0		64.7
-11			1.8	57.3		59.1
-15			15.7	48.6		64.3
-19		3.2	2.7	56.1	1.0 @ 14	63.0
-24	0.5	0.9		46.7		48.1
-25	1.8		2.1	54.3		58.2

[a] Chromatography was done with the *n*-butanol/pH 7.0 K_2HPO_4-H_3PO_4 system on Whatman No. 1 paper at 24 to 26°. Each sample was 4 μg.

[b] The R_f values are the average of 6 to 10 determinations.

[c] Denotes commercial samples.

[d] Only a trace amount was present.

[e] Sample 8 streaked the entire length of the sheet.

Chromatography and Instrumental Analysis of Fluorescein Isothiocyanates. Chromatography of the fluorescein isothiocyanates was complicated by solubility difficulties. They are too insoluble in most nonreactive solvents. While fluorescein isothiocyanates will react with both methanol and *n*-butanol to form stable thiocarbamates, the reaction with *n*-butanol is relatively slow.

Tables 3 and 4 show the R_f values and areas for the spots of fluorescein isothiocyanate samples that were dissolved in *n*-butanol and chromatographed immediately on paper buffered with pH 7.3 K_2HPO_4-H_3PO_4. Fluorescein isothiocyanate I appeared as a spot at R_f 46 and its butyl thiocarbamate at R_f 64, while isothiocyanate II had an R_f of 55 and butyl thiocarbamate II appeared at R_f 80. The identity of the spots was established by chromatographing fresh and aged samples. Samples kept at 24 to 27° for 4 months had their single predominant spot at R_f 64 (isomer I) and 80 (isomer II), which are accepted as being due to the butyl thiocarbamates. The 4-month samples also had significant yellow spots at R_f 92, and isothiocyanate I samples produced significant spots at R_f 30

TABLE 3

AREAS CALCULATED FOR COMPONENTS OF FLUORESCEIN ISOTHIOCYANATE I SAMPLES FROM FLUORESCENCE PROFILE CURVES[a]

Sample No.	Areas in cm² at R_f[b]						Total A (cm²)
	03	11	29	46	64	Misc.	
NCS-1[c]	4.2	[d]	[d]	70.5	12.9		87.6
-5[c]	4.9	1.1	1.3	41.7	22.4	1.3 @ 80	72.7
-6[c]	9.0	2.4	3.5	13.4		43.8 @ 55[e]	72.1
-7[c]	5.8	4.3	3.4	8.0	5.4	3.3 @ 55	30.2
-9[c]	11.7	3.4	2.9	28.6	3.5	1.9 @ 59	52.0
-11[c]				10.3	4.8	14.4 @ 39	32.7
-14	7.3			48.7	30.6	1.0 @ 09	89.8
						2.2 @ 21	

[a] Chromatography was done with the *n*-butanol/pH 7.3 K_2HPO_4-H_3PO_4 system on Whatman No. 1 paper at 24 to 26°. Each sample was 2 μg.

[b] The R_f values are the average of 6 determinations.

[c] Denotes commercial samples.

[d] Only a trace amount was present.

[e] The R_f 55 spot could not have been due to isothiocyanate II, since no spot was found at R_f 80.

(corresponding in position and color to aminofluorescein I). Fresh samples had their strongest spots at R_f 46 (isomer I) and 55 (isomer II), with streaks leading to weak spots in the butyl thiocarbamate positions.

TABLE 4

AREAS CALCULATED FOR COMPONENTS OF FLUORESCEIN ISOTHIOCYANATE II SAMPLES FROM FLUORESCENCE PROFILE CURVES[a]

Sample No.	Areas in cm² at R_f[b]						Total A (cm²)
	11	21	55	60–70	80	Misc.	
NCS-2[c]		[d]	38.2	7.4	6.6	@ 03[d]	52.2
-3[c]	4.0	1.2	25.8	7.2	7.4	1.7 @ 03	47.3
-4[c]		1.3	35.3	7.9	4.1	@ 00[d]	48.6
-8[c]	2.3	1.5	15.7	3.3	3.8	1.3 @ 03	27.9
-15		1.9	14.9	5.1	28.4		50.3

[a] Chromatography was done with the *n*-butanol/pH 7.3 K_2HPO_4-H_3PO_4 system on Whatman No. 1 paper at 24 to 26°. Each sample was 2 μg.

[b] The R_f values are the average of 6 determinations.

[c] Denotes commercial samples.

[d] Only a trace amount was present.

Samples kept at −4° for 6 weeks had isothiocyanate spots and corresponding butyl thiocarbamate spots that were of approximate equal intensity when examined by fluoroscanning.

Table 5 shows some data obtained by completely converting the iso-

thiocyanates to their methyl thiocarbamates by allowing the methanol solutions to stand at room temperature for 8 hr before spotting and chromatographing. The reaction with methanol was complete under these conditions, as shown by the absence of a spot at R_f 55 for isothiocyanate II samples; methyl thiocarbamate II had an R_f of 67. On the basis of R_f alone, fluorescein isothiocyanate I (R_f 46) and fluorescein methyl thiocarbamate I (R_f 48) are indistinguishable. However, when methanolic

TABLE 5

AREAS CALCULATED FROM FLUORESCENCE PROFILE CURVES FOR COMPONENTS OF SELECTED FLUORESCEIN SAMPLES[a,b]

Sample No.	Areas in cm² at R_f[c]						Total A (cm²)
	03	30	48[e]	67[f]	82	Misc.	
1[d]	2.4		114.3				116.7
2[d]				47.9			47.9
6[d]	8.3	3.5	66.9		4.3	[g]	83.0
7[d]	3.8	2.9	27.1	7.1	1.8		42.7
9[d]	11.2	2.5	52.3		3.4		69.4
11[d]	[h]		12.8		1.9	11.2 @ 39	25.9
14	7.6	2.5	78.1	15.3	[h]		103.5
15				28.2	18.8		47.0
NH_2-2					5.3		5.3

[a] Fluorescein isothiocyanate samples which were converted to their methyl thiocarbamate derivatives, before chromatographing, by reaction with methanol.

[b] Chromatography was done with the *n*-butanol/pH 7.3 K_2HPO_4-H_3PO_4 system on Whatman No. 1 paper at 24 to 26°. Each sample was 4 μg.

[c] The R_f values are the average of 6 determinations.

[d] Denotes commercial samples.

[e] The methyl thiocarbamate derived from isomer I.

[f] The methyl thiocarbamate derived from isomer II.

[g] A spot visible at R_f 55 had its profile curve merged with that of the spot at R_f 48.

[h] Only a trace amount was present.

solutions of isothiocyanate were chromatographed immediately, a trace of butyl thiocarbamate resulting from a slow reaction of isothiocyanate with developer was seen at R_f 64; no butyl thiocarbamate could be detected after 8 hr in methanol, indicating that all the isothiocyanate had reacted with methanol. In addition, when fluoroscans were obtained on the developed chromatograms from immediate and 8-hr samples, the methyl thiocarbamate I produced a profile curve with 2.5 times greater area than the isothiocyanate I (immediate sample). These differences could be easily detected by visual inspection.

Instrumental Comparison of Related Fluorescein Derivatives. Table 6 enables a comparison to be made of the areas under the fluorescence profile curves of all types of compounds examined in this study. Only the

TABLE 6
COMPARISON OF FLUORESCENCE PROFILE CURVES OF RELATED FLUORESCEIN DERIVATIVES[a]

Sample $C_{20}H_{11}O_5$-	A (cm^2) major spot	Isomer
NO_2-4	0.7	I
NO_2-5	1.0	II
NH_2-12	6.1	I
NH_2-11	2.4	II
NCS-1	29.9	I
NCS-4	16.9	II
NHC(S)OBu-1	46.7	I
NHC(S)OBu-4	20.4	II
NHC(S)OMe-1	73.9	I
NHC(S)OMe-4	21.1	II

[a] Chromatography was done with the *n*-butanol/pH 7.3 K_2HPO_4-H_3PO_4 system on Whatman No. 1 paper at 24 to 26°. Each sample was 2 μg.

area for the spot identified as the predominant substance has been tabulated.

DISCUSSION

A variety[6] of chromatography systems was examined before the system using *n*-butanol and dibasic potassium phosphate-phosphoric acid buffer was selected for this study. Another system that gave good resolution for the aminofluoresceins was *n*-butanol and aqueous oxalic acid at pH 1.1.[6]

n-Butanol-4 *N* ammonium hydroxide (5) and ethanol-ammonium hydroxide (6, 7), which have been applied to chromatography of other xanthene dyes, did not give enough mobility to either amines or isothiocyanates. The butanol-acetic acid developer of Lima and Pieroni (8) moved most components to the solvent front.

The R_f values in individual experiments reported varied by no more than ±4 units from the mean R_f values tabulated. In most cases the

[6] All chromatography was done on Whatman No. 1 paper. Unbuffered, ascending strips were generally useless. With *n*-butanol above pH 8.0 the fluoresceins were too immobile. Between pH 2.0 and 6.0 amines and isothiocyanates moved with the solvent front with butanol and ethyl acetate. *n*-Butyl acetate produced streaking at pH 1.1 and 2.0. The resolution was poor with *n*-butanol at pH 8.0 and with ethyl acetate at all pH values in the range 1.1 to 7.5. Methyl isobutyl ketone gave poor resolution and/or streaking when used as a developer between pH 1.1 and 8.0. The only system that showed worthwhile utility for the amines in addition to the one reported in detail involved the development of pH 1.1 (2.5% aqueous oxalic acid) paper with *n*-butanol. Aminofluorescein I had its major spot near R_f 55, while that of isomer II appeared at 70. However, this pH 1.1 system showed considerable variation, the spots were quite unsymmetrical, and the amines appeared to undergo decomposition. Also, it did not resolve the isothiocyanates.

variation was only ±2 units and the R_f values from fluoroscanning coincided with those determined visually. As an aid to obtaining reproducible results, it was necessary to wash the chromatography tanks and add fresh water saturated with *n*-butanol after 4 to 8 weeks of continuous use.

In spite of all efforts to obtain an identical R_f value for a given spot on every chromatogram, fluctuations were unavoidable. The R_f value of aminofluorescein I varied between the limits R_f 17 to 45 using paper treated with pH 7.0 buffer. Other amine or isothiocyanate spots appeared at related higher or lower values. Even in these cases, however, the pattern of spots from any series of samples was recognized as being the same as that obtained when the amine spots appeared at R_f 32 (I) and 82 (II).

In practice, when the R_f of aminofluorescein I fell below 20, the corresponding decreases for isothiocyanate I and II brought the two spots so close together that they were indistinguishable on the basis of R_f alone. To circumvent this, before fluoroscan analyses were done, sheets from the same lot of paper were buffered at pH 7.0, 7.1, 7.2, 7.3, and 7.5, and several samples of aminofluorescein I were chromatographed on each. The pH selected for actual analysis was that which permitted aminofluorescein I to have a mobility close to 30 (26 to 34). As a result of such a preliminary determination, in this study, the aminofluoresceins were chromatographed on paper buffered at pH 7.0, while the isothiocyanates and their alkyl thiocarbamate derivatives were examined using pH 7.3 paper.

The R_f value of the major spot of either isomer of aminofluorescein makes their identification simple, and enables the presence of the opposite isomer to be detected. Of the four aminofluorescein I samples from commercial sources listed in Table 1, probably all contain small amounts of isomer II, as indicated by the spot at R_f 82. Of these samples, No. 10 appears to be quite inferior, with only one-third of its fluorescence at R_f 32.

Of the six aminofluorescein II samples from commercial sources in Table 2, it appears that four probably contained some isomer I, as indicated by the spot at R_f 32. There was considerable variation in the purity of these commercial samples, with No. 8 showing no resolved spot at R_f 82. Amine No. 6 showed slightly more than one-half of the fluorescence area at R_f 82 shown by amine No. 3, which appears to be the best sample of the series. Curiously, sample No. 3 erroneously had been labeled isomer I by the supplier.

The absence of any resolved spot at R_f 22 in any of the amine I or amine II chromatograms makes it appear unlikely that any of the amines studied were contaminated with the nitrofluorescein intermediate. The

most prominent contaminant was the R_f 56 substance found in all but one of the isomer II samples with resolved spots.

The best sample of aminofluorescein I (No. 14), from Table 1, had a fluorescence area of 109.2 cm^2 at R_f 32 as compared with an area of 74.6 cm^2 for the best aminofluorescein II sample (No. 3). This finding illustrates the fact that isomer I derivatives, with the exception of nitrofluorescein I, invariably showed greater fluorescence than the corresponding isomer II derivatives. Such relationships are shown by the data in Table 6.

Generally, the relative composition of a sample can be estimated by visual inspection of the chromatograms, so a practical evaluation of amine or isothiocyanate samples can be made without instrumental analysis. An alternative method to fluoroscanning, which would give semiquantitative data, would be to elute the resolved zones and examine the eluates spectrophotometrically.

Fluorescein isothiocyanate samples were dissolved in both *n*-butanol and methanol. Immediate chromatography of either set gave a complex pattern. Presumably, the complex pattern resulted primarily from a slow reaction of isothiocyanate with butanol developer to produce butyl thiocarbamate. The conversion to methyl thiocarbamate of either isomer, which is complete within 8 hr at room temperature, simplified this chromatographic analysis. The reason for the variation in the extent to which the different isothiocyanate samples were converted to their butyl thiocarbamates (Tables 3 and 4) is unknown.

Samples NCS-7 and 11, which were undesignated by their suppliers, were shown to contain isomers I and II (No. 7) and I (No. 11).

Four of the isothiocyanates had significant, isolated, unidentified spots:

(*1*) Sample NCS-6 had a prominent spot at R_f 55 regardless of the solvent used; in the butanol sample it was the predominant spot. This substance was unaffected by either alcohol, so it is doubtful that it is an isothiocyanate. Its fluorescence was too great to be the R_f 56 contaminant common in aminofluorescein II samples.

(*2*) Sample No. 11 had approximately 43% of its fluorescence at R_f 39 when either solution was analyzed. This substance appeared to be the major component in the sample and seemed to be nonreactive with methanol or butanol.

(*3*) Fluoroscanning the strip from the methanol solution of No. 14 showed 15% of the fluorescence at R_f 67, which suggested the presence of a substantial amount of isomer II. However, chromatography of the butanol sample did not show any butyl thiocarbamate II (R_f 80). The nature of this R_f 67 artifact is unknown.

(*4*) The methanol sample of NCS-15 had 40% of its fluorescence at R_f

82. The area recorded was 3.5 times the area found for 4 μg of NH_2-2 fluoroscanned in the same sequence so it could not be aminofluorescein II.

The data presented here indicate clearly that most of the samples of aminofluorescein and fluorescein isothiocyanate examined contained substantial amounts of impurities. Although it is not known for certain why the amines and isothiocyanates are not pure, it is suspected that one of the critical steps in their preparation is in the reduction of nitrofluorescein to aminofluorescein.

Although we have some limited evidence correlating labeling efficiency with our chromatographic results, a careful study of this correlation is yet to be made.

The chromatography described here can be used, with practice, to evaluate samples of fluorescein isothiocyanate semiquantitatively by inspection. The use of these systems should be of considerable value to workers attempting to make purer preparations of these protein-labeling substances. It is significant that isomer I derivatives yield two to four times the fluorescence per microgram as the corresponding isomer II derivatives. Because of this, and since each member of the isomer I series is easier to obtain in a purer condition, isothiocyanate I should be the labeling agent of choice. The fact that the methyl thiocarbamates are more strongly fluorescent than the *n*-butyl derivatives supports the speculation that improvements in labeling agents may be achieved by further modifying the group which participates in conjugation, or through the synthesis and use of fluorescein isothiocyanate isomers that are different from those obtained by the synthesis of Coons and Kaplan (1) as modified by Riggs *et al.* (2).

SUMMARY

1. The paper chromatography systems using Whatman No. 1 paper buffered at pH 7.0–7.5 and *n*-butanol as the developing solvent can be used for the qualitative and quantitative evaluation of nitro- and aminofluorescein and fluorescein isothiocyanates.

2. A study has been made of the chromatographic behavior of fluorescein isothiocyanates I and II as their corresponding methyl and *n*-butyl thiocarbamates.

3. A novel instrumental method of analyzing the developed chromatograms for fluorescence is described. Data obtained with this technique show that the amine and isothiocyanate isomer I have a greater fluorescence per microgram than their counterparts from the isomer II series. This indicates fluorescein isothiocyanate I is superior to II for fluorescent antibody work.

4. The chromatographic methods developed could form the basis of

assay procedures that may lead to more uniform commercial products. Most of the commercial samples examined contained a number of components.

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Determination of Ethanolamine and Serine in Phospholipids[1]

KARL H. SLOTTA AND J. K. POWERS

From the Department of Biochemistry, University of Miami, School of Medicine, Miami, Florida

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INTRODUCTION

When we recently investigated the blood-clotting activity of individual and combined phospholipids (1), PE[2] and PS in naturally occurring phospholipid mixtures had to be determined. These mixtures only showed the high activity of the "natural lipid activator" from platelets if they contained at least small amounts of PS. In soybean cephalin, however, which strongly promotes blood clotting, PS could often not be detected (2). Thus the necessity arose to elaborate a method for the exact determination of smallest amounts of PS and also PE. The various methods described in the literature proved inadequate for this purpose. The usual approach is to hydrolyze the phospholipid sample, to separate ethanolamine and serine, and to determine their quantities. In his excellent survey of the abundant earlier literature McKibbin (3) evaluated one method as "sufficiently satisfactory." Dittmer *et al.* (4) stated that "an improved method of hydrolysis . . . is obviously needed."

Since then a new technique has been suggested by Magee *et al.* (5) and the remarkable method of Dawson (6), which even permits separate identification of the ethanolamine and serine originating from the diester and the plasmalogen part of the phospholipid mixtures, was published. In the present paper we describe a simple and rapid technique for the determination of ethanolamine and serine from 5- to 15-mg samples of phospholipid. This method gives consistent results when tested on synthetic and native phospholipids of animal and vegetable origin.

[1] This investigation was supported by grant H-4889 from the National Institutes of Health, Bethesda, Maryland.

[2] The abbreviations used are: PE = phosphatidyl ethanolamine, PS = phosphatidyl serine, OPE = *O*-phosphoethanolamine, OPS = *O*-phosphoserine.

METHODS

I. Apparatus and Reagents

a. Two columns with the following dimensions: 14 mm o.d., 11.6 mm i.d., 120 to 200 mm length, with a 40 × 50 mm o.d. tube on one end. The first column with 4 ml (wet volume) Dowex 50 W-X8, 100–200 mesh resin (J. T. Baker, Phillipsburg, N. J.) is washed with 7.5 ml 1 *N* sodium hydroxide followed by water until neutrality. Washing is continued with 3 ml 3 *N* hydrochloric acid and water again to neutrality. With 5 ml 10% acetic acid the effluent of the column is brought to pH 2. The second column containing 4 ml (wet volume) of the same resin is treated like the first one with sodium hydroxide and water and brought to pH 5 with 10 ml 0.2 *M* acetate buffer.

b. 10% (v/v) acetic acid (1.71 *N*, pH 2.3).

c. 0.2 *M* sodium acetate buffer (pH 5) (prepared from 3 vol 0.2 *M* acetic acid plus 7 vol 0.2 *M* sodium acetate).

d. 10% sodium acetate (pH 7.2).

e. Ninhydrin solution for staining: 300 mg ninhydrin (Consolidated Midland Corporation), 90 ml acetone, 10 ml 2,4-lutidine (pract.); stored in refrigerator.

f. Ninhydrin reagent for quantitative determination: 500 mg ninhydrin and 50 mg stannous chloride (dried in a vacuum desiccator) are placed in a 250-ml Erlenmeyer flask; 70 ml peroxide-free methyl Cellosolve (= 2-methoxyethanol) and 25 ml 4 *N* acetic acid are added, and carbon dioxide is bubbled through for 2–3 min. The bubbling is continued, and 25 ml 3 *N* sodium hydroxide is poured slowly in over a period of 5 to 6 min. (Care must be taken not to add the sodium hydroxide too fast.) This reagent is stored under carbon dioxide and must be prepared fresh daily.

g. Standard solutions. Either ethanolamine or OPE can be used for one standard curve, and serine or OPS for the other. Ethanolamine (= 2-aminoethanol) (Eastman) must be redistilled. The fraction at 170–174°C is used after titration with bromothymol blue as indicator. Serine, OPE, or OPS was purchased from California Corporation for Biochemical Research.

II. Preparation of Samples

The phospholipids must be freed of amino acids. This could not be completely accomplished by dialysis or by chromatography on cellulose powder column. The treatment of the phospholipid dissolved in chloroform-methanol (2:1) with a salt solution was found preferable (7). Then 100 and 200 μg in a few μl of chloroform are chromatographed on a paper

disk (20-cm diameter) with chloroform-methanol-water (80:20:0.5). Only those samples should be hydrolyzed which show no trace of amino acids on the point of application after staining with ninhydrin.

III. Hydrolysis

The total amino nitrogen in the amino acid-free sample is analyzed by the method of Lea and Rhodes (8) and the quantity of phospholipid to be hydrolyzed is based on this determination. The sample should contain not more than 8 μmoles of amino nitrogen (generally corresponding to 5–15 mg of naturally occurring phospholipid mixtures). The substance is weighed into a 13 × 100 mm (Wassermann) tube on a micro or semi-micro balance and dissolved in 0.3 ml chloroform; 50 mg barium hydroxide [$Ba(OH)_2 \cdot 8H_2O$], 0.25 ml water, and 0.25 ml ethanol are added. The mixture is heated under reflux (rubber stopper and 50-cm long glass tube) in a 65–70°C water bath for 20 min. The tube is removed and after adding 0.2 ml 6 *N* hydrochloric acid the contents are refluxed for 20 min more. After cooling, the fatty acids are removed by washing and centrifuging twice with 1-ml portions of petroleum ether (b.p. 30–60°). The solvent is removed with a fine pipet and washed with 0.3 ml water, and must give a negative ninhydrin test on paper. The wash water is added to the hydrolyzate.

IV. Column Chromatography

Figure 1 shows the flow of the different solutions through the two small columns and the collection of the four eluents into the graduated cylinder and the volumetric flask. The columns and the simple equipment guarantee a complete separation of the four split products widely different in acidity and adsorption affinity, if the various pH grades and quantities of eluates are strictly controlled as described.

The entire hydrolyzate is quantitatively transferred to the first (4 ml) column, using small portions of 10% acetic acid up to a total of 2 ml. The effluent is allowed to drain to the top of the resin, and 10 ml of pH 5 acetate buffer is added. This 12-ml effluent containing OPS is set aside in a 50-ml graduated cylinder. The elution is continued with 16 ml of pH 5 acetate buffer. This effluent, which contains OPE, is collected in a 50-ml volumetric flask. Another 2 ml of the acetate buffer is added and discarded.

At this point, the second column (pH 5) is placed so that the effluent of the initial column will drain into it. To remove the serine and ethanolamine from the top column, 2.0 ml of 10% sodium acetate solution is added to it, and the corresponding effluent from the lower column is dis-

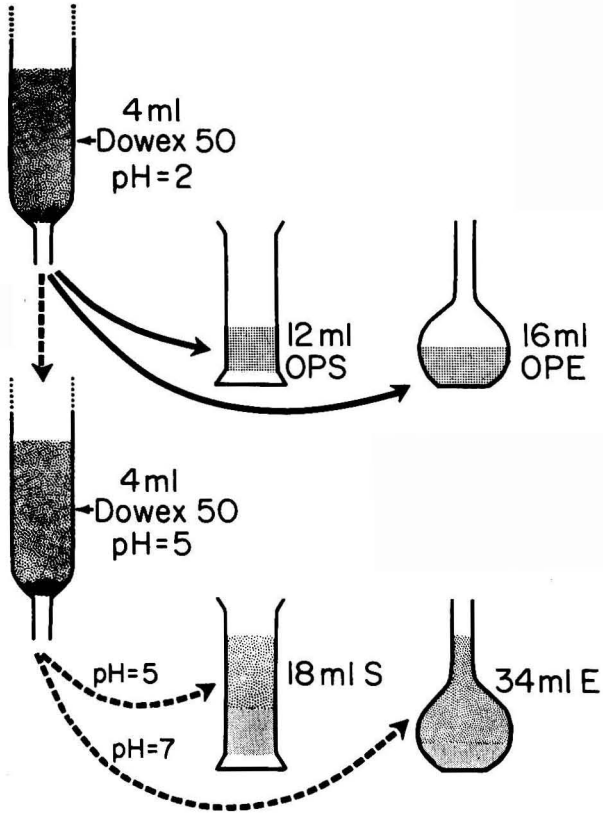

FIG. 1. Flow diagram of the separation procedure.

carded. pH 5 acetate buffer (18 ml) is added to the top column and the effluent from the lower one is collected in the 50-ml graduated cylinder already containing OPS, to bring the volume up to exactly 30 ml.

The 50-ml volumetric flask, already containing OPE, is now used for the collection of ethanolamine, which is eluted by adding 10% sodium acetate to the top column until an exact total volume of 50 ml of OPE and ethanolamine is reached. While both columns are in operation, flow rates should be adjusted with rubber tubes and screw clamps in such a way that the liquid layer on the top of the lower column does not exceed 1 cm.

V. Quantitative Determination

Aliquots (2-ml) of the serine and the ethanolamine fractions as well as 2 ml water for the blank are pipetted into 18 × 150 mm test tubes; 7 ml

of the ninhydrin reagent is added to each tube. This determination should be performed in triplicate. Tubes are placed in a vigorously boiling water bath, stoppered firmly after 1 min, and boiled for 20 min. After removal, they are cooled in an ice bath for 2 min. The OD's are measured at 570 mμ in a Beckman spectrophotometer, the micromoles determined from the standard curve, and the results averaged. A blank experiment using exactly the same quantities of barium hydroxide, ethanol, water, and hydrochloric acid is run on the column, and the same volumes of effluents are collected. With known solutions of serine or OPS and ethanolamine or OPE in μl quantities together with 2 ml of the respective effluents, two standard curves are prepared using water as a blank (Fig. 2).

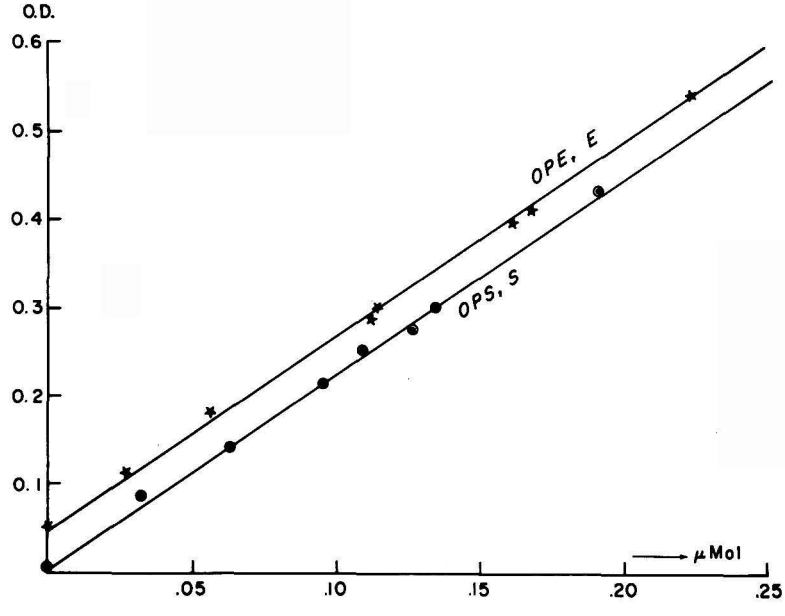

FIG. 2. Standard lines obtained with the four synthetic substances and the two corresponding effluents from the columns.

RESULTS

The analytical procedure outlined above has been applied to three types of material:

1. A mixture of synthetic OPS, OPE, serine, and ethanolamine before and after hydrolysis.

2. A preparation of calf brain phospholipids with approximately equal

amounts of PE and PS, obtained by ether extraction from the acetone powder of calf brain (9).

3. Two preparations of soybean cephalin,[3] both as active as the calf brain preparation in the Thromboplastin Generation Test of Biggs, Douglas, and McFarlane (10) but containing much smaller quantities of PS. The quantitative determination of PS in the soybean phospholipids seems particularly important because Tookey and Balls (2) could not detect any trace of PS in soybean phospholipids, whereas Williams (11) found "varying quantities of PS, PE or both compounds" in all blood clot promoting fractions of soybean cephalin.

In order to determine whether the four ninhydrin positive split products of phospholipids could be completely separated by means of the method described above, standard solutions of 68 μg OPS, 133 μg serine, 78 μg OPE, and 470 μg ethanolamine were used as such and after hydrolysis. The effluents from the column were collected in 1-ml samples, and after reaction with ninhydrin the OD at 570 mμ was measured. As Fig. 3 shows, the quantity of phosphorylated products diminished after hydrolysis but they never completely disappeared. Corresponding experiments with native animal as well as vegetable phospholipids gave similar results. A sharp separation of the four split products occurred in each case, the relative quantities of OPS and S as well as of OPE and E varied according to differences in heating and other conditions.

Table 1 contains the analyses of mixtures of synthetic OPS, serine, OPE, and ethanolamine, simulating the analyses of hydrolyzates of phospholipids. Table 2 summarizes the data obtained from two different analyses of soybean cephalin. Table 3 contains the results of the analyses of three native phospholipids. In all three cases the first analyses give the values for the four split products separately; in the second analyses two and two are combined in the manner recommended. The data show that the results are practically identical and in all cases the sum of the ethanolamine nitrogen and serine nitrogen was equal to the total amino nitrogen previously determined by the method of Lea and Rhodes (8).

DISCUSSION

Hydrolysis

The native phospholipids are generally mixtures of phospholipids with two fatty acid esters and those (= plasmalogens) with one vinyl ether and one fatty ester group. The former can be split very easily by mild alkaline but not by acid, the latter by weak acid but not by alkaline,

[3] We are grateful to Central Soya Co., Chicago, Ill. for the gift of soybean cephalin and to American Lecithin Co., Woodside, N. Y., for a sample of Inosithin.

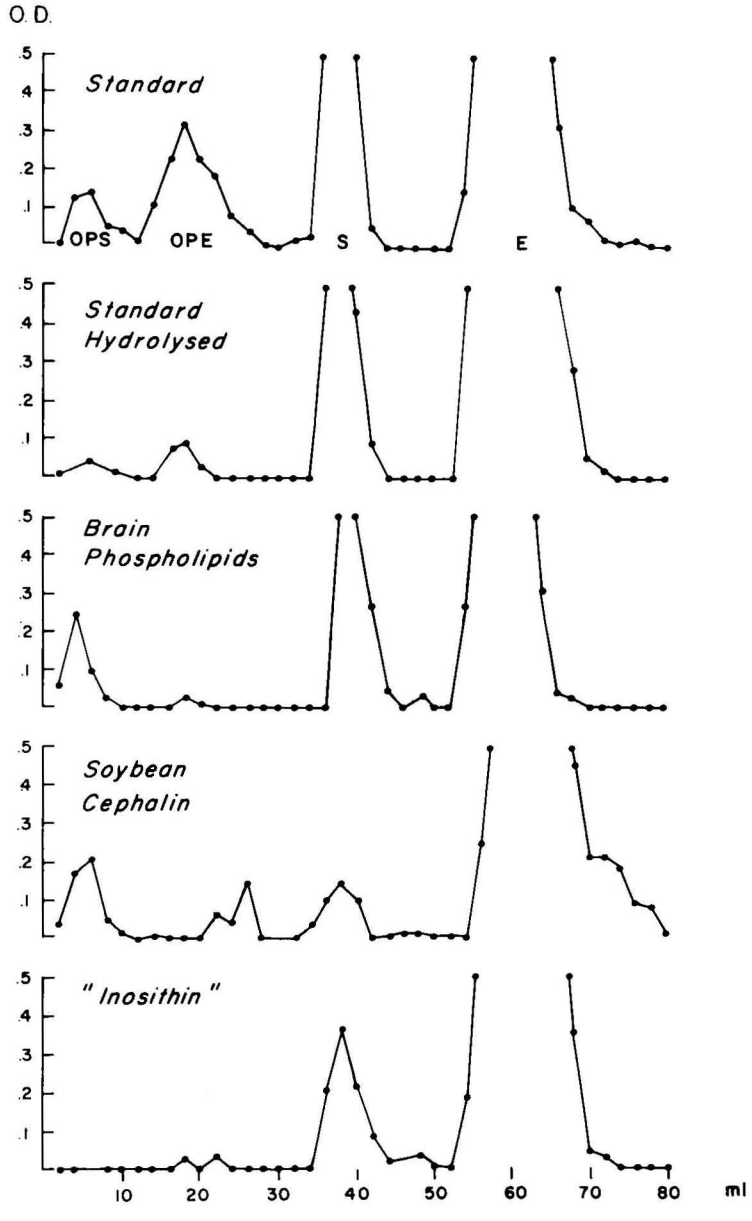

FIG. 3. Separation of the four ninhydrin-positive products from phospholipid hydrolyzates on Dowex columns.

TABLE 1
ANALYSIS OF STANDARD MIXTURES

Sample No.	Compound	μM	Quantity recovered		
			Average OD[a]	μM	Total μM
1	OPS	0.359			
	S[b]	1.646			
	Total	2.005	0.289	0.127	1.905
	OPE	0.567			
	E[b]	2.294			
	Total	2.861	0.300	0.113	2.825
2	OPS	0.367			
	S	1.265			
	Total	1.632	0.234	0.103	1.545
	OPE	0.556			
	E	3.434			
	Total	3.990	0.410	0.162	4.05
3	OPS	0.359			
	S	1.646			
	Total	2.005	0.295	0.130	1.95
	OPE	0.567			
	E	2.294			
	Total	2.861	0.319	0.121	3.025

[a] Color yield from: 2 ml aliquots of OPS + S solution (= 1/15 of total), 2 ml aliquots of OPE + E solution (= 1/25 of total).

[b] Additional abbreviations in the tables are: E = ethanolamine, S = serine.

TABLE 2
ANALYSIS OF 11.7 MG (a) AND 16.35 MG (b) OF SOYBEAN CEPHALIN

Sample	Compound	Average OD[a]	μM	μg	Total μg	%
(a)	S	0.063	0.026	2.73	40.95	0.349
	E	0.226	0.080	4.88	122.0	1.04
(b)	S	0.080	0.034	3.57	53.55	0.328
	E	0.302	0.114	6.95	173.85	1.06

[a] Color yield from: 2 ml aliquots of OPS + S solution (= 1/15 of total), 2 ml aliquots of OPE + E solution (= 1/25 of total).

hydrolysis. Therefore, native phospholipid mixtures must be hydrolyzed with both alkali *and* acid as in Dawson's procedure to obtain completely water-soluble split products (6). Heating of phospholipids in aqueous 2 *N* or 6 *N* sodium hydroxide (12) destroyed much of the serine present in native phospholipids (4). Heating in sealed tube or boiling under reflux

TABLE 3

DETERMINATION OF FOUR SPLIT PRODUCTS INDIVIDUALLY AND IN COMBINATION

Lipid	Amt. hydrolyzed (mg.)	Total μM OPS	Total μM S	μM S + OPS	Corresp. mg S	% S	Total μM OPE	Total μM E	μM E + OPE	Corresp. mg E	% E
Brain phospholipids	9.874	0.138	0.981	1.119	0.117	*1.19*	0.056	3.247	3.3	0.201	*2.04*
	14.25	—	—	1.69	0.177	*1.24*	—	—	4.55	0.277	*1.94*
Soybean cephalin	15.26	0.102	0.342	0.444	0.047	*0.31*	0.072	2.431	2.50	0.153	*1.0*
	16.35	—	—	0.510	0.053	*0.33*	—	—	2.85	0.174	*1.06*
Inosithin	23.7	0.108	0.063	0.171	0.018	*0.076*	0.184	4.32	4.5	0.274	*1.16*
	20.13	—	—	0.135	0.014	*0.070*	—	—	3.92	0.239	*1.19*

with 1 *N* (13), 2 *N* (5), or 6 *N* (4, 14) aqueous hydrochloric or sulfuric acid for 3 to 48 hr causes less destruction but 9–16% of the nitrogen is lost as ammonia. The milder hydrolysis with methanolic hydrochloric acid has the disadvantage that the serine methyl ester formed must then be hydrolyzed with alkali (15). Since the phospholipids are insoluble in water and their split products insoluble in chloroform, alcohol must be present as a "bridge" during the hydrolysis. In chloroform-alcohol-water mixture the hydrolysis with base as well as with acid is completed after 20 min at 65–70°C without loss of nitrogen or formation of dark tar-like substances.

If the hydrolysis is carried out under even milder conditions and at 37°C, glycerol remains esterified with the phosphoric acid portion of the molecule (6). However, in various experiments, apparently under the same conditions, we sometimes found (by paper chromatography) OPE and OPS instead of glycerol phosphoryl ethanolamine and glycerol phosphoryl serine. This is why we prefer to use more concentrated solutions and a higher temperature for the splitting of the phospholipids to serine, ethanolamine, OPS, and OPE.

All our efforts to find hydrolyzing methods which yield only serine and ethanolamine failed. By paper chromatography a small, but for quantitative analysis very significant, amount of serine and ethanolamine was always found in the form of OPS and OPE. These phosphoryl derivatives can be split further only under conditions which partly destroy the desired products. From the hydrolyzate of brain cephalin (2 *N* hydrochloric acid, 20 hr under reflux) Ägren isolated 18% of the total ethanolamine in the form of OPE and 3.8% of the total serine as OPS (16). With our hydrolysis method, the percentage of the phosphoryl derivatives formed is much lower, but not negligible.

Disregard for OPS and OPE in the analysis seems to be the main reason for the high "undetermined" nitrogen in animal tissue lipids (17). The same is true for unexpectedly low serine and ethanolamine values, when the formation of the phosphoryl derivatives in the hydrolysis (5) is ignored.

When we hydrolyzed the phospholipids with sulfuric acid and neutralized the hydrolyzate with barium carbonate according to Magee *et al.* (5) we found appreciable quantities of ninhydrin-positive substances in the barium sulfate precipitate. This proves that a loss of amino nitrogen containing substances cannot be avoided by this technique. No satisfactory results were obtained by hydrolysis with sodium hydroxide and hydrochloric acid, with potassium hydroxide and perchloric acid, or with triethylamine and trichloroacetic acid. In solid barium hydroxide and hydrochloric acid we found the most reliable agents, which under the

related conditions split all PE and PS mostly to ethanolamine and serine; only small amounts of OPS and traces of OPE are by-products.

Separation

The separation of the two main and the two by-products was first tried by chromatography on Whatman No. 1 and Whatman No. 3 paper, based on earlier procedures (5, 14). The results were good with *n*-butanol-acetic acid-water (40:10:15) as solvent, followed by spraying the paper cuttings with 1% potassium hydroxide in methanol and extracting them directly in the tubes with the ninhydrin reagent in a boiling water bath. If ethanolamine or serine was present in extremely small amounts, part of the hydrolyzate was evaporated in high vacuum to dryness, the residue dissolved in a very small volume of water, and an aliquot chromatographed; the concentration of the split product thus obtained became sufficient for determination. The separation of the split product, however, is not very good when the ratio of ethanolamine to serine or serine to ethanolamine is of the order 10:1 because of overloading of the paper. The separation on paper is more difficult and less exact than on columns; however, these experiments indicated that no other amino acids were present in our hydrolyzates. Artom (18) and Spiro and McKibbin (19) used Permutit and Ågren (16) and Dittmer *et al.* (4) Dowex resin column for this purpose. Since the separations with this resin were perfect, no others were tried out.

Determination

We found that the reaction of the four split products, OPE, OPS, serine, and ethanolamine, with ninhydrin in acetate buffer is complete after heating to approximately 100°C for 20 min. All four substances have the same molar extinctions under the described conditions. By plotting the OD of the reaction products of OPS, OPE, serine, and ethanolamine against μmoles of these substances, a straight line results (Fig. 2, lower line).

It was necessary to use a higher concentration of methyl Cellosolve than usual to overcome cloudiness, which otherwise occasionally appeared. We further use stronger buffer solution to assure that the ninhydrin reaction is performed at about pH 5.

Any trace of ammonia in the column or in the reagents appears in the ethanolamine fraction. Therefore, the columns must be washed just before use. Bottles of 6 *N* hydrochloric and glacial acetic acid are stored in a desiccator over acid-soaked silica gel. Since it is not feasible to remove the ammonia even from the best commercially available concentrated hydrochloric acid (usually 3 μg/ml), we recommend checking

the reagents and the procedure by determining the ethanolamine standard line. This line is always slightly higher due to the trace of contaminating ammonia from the acids used (Fig. 2, upper line).

SUMMARY

A mild alkaline and acid hydrolysis is performed to split phosphatidyl ethanolamine and phosphatidyl serine in samples of 5–15 mg of native phospholipid mixtures. The two main split products, ethanolamine and serine, and their two phosphorylated derivatives are separated on two small Dowex 50 columns and the quantities of the amino group containing compounds analyzed by means of modified ninhydrin reaction. With this method the ethanolamine and serine content of samples with 4–8 μmoles phosphatidyl ethanolamine and phosphatidyl serine, respectively, can be determined with an error of about 3–5%.

ACKNOWLEDGMENTS

The skillful technical assistance of Mr. Julian de Velasco and Mr. W. M. McGaw is gratefully acknowledged.

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ANALYTICAL BIOCHEMISTRY 4, 81–83 (1962)

SHORT COMMUNICATION

Metal-Free Tube Cap Assemblies for the Spinco Model L Preparative Ultracentrifuge[1]

In the course of studies on copper proteins in brain and in liver, preparative high-speed centrifugation has sometimes been required (1, 2). It therefore became most desirable to have available a method for use of the Spinco Model L preparative ultracentrifuge, previously requiring metal tube cap assemblies, which would assure the absence of contact of the sample with extraneous metal during centrifugation. The ability to use this apparatus under metal-free conditions should be of advantage not only in studies of other metalloproteins but also in eliminating the possible toxic effects of metals such as copper during the preparation of certain enzymes.

Coating metal cap assemblies with plastic (e.g., polyurethane) has been unsatisfactory in that the plastic may chip and expose the centrifuged material to metal surfaces. Our initial attempts to construct all-plastic tube cap assemblies entirely of nylon or entirely of Lucite were also unsatisfactory. With nylon alone, the sides of the crown piece were sufficiently flexible to allow lateral bending away from the gasket with resultant excessive leakage from the tube during centrifugation. With Lucite alone, the shank of the stem piece was not strong enough to withstand high gravitational forces and cracked away from the nut allowing the stem piece to sink to the bottom of the tube.

These difficulties have been overcome by constructing the stem piece of nylon and the crown piece of Lucite. Modifications in the cap assemblies which have been made to increase strength are shown in Fig. 1. For the nylon stem piece, these changes include use of a solid rather than a hollowed base, a tap in the shank which is blind rather than through and of smaller diameter than that in the metal stem, increase in outer diameter of the shank, and increase in the number of threads per inch on the shank. For use with the smaller tubes (Spinco No. 303404 and No. 303369) the

[1] This work was supported by research grant B-1733(C_3) from the National Institute of Neurological Diseases and Blindness, United States Public Health Service.

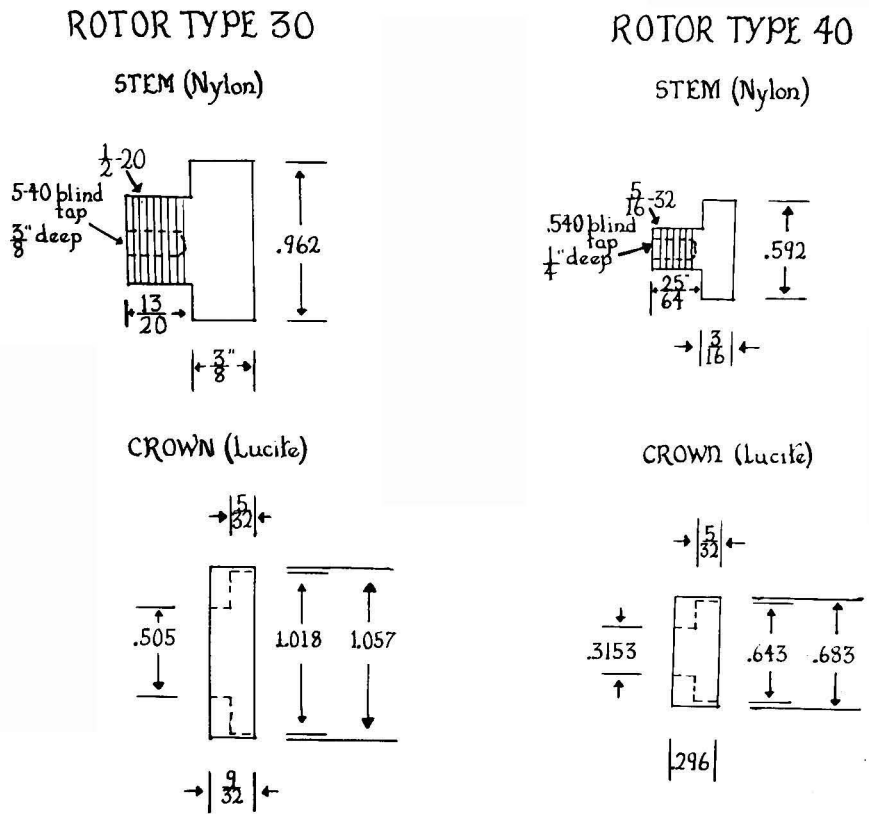

FIG. 1. Dimensions (in inches) of plastic tube cap assemblies for use with the Spinco Model L Type 30 and Type 40 Rotors.

length of the stem piece inside the tube can also be increased to prevent tube collapse when centrifuging small volumes such as 1.0 ml. For the Lucite crown piece changes included increase in the diameter of the opening for the shank of the stem piece and increase in the thickness of the base plate. In the case of cap assemblies for the relatively small tubes used in the Type 40 rotor, changes in dimension were minimal but, for the larger tubes used in the Type 30 rotor, it was necessary to increase the outer diameter of the threaded shank of the stem piece by more than 50% and to use a nut of ¾ in. outer diameter. Use of soft gum gaskets and of stainless steel rather than coated nuts has been found satisfactory. All portions of the cap assemblies (except the steel nut which does not contact the sample) can be rendered metal-free by cleaning in detergent followed by soaking in ¼ *N* HCl overnight and exhaustive rinsing with water redistilled over glass.

The nylon-Lucite tube cap assemblies herein described have been in use in this laboratory for the past six months. They have proved satisfactory with regard to durability in centrifugations at maximum rated rotor speeds providing more than $100{,}000 \times g$ at the bottom of the tube, as well as to absence of excessive tube leakage and to elimination of contamination with extraneous metal during centrifugation.

ACKNOWLEDGMENT

The authors wish to thank Mr. Ralph French of Laboratory Associates, Inc., Belmont, Massachusetts, for assistance in the production of the tube cap assemblies herein described.

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HUNTINGTON PORTER
JANET JOHNSTON

Department of Neurology
New England Center Hospital, and
Department of Medicine (Neurology)
Tufts University School of Medicine
Boston, Massachusetts
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Biochemical Applications of Gas Chromatography

By H. P. BURCHFIELD
Southwest Research Institute, San Antonio, Texas
and ELEANOR E. STORRS
Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York

March 1962, 680 pp., $22.00

This book fulfills a need for a text that supplies both theoretical background and details of the applications of gas chromatography to biochemical problems. Specific procedures are supplied together with enough background information to enable the experimentalist to improvise adaptations without an extensive study of the literature. Although the work is organized along biochemical lines, information is included that will be of value to workers in other fields, such as air pollution and petroleum chemistry.

Gas Chromatography is the technique described most extensively in this book, but it is not the only one. Detailed methods for sample collection are given for each group of compounds, and prefractionation of samples by liquid-solid chromatography and ion exchange techniques is described. Methods are provided for the preparation of stationary liquids, subtraction of unwanted compounds from the gas stream, and synthesis of volatile derivatives of non-volatile compounds.

A special feature is the adoption of a standard form for tabulating conditions for chromatography. Most of the methods were checked for accuracy and updated by the original authors; therefore, many of the methods are more complete than in the original literature. Extensive tables of retention values have been summarized from a variety of sources, and indexes are included to provide easy access to the contents.

This book is the only complete experimental text presently available in the field.

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Fluorometric Determination of Isonicotinic Acid Hydrazide in Serum

MARJORIE T. HEDRICK, JOHN W. RIPPON,[1] LUCILE E. DECKER, AND JOSEPH BERNSOHN

From the Research Service, Veterans Administration Hospital, Hines, Illinois, and Northwestern University Medical School, Chicago, Illinois

Received October 30, 1961

Research into the mode of action of INH[2] and the evaluation of treatment efficacy in patients receiving the drug have been hampered by lack of a simple, sensitive method for its determination. Currently, the procedure in general use for serum is the bioassay method (1). This requires much manipulation and 10 days to complete. Present colorimetric methods include differential solvation (2), and the formation of colored addition products (3, 4) or the cyanogen bromide derivative (5). In one recent method (6) a cation-exchange resin was utilized to separate INH from AINH. INH was hydrolyzed with HCl to form INA, which was then reacted with chloramine T and barbituric acid to form a colored product (7). A microfluorometric procedure (8) has been reported in which INH is hydrolyzed with NaOH and the resultant hydrazine measured. Peters (9) has utilized differential solvent extraction followed by fluorescent measurement of an alkaline cyanide derivative of INH.

Fluorometric procedures in general are more sensitive than colorimetric methods. The fluorometric properties of INA and INH after treatment with NaOH, H_2O_2, and heat have been reported elsewhere (10). In the present study, use was made of the selective adsorption of INH from a protein-free serum filtrate on a cation-exchange resin, followed by elution, and subsequent treatment with H_2O_2 and heat at a neutral pH to form a fluorescent product.

[1] Present address: Department of Biology, Loyola University, Chicago, Illinois

[2] The abbreviations used include: isonicotinic acid hydrazide, INH; isonicotinic acid, INA; acetyl derivative of INH, AINH; isopropyl derivative of INH, IPINH; nicotinic acid, NA; nicotinamide, NAM; diphosphopyridine nucleotide, DPN; triphosphopyridine nucleotide, TPN; ethylenediamine tetraacetate Na_2, EDTA; tris(hydroxymethyl)aminomethane, Tris.

METHOD

Preparation of Serum

Serum from fasting patients was collected at zero time and at 2- and 6-hr intervals following oral administration of INH (4 mg/kg body weight). A protein-free filtrate of serum was prepared by the method of Somogyi, in which 2 ml of serum was added to 2 ml of 10% $ZnSO_4 \cdot 7H_2O$ and 14 ml of water.[3] The mixture was allowed to stand for 5 min; 2 ml of 0.57 *N* NaOH was added, and the extract was centrifuged. After centrifugation 0.1–0.2 ml of a 5% EDTA solution was added to a 15-ml aliquot of the supernatant, and the pH was adjusted to 6.5 ± 0.3 with 1–3 drops of 0.1 *N* NaOH. All pH determinations were made using a pH meter with glass electrodes. The pH-adjusted solution was then placed on a cation-exchange column to adsorb the INH.

Preparation of Columns

The Amberlite XE-64 resin[4] was stirred in water for 20 min, allowed to settle for 3 min, and decanted. This process was repeated 5 times in order to remove most of the finer particles of resin. The water suspension of the remaining resin was added to base-exchange tubes (5–7 mm column diameter, fitted with short lengths of latex tubing and small screw clamps) until a resin bed of 20–30 mm depth was formed. The column was washed with 1 ml of 0.5 *N* HCl (to remove any substances which might cause fluorescence in the resin blank), and then washed with water until chloride free. It was found convenient in this laboratory to complete the HCl treatment after the columns were filled; however, this could be done by batch process if desired.

Adsorption and Elution of INH

The pH-adjusted filtrate-EDTA solution (approximately 15.5 ml) was allowed to flow through the column at a rate of 10–12 drops/min, and the resin then was rinsed with 3 ml of water. The effluent from these steps was discarded.) A 20-ml beaker was placed under the column and the INH eluted by passing 1 ml of 0.75 *N* HCl through the column followed by two 1-ml water washes. The pH of these eluates was adjusted to 7 by adding approximately 0.6 ml of 1 *N* NaOH.

[3] All of the water was prepared by passing distilled water through a mixed ion-exchange resin bed. Avoid polyethylene containers, which may impart fluorescence to reagents.

[4] Amberlite XE-64, Rohm and Haas Co., a pharmaceutical grade of Amberlite IRC-50 (H^+) form, carboxylic cation resin, finely pulverized, 100–325 mesh.

Fluorometric Analysis

To the pH-adjusted eluate, 0.1 ml of 1 *M* Tris·HCl buffer (Sigma 121), pH 8.7, was added and enough water to make a final volume of 5 ml. A 2-ml aliquot of this solution was placed in a Folin-Wu sugar tube, and 0.2 ml of cold 30% H_2O_2 solution was added. The pH of the reaction mixture dropped to approximately 7 after this addition. The tubes were placed in a boiling water bath for 30 min, then removed and allowed to stand in cold water for 15 min; fluorometric readings were taken within 60 min. After oxidation, the pH should be between 6.5 and 7.5 in order to obtain maximum fluorescence. Known amounts of INH were added to serum for recovery studies, and standard solutions of INA[5] and INH[6] served as controls for the various steps of the procedure.

The solutions were read using $\frac{3}{16}$-in. slits in No. 2 and No. 5 positions with activation wavelength set at 320 mμ and fluorescent wave length set at 415 mμ in an Aminco-Bowman spectrophotofluorometer equipped with a Moseley Autograf Recorder. The instrument was standardized daily using 0.02 μg/ml quinine sulfate solution (11), by setting the fluorometric reading at 50% transmission with the meter multiplier at 0.1 using activation and fluorescent wavelengths of 350 and 455 mμ, respectively. All of the fluorescent wavelengths reported in this paper are those obtained experimentally with this instrument and were not further corrected.

Paper Chromatography

In an attempt to identify the fluorescent compound obtained from INA and INH, paper chromatography was utilized to separate the components of the reaction mixture. Various concentrations of INA or INH were heated in 0.02 *M* Tris, pH 8.7, and H_2O_2, cooled, and aliquots spotted on Whatman No. 1 paper. The R_f values of the compounds were determined by descending chromatography in: *n*-butanol: water: glacial acetic acid, 50:10:2 (12); Pollard's solvent (water: concentrated HCl: diethyl ether: methanol, 15:4:50:30) (13); isopropanol: water, 85:15 (14); and pyridine: water, 2:1 (15). All papers were first viewed with a Mineralight ultraviolet lamp wih a maximum wavelength of 2537 A, and spot tests were carried out on some papers as described in the section on results.

Absorption spectra were determined using a Beckman DB spectrophotometer. The fluorescent spots of the oxidized product were cut from

[5] INA was obtained from California Corporation for Biochemical Research.

[6] The authors wish to thank Hoffmann LaRoche, Inc. for donating IPINH (Marsilid Phosphate), INH (Rimifon), and N^1-acetyl-N^2-isonicotinylhydrazine.

papers developed in Pollard's solvent and eluted with hot water. An equivalent amount of paper was cut from the solvent-exposed background area, where no spots appeared, and treated with hot water in the same way. This served as a "blank" against which each eluate was balanced while being scanned. Dilutions were made in either 0.02 *M* Tris or 0.1 *N* NaOH to determine the effect of pH on the absorption maxima.

RESULTS AND DISCUSSION

Fluorometric Studies

Figure 1 shows the typical activation and fluorescent spectra of the oxidized product formed from INA and INH by treatment with H_2O_2 and heat in the presence of 0.02 *M* Tris. The maximum fluorescence occurred at an activation wavelength of 320 mμ and a fluorescent wavelength of 415 mμ. When 0.02 *M* acetate buffer, pH 5.5, was used in place

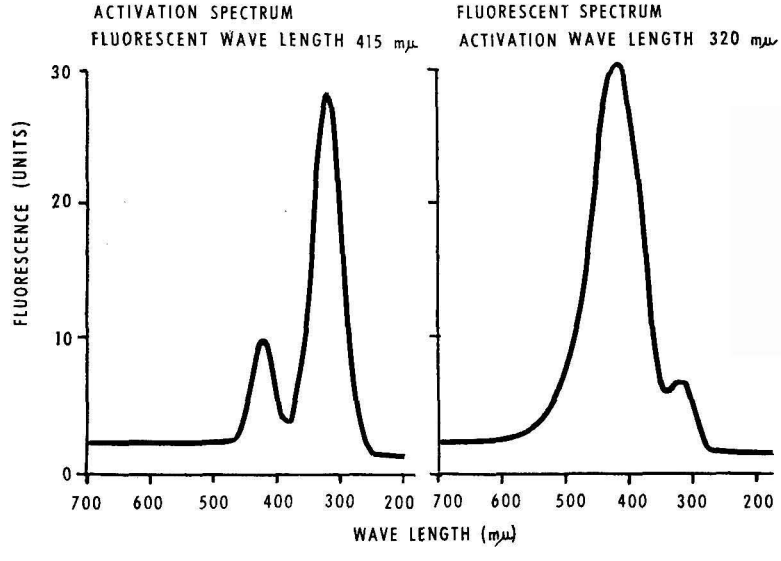

FIG. 1. Activation and fluorescent spectra of compound formed from INA or INH after H_2O_2-heat treatment in presence of 0.02 *M* Tris.

of Tris there was a shift in the activation maximum to 328 mμ, but no change in the fluorescent maximum or in degree of fluorescence. In acetate buffer the optimal pH for maximum fluorescence was between 5.5 and 6.5, while in Tris the pH optimum (after the addition of H_2O_2) was between 6.5 and 7.5. Figure 2 shows the effect of pH on the fluorescence

obtained from INH by heating with H_2O_2 and 0.02 *M* Tris. The pH of the Tris solutions fell considerably upon the addition of H_2O_2; thus it was necessary to use pH 8.7 buffer in order to maintain a pH of 6.5–7.5 after H_2O_2 was added. Tris was chosen in preference to acetate because the fluorescence of the samples could be read 15 min after heating, while, with acetate, longer standing was required for maximum

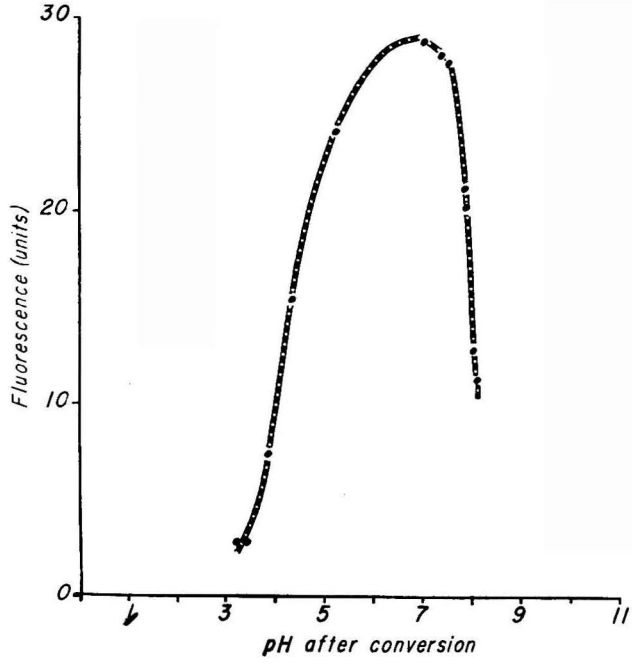

FIG. 2. Effect of final pH on fluorescence after treatment of INH with H_2O_2-heat (2 μg/ml).

readings (at least 45 min). When 0.02 *M* phosphate buffer, pH 7–8, was used with H_2O_2 and heat for INH there was little if any fluorescence.

In all cases equivalent molarities of INA and INH gave the same fluorescent readings and activation and fluorescent maxima under the conditions of this reaction. Table 1 shows the relative intensity of the fluorescence of the oxidized product formed from various concentrations of INA compared with the molar equivalent of INH after treatment with H_2O_2 and heat at pH 7 in 0.02 *M* Tris. The proportionality of fluorometric readings with INH concentration is shown in Fig. 3. When INH was added to serum, recoveries of 76–106% were obtained, based on the readings obtained from the same amount of INH adsorbed and eluted

TABLE 1
FLUORESCENCE OF COMPOUND FORMED FROM INA OR INH BY OXIDATION WITH H_2O_2 AND HEAT IN 0.02 *M* TRIS
(final pH 7.0)

INA or INH[a] (μg/ml)	Fluorescence (units)	
	INA	INH
0.05	0.64	0.64
0.10	1.21	1.42
0.20	2.07	2.04
0.40	4.92	5.16
1.00	10.94	10.82
1.60	18.12	19.16
2.00	22.54	22.20

[a] Molar equivalent of INA.

from the resin in a solution without serum but with the addition of comparable amounts of $ZnSO_4$, NaOH, and EDTA (Table 2). Of the many protein precipitation methods tried it was found that the Somogyi $Zn(OH)_2$ procedure gave the best results. However, some zinc was eluted from the column which caused a precipitate in the final heating process.

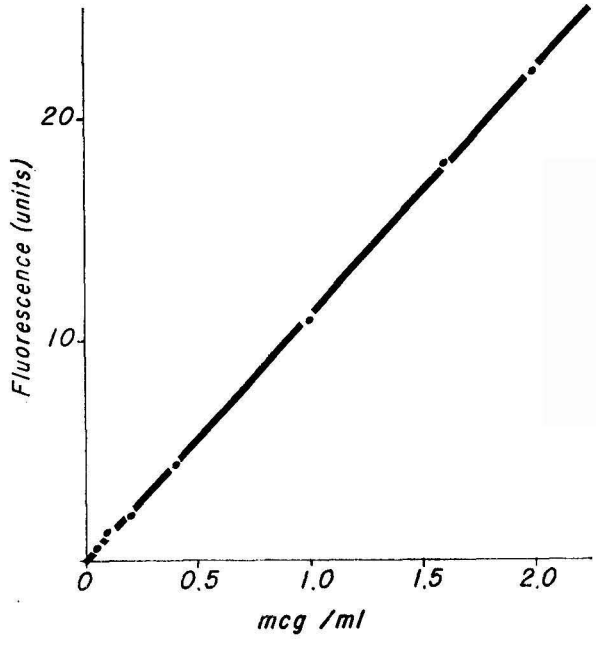

FIG. 3. Standard curve for INH after H_2O_2-heat in 0.02 *M* Tris (final pH 7).

TABLE 2
RECOVERY OF INH FROM SERUM

INH[a] added to serum (μg/ml)	Fluorescence (units)	Recovery (%)
0.5	3.07	76
1.0	6.96	101
1.5	11.52	95
2.0	16.36	106
2.5	19.36	104

[a] Molar equivalent of INA.

This was eliminated by adding EDTA to the protein-free filtrate prior to placing the filtrate on the column. The effect of varying concentrations of EDTA on final fluorescence of the oxidized product is shown in Table 3.

TABLE 3
EFFECT OF EDTA CONCENTRATION ON FLUORESCENCE OF INH ADDED TO SERUM

Amount of EDTA added/ 15 ml filtrate (mg)	Fluorescence (units)
200	6.39
100	8.28
70	6.70
40	7.45
10	13.23
5	15.11
2	6.80

Table 4 shows the effect of H_2O_2 and heat on the fluorescent properties of INA, INH, and some other pyridine derivatives. It should be noted that, although all of the compounds exhibited at least a small amount of fluorescence (either native to the compound or due to other organic contaminants in the solid), the fluorescence was enhanced many-fold by the H_2O_2 and heat treatment (80 times for INA, and even more for the other compounds). The products formed from NA, NAM, DPN, and TPN after heating with H_2O_2 and 0.1 *M* acetate buffer, showed maximum fluorescence at a pH of 4.0, 4.3, 3.7, and 4.0, respectively. These fluorescent compounds were characterized by an activation peak at 330 mμ and a fluorescent peak at 390 mμ.

The oxidized product formed from NAM was the only one of the four compounds described above which showed appreciable fluorescence under the conditions described for the determination of INH. Since NAM was

adsorbed and eluted from XE-64 (Table 5), it might interfere with the determination of INH in serum.

Correction was made for any possible interfering fluorescence from NAM or other substances by treating the zero-time readings as "serum blanks" and subtracting them from the 2- and 6-hr readings. It should be noted that the patient must not ingest any food or vitamins until all of the blood samples have been withdrawn if the "serum blank" is to be accurate. If the patient is receiving IPINH therapy this drug will cause a high "serum blank."

TABLE 4

FLUORESCENT PROPERTIES OF PYRIDINE DERIVATIVES BEFORE AND AFTER OXIDATION WITH H_2O_2 AND HEAT[a]

		Before oxidation			After oxidation			
Compound[b]	pH	Activ. (mμ)	Fluor.[c] (mμ)	Fluor. (units)	Buffer[d]	Activ. (mμ)	Fluor. (mμ)	Fluor. (units)
INA	5	330	405	14.48	0.02 *M* Tris·HCl	320	415	22.54
INH	7	330	405	1.37	0.02 *M* Tris·HCl	320	415	22.20
AINH	6	290	415	2.32	0.02 *M* Tris·HCl	320	430	16.98
IPINH	5	330	415	1.00	0.02 *M* Tris·HCl	320	415	22.31
NA	4	330	390	10.88	0.1 *M* acetate	330	390	23.05
NAM	7	330	390	3.14	0.1 *M* acetate	330	390	31.22
DPN	4	303	390	6.05	0.1 *M* acetate	330	390	29.58
TPN	4	300	390	5.02	0.1 *M* acetate	330	390	30.10

[a] All compounds were heated for 30 min except DPN and TPN, which required 70 min.

[b] Measurements were made on 0.8 μM/ml before oxidation and 0.016 μM/ml after oxidation.

[c] Instrument standardized with quinine sulfate (0.02 meq/ml in 0.1 *N* H_2SO_4). Activation = 350 mμ, fluorescence = 455 mμ.

[d] Tris buffer (final pH 7.0), acetate buffer (final pH 4.0) used.

The high selectivity of the XE-64 resin as prepared and used for INH is demonstrated in Table 5. When INA was placed on a column of this resin it was not adsorbed, but quantitatively recovered from the first effluent and effluent wash (which was discarded when determining INH). AINH, although adsorbed on the resin, was not eluted with the small amount of HCl used for elution of INH. The present study was not extended to include the quantitative measurement of AINH beyond establishing the optimal pH for the H_2O_2 heat treatment (Table 4). It is of importance to note that the proposed method does not measure this derivative of INH which interferes in some of the techniques developed in the past. Another advantage of this method is that no prior purification of solvents or chemicals is required, other than washing the resin.

The levels of INH found in the serum of patients were 0.8–3.9 μg/ml,

TABLE 5
COMPARATIVE FLUORESCENCE OF VARIOUS COMPOUNDS[a]

Compound tested[b]	Fluorescence (units)
Isonicotinic acid hydrazide	15.44
Isonicotinic acid	0.90
N^1-Acetyl-N^2-isonicotinylhydrazine	1.12
Isopropylisonicotinylhydrazine	21.20
Nicotinic acid	1.04
Nicotinamide	11.81
Riboflavin	0.82
Pyridoxine·HCl	0.00
Thiamine·HCl	0.14
p-Aminobenzoic acid	0.00
Benzoic acid	0.00
Phenylalanine	0.00
Tryptophan	0.12
Tyrosine	0.14
Phenol	0.00
Pyridine	0.13
p-Aminosalicylic acid	0.32

[a] Compounds were oxidized and adsorbed and eluted from XE-64 resin as described in text.

[b] Ten micrograms of compound placed on column; 5 ml eluate volume.

2 hr following drug administration; and 0–1.3 μg/ml, 6 hr following the drug. This is within the range of values reported where the bioassay technique was employed (1).

Paper Chromatography

The R_f values of some pyridine compounds after paper chromatography are shown in Table 6 and Fig. 4. The unoxidized INA, INH, NA, NAM, and DPN showed up as dark spots under the ultraviolet light, while the oxidized products showed a bluish-white fluorescence. It was thought that the oxidized product formed from INA might be the pyridine *N*-oxide of INA, since this compound is known to be formed by H_2O_2 oxidation of INA (16). However, comparison of the R_f values and fluorescent spectra of synthetic pyridine *N*-oxide of INA with the oxidized product showed that the two compounds were not the same. It seems possible that the pyridine *N*-oxide of INA is an intermediate in the reaction, which then undergoes rearrangement or further oxidation to the unidentified oxidized product (17).

Spot tests were carried out on the chromatograms after viewing under ultraviolet light. The spot from the reaction mixture at the R_f corresponding to known INA gave a light blue color when treated with cyano-

TABLE 6

R_f VALUES FOR SOME PYRIDINE COMPOUNDS BEFORE AND AFTER OXIDATION

Compound	Pollard's solvent	Isopropanol: water	*n*-Butanol: water: acetic acid	Pyridine: water
		Before Oxidation		
INA (1)[a]	0.64	0.39	0.59	0.71
INH (2)	0.34	0.60	0.60–0.72	0.79
AINH (3)	—	—	—	0.86
IPINH (4)	—	—	—	0.87
NA (5)	0.72	0.33–0.47	0.69	0.85
NAM (6)	0.61	0.68	0.61	0.63
DPN (7)	0.32	—	—	0.76
2,6-Dihydroxy-INA	0.70	0.69	0.51	—
6-Hydroxy-NA	—	0.18–0.53	0.56	—
INA *N*-oxide	0.78	0.12–0.40	0.29–0.44	—
INH *N*-oxide	0.79	0.12–0.46	0.27–0.43	—
NA *N*-Oxide	0.79	—	0.20–0.38	—
NAM *N*-oxide	0.63	0.35	0.28	—
		Oxidation Product		
INA (8)[b]	0.80	0.44–0.50	0.35	0.80
INH (9)[b]	0.81	0.45–0.52	0.34	0.80
AINH (10)[b]	—	—	—	0.81
IPINH (11)[b]	—	—	—	0.81
INH[c]	0.81	0.43	0.31	—
NA (12)[c]	0.83	0.17	0.09	0.75
NAM (13)[c]	0.75	0.51	0.51	0.86
DPN (14)[c]	0.74	—	—	0.85

[a] Numbers in parentheses refers to code in Fig. 4.

[b] Tris buffer (final pH 7) used.

[c] Acetate buffer (pH 4.0–5.5) used.

gen bromide vapor followed by benzidine, a bright yellow color with picryl chloride (18), and red-blue with Nielsch's chloramine T reagent (7), indicating that when large initial quantities of INA (250 μg/ml) were present in the reaction some of the INA remained unreacted. The fluorescent spot of the oxidized product gave no color with diazotized sulfanilic acid (19) or $FeCl_3$ in ethanol. Several hydroxy-substituted pyridine compounds yield a chromogenic product with $FeCl_3$ (20).

Ultraviolet Spectrophotometry

Figure 5 shows the absorption spectra of the aqueous eluate of the fluorescent spots which appeared at an R_f value of 0.8 in Pollard's solvent. The concentration of the undiluted eluate was estimated to be

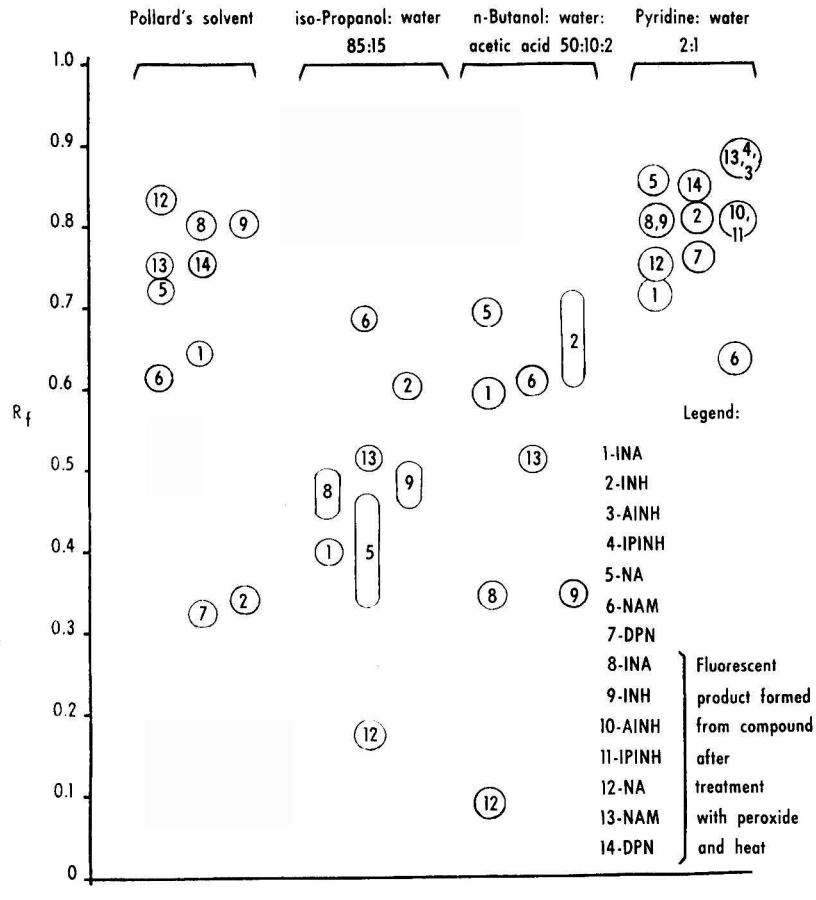

FIG. 4. R_f values of some pyridine compounds before and after H_2O_2-heat using descending chromatography in four different solvents on Whatman No. 1 paper.

70 μg/ml (INA equivalent), based on the measurement of fluorescence. The ultraviolet absorption maxima were similar at a constant pH whether the fluorescent compound had been formed from INA or INH. Maximum absorption occurred at 220 mμ with a secondary peak at 264 mμ at pH 2.7, 209 and 265 mμ at pH 6.5, and 218 and 269 mμ at pH 13.0 for the oxidized product formed from INA; and for the product of INH, 223 and 264 mμ at pH 2.4, 208 and 267 mμ at pH 6.5, and 218 and 269 mμ at pH 13.0. A slight maximum was observed between 305 and 340 mμ in each case. There are absorption maxima present in the oxidized product which do not correspond to the spectra of either unreacted INA or INH (Fig. 6) or to the pyridine *N*-oxide of INA, or citrazinic acid (2,6-dihydroxyisonicotinic acid).

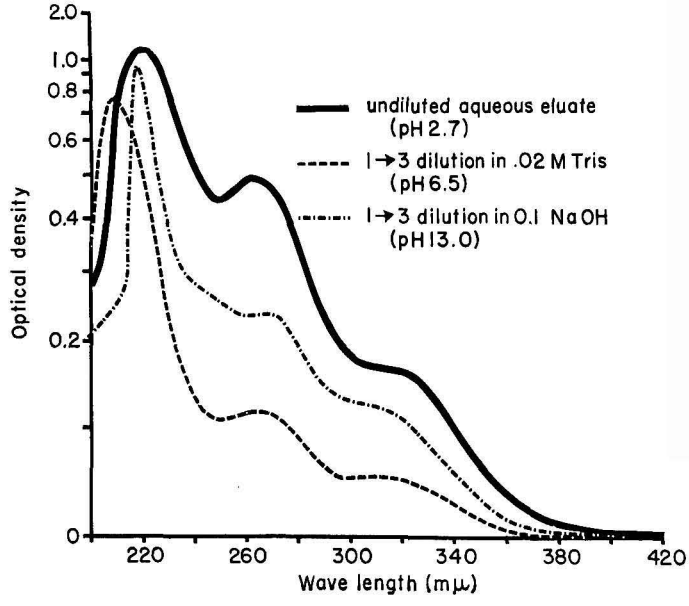

FIG. 5. Absorption spectra of fluorescent compound formed from INH by H_2O_2-heat in 0.02 *M* Tris. The compound was eluted after chromatography in Pollard's solvent. The same spectra was obtained from INA under similar conditions.

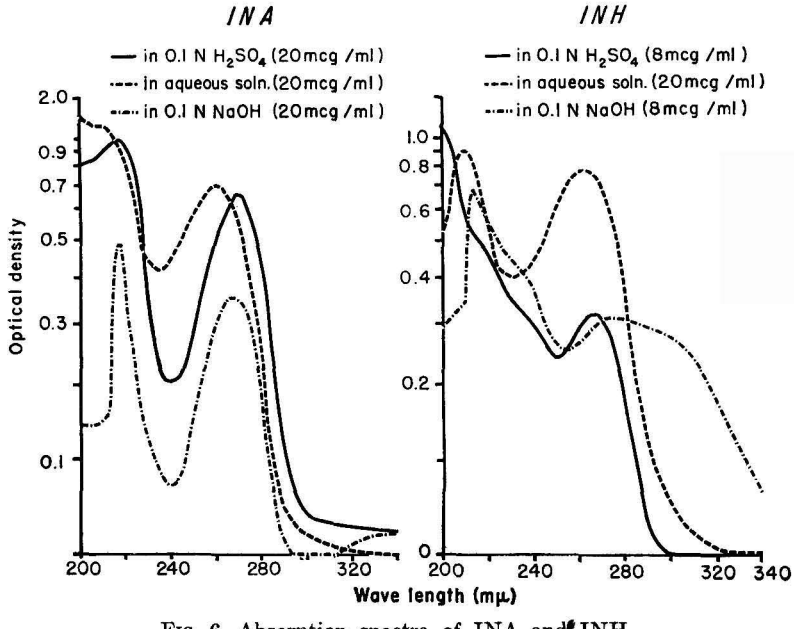

FIG. 6. Absorption spectra of INA and INH.

Other Pyridine Analogs

Citrazinic acid prepared by fusion of urea with citric acid (21) did not have the same R_f value (Table 6) nor the same activation or fluorescent maxima (350 and 440 mμ, respectively, after neutralization to pH 6.5 with NaOH) as the oxidized product prepared from INA. When 6-hydroxynicotinic acid was chromatographed it had a different R_f value than that of the oxidized product prepared from NA. However, 6-hydroxynicotinic acid did have activation and fluorescent maxima (330 and 390 mμ, respectively) similar to the oxidized product of NA.

The fluorescent product formed from NA had a different R_f value than that of the reacted NAM, and only 75% as much fluorescence (Table 4). Paper chromatography of heated H_2O_2 reaction mixtures of NA and NAM showed that the fluorescent product formed did not appear at the same R_f as the pyridine N-oxide synthesized (16, 17) from NA and NAM, respectively.

The data from paper chromatography would seem to indicate that the same fluorescent compound was formed from DPN as from NAM after treatment with H_2O_2 and heat in the presence of 0.1 M acetate buffer, pH 4 (Table 6). This fluorescent product did not appear to be the same compound as that obtained by Lowry and colleagues (22) after treating pyridine nucleotides with H_2O_2 and NaOH, since the latter compound had an activation maximum at approximately 365 mμ and a fluorescent maximum at 460 mμ. Their compound was reported to have zero fluorescence at pH 6, while the oxidized product formed in this laboratory from DPN or NAM by H_2O_2-heat treatment in 0.1 M acetate buffer at pH 6 exhibited 40% of the fluorescence of the product formed at the optimal pH of 4. Furthermore, the fluorescence dropped to 17% of the optimal at pH 7, for the oxidized product formed from NAM, DPN, and TPN.

SUMMARY

The method herein described for separation of INH by means of an ion-exchange column and subsequent measurement of the fluorescent compound formed by H_2O_2 and heat is quantitative for the measurement of INH in serum in concentrations of 0.05 to 10 μg INH/ml of serum. Neither AINH nor INA interfere and any possible interference of NAM is eliminated by means of a serum blank. IPINH may also be determined by this method; thus, the presence of IPINH in serum or tissues will interfere with INH determination. A distinct advantage of the method is that no special purification of chemicals or solvents is needed, and that it involves no losses in solvent extraction procedures.

Several other pyridine derivatives yield highly fluorescent products when treated with H_2O_2 and heat. The conditions necessary for their reaction and the properties of the fluorescent compounds formed are described.

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A Method for Liquid Scintillation Counting Utilizing Ultrasonic Extraction

JANOS SCHULZE AND F. A. LONG

From the Department of Chemistry, Cornell University, Ithaca, New York

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INTRODUCTION

Liquid scintillation counting, although generally very attractive (1), offers some difficulties when one wishes to make quantitative studies on labeled organic compounds in aqueous solutions. The difficulty is that the counting medium for best results must normally be a nonaqueous phase and the usual extraction procedures are distinctly time consuming. As a typical example, in a kinetic study of acid-catalyzed aromatic tritium exchange (2), a six-step procedure was followed: (*1*) taking an aliquot of the aqueous solution, (*2*) quenching and extracting in a separatory funnel, (*3*) separating the aqueous layer from the organic solvent, (*4*) drying, (*5*) transferring a measured portion of the dried organic solution of the labeled compound to a vial which contains the scintillation solution, and (*6*) counting.

We wish to report on a new and simpler method based on the application of a novel extraction procedure. By the use of ultrasonics the extraction of the organic compound can be accomplished in the counting vial itself with the scintillation solution already present and the resulting two-phase mixture can be counted directly. Thus the only necessary steps are: (*1*) taking an aliquot, (*2*) ultrasonic extraction, and (*3*) counting. For quantitative work with this method it is desirable that there be a partition ratio of five or larger for partition of the substance of interest from the aqueous phase to the organic scintillation medium (usually a toluene or xylene solution). In practice this is not a very restrictive condition.[1]

METHOD

Equipment and Material Used

A Disontegrator ultrasonic cleaner (Ultrasonic Industries, Inc., Albertson, L. I., N. Y., Generator Model No. G-40C1) and a Tri Carb Liquid

[1] For certain types of complex systems, difficulties of coextraction of other radioactive materials can arise. As usual, these must be assessed and corrected for on a more or less individual basis.

Scintillation Counter (Packard Instrument Company, La Grange, Illinois) equipped with an automatic sample changer were used for the experiments. The scintillation solution was prepared by dissolving 4.0 gm PPO (2,5-diphenyloxazole) and 0.1 gm POPOP (*p*-bis[2-(5-phenyloxazolyl)]-benzene) in 1000 ml xylene.

To prepare azulene-H^3(1), 100 mg azulene was added to 15 ml ether and a mixture of 1 ml tritiated water (25 mc/gm) with 1 ml 70% $HClO_4$ to give a homogeneous solution. After 5 min this solution was diluted to 250 ml. Extraction, chromatographic purification on alumina, and sublimation gave tritiated azulene with a specific activity of 3.3×10^6 disintegrations per minute per milligram. Tritiated water was supplied by Volk Radiochemical Company, Chicago 40, Illinois.

Description of the Procedure

A sample of 1 to 3 ml of aqueous solution of the labeled organic compound is placed in a counting vial which already contains 5 ml of scintillation solution and, in the case of kinetic experiments, the quenching solution.

The vial is partially immersed for 30 sec in the water tank of an ultrasonic generator. The vial should not be held by hand but should be raised and lowered repeatedly to help emulsification. It is advisable to keep it at a 45° angle in order to avoid losses due to scattering of droplets on the cap of the vial.

If the vial is subjected to counting immediately after the emulsification process the count is frequently somewhat higher than the final figure, presumably because of interference from the finely dispersed water phase. This effect diminishes as the emulsion breaks up and has typically vanished in two to three hours. If necessary, this separation time can be further reduced by a short centrifugation of the emulsified samples.

DISCUSSION

Comparison with the Conventional Procedure

Separate comparisons of the reproducibility of the "separatory funnel" and "ultrasonic" methods shows them both to give results with a similar standard deviation of about 1%. (*a*) Ten identical tritium samples (2 ml aqueous solution of azulene-H^3(1) and 5 ml scintillation solution) with a radioactivity of about 110,000 dpm were counted by the new procedure. Counting for 5 min gave 88,226 $\pm$ 1171 counts. The standard deviation of the measurement was 1.3%. (*b*) A group of three tritium samples of the same radioactive strength as in the previous experiment was counted with the "funnel" method. Ten ml of the aqueous azulene-H^3(1) solution was

extracted with 10 ml xylene. Two ml of the dried xylene solution, with 5 ml of the scintillation solution added, was counted, resulting in 80,906 $\pm$781 counts/5 min. The standard deviation was 0.9%. The volumes employed for these two comparisons were such that, assuming equal counting efficiency, the recorded counts per minute should be the same. It is of interest that this is very nearly true.

A different sort of comparison concerns the application of the methods to kinetic measurements. As an example, the acid-catalyzed tritium loss of azulene-H^3(1) in 0.005 *M* HCl and at an ionic strength of 0.1 was measured at 25°C. The results obtained by both methods agreed very closely. (*a*) Five ml HCl/KCl solution and 45 ml aqueous azulene-H^3(1) solution were placed in a 50-ml volumetric flask. Three-ml aliquots (nine in all) were taken and submitted to the six-step procedure of the "funnel" method. (*b*) One ml HCl/KCl solution and 9 ml aqueous azulene-H^3(1) were placed in a 10 ml volumetric flask. A similar set of 1-ml aliquots was taken and was extracted and counted with the "ultrasonic" method. In both cases log $(c - c_\infty)$ was plotted against time, where c stands for number of counts per minute. The resulting first-order rate constants for the detritiation were 9.45×10^{-4} sec^{-1} and 9.25×10^{-4} sec^{-1}, respectively.

Influence of Radioactivity in the Water Layer

If the sampling operation leads to a water layer with a high concentration of radioactivity, a significant contribution to the total count can arise and must be corrected for. A particular example is the production of the species HTO in a detritiation experiment of the sort discussed earlier. Some idea of the possible magnitude of this effect for the case of tritium is illustrated by the data from the following simple experiment. Two ml of tritiated water, with a radioactivity equivalent to 48,000 counts/min at standard counting efficiency, was added to 5 ml of a xylene scintillation solution and emulsified by the ultrasonic method. Counting gave an average of 1710 counts/min, compared to a normal background of 20. The implication is that, due to this HTO contribution, the "infinity time" extraction for a detritiation kinetic experiment which had started with a count per minute of about 50,000 could lead to a final count which was 3.5% of the initial count rather than to the proper value of zero. Needless to say contributions of this sort can be determined *a priori* and corrected for but they will need consideration for each sort of experiment.

SUMMARY

Details are given on a new, rapid method for the extraction and counting of aqueous solutions of isotopically labeled organic molecules. Appli-

cation of the method to kinetic measurements, such as acid-catalyzed detritiation, is illustrated and discussed.

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Use of Ion-Exchange Resins to Classify Plant Nitrogenous Compounds in Beet Molasses

J. B. STARK

From the Western Regional Research Laboratory,[1] *Albany, California*

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Although numerous ion-exchange methods are available for fractionating specific classes of nitrogenous compounds, such as amino acids, into individual members (1) little has been published on ion-exchange separation of mixtures of nitrogenous compounds into related groups. This paper describes ion-exchange procedures that can be used to separate nitrogenous compounds in plant extracts into several classes, thus simplifying their identification, determination, and evaluation. The method separates the following groups: highly basic compounds; neutral amino acids; dibasic amino acids; basic purines, pyrimidines, and nucleosides; acidic purines, pyrimidines, and nucleosides; acidic compounds; and very weakly acidic or neutral compounds. The method described here is an expansion of one presented earlier (2), and some aspects of it have been given in patents (3). The method can be used simultaneously for the separations described and for the determination of average equivalent weight and total weight of acids (4).

METHOD

Use Dowex 50-X8,[2,3] 20–50 mesh, in the hydrogen form as the cation-exchange resin. Regenerate the resin in 5-liter lots in a column, using 6 liters of 3 *N* hydrochloric acid per liter of resin, wash the resin with distilled water until the effluent is chloride free, air-dry it, and store until needed.

Use Dowex 1-X8,[3] 20–50 mesh, as the anion-exchange resin. Regenerate the resin in 5-liter lots in a column, using 10 liters of 2 *N* sodium hydroxide per liter of resin, wash with distilled water until the effluent

[1] A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

[2] Reference to a company or product name does not imply approval or recommendation of the product by the Department of Agriculture to the exclusion of others that may be suitable.

[3] Dow Chemical Company, Midland, Michigan.

is nearly colorless to phenolphthalein, air-dry, and store until needed. If the anion resin is to be used in an acid carbonate form, prepare it just prior to use by passing carbon dioxide into a stirred water slurry of the hydroxide form of the resin until the supernatant becomes slightly acid. Drop pieces of solid carbon dioxide into the resin slurry to hasten the conversion of the resin to the acid carbonate form.

Use four ion-exchange columns for the fractionation. Each column consists of a glass tube 60 cm long and 4 cm in diameter stoppered at one end with a one-hole rubber stopper. A glass outlet tube, flush with the small end of the stopper extends two inches beyond the large end of the stopper and is connected to rubber tubing. Maintain a flow rate of approximately 16 bed volumes per hour by use of a screw clamp attached to the rubber tubing. A circle of fine-mesh nylon bolting cloth held in by the stopper can be used to cover the outlet tube and prevent loss of resin.

Fill each column with 400 ml of wet resin. Wash the resin with a liter of water immediately prior to use to remove any decomposition products formed on standing. When the acid carbonate form of the resin is used, wash with saturated aqueous carbon dioxide.

Pass the load (A, Fig. 1),[4] 50 gm of molasses diluted to 200 ml with distilled water, through the column of cation exchanger, D-50 in the hydrogen form; discard the first 100 ml of effluent—this portion does not contain any substances present in the load. The breakthrough of the load can be detected by passing the effluent through a small cell equipped with pH electrodes. When the pH of the effluent drops 0.3 from its initial value, start collecting. After loading, wash the column with distilled water (a). The level of the wash water should be about 1 cm above the level of the resin. Collect the first 2 liters of effluent (B) in a 2-liter graduated cylinder. At this time the pH of the effluent should be 3.1 or slightly greater. If it is less, continue washing. Determine the total volume of the effluent. Save a 100-ml aliquot for nitrogen, solids, and other analyses.

Pass the remainder of the cation-exchange resin effluent (B) through a column of Dowex-1, hydroxide form. After loading the column wash with 2 liters of water (b), concentrate the effluent (C) containing neutral compounds to less than 1 liter and then make up to volume in a liter volumetric flask for analysis. Elute the column with saturated aqueous carbon dioxide (c). Sugars retained on the column will be eluted in the first fractions and will be followed by pyrimidines and similar compounds (D). Absorbance of UV light (260 mμ) will verify the presence of pyrimidines and a drop in absorbance will indicate completion of

[4] Upper and lower case letters refer to effluents and eluants in Fig. 1.

elution. Total purines, pyrimidines, and nucleosides may be estimated from a spectrophotometric curve for each fraction, or the individual compounds may be determined after further separation by paper chromatography (5). The more acidic compounds can be eluted with ammonium carbonate, sodium carbonate, sulfuric acid, or a number of other eluants (d). The choice will depend on subsequent treatment of the effluent (E).

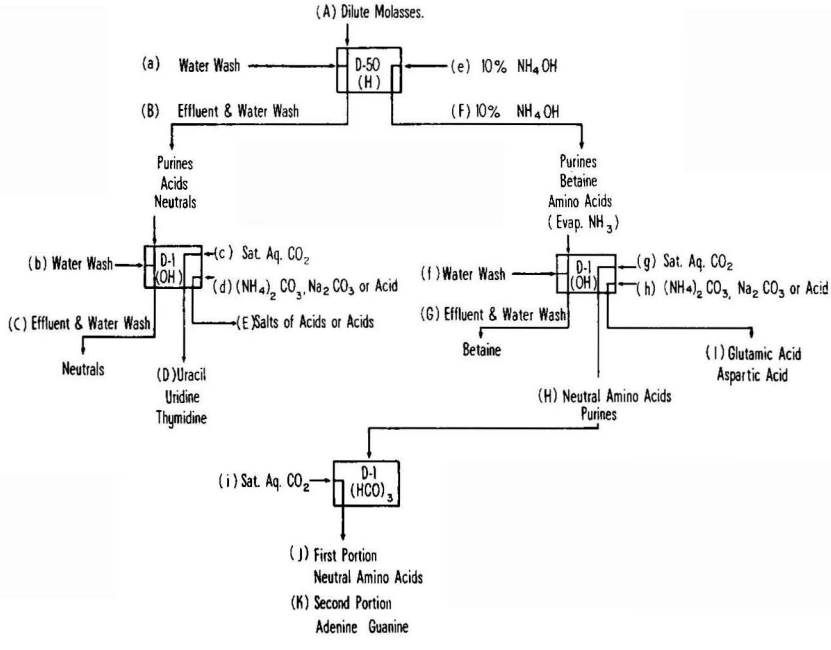

FIG. 1. Separation of nitrogenous compounds into classes.

After loading (A) and washing (a) the cation resin (D-50), elute with 3 liters of 10% ammonium hydroxide (e) made by diluting 600 ml of 28% ammonia with water to 3 liters. Concentrate the eluate (F) until there is no odor of ammonia and then dilute to exactly 2 liters. Take a 100-ml sample for nitrogen, solids, and other analyses. If removal of ammonia present as salts is desired, dilute the sample with 2 or 3 volumes of water and 5 ml of 1 *N* sodium hydroxide, and concentrate slowly for 3 or 4 hr. If the pH is below 7 after this treatment, add more sodium hydroxide and repeat the process. Make the ammonia-free eluate up to the original 100 ml. If ash or solids determinations are desired, use an untreated portion of the sample.

Concentrate the remaining 1900 ml of eluate (F) to approximately 250 ml and load on a column of D-1, hydroxide form. Wash the column

with 2–3 liters of water (f). Any ammonia still present in the load (F) will be found in the wash and effluent (G) and will be evaporated during the concentration of the effluent. Elute the D-1 resin column with 3 or more 1-liter portions of saturated aqueous carbon dioxide (g) prepared by bubbling carbon dioxide gas through cool distilled water until the pH is 4.0–4.1. Amino acids will appear in the eluate (H) when its pH drops below 7. Purines and similar compounds will appear as the elution continues. Total purines can be estimated or the individual compounds determined as described previously for the estimation and determination of pyrimidines (5). It should be pointed out that purines, pyrimidines, or nucleosides may be present in fractions D or H depending on their reactivity toward the ion-exchange resins (5).

After removal of the betaine and neutral amino acid fractions, elute the anion resin with 3 liters of 1 *N* sodium carbonate (h). The eluate contains the more acidic nitrogen compounds such as glutamic acid (I). Ammonium carbonate or acids may be selected as eluants for these compounds if it is desirable.

Concentrate the fractions of (H), containing both neutral amino acids and purines to 200–300 ml, load on a column of D-1 resin (acid carbonate form), and follow directly with the eluant, saturated aqueous carbon dioxide (i). The amino acids (J) are eluted very quickly, with the exception of the aromatic amino acids, which lag slightly behind. Purines (K) are released by further elution. A better fractionation can be obtained by using a finer mesh resin in this column than is specified for the other columns. The resin does not need regeneration after use, but may be reused immediately since it is in the desired acid carbonate form.

RESULTS

The effluent (B) from the cation resin, D-50, contains hydrochloric, lactic, pyrrolidonecarboxylic, and many other acids. In addition, uridine and similar acidic substances as well as neutral compounds are present in the effluent. Fraction (C) from the D-1 column contains carbohydrates and very small amounts of nitrogen compounds. The latter may be basic decomposition products of the resin or may be compounds present in the plant extract that are too weakly acidic or basic to be retained by either the anion or cation resins. Eluate (D), the pyrimidine fraction, contains the slightly acidic purines, pyrimidines, and nucleosides that pass through the cation-exchange resin and are eluted from the anion-exchange resin by aqueous carbon dioxide. Eluate (E) contains salts of the acids from effluent (B).

Eluate (F) contains many basic and amphoteric compounds such as

betaine, serine, adenine, and glutamic acid. Ammonia is present in the free state and in the form of salts, but since little potassium or sodium is eluted the eluate (F) is nearly ash free. Effluent (G) contains the more basic compounds, such as betaine, that are not retained by the anion-exchange resin. Neutral amino acids and purines retained by the resin are eluted by aqueous carbon dioxide. This eluate (H) is fractionated into amino acids and purines. Dibasic amino acids are eluted from the anion resin by ammonium carbonate, sodium carbonate, or acids and appear in eluate (I).

The tables present data obtained from the separation of nitrogenous compounds in a sample of California straight house sugar beet molasses.

The preliminary separation on Dowex-50 resin divided the nitrogenous compounds into two main groups (Table 1) with about 60% of the

TABLE 1
SEPARATION OF NITROGENOUS COMPOUNDS INTO ACIDIC AND BASIC GROUPS

Sample	Nitrogen (gm/100 gm molasses)			
	Total	Amino	Amide	Ammonia
Original load	1.85[a]	0.30	0.07	0.01
Cation eluate (basic)	1.08	0.26	—	—
Cation effluent	0.78[a]	0.03	—	—

[a] Including nitrate nitrogen.

nitrogen in the basic fraction and the remainder in the acid and neutral fraction. The recovery of total and amino nitrogen is good.

The recovery of nitrogenous acidic substances is relatively poor if aqueous carbon dioxide and sodium carbonate are used as the only eluants (Table 2) for the fractionation of acidic and neutral compounds on Dowex-1. More nitrogen can be recovered if sulfuric acid is used as a

TABLE 2
SEPARATION OF ACIDIC NITROGENOUS COMPOUNDS

Fraction	Gm/100 gm molasses	
	Total N	Pyrimidine bases[a]
Neutral	0.02	—
Acid by difference	0.76	—
Effluents		
1st CO_2	0.00	—
2nd CO_2	0.019	0.044
3rd CO_2	0.002	0.004
Na_2CO_3	0.51[b]	—

[a] Calculated as uridine from spectrophotometric curves.

[b] Some nitrogen-containing color bodies remain on the column.

third eluant. If one desires only to separate the neutral from the acidic compounds, a weak anion-exchange resin such as Duolite A-4[5] may be used. In this case the resin is eluted with ammonium hydroxide which is then removed by evaporation in the presence of sodium hydroxide

TABLE 3
SEPARATION OF BASIC NITROGENOUS COMPOUNDS

Effluent	Gm/100 gm molasses		
	Total N	Amino N	Purines[a]
Betaine	0.58	0.00	—
1st CO_2	0.002	—	—
2nd and 3rd CO_2	0.25	0.16	0.088
4th CO_2	—	—	0.005
5th CO_2	0.010	—	—
6th CO_2	0.002	—	—
Na_2CO_3	0.064	—	—

[a] Calculated as adenine from spectrophotometric curves.

equivalent to the anions present in the solution. Many of the complex colored compounds formed in sugar beet molasses during processing cannot be eluted from a strong anion-exchange resin and must be isolated in this manner.

Table 3 shows the results of separating the basic nitrogen compounds. The betaine fraction is separated from the less basic amino acids since it is not absorbed on the resin. The neutral amino acids fraction con-

TABLE 4
SEPARATION OF AMINO ACIDS FROM PURINES

	Gm/100 gm molasses		
	Total N	Amino N	Purines[a]
Load	0.25	0.16	0.088
Effluent			
1st CO_2	0.21	0.17	0.022
2nd CO_2	0.036	0.014	0.043
3rd CO_2	0.007	—	—

[a] Calculated as adenine from spectrophotometric curves.

tains most of the purine bases. The quantities estimated compare favorably with those found by analysis for the individual compounds (5).

The combined second and third carbon dioxide eluates (Table 3) were fractionated to separate amino acids and purines (Table 4). For a separation better than that illustrated in the table smaller fractions

[5] Chemical Process Co., Redwood City, California.

of the eluate may be taken. The amino acids are in the first fraction and the majority of the purine bases in the second fraction.

SUMMARY

The method presented for separating nitrogenous compounds into several classes depends on the reactivity of the compounds to ion-exchange resins and various eluants. The method may be used to determine nitrogen distribution and to remove undesirable substances prior to other chromatographic procedures or to isolate specific compounds. It has been used to determine quantitative variations of different classes of nitrogenous compounds in sugar beet molasses and should prove valuable in survey studies of the composition of other plant materials.

Aqueous carbon dioxide is a satisfactory eluant for a number of compounds and has the advantage of leaving no residue on concentration.

ACKNOWLEDGMENT

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Identification Studies on Some Minor Flavonoid Constituents of the Grapefruit

WILLIAM J. DUNLAP AND SIMON H. WENDER

From the Chemistry Department, University of Oklahoma, Norman, Oklahoma

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Although the principal flavonoids of the various common species of citrus fruits have been known for many years, knowledge concerning flavonoid compounds present in smaller quantities in these fruits has until recently remained meager. Recent investigations have provided information regarding the minor flavonoid constituents of the lemon (*Citrus limon*) (1–4) and of the sweet orange (*Citrus sinensis*) (5, 6). Little information, however, regarding the flavonoids of the grapefruit (*Citrus paradisi*) had previously been presented. Only poncirin, a 7-rhamnoglucoside of isosakuranetin (5,7-dihydroxy-4′-methoxyflavanone) (7), and the principal flavonoid of the grapefruit, naringin, which is a 7-rhamnoglucoside of naringenin (4′,5,7-trihydroxyflavanone) had been reported in this fruit. This paper reports a study of some of the minor flavonoid constituents of the grapefruit. The isolation and identification of neohesperidin, a 7-rhamnoglucoside of hesperetin (3′,5,7-trihydroxy-4′-methoxyflavanone), and rhoifolin, a 7-rhamnoglucoside of apigenin (4′,5,7-trihydroxyflavone), from a commercially produced grapefruit flavonoid preparation containing mostly naringin (8), and of kaempferol (4′,5,7-trihydroxyflavonol) from the hydrolysis products of this preparation, are described. The isolation and identification of an isosakuranetin-7-rhamnoglucoside from this source and a comparison of this compound with an isosakuranetin-7-rhamnoglucoside previously isolated from the orange (6) are also presented.

METHODS AND RESULTS

Studies on Flavonoid Glycosides of a Grapefruit Flavonoid Preparation

A commercial naringin preparation, Sunkist Product No. 610E2, produced from grapefruit peel and pulp (8) was subjected to column chromatography, using Magnesol (Food Machinery and Chemical Corp., N. Y.) as adsorbent (6). In a typical experiment 6 gm of naringin product, dissolved in warm methyl alcohol and filtered to remove some

insoluble brown material, were adsorbed on an 8 × 70 cm Magnesol (dry-cleaning grade) column. Upon elution of the column with water-saturated ethyl acetate, a number of zones, mostly fluorescing blue, yellow-green, or yellow-brown, were developed. Four of these zones, designated according to the order in which they moved off the column as fractions C-2, -3, -5, and -6, contained flavonoids other than naringin, and these fractions were chosen for further study.

Fraction C-5 consisted mostly of a narrow yellow-brown fluorescing zone mixed with the trailing edge of the major zone on the column, naringin. Rechromatography of C-5 on a 4.5 × 45 cm Magnesol column removed most of the naringin, and also yielded three other fractions, designated in the order which they moved off the column as fractions C-5A, C-5B, and C-5C.

Neohesperidin. Fraction C-5A, which consisted mostly of a narrow yellow-green fluorescing zone, was further purified by rechromatography on two successive Magnesol columns. The resulting small quantity of purified C-5A gave color reactions and an ultraviolet absorption spectrum (maxima 284 and 332 mμ, minima 247 and 311 mμ) typical for a flavanone glycoside. The R_f values of this compound in *n*-butyl alcohol-acetic acid-water (6:1:2, v/v/v, hereafter called BAW), 15% acetic acid, and distilled water were identical with those of cochromatographed authentic neohesperidin (0.51, 0.77, 0.52, Whatman No. 1 paper, descending chromatography).

A small portion of fraction C-5A was hydrolyzed by refluxing for 3 hr with 3% hydrochloric acid, and the resulting aglycone was extracted from the aqueous hydrolysis mixture with ethyl acetate. This aglycone was cochromatographed with reference flavanone aglycones in BAW, 60% acetic acid, and nitromethane-benzene-water (3:2:5, v/v/v, hereafter called NBW) solvent systems, and the R_f values obtained (0.92, 0.79, 0.92) were found to agree with those of the reference authentic hesperetin. The aglycone was then degraded with aqueous potassium hydroxide and the degradation products were identified by paper chromatography (9) as isoferulic acid (3-hydroxy-4-methoxycinnamic acid) and phloroglucinol, thus confirming the aglycone of C-5A as hesperetin. The aqueous portion of the fraction C-5A hydrolysis mixture was found, by paper chromatography with reference sugar samples, to contain rhamnose and glucose.

The ultraviolet absorption spectrum of fraction C-5A was not affected when solid anhydrous sodium acetate was added to an alcoholic solution of this compound. This showed that the molecule did not possess a free 7-OH group (3), and indicated that fraction C-5A was a 7-glycoside. When this fraction was incubated with an enzyme preparation which

selectively hydrolyzes rhamnose from flavonoid rhamnoglucosides (10), rhamnose and a glucoside which was chromatographically identical with the hesperetin-7-glucoside produced from authentic neohesperidin were obtained. Hence fraction C-5A was confirmed as a 7-rhamnoglucoside of hesperetin, neohesperidin.

Rhoifolin. Fraction C-5B, which consisted mostly of a yellow-brown fluorescing zone, was further purified by rechromatography on two more Magnesol columns. The yellow solid material thus obtained produced spots on paper chromatograms which were brown in ultraviolet light and yellow in ultraviolet light after spraying with 1% alcoholic aluminum chloride. These colors and the R_f values for this fraction in BAW (0.52), 15% acetic acid (0.54), and distilled water (0.14) were identical with those of authentic cochromatographed rhoifolin (apigenin-7-rhamnoglucoside). The ultraviolet absorption spectrum of fraction C-5B had maxima at 268 and 333 mμ and minima at 248 and 292 mμ, and was identical with the spectrum produced by authentic rhoifolin. The aglycone produced by acid hydrolysis of fraction C-5B was found to give R_f values corresponding to those of cochromatographed apigenin in BAW (0.89), 60% acetic acid (0.66), and NBW (0.48), and the sugars thus produced were found by paper chromatography to be rhamnose and glucose. Fraction C-5B was, therefore, rhoifolin. [Commercial naringin has been found to contain 2–5% of this compound, and a method for preparing larger quantities of the glycoside from this source has been developed (11).]

Fraction C-5C. Fraction C-5C, a faint yellow-brown fluorescing zone which appeared on the column in the trailing edge of the rhoifolin zone, was not obtained in sufficient quantity and purity for identification studies. Its appearance on paper chromatograms indicated it was probably a flavone glycoside similar to rhoifolin, with R_f values of 0.46, 0.39, and 0.07 in BAW, 15% acetic acid, and distilled water.

Isosakuranetin-7-rhamnoglucoside. Fraction C-3 contained mostly a yellow-green fluorescing zone moving off the column just ahead of and mingled with the leading edge of the major zone, naringin. By successive rechromatography on four more Magnesol columns, fraction C-3 was finally separated from naringin and purified. Studies of this fraction by the procedures described for fraction C-5A established it to be a 7-rhamnoglucoside of isosakuranetin, probably poncirin, as previously reported in the grapefruit by Horowitz (7). Fraction C-3 was different from the isosakuranetin-7-rhamnoglucoside which we had previously isolated from the sweet orange (6). The R_f values of fraction C-3 (0.61 in BAW, 0.81 in 15% acetic acid, and 0.57 in distilled water) differed somewhat from those of the compound from the orange (0.59,

0.79, and 0.51) when the two compounds were cochromatographed; the melting point of fraction C-3 was about 210°, compared with 207–208° for the compound from the orange, and the mixed melting point of the two compounds was depressed; and fraction C-3 appeared to be bitter, while the isosakuranetin-7-rhamnoglucoside from the orange was not. When the two compounds were incubated with the previously mentioned rhamnosidase enzyme preparation, both yielded rhamnose and isosakuranetin glucosides, and these glucosides were chromatographically identical. Hence it appeared that the difference between the isosakuranetin-7-rhamnoglucosides from the grapefruit and sweet orange was in the rhamnose-glucose linkages in the sugar moieties of the molecules. The relationship between the two compounds was probably analogous to that between hesperidin and neohesperidin.

A Naringenin Rhamnoglucoside. Fraction C-2 contained a yellow-green fluorescing zone which moved off the column ahead of the isosakuranetin-7-rhamnoglucoside (C-3) fraction. This fraction was not obtained in pure form, even after extensive chromatography. Studies of the impure fraction C-2 (R_f values: 0.76, 0.67, and 0.39 in BAW, 15% acetic acid, and distilled water) indicated that it probably contained naringenin, rhamnose, and glucose, but it was definitely not identical with naringin.

Fraction C-6. Fraction C-6 contained a narrow green fluorescing zone moving off the column after the rhoifolin and C-5C fractions. The quantity of this compound obtained was insufficient for identification, but its appearance on paper chromatograms and its ultraviolet absorption spectrum indicated that it was a flavanone glycoside with R_f values of about 0.42, 0.74, and 0.56 in BAW, 15% acetic acid, and distilled water.

Studies on Aglycones Produced by Hydrolysis of a Grapefruit Flavonoid Preparation

Eighteen grams of the commercial naringin preparation, Sunkist Product No. 610E2, were hydrolyzed by refluxing with 2 liters of 3% hydrochloric acid for 4 hr, and the resulting aglycones were extracted with ethyl acetate and chromatographed on a 6 × 65 cm Magnesol column. The first fractions eluted from this column with water-saturated ethyl acetate were found by subsequent paper and silicic acid column chromatography to contain mostly naringenin, with some isosakuranetin. The third fraction eluted, which included a zone from the column that was yellow in visible light and that varied in fluorescence from yellow-green to yellow-brown, contained naringenin and other substances, some of which appeared possibly to be flavone and flavonol aglycones.

This third fraction was chromatographed on a 6 × 71 cm silicic acid

column employing 25% acetone-75% benzene as solvent (12). A wide brown fluorescing zone which moved down this column was collected as two fractions, and paper chromatograms revealed that the second fraction contained a substance which resembled the flavonol aglycone kaempferol in fluorescence and R_f values. Successive rechromatographing of this second fraction through two smaller silicic acid columns, employing 16% acetone-84% benzene as solvent, yielded a fraction containing apigenin and a fraction that gave a spot on paper chromatograms which was pale yellow in visible light and fluoresced yellow in ultraviolet light, bright yellow when exposed to ammonia fumes, and yellow-green after spraying with alcoholic aluminum chloride. The fluorescence and R_f values of this compound in BAW (0.88), 60% acetic acid (0.56), and NBW (0.50) were identical with those of cochromatographed authentic kaempferol. The ultraviolet absorption spectrum of this fraction had maxima at 268 and 366 mμ, which corresponded to the absorption maxima for authentic kaempferol. Thus the presence of kaempferol in the grapefruit was established. The kaempferol, which probably occurs in the fruit as a glycoside, appeared to be present in the naringin preparation in very low quantity, which probably accounted for the failure to isolate it in the previously described glycoside studies.

SUMMARY

The following flavonoid compounds were isolated and identified from a commercial naringin product prepared from grapefruit: neohesperidin, isosakuranetin-7-rhamnoglucoside, rhoifolin, and kaempferol. The isosakuranetin-7-rhamnoglucoside was compared with an isosakuranetin-7-rhamnoglucoside previously isolated from the orange, and the two compounds were found to be different, the difference apparently being in the rhamnose-glucose linkages of the sugar moieties.

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A Procedure for the Microdetermination of Formic Acid in Periodate Oxidation Mixtures

DAVID H. RAMMLER[1] AND JESSE C. RABINOWITZ

From the Department of Biochemistry, University of California, Berkeley, California

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INTRODUCTION

Periodate oxidation has been used for the structural analysis of carbohydrates and carbohydrate derivatives (1, 2). Most of these structural studies have been based on the determination of periodate consumption using titrimetric methods. The use of a spectrophotometric method for determining periodate consumption (3) has found wide application in the biochemical field because of the facility and sensitivity of the method. The present communication describes a procedure for the determination of formic acid formed during the spectrophotometric determination of periodate consumption.[2]

Formic acid formed in sodium periodate oxidation mixtures is determined using the crystalline enzyme formyl-tetrahydrofolate synthetase (5). The enzyme catalyzes the quantitative conversion of formic acid to 10-formyltetrahydrofolic acid according to Eq. (1):

$$\text{HCOOH} + \text{ATP} + \text{tetrahydrofolic acid} \rightleftharpoons \text{10-formyltetrahydrofolic acid} + \text{ADP} + \text{P}_i \quad (1)$$

Conditions are described where the determination of formic acid is unaffected by periodate and iodate ions. The product is determined spectrophotometrically by measuring the increase in absorption at 350 mμ resulting from the conversion of 10-formyltetrahydrofolic acid to 5,10-methenyltetrahydrofolic acid according to Eq. (2):

$$\text{10-formyltetrahydrofolic acid} + \text{H}^+ \rightleftharpoons \text{5,10-methenyltetrahydrofolic acid} + \text{H}_2\text{O} \quad (2)$$

MATERIALS

Tetrahydrofolic acid was prepared by catalytic reduction of folic acid (California Corporation, reagent grade) as described previously (6).

[1] Present address: Institute of Neurological Diseases and Blindness, National Institutes of Health, Bethesda, Maryland.

[2] The amount of formaldehyde formed in the periodate oxidation under the conditions described here has been determined in certain instances using the method described by Frisell and Mackenzie (4).

Formyl-tetrahydrofolate synthetase was prepared in crystalline form from *Clostridium cylindrosporum* as previously described (5).

Sodium metaperiodate and potassium metaperiodate were obtained from G. Frederick Smith Chemical Co., Columbus, Ohio. Potassium iodate was a product of J. T. Baker Chemical Co., Phillipsburg, N. J.

EXPERIMENTAL

Spectrophotometric Determination of Periodate Reduction Solutions

Sodium Metaperiodate. The aqueous stock solution of sodium metaperiodate made by dissolving 534.7 mg in 100 ml of water, contains 25.05 μmoles/ml. This reagent is stable for several months when stored in the dark.

Potassium Metaperiodate. A saturated aqueous solution is prepared by dissolving 0.75 gm of potassium metaperiodate in 100 ml of hot water. The solution is cooled in ice and the precipitated material is removed by filtration through a sintered glass funnel. The concentration of periodate in the filtrate is determined spectrophotometrically. This solution is stable in the dark.

Adenosine. An aqueous solution was prepared containing approximately 1.5 μmoles/ml. The exact concentration was determined from the absorbancy at 259 mμ, using a molar extinction coefficient of 15,000.

Method A: In this method, it is not necessary to use an exact molar extinction coefficient for the periodate solution used. The concentration of periodate is determined at the time of the assay by oxidizing an aliquot of a standard adenosine solution. This method also overcomes the necessity of correcting for the absorption of the variable amounts of iodate which are formed in the oxidation.[3]

In order to determine the exact amount of periodate to use, an amount of the periodate solution is added to one of two duplicate cuvettes containing 2.0 ml of 0.1 *M* potassium phosphate buffer at pH 6.8 and 1.0 ml of water to give an absorbancy of 0.8 to 0.9 at 235 mμ, using the other cuvette as a reference. For kinetic studies, the volume of periodate added should be small in order that there is no appreciable error in the initial volume of buffer. When the reaction is carried out at pH 7 or greater, it is preferable to use potassium metaperiodate because of the low solubility of sodium metaperiodate at these pH values. The absorbancy of the periodate containing cuvette is recorded and is termed "A_{initial}."

To determine the periodate consumption, two duplicate cuvettes are

[3] The spectrum of the oxidized compound must be determined after the oxidation in order to insure that no alteration of the chromophore has occurred. If the spectra has changed, the appropriate corrections should be made.

prepared by mixing 2.0 ml of 0.1 *M* buffer and 1.0 ml of water. To one is added 0.1 ml of an adenosine solution containing 1.5 μmoles/ml. The concentration of the adenosine is accurately determined by its absorbancy at 259 mμ. The same amount of adenosine is added to the reference cuvette, and the wavelength changed to 235 mμ or any other used to follow the periodate consumption. There should be no difference in absorbancy between the two cells. The exact amount of periodate previously determined to give the A_{initial} value is added to the reaction cuvette. After approximately 3 to 5 min, the absorbancy at 235 mμ has decreased to a constant value, A_{final}. ΔA is obtained by subtracting A_{final} from A_{initial}. The concentration of adenosine used divided by the ΔA gives the "periodate titer." A similar experiment is carried out using the unknown substance instead of adenosine,[4] and ΔA_{exptl} times the periodate titer yields the concentration of periodate consumed.

Method B: Two duplicate reaction mixtures are prepared in cuvettes with a 1-cm light path containing an aliquot of the unknown solution, 0.75 ml of sodium periodate (18.8 μmoles), and sufficient 1 *M* sodium acetate buffer at pH 4.3 to bring the volume to 3.0 ml. Because of the possibility of a light-catalyzed oxidation of formic acid by excess periodate (3, 7), one cuvette is placed in the dark and is used for measuring the amount of formic acid and formaldehyde formed during the reaction. The absorbancy of this cuvette should be determined only at the beginning and at the end of the reaction. The other cuvette is used to determine when the periodate oxidation is complete. The initial absorbancy of the solution at 300 mμ, determined against a reference cuvette containing only the buffer and an aliquot of the unknown compound, should be 1.0 or less. The amount of oxidizable material used should be such that the absorbancy is reduced to not less than 10% of the initial value after completion of the reaction. The amount of material used for the analysis will therefore vary depending on the number of bonds labile to periodate oxidation, and an amount should be used which will utilize no more than 17 μmoles of periodate. The decrease in absorbancy at 300 mμ is then determined as a function of time.

Calculations. The absorbancy of sodium periodate in 1 *M* sodium acetate buffer at pH 4.3 follows Beer's Law. The molar extinction coefficient of sodium periodate at 300 mμ is 157.9. An absorbancy change of 0.5 for a 3-ml sample in a cuvette with a 1-cm light path corresponds to the disappearance of 9.5 μmoles of sodium periodate. The product of the reduction, sodium iodate, absorbs about 3% of the total light at this

[4] An amount of sample should be used which gives approximately the same ΔA as was obtained using the standard adenosine solution in order to eliminate the need to correct for the absorption of the iodate formed.

wavelength, and the total amount of sodium periodate which has disappeared under these conditions is therefore 9.78 μmoles.

Determination of Formic Acid

Solutions

I. Triethanolamine buffer (1.0 *M*, pH 8.0). Dilute 149.2 gm of 2,2′,2″-nitrilotriethanol in 750 ml of water. Adjust to pH 8.0 with concentrated HCl and dilute to 1 liter.

II. *dl*-Tetrahydrofolic acid (ca. 0.01 *M*). Dissolve 56 mg of *dl*-tetrahydrofolic acid diacetate in 10 ml of 1 *M* mercaptoethanol. Neutralize with 2 *N* KOH to pH 7.0 (about 7 to 10 drops).

III. ATP (0.05 *M*). Dissolve 151.2 mg of $Na_2ATP \cdot 4H_2O$ in 4 ml of water, neutralize, and dilute to 5.0 ml.

IV. Magnesium chloride (0.1 *M*).

V. Sodium formate, pH 7. Standard solution containing 1.0 μmoles/ml.

VI. Formyl-tetrahydrofolate synthetase, 90,000 units/ml. Use 500 to 1,000 units per test (10 μl). The enzyme should be stored as a crystalline suspension in ammonium sulfate at 0 to 4° and should not be frozen.

Procedure. The following solutions are pipetted into a Pyrex test tube, 11 × 100 mm:

0.10 ml triethanolamine buffer (Solution I)
0.10 ml ATP (Solution III)
0.10 ml $MgCl_2$ (Solution IV)
0.20 ml *dl*-tetrahydrofolic acid (Solution II)
0.50 ml volume composed of water and varying amounts of the periodate reaction mixture obtained in Method A or B containing from 0.01 to 0.10 μmoles of formic acid.[5]

A reference tube is prepared with all the reagents included except the test solution containing formic acid.

The procedure can be simplified by making a mixture containing one part triethanolamine buffer, one part ATP, one part $MgCl_2$, and two parts of tetrahydrofolic acid solution, and using 0.5 ml of this mixture per incubation tube.

The enzymic reaction tubes are placed in a 37° water bath for about 2 min and the reaction is started by adding 500 to 1,000 units (10 μl of the crystalline enzyme solution) of formyl-tetrahydrofolate synthetase to each tube. The tubes are incubated for 10 min at 37°. After this time, 2.0 ml of 2% perchloric acid is added. The absorbancy at 350 mμ is determined 10 to 30 min after the addition of the acid, using the "blank" tube as a reference.

[5] Although excess sodium periodate interferes with the enzymic determination of formic acid, up to 0.5 ml of the reaction mixture of Method B may be used in the enzymic assay.

Calculations. The reaction is stoichiometric under the conditions described, and the molar absorption coefficient of the 5,10-methenyltetrahydrofolic acid is 24.9×10^3. Therefore the micromoles of formic acid per milliliter of test solution $= E/8.3 \times v$, where E is the absorbancy of the test solution and v is the volume of the test aliquot in milliliters.

RESULTS

Inhibition of Enzyme Activity. The effect of sodium metaperiodate and potassium iodate on the enzymic analysis of formic acid is illustrated in Table 1. The enzyme is not inhibited by potassium iodate in concen-

TABLE 1
EFFECT OF SODIUM METAPERIODATE AND POTASSIUM IODATE ON ENZYMIC DETERMINATION OF FORMIC ACID

Amt. added (μmoles)	Additions	
	Periodate	Iodate
	Absorbancy at 350 mμ[a]	
0	0.540	0.515
0.18	0.545	—
0.30	—	0.515
1.7	0.545	—
2.5	—	0.515
3.4	0.545	0.515
5.0	0.555	—
8.5	0.605	—
9.5	0.705	—

[a] Absorbancy was determined under the conditions of the formate assay as described in Methods. The assay system contained 0.065 μmole of formate and the amount of sodium metaperiodate shown or 0.062 μmole of formate and the amount of potassium iodate indicated.

trations as large as 5 μmoles/ml of enzyme reaction mixture. Sodium metaperiodate, in concentrations larger than 3.6 μmoles/ml of enzyme reaction solution, causes an apparent increase in the amount of formic acid. The increase may be due to the oxidation of substances in the enzyme assay mixture by the excess periodate. The enzyme is inhibited by sodium acetate buffer in concentrations greater than 1.5 *M*. Formaldehyde, cyclohexylamine,[6] and adenine in concentrations of 0.25, 0.76, and 0.1 μmole/ml, respectively, of enzyme reaction mixture did not cause inhibition.

Oxidation of Substances of Known Structure. A number of carbohydrates and carbohydrate derivatives of known structure were subjected

[6] It has been observed that amine salts of phosphate esters of carbohydrate derivatives occasionally interfere with the spectrophotometric determination of periodate consumption.

TABLE 2

FORMATE FORMATION IN PERIODATE OXIDATION OF CARBOHYDRATES AND CARBOHYDRATE DERIVATIVES

	Spectroscopic assay[a]							Titrimetric assay[b]						
		Metaperiodate consumed			Formate formed				Metaperiodate consumed			Formate formed		
Compound[c]	Amt. of substrate (μmoles)	Theory (μmoles)	Found (μmoles)	% theory	Theory (μmoles)	Found (μmoles)	% theory	Amt. of substrate (μmoles)	Theory (μmoles)	Found (μmoles)	% theory	Theory (μmoles)	Found (μmoles)	% theory
Ethylene glycol[d]	7.52	7.52	7.40	98	0.00	0.00	—	15.04	—	14.9	99	0.00	0.00	—
Glycerol[e]	3.64	7.27	7.05	97	3.64	3.00	83	7.26	14.52	14.32	98	7.26	8 0	110
Threitol 4-phosphate[f]	—	—	—	—	—	—	—	4.8	9.6	9.6	100	4.8	4.5	94
D-Arabitol[g]	1.88	7.50	7.35	98	5.6	5.3	95	3.75	15.0	15.0	100	11.2	11.0	98
D-Ribitol[h]	1.89	7.6	7.4	97	6.2	5.7	92	—	—	—	—	—	—	—
D-Sorbitol[i]	1.52	7.6	7.4	97	6.0	5.5	92	3.03	15.2	14.4	96	12.1	12.6	104
D-Mannitol[j]	0.6	3.0	2.9	97	2.4	2.2	92	—	—	—	—	—	—	—
(−)-Inositol[k]	1.25	8.7	8.5	97	6.3	5.6	89	2.49	17.5	18.6	106	14.9	14.5	97
D-Ribose[l]	1.81	7.34	7.2	97	7.34	7.2	98	—	—	—	—	—	—	—
D-Glucose[m]	1.38	6.93	7.0	101	6.9	6.9	100	6.9	34.5	33.3	97	34.5	32.0	93
Deoxyadenosine[n]	0.10	0.0	0.0	—	0.0	0.0	—	—	—	—	—	—	—	—
Adenosine[n]	0.10	0.10	0.09	90	0.0	0.0	—	—	—	—	—	—	—	—
Adenosine[n,o]	0.174	0.174	0.175	100	0.0	0.0	—	—	—	—	—	—	—	—

[a] Periodate consumption was determined by Method B and formate formation was determined as described in Methods.

[b] The method used (8) is based on the procedure described by Dyer (2). An aliquot of the compound to be oxidized was placed in a 10-ml volumetric flask containing 5 ml of water. To the solution was added 1.00 ml of 0.1000 *M* sodium metaperiodate and the volume was adjusted to 10.0 ml. At various time intervals, a 1.0-ml aliquot of the solution was removed and added to 25 ml of "quenching solution." (The latter solution is prepared just prior to use by mixing 25 ml of starch solution, 200 ml of 0.0125 *N* Na_2AsO_3, and 20 ml of 4% sodium iodide. The solution is diluted to 500 ml with 1 *M* sodium phosphate buffer, pH 7.0.) After 15 min, this solution was titrated to the starch end point with standard iodine solution. A second aliquot was removed and treated with 25 μl of ethylene glycol to remove excess periodate, and assayed for formic acid as described in the methods.

[c] All carbohydrates and carbohydrate derivatives were examined chromatographically and shown to be homogeneous in two solvent systems. Samples were dried to constant weight prio. to use.

[d] Product of Eastman Organic Chemicals Dept.; b.p. 195–197°.

[e] Product of Baker and Adamson Allied Chemical and Dye Corp., heated to 190° and cooled over P_2O_5.

[f] Prepared by an unpublished procedure (D. H. Rammler).

[g] Prepared by reduction of D-arabinose with sodium borohydride.

[h] Prepared by reduction of D-ribose with sodium borohydride.

[i] Obtained from Atlas Powder Co., Wilmington, Del.

[j] Product of Pfanstiehl Chemicals, Waukegan, Ill.

[k] Gift of C. E. Ballou.

[l] Gift of Hoffman-LaRoche Co.

[m] Product of Baker and Adamson Allied Chemical and Dye Corp

[n] Product of California Corp., Los Angeles, Calif.

[o] Periodate consumption determined by Method A.

to periodate oxidation according to Method B, and aliquots of the reaction mixtures were removed and analyzed for formic acid by the method described. The results of these analyses are summarized in Table 2, and demonstrate the accuracy of the formic acid analysis by this procedure on small samples of a variety of carbohydrates and carbohydrate derivatives. Analyses were also done by the titrimetric periodate method in order to augment the spectrophotometric analysis and also to demonstrate the application of the formate analysis to reaction mixtures obtained after titrimetric analysis.

A study of the relative rates of periodate consumption and formic acid formation in the periodate oxidation of D-glucose is illustrated in Fig. 1. It was found that the rate of formate formation is identical to the

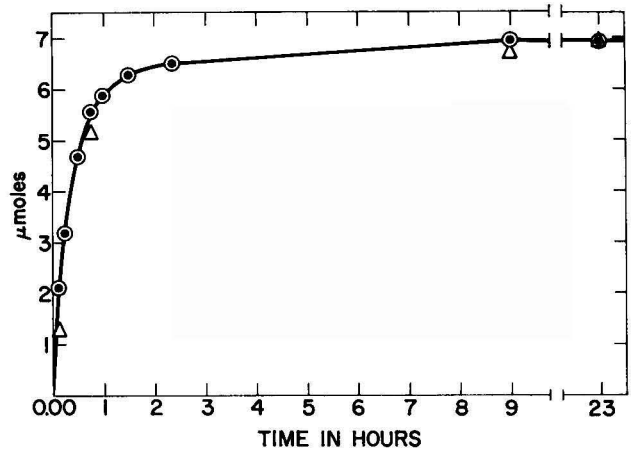

FIG. 1. Kinetics of periodate oxidation of glucose: 1.38 μmoles of glucose was oxidized as described in Method B; aliquots were removed at the times indicated and were analyzed for periodate consumption (⊙) and formate formation (△).

rate of periodate consumption and the ratio of formate formed to periodate consumed is 1.0. In view of other evidence for the formation of formyl esters of D-glucose during periodate oxidation (9), it appears that any formyl esters of D-glucose formed by the periodate oxidation are hydrolyzed during the enzymic determination of formic acid.

SUMMARY

A procedure is described for the enzymic assay of formic acid formed during the periodate oxidation of carbohydrates and carbohydrate derivatives. The enzymic assay of formic acid is based on the action of formyl-tetrahydrofolate synthetase obtained from *Clostridium cylindrosporum*. Aliquots of the mixture used for the spectroscopic determination

of the periodate consumption may be used directly in the enzymic assay. The results obtained using this method on a number of compounds of known structure are given.

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A New System for Paper Chromatography of Polar Steroids

JOSEPH C. TOUCHSTONE, M. KASPAROW, AND T. MURAWEC

From the Department of Obstetrics and Gynecology and Harrison Department of Surgical Research, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

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Many paper chromatographic solvent systems have been developed for separation of steroids. Seldom is any one system sufficient for separation of a number of steroids of wide degrees of polarity. The systems of Bush (2) and Eberlein and Bongiovanni (3) lack capacity and require control of temperature due to the use of low-boiling solvents. Zaffaroni-type systems (1) have greater capacity but usually require longer development times. Experience with a number of solvent systems for polar steroids and estrogens indicated that there was need of other systems for faster separation along with capacity. The isopropyl ether-ethylene glycol system described here was developed initially for separation of estriol from blood extracts and was subsequently shown to be of value in separation of other steroids, particularly those of the corticoid series. This system also proved of advantage in that cleaner papers resulted after development and lower background was found after treatment with color reagents, particularly ferric chloride-potassium ferricyanide as used for estrogens. With this procedure 0.5 μg of estriol was clearly visualized on the paper. The relative mobilities of a number of steroids in the new system are reported along with the mobilities in the toluene-propylene glycol and toluene-ethylene glycol (4) systems for comparison.

METHODS

The solvents used were of reagent grade. Isopropyl ether was redistilled after treatment with ferrous sulfate. A mixture of isopropyl ether and a small amount of ethylene glycol was shaken at room temperature. The solvent system so prepared was stable for several weeks. The top layer served as the mobile phase and was used to saturate the chromatography chamber. The paper strips (Whatman No. 1) were drawn through a trough containing ethylene glycol and then blotted between sheets of filter paper. The steroids were then spotted on the starting line and the

strips developed in the usual Zaffaroni technique, preferably in cylindrical tanks 24 in. in height. Development was carried out for a predetermined length of time. For long development times requiring repeated addition of solvent a hole in the top of the tank is required. For estriol a development time of 24 hr resulted in a movement of approximately 5 cm, depending upon the temperature. For corticosterone 60 hr was required. Faster development occurred at higher temperatures. However, too high room temperatures caused erratic results. After development, the strips are dried in air, preferably in a dark room, and the steroids detected by ultraviolet illumination or appropriate color tests.

RESULTS

Table 1 indicates that estriol has a much greater mobility in the new system when compared to its mobility in toluene-propylene glycol or toluene-ethylene glycol systems developed in the conventional manner.

TABLE 1
MOBILITIES OF STEROIDS ON PAPER CHROMATOGRAMS

Steroid	IPE-EG[a]	Time[b]	T-EG[a]	Time	T-PG[a]	Time
6α-Hydroxycortisol	0.15	—	0.05	—	—	—
6β-Hydroxycortisol	0.15	120	0.06	—	—	—
20α-F glycol[c]	0.32	96	0.25	168	0.28	168
20β-F glycol[d]	0.45	96	0.29	168	0.34	168
Tetrahydrocortisol[e]	0.88	72	0.27	96	0.35	168
Cortisol	1.0	72	1.0	50	1.0	96
Cortisone	1.2	72	2.5	50	2.3	96
Estriol	1.4	72	0.15	168	0.18	168
Tetrahydrodehydrocorticosterone	2.1	65	16.2	7	8.2	16
Corticosterone	2.7	65	20.5	7	11.9	16
11-Desoxycortisol	3.5	65	16.7	7	8.5	16
11β-Hydroxyandrostenedione	8.0	7	50.9	3[f]	29.4	3
Desoxycorticosterone	11.4	16	87.3	3	58.0	3
17α-Hydroxyprogesterone	17.0	7	74.5	3	44.1	3
Estradiol	19.0	7	11.2	10	4.8	16
Androstene-3,17-dione	21.7	3[f]	90.0	3	—	—
Estrone	24.0	3	[g]	—	20.0	5
Progesterone	35.0	3	[g]	—	94.1	3

[a] Systems: IPE-EG, isopropyl ether-ethylene glycol. T-EG, Toluene-ethylene glycol. T-PG, Toluene-propylene glycol.

[b] Time in hours required for movement of approximately 5–15 cm.

[c] 4-Pregnene 11β, 17α, 20α, 21-tetrol-3-one.

[d] 20β epimer of *c*.

[e] The designation tetrahydro refers to the steroid with ring A reduced and a 3α-hydroxyl substituent.

[f] Solvent front reached the end of the strip in this time.

[g] Migrates with the solvent front.

Estriol moved 5 cm from the starting line in 18 hr while in the two older systems almost 5 days would be required for similar movement. It is faster than cortisone in the new system while in the older systems, as noted in the table, it is almost ten times slower. Figure 1 shows results of

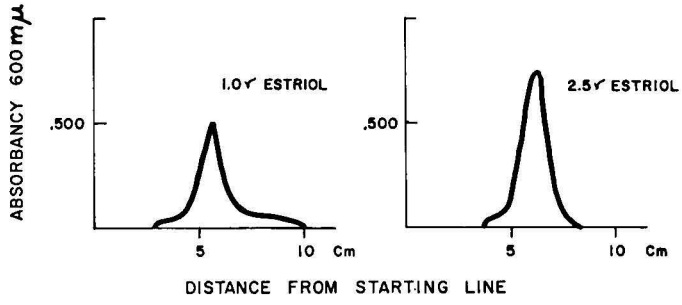

FIG. 1. Chromatography and densitometry of estriol. Developed 18 hr in isopropyl ether-ethylene glycol and color developed with ferric chloride-potassium ferricyanide reagent, then scanned at 600 mμ.

plotting density versus distance from the starting line after chromatography of 1 and 2.5 μg of estriol. Color was developed with the ferric chloride-potassium ferricyanide reagent and the strip was scanned in a Beckman spectrophotometer at 600 mμ using an adapter as described by Tennant *et al.* (5). Estrone and estradiol separate well, and clear color zones are developed in the same way. Estradiol in the new system is faster than desoxycorticosterone while in toluene-propylene glycol it is slower. Corticosterone (B) and Substance S and cortisone are well separated in the isopropyl ether-ethylene glycol system (Fig. 2). Substance S and B

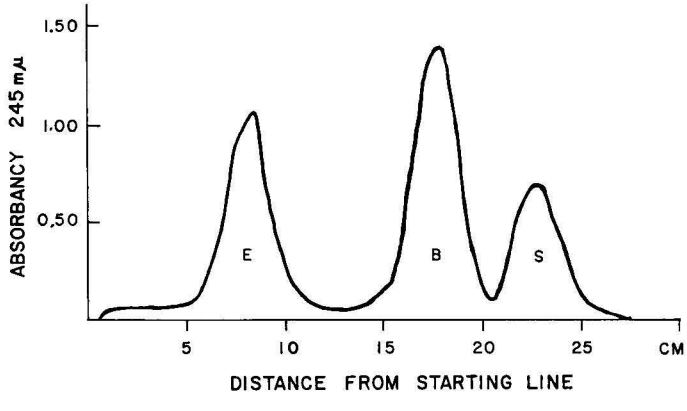

FIG. 2. Separation of cortisone, corticosterone, and 11-deoxycortisol. Developed 60 hr in isopropyl ether-ethylene glycol and scanned at 245 mμ.

separate well in the methylcyclohexane-dimethylformamide system previously reported by this laboratory (6) in 140 hr while only 65 hr gives a comparable separation in the new system.

There is an advantage in the use of the new system for separation of others of the adrenal steroids, as greater mobility is obtained without loss of sharpness of separation. This is illustrated in Fig. 2. There appears to be some specificity for the chromatography of the estrogens since their relative mobility with isopropyl ether as the mobile phase was much greater than that of the other steroids tested. The system has been successfully used in the separation of estriol from blood extracts.

SUMMARY

The new paper chromatographic system, isopropyl ether-ethylene glycol system, provides an improved and faster separation of steroids, particularly estrogens in paper chromatograms. The mobilities of a number of steroids are listed.

ACKNOWLEDGMENTS

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ANALYTICAL BIOCHEMISTRY 4, 128–131 (1962)

Quantitative Carbon-14 and Tritium Assay of Thin-Layer Chromatography Plates

FRED SNYDER AND NELSON STEPHENS

From the Medical Division,[1] Oak Ridge Institute of Nuclear Studies, Oak Ridge, Tennessee

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Thin-layer chromatography (TLC) (1) is an extremely useful tool in the isolation of lipids from animal tissues. Its many advantages for qualitative, quantitative, and preparative work in accomplishing difficult separations, sometimes in minutes, have recently been reviewed by Mangold (2). We are using thin-layer chromatography to study the incorporation of C^{14}-labeled metabolites into lipids, and have developed this technique for the quantitative recovery of radioactivity in fractions separated on Silica Gel G. No elution is necessary. The procedure is therefore ideal for isolating and measuring multiple tagged compounds (e.g., H^3, C^{14}, P^{32}) and for rapidly checking the radiopurity of commercial compounds. Its speed and counting efficiency are high, and it offers quantitation that is difficult or impossible to achieve by other detecting systems.

EXPERIMENTAL

Quantitative measurements were made on commercially available C^{14}- and H^3-labeled lipids. They were separated on TLC plates by the method of Malins and Mangold (3), using a solvent system of petroleum ether: diethyl ether:glacial acetic acid (90:10:1 vloumes), and were visualized by iodine vapor and by dichlorofluorescein under ultraviolet light. Five or six areas were marked off between the origin and the solvent front, and the specific areas of the thin-layer adsorbent containing the lipid components were carefully scraped with a Plexiglas spatula into a Tri-Carb counting vial. The scrapings were suspended in 15 ml of a 4% (w/w) Cab-O-Sil[2] (4) toluene scintillation solution containing 5 gm PPO[3] and 0.3 gm dimethyl POPOP[4] per liter of toluene. The radioactivity in each

[1] Under contract with U. S. Atomic Energy Commission.

[2] Cab-O-Sil, thixotropic gel powder, available from Packard Instrument Co., La Grange, Illinois.

[3] PPO, 2,5-diphenyloxazole, available from Packard Instrument Co.

[4] POPOP, *p*-bis[2-(4-methyl-5-phenyloxazolyl)]benzene, available from Packard Instrument Co.

vial was determined in a Packard Tri-Carb liquid scintillation spectrometer. The counting vial was assayed at 10°C. Equilibration of the tritium samples for 30 min at this temperature was required for stable counting rates; temperature equilibration was unnecessary for the carbon-14 samples. An internal standard of C^{14}-toluene or H^3-toluene was used to evaluate the quenching characteristics of the samples.

A possible error in working with gels thickened further by the addition of silica is that some of the radioactivity can be trapped underneath the cap of the counting vial after shaking. This can be avoided by capping the vials with Scotch tape trimmed to the circumference of the opening before shaking and assay. If phosphatides are assayed according to this method, polyethylene vials should be used, for adsorption of some polar lipids on the glass surface can occur.

RESULTS AND DISCUSSION

The poor radiopurity of a commercially available C^{14}-labeled tripalmitin used in these experiments is indicated by the autoradiogram pictured in Fig. 1. Quantitative assay of the radiopurity was done as described and the results are reported in Table 1. The Silica Gel G had no quenching properties (up to at least 100 mg). The excellent recovery and reliability of the method is shown by the recovery of radioactivity from eight samples ($99 \pm 2.2\%$) of C^{14}-tripalmitin separated and assayed by this method. The data demonstrate that this procedure can quickly answer any questions on the quantitative radiopurity of a compound; the entire separation and radioassay procedure takes less than 1 hr. The technique has also been successful in demonstrating the incorporation of radioactivity into a given fraction when tissue homogenates were applied directly to a plate.

Scintillation liquids without Cab-O-Sil can be used, providing the radioactive material is soluble in the solvents. The advantage of the Cab-O-Sil system, however, is that the scrapings are suspended, which makes it immaterial whether the labeled materials (except the fatty acids) are eluted or remain on the silica particles. The acidic condition of the silica as used in the procedure is absolutely essential for counting fatty acids; when acetic acid is not used in the TLC separation, 50 μl of acid can be added directly to the counting vial. If adsorption of long-chain fatty acids on silica gel is allowed to occur in the vial, a decrease in the counting efficiency owing to self-absorption of energy will result; on the other hand, the counting of mixed C^{14}-labeled phospholipids (from Euglena) or of the unidentified polar lipids present in the impure C^{14}-tripalmitin has the same efficiency whether the radioactivity is adsorbed on particles or remains in solution.

TABLE 1

RECOVERY OF CARBON-14 AND TRITIUM FROM THIN-LAYER CHROMATOGRAPHY PLATES (COUNTS/MIN IN THOUSANDS)

Compound	Total radioactivity applied	Radioactivity recovered							Per cent recovery
		Area 1	Area 2	Area 3	Area 4	Area 5	Area 6	Total	
Tripalmitin-$C^{14}OOH$									
a	64.2	9.0	6.0	4.2	41.8	0.53	0.52	63.0	97
b	46.9	7.6	4.4	2.6	31.7	0.39	0.03	46.7	100
c	105	15.8	10.2	6.9	68.5	0.78	0.62	103	98
d	161	26.6	15.3	9.7	109	1.6	0.05	162	101
Oleic acid-1-C^{14}	86.0	19.8	2.9	62.1	1.1	0.11	0.33	86.3	100
Stearic acid-1-C^{14}									
a	42.6	2.9	1.6	40.0	0.13	—	—	44.6	105
b	429	19.1	14.7	381	1.5	0.49	—	417	97
Stearic acid-9,10-H^3									
a	34.2	5.2	3.7	1.7	23.3	1.2	—	35.1	103
b	126	17.8	14.5	10.3	83.2	4.0	—	130	103
Palmitic acid-9,10-H^3									
a	46.1	2.9	1.8	40.4	0.47	1.0	—	46.6	101
b	102	7.4	3.8	88.1	1.4	1.7	—	102	100

The mean recovery value and the standard deviation for all of the samples in the table was 100 ± 2.6%. Area 3 represents pure fatty acid and Area 4 represents the pure tripalmitin.

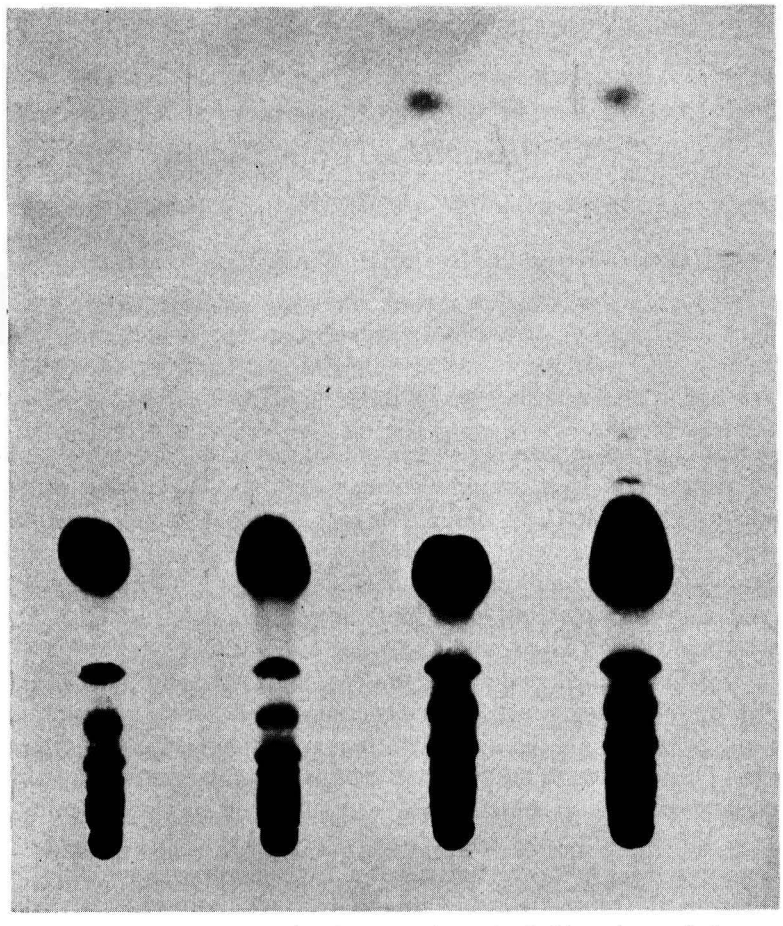

FIG. 1. Autoradiogram of C^{14}-carboxy-labeled tripalmitin after thin-layer chromatography. The four samples represent four different specific activities of the same sample. The quantitative radioassay of this compound is shown in Table 1.

SUMMARY

A fast and reliable technique for recovering essentially 100% of total carbon-14- and tritium-labeled lipids separated on thin-layer chromatography (TLC) plates is described. Cab-O-Sil, a gelling agent, was used to suspend the TLC adsorbent in a liquid scintillator solution, and for this reason the procedure requires no elution steps.

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ANALYTICAL BIOCHEMISTRY 4, 132–142 (1962)

Quantitative Paper Chromatography of $C_{21}O_5$ and $C_{21}O_6$ Corticosteroids from Guinea Pig Urine Extracts[1]

SHLOMO BURSTEIN AND HOWARD L. KIMBALL

From the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts

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The utility of the guinea pig as a laboratory animal for urinary corticosteroid studies has been amply demonstrated (1–11). Guinea pig urine contains a number of $C_{21}O_5$ and $C_{21}O_6$ corticosteroids which are excreted in the free (unconjugated) form extractable directly with ethyl acetate (5, 12) and therefore easily determinable. Cortisol (5), 6β-hydroxycortisol (7), and 2α-hydroxycortisol (13) have been previously identified among several other urinary $C_{21}O_5$ and $C_{21}O_6$ corticosteroids of unknown nature (8, 9) and determined under various physiological conditions (14, 15). Because of the difficult task of determining the separate steroids in the urine of single animals the determination of corticosteroid excretion patterns has been limited to studies with *groups* of similarly treated animals, the urinary extracts of which have been subjected to paper chromatography in different systems before and after acetylation (8, 9, 14). The procedure used was laborious and not free from serious unavoidable losses. Furthermore, the restriction of having to work with groups of animals put serious limitations on the design of the physiological experiments and their interpretation. The purpose of this study was to devise a simple paper chromatographic procedure for the determination of $C_{21}O_5$ and $C_{21}O_6$ reducing corticosteroid patterns in the urine of individual guinea pigs. The method, which involves a single chromatographic separation in an ethylene chloride-methanol-water system, reaction with blue tetrazolium, and direct formazan color measurement on paper, was found suitable for the routine determination of $C_{21}O_5$ and $C_{21}O_6$ corticosteroids in the urine of individual guinea pigs.

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MATERIALS AND METHODS

Methanol, sodium hydroxide, sodium bicarbonate, and *glacial acetic acid* were all Mallinckrodt, *Analytical Reagent.*

1,2-Dichloroethane, b.p. 83–84°, and *o-dichlorobenzene,* b.p. 179.5–180.5°, were purchased from Matheson Coleman and Bell. The ethylene chloride was shaken with magnesium oxide, filtered, and redistilled in an all-glass apparatus.

Blue tetrazolium reagent. Blue tetrazolium (BT), 3,3′-(3,3′-dimethoxy-4,4′-biphenylene)bis(2,5-diphenyl-2H-tetrazolium) chloride, was purchased from Dajac Laboratories, Borden Chemical Company, Philadelphia, Pa. The material was further purified by treating with decolorizing carbon. BT (2 gm) was dissolved in 30 ml methanol and boiled with 0.5 gm Nuchar (C190-N, Fisher Scientific) for a few minutes and filtered. The filtrate was evaporated to dryness and the residue triturated with hexane and dried. A 1% solution of BT in water was kept in the refrigerator without deterioration for several months. Just before use 2 vol of the solution was mixed with 1 vol of 10% aqueous NaOH.

Formazan color development. Papers were pulled through the alkaline BT reagent described above and placed on flat glass plates for 20 min for full color development. The papers were then immersed in 2% aqueous acetic acid to wash off excess BT and hung up to dry. When dry the papers were rinsed in methanol, which removed most of the pink color in the formazan zones, leaving the blue formazan on a nearly colorless background.

Filter paper for chromatography. Whatman No. 2 or No. 1 (which gave slightly faster runs) was used without prior washing.

Guinea pig urine extracts. Guinea pig urine was collected in glass cages. Larger and more uniform urine volumes were obtained by substituting a constant quantity of cucumber and lettuce for the water. After dissolving 20% (w/v) NaCl in the urine, it was extracted twice with 3 vol of ethyl acetate. The ethyl acetate was washed 3 times with (5% of ethyl acetate volume) 0.1 *N* NaOH, and 3 times with water, and the solvent was removed under reduced pressure.

Chromatography tanks. Pyrex cylindrical jars of 30.5-cm o.d. and 120-cm height were ordered from Corning Glass Works. Stainless-steel platforms (8437) to support the solvent trays (8434) and glass plates (8435) were all purchased from Will Corporation.

Apparatus and densitometry. Formazan color densities were measured with a Photovolt recording densitometer consisting of the following units: Photometer model 52-C, Varicord Variable Response Recorder model 42-A, and Integraph Integrator model 49. A 595-mμ filter and an aperture

2 mm in diameter were used. A linear relationship of concentration and integrated color density was obtained with response 5 of the recorder. The colors were measured by setting the photometer and recorder to 100% transmission at the areas of the strips not containing formazan zones.

EXPERIMENTAL AND RESULTS

Development of Paper Chromatographic System

Systems for separating polar steroids have been described (16–23), but none of the two-phase volatile systems was satisfactory for separating the $C_{21}O_5$ and $C_{21}O_6$ reducing corticosteroids from guinea pig urine in a single chromatogram. In these systems the $C_{21}O_6$ steroids either did not move from the starting line or ran too fast without affording good separation.

Because of the limited supply of pure $C_{21}O_6$ corticosteroids most of the early experimentation was done with guinea pig urine extracts.

The relatively good properties of the chloroform-formamide system used previously for the separation of $C_{21}O_5$ and $C_{21}O_6$ corticosteroids in guinea pig urine (7) pointed to the possibility of substituting the formamide with an aqueous alcohol. A promising system (found in collaboration with Dr. Leonard R. Axelrod at the Southwest Foundation for Research and Education, San Antonio, Texas), tested in 60-cm high chromatography jars, consisted of 5 vol of methylene chloride shaken with 1 vol of 60% aqueous methanol. The chromatography tank was saturated only with mobile phase. The papers were dipped in the stationary phase (as described by Schenker *et al.* (18) and Pechet (19) just before steroid application, blotted between filter paper and developed with the lower phase. Despite good resolution this system was not reproducible. Better reproducibility was found with the system consisting of 5 vol of ethylene chloride saturated with 1 vol of methanol:water 3:2 (v/v). To increase the resolution, chromatography was attempted in 120-cm high tanks [as described by Fukushima *et al.* (22)]. However, with both phases in the tank, an irregular movement of mobile phase occurred and no chromatography resulted. When developed in a 120-cm high tank *containing only the lower phase* and the papers (dipped in water and blotted prior to placing in the chromatography tanks) equilibrated for 24 hr, a good resolution of the $C_{21}O_5$ and $C_{21}O_6$ corticosteroids was achieved, but uniform zones were not consistently obtained.[2] In a more systematic effort

[2] Because of the difficulties encountered in the beginning with the chromatography in the longer tanks in the presence of both the mobile and the stationary phases and of the promising results obtained in the presence of the mobile phase alone, our efforts were directed primarily at the development of "single-phase" systems. At the end of this study some success was also achieved in the long tanks in the presence of both phases when mobile phase was run on 15-cm wide papers for 24–48 hr prior to paper equilibration.

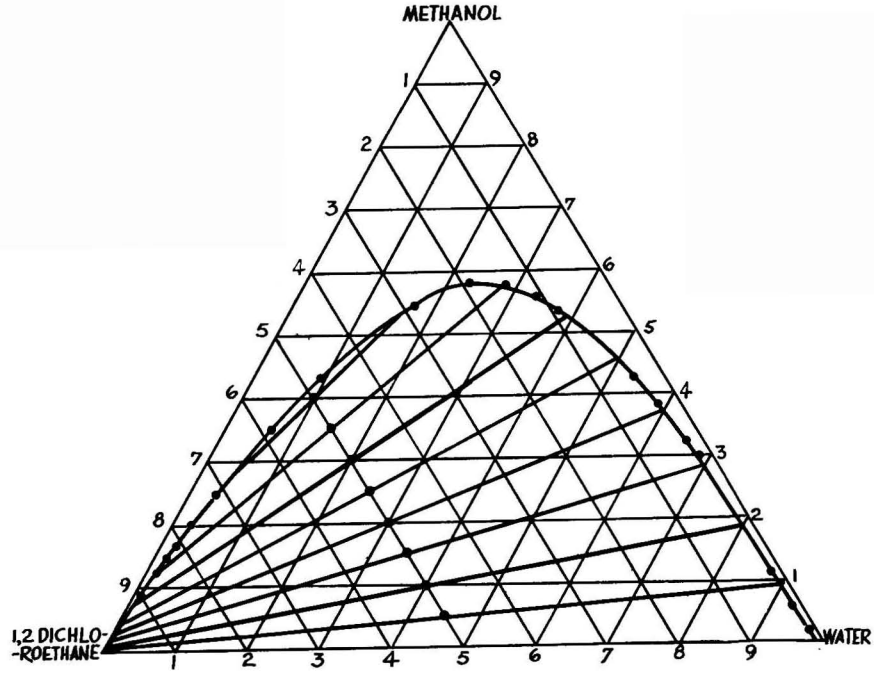

FIG. 1. Ethylene chloride-methanol-water phase diagram, 26 ± 1°C. The dots inside the partially miscible region represent the compositions from which the points of the lower phases of the tie lines have been obtained. The dots on the curve were obtained by determining the range of miscibility of the ternary system.

to find a better running solvent, the phase diagram of the ethylene chloride-methanol-water system was constructed (Fig. 1). This was done by determining the range of miscibility of the ternary system and the composition of the lower phase by gas chromatography. The best results were obtained with systems giving a mobile phase containing 1–2% methanol in ethylene chloride. Such a solvent mixture obtains when the mobile phase of the ethylene chloride:60% aqueous methanol 5:1 system described above is placed in a 120-cm high tank, because of methanol evaporation into the atmosphere of the tank. This partly explains the good running properties of this system when only mobile phase is placed in the tank.

After considerable experimentation an empirical procedure was developed which afforded a good resolution among the $C_{21}O_5$ and $C_{21}O_6$ corticosteroids and fairly straight zones.

Chromatographic Procedure

The mobile phase consisted of ethylene chloride containing 2% methanol (by volume) shaken with water until saturated. One-half liter of this

mixture is poured into the bottom of the tank and vigorously shaken. A tray containing 300 ml of saturated aqueous $NaHCO_3$ solution is placed at the bottom of the tank and in it is suspended from the top a 15-cm strip of Whatman No. 2 paper. Another 6″-wide strip of paper (long enough to reach almost to the bottom of the tank) is suspended from the top of the tank and 100 ml methanol is poured on this paper (to facilitate evaporation); the tanks are left to equilibrate for at least 24 hr. Running of mobile phase on 15-cm wide papers hastened the equilibration of the tanks.

Once in good running order the tanks gave good results for periods as long as 3 months. The mobile phase at the bottom of the tanks was replenished, when it became low, with ethylene chloride containing 2% methanol.

The tanks were kept in a thermostatically controlled cabinet at 31 ± 1°C. Urine extracts (or cortisol standards) were streaked with a micropipet across 2.5-cm wide papers without depositing on the very edges of the papers. The papers were dipped in distilled water before material application and were left to equilibrate between two glass plates in the solvent trays for 24 hr. Mobile phase was added through a hole in the cover of the tanks and chromatography allowed to proceed for 9–12 hr.

Direct Determination of Formazan Colors on Paper

Direct color measurement of reducing corticosteroids on paper has been reported by several investigators (24–28). In general agreement with Bush and Gale (29) it was found that blue tetrazolium from commercial sources did not give pure intense blue colors when chromatograms containing reducing corticosteroids were developed with BT (see Methods and Materials) but contained to a variable extent a red-pink pigment. The relative concentration of the red pigment seemed to depend on the chromatographic system used and also on the age of the system. Attempts to prepare pure BT, which, according to Bush and Gale, did not give rise to the red color, were not successful in our hands.

During the study of various paper partition systems with reducing corticosteroids, it was discovered that when 1,2-dichlorobenzene was used as a component of one of the mixtures an intense blue formazan with only little red in it was obtained on reaction with BT. The presence of 1,2-dichlorobenzene in the papers during reaction with BT was responsible for this effect. These results suggested the development of an improved BT reaction on paper, especially since it seemed that in addition to a great improvement in hue there was also a considerable color intensification. The method finally developed for $C_{21}O_5$ and $C_{21}O_6$ reducing corticosteroids consisted of pulling the papers through a 20% solution (v/v) of 1,2-dichlorobenzene in hexane and blotting between filter paper sheets before

developing with BT. A similar intensification of color was also obtained with the more expensive meta isomer (1,3-dichlorobenzene). Dipping in saturated hydrocarbons such as hexane or isooctane did not promote the blue formazan formation. The aromatic hydrocarbons benzene, toluene, or xylene improved the hue of the formazan formed but did not lead to the intense colors produced in the presence of *o*-dichlorobenzene.

A typical curve obtained with cortisol with the Photovolt recording densitometer is shown in Fig. 2. Included in this figure is also a curve

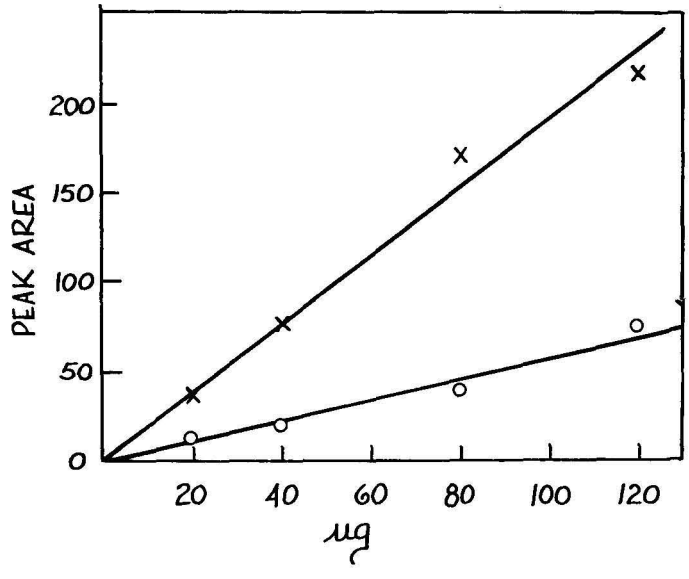

FIG. 2. Regression lines of peak area (arbitrary units) on cortisol concentration, following chromatography in the ethylene chloride-methanol-water system: ○—○ without 1,2-dichlorobenzene treatment, ×—× papers dipped in 1,2-dichlorobenzene and blotted before reacting with BT. The peak areas in these experiments have been determined by cutting out and weighing the steroid peaks obtained with the Photovolt recording densitometer.

obtained without 1,2-dichlorobenzene treatment. The formazan colors obtained following 1,2-dichlorobenzene treatment did not fade more than 10% in 24 hr. Only an apparent fading (which is probably due to light reflection phenomena) occurred upon visual examination when the papers dried, since the "strength" of the color to the eye could be brought back by dipping in water.

Resolution

A diagrammatic presentation of a chromatogram obtained with a guinea pig urine extract of a single animal is shown in Fig. 3*A*. Zones III, VI, and VII had the mobilities of 6β-hydroxycortisol, 2α-hydroxycortisol,

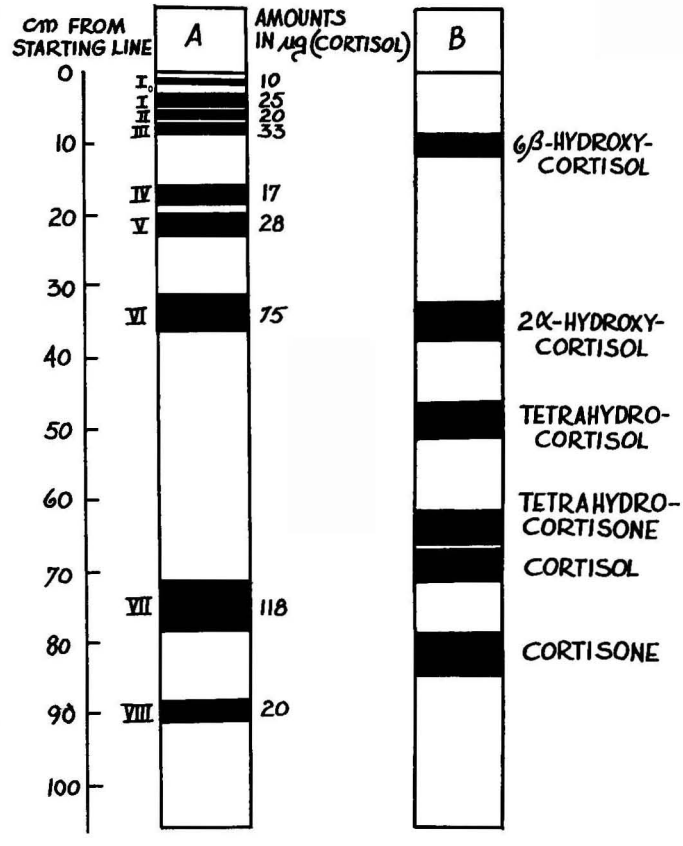

FIG. 3. Diagrammatic presentation of paper chromatograms (on 2.5-cm wide Whatman No. 2) of a urinary extract from a single guinea pig (*A*) and a mixture of 6β-hydroxycortisol, 2α-hydroxycortisol, tetrahydrocortisol (3α,11β,17α,21-tetrahydroxypregnane-20-one), tetrahydrocortisone (3α,17α21-trihydroxypregnane-11,20-dione), cortisol, and cortisone (*B*) run in ethylene chloride-methanol-water system. Chromatograms *A* and *B* were run on two different occasions *in different tanks* for approximately 10 hr.

and cortisol, respectively, previously isolated from guinea pig urine (7, 13). In addition to the as yet unidentified zones I_0, I, II, IV, and V, several other reducing zones, in smaller concentrations, were noted in certain guinea pig urine extracts. The separation of 6β-hydroxycortisol, 2α-hydroxycortisol, tetrahydrocortisol, tetrahydrocortisone, cortisol, and cortisone is shown in Fig. 3*B*. Although the sequence of the steroids on the chromatograms was constant the R_f values showed some variability especially when chromatography was done in different tanks, as can be seen in Fig. 3. The R_f values of zones I–VIII, determined in a different

experiment, using Sudan III to locate the solvent front, were approximately 0.07, 0.08, 0.09, 0.22, 0.28, 0.38, 0.77, and 0.92. Although there was a fairly good resolution among the steroids of the polarity of 6β-hydroxycortisol by running for approximately 10 hr, a much better separation could be achieved by running the chromatograms for 48–72 hr. Occasionally (especially in heavy extracts) the separability of zones I–III was not good, but it was still possible to obtain information as to the total amount of I–III present and a rough estimate as to relative concentrations, which then could be used to decide which extracts should be rerun for longer times for a more accurate determination.

Precision

The precision of the method depended on several variables not easily controllable. The most important factor, as pointed out by Bush (27),

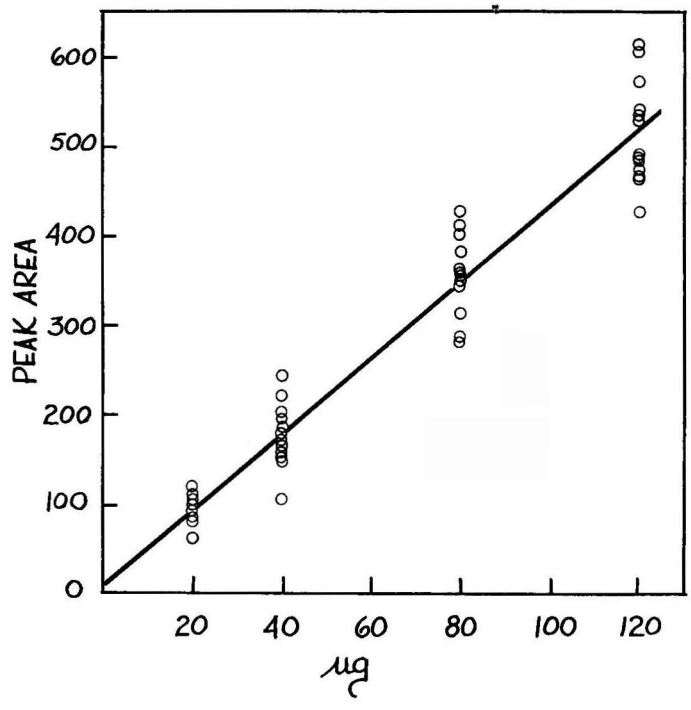

FIG. 4. Regression line of peak area (y) (arbitrary units) (determined by the Photovolt densitometer and integrator) on cortisol concentration (x) obtained following chromatography on 2.5-cm wide papers in the ethylene chloride-methanol-water system described under Methods (15 determinations at approximately weekly intervals). $y = 4.3x + 8.3$, correlation coefficient 0.98, standard error of estimate $S_y = 33$.

was the uniformity of the zones. Curved bands and especially bands in which the distribution of the material was not even in respect to the width of the paper, as well as extremely narrow zones, caused the greatest errors. The results obtained in 15 determinations made on different days at approximately weekly intervals are given in Fig. 4. The means ±S.D. for 20, 40, 80, and 120 μg cortisol were 94 ± 15, 177 ± 33, 357 ± 42, and 513 ± 52, respectively, with a mean coefficient of variation of ±14%. Determinations done on the same day gave a mean coefficient of variation less than ±10% with a smaller spread at the higher concentrations. Since unknowns were always run with a set of standards, the precision achieved was roughly 10% (coefficient of variation).

DISCUSSION

The method presented allows the routine paper chromatographic separation and determination of 6β-hydroxycortisol, 2α-hydroxycortisol, and cortisol in addition to other as yet unknown corticosteroids in urinary extracts from individual guinea pigs.

Although chromatographic tanks prepared by the procedure outlined above gave reproducible results over long periods of time, it must be pointed out that the factors responsible for good chromatograms cannot as yet be fully defined and tanks had to be tested before putting them to routine use. In general, tanks 120 cm in height were much harder to construct than those 60 cm high. Tall tanks in which good runs were not obtained (very slow and irregular solvent movement on the papers) could be corrected by shaking the solvent at the bottom of the tank vigorously and running mobile phase on 15-cm wide papers. It seemed that proper saturation of the tanks was the most important factor.

A roughly three-fold increase in the formazan color intensities was achieved by dipping the papers (before reaction with blue tetrazolium) in 1,2-dichlorobenzene. This treatment also resulted in intense blue colors with much less red pigment than found without the 1,2-dichlorobenzene treatment. Not only was the sensitivity increased with 1,2-dichlorobenzene, but it seemed that in certain crude extracts its use counteracted the effect of inhibitory substances which otherwise did not allow the formazan color to develop. Because of the relatively large solubility of the formazan in this solvent, it would appear that the presence of the 1,2-dichlorobenzene may increase the solubility of the transition state complex and thereby decrease the activation energy of the formation of the fully reduced formazan. The effect of 1,2-dichlorobenzene was also shown by 1,3-dichlorobenzene but not by saturated hydrocarbons, in which the formazan is considerably less soluble. Treating papers with aromatic

hydrocarbons gave a bluer formazan which was, however, appreciably less intense than that obtained following 1,2-dichlorobenzene treatment.

The over-all precision achieved (mean coefficient of variation 10%) was lower than that claimed by Bush and Willoughby (26) or reported by Kittinger (28). The use of the taller tanks in the present study with the longer distances traveled by the steroids may be, in part, responsible for the lower precision achieved because of the greater probability of the formation of imperfections in the zones.

While much remains to be desired in increasing the precision of the method, it has proved to be of sufficient reproducibility for the determination of corticosteroid excretion in individual guinea pigs under a variety of physiological conditions. This is possible because of the even larger variability exhibited by the animals. The results of these and other physiological studies will be reported elsewhere.

SUMMARY

The development of a quantitative paper chromatographic method for the routine determination of reducing $C_{21}O_5$ and $C_{21}O_6$ corticosteroids from guinea pig urine extracts by means of a single chromatographic step and direct densitometry following reaction with blue tetrazolium has been described.

Among a number of paper chromatographic systems tried, the system 1,2-dichloroethane-methanol-water was found to give the most suitable results. The phase diagram of this ternary system was constructed and a number of compositions and other variables affecting the resolution have been studied. An empirical system with a mobile phase consisting of 1–2% methanol in ethylene chloride saturated with H_2O was found to give a good resolution and reproducibility.

Treating the papers with 1,2-dichlorobenzene prior to reaction with blue tetrazolium caused the formation of a much bluer formazan with an absorbancy of roughly three times that obtained without treatment. The all-over precision achieved following cortisol (20–120 μg) deposition on paper, chromatography, and densitometry was on the average 10% (coefficient of variation).

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Determination of Carbonyl Acids Formed upon Periodate Oxidation.[1] I. Assay Procedure

ELLIOT JUNI AND GLORIA A. HEYM

From the Department of Microbiology, Emory University, Atlanta, Georgia

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INTRODUCTION

Periodic acid oxidation of certain organic compounds such as tartrate and gluconate are known to involve the intermediary formation of glyoxylate (1, 2):

$$HO_2C—CHOH—CHOH—CO_2H \xrightarrow{+HIO_4} HO_2C—CHO + CHO—CO_2H$$

$$CH_2OH—CHOH—CHOH—CHOH—CHOH—CO_2H \xrightarrow{+4\ HIO_4} HCHO + 3\ HCO_2H + CHO—CO_2H$$

Under many of the usual conditions employed in these oxidations, glyoxylate is further degraded to formate and CO_2 (3, 4). The present study was the result of a search for a quantitative chemical method for the analysis of microgram quantities of gluconate in biological systems. The successful application of periodate oxidation to this problem necessitated the determination of conditions under which oxidation to carbonyl acids takes place fairly rapidly with little or no further degradation of the newly formed carbonyl acids. Once this was achieved, methods for the removal of excess periodate and subsequent estimation of carbonyl acids were devised.

Glyoxylate is also known to be an intermediate in some of the so-called "overoxidations" which several compounds undergo in the presence of periodate (5). Application of the new procedure to this reaction has made possible the quantitative analysis of certain oligosaccharides.

Reduction of compounds such as hydroxypyruvate and 2-ketogluconate results in products which are oxidized to glyoxylate by periodate, and these materials can therefore be determined by this procedure. Prior reduction also has the advantage of eliminating interference by carbonyl acids present before periodate oxidation. It is consequently possible to

[1] This investigation was supported in part by a grant from the U. S. Public Health Service (E-1545).

determine α-acetolactate in the presence of large excesses of pyruvate and acetoin.

Phosphorylated sugar acids, such as 6-P-gluconate,[2] may also be determined by this procedure.

The method described also provides a means for the kinetic study of the periodate oxidation of compounds for which carbonyl acids occur as intermediates. The procedure used for the estimation of carbonyl acids is a modification of the method of Friedemann and Haugen (6), and can also be employed with some advantage for the routine determination of carbonyl acids usually found in biological systems. A macro method for the detection of glyoxylate resulting from the action of periodic acid on organic compounds has been reported by Fleury and Dizet-Joly (7).

The present paper describes the details of the assay procedure and also outlines a modification of the Friedemann and Haugen method for determination of carbonyl acids in the absence of periodate. Studies dealing with the periodate oxidation of sugar acids, sugar acid lactones, monosaccharides, disaccharides, aldobionic acids, hydroxypyruvate, tartronic semialdehyde, tartrate, and α-acetolactate are presented in a second paper (8).

MATERIALS AND METHODS

Chemicals

The suppliers of the various compounds used were: J. T. Baker, sulfuric acid, ethyl acetate; Distillation Products Industries, thioacetamide, *p*-nitrophenylhydrazine hydrochloride, ethyl acetate; California Corp. for Biochemical Research, thioacetamide; Merck, ethyl acetate; Metal Hydrides, Inc., sodium borohydride; Pfanstiehl Laboratories, Inc., sodium D-gluconate; Nutritional Biochemicals Corp., sodium pyruvate; Matheson Coleman and Bell, thioacetamide; Mallinckrodt, sodium carbonate, ethyl acetate; G. Frederick Smith Chemical Co., paraperiodic acid, sodium meta periodate; Sigma Chemical Co., Tris, "Sigma 7–9" grade, sodium glyoxylate.

Chemical and Physical Methods

Carbonyl acids used as standards were assayed by oxidation with ceric sulfate (9). Absorbancies of solutions of carbonyl acid *p*-nitrophenylhydrazones were measured in a Beckman Model DU Spectrophotometer at 390 mμ using rectangular cells having a 1-cm light path. Ethyl acetate was transferred during several steps of the assay procedure using a

[2] The following abbreviations are used: 6-P-gluconate, 6-phosphogluconate; Tris, tris(hydroxymethyl)aminomethane.

pipettor (Propipette, Instrumentation Associates, New York). Extraction between ethyl acetate and aqueous phases was greatly facilitated by the use of an orbital mixer. The orbital mixer used in these studies was made by mounting an orbital sander (Model 1, Speedway Manufacturing Co., La Grange Park, Ill.) with the rotating platform facing upward. By placing a test tube in an indentation in the center of the rubber mat covering the rotating platform, an excellent mixing action is obtained. A commercially available orbital mixer (Vortex, Jr., Scientific Industries, Inc., Springfield, Mass.) was also found to be satisfactory. A conventional thermostatically controlled electrically heated water bath set at 30°C was used for all incubations.

ASSAY PROCEDURE

Reagents

1. *Sulfuric Acid, 10 N Aqueous Solution.* To 72.2 ml of distilled water is added 27.8 ml of reagent-grade sulfuric acid.

2. *Periodic Acid or Sodium Periodate, 0.1 M Aqueous Solution.* Dissolve 2.28 gm of paraperiodic acid (H_5IO_6) or 2.14 gm of sodium meta periodate ($NaIO_4$) in distilled water to 100 ml. Store in a brown bottle in the dark.

3. *Thioacetamide, 0.867 M Aqueous Solution.* Dissolve 650 mg of thioacetamide in distilled water to 10 ml. Prepare fresh each day.

4. *p-Nitrophenylhydrazine Reagent, 0.1 M Solution in 10 N Sulfuric Acid.* Dissolve 380 mg of *p*-nitrophenylhydrazine hydrochloride in distilled water to 10 ml and filter through Whatman No. 1 filter paper. Add a volume of 20 *N* H_2SO_4 equal to the volume of the filtrate. Prepare fresh each day and store at 25–30°C to prevent crystallization of *p*-nitrophenylhydrazine hydrosulfate.

5. *Ethyl Acetate.* Reagent grade.

6. *Tris, 0.5 M Aqueous Solution.* Dissolve 12.1 gm of Tris in distilled water to 200 ml.

7. *Sodium Borohydride, 1.0 M Aqueous Solution.* Dissolve 380 mg of $NaBH_4$ in distilled water to 10 ml. Filter twice through Whatman No. 1 filter paper. Prepare fresh each day.

8. *Sodium Gluconate Standard, 0.0001 M Aqueous Solution.* Stock 0.1 M solution is prepared by dissolving 1.09 gm of sodium gluconate in distilled water to 50 ml. The 0.0001 *M* solution of sodium gluconate is prepared daily by dilution from the 0.1 *M* stock solution. The stock solution is stored at 3°C.

9. *Sodium Pyruvate Standard, 0.0001 M Aqueous Solution.* Stock 0.1 *M* solution is prepared by dissolving 550 mg of sodium pyruvate in distilled water to 50 ml. The 0.0001 *M* solution of sodium pyruvate is prepared

daily by dilution from the 0.1 *M* stock solution. The stock solution is stored at 3°C.

Method

1. *Periodate Oxidation.* To an 18 × 150 mm Pyrex test tube are added 1.0 ml of 10 *N* H_2SO_4 and exactly 1.0 ml of 0.1 *M* paraperiodic acid or 0.1 *M* sodium meta periodate. The contents are mixed thoroughly. The maximum sample volume employed is 2.0 ml. When smaller samples are used, sufficient water is first placed in the tube to make a total volume of 4.0 ml when the sample is finally added. All samples are run in duplicate and duplicate reagent blanks, containing 2.0 ml of water in place of sample, are always prepared for each assay. For samples giving rise to glyoxylate, sodium gluconate may be used as a standard. Sodium pyruvate is the standard when pyruvate is the carbonyl acid expected after periodate oxidation. After addition of a neutral or slightly alkaline sample, containing 0.02 to 0.2 μmole of a carbonyl acid precursor, the contents of each tube are mixed thoroughly and placed in a 30°C water bath.

2. *Termination of Periodate Oxidation.* After incubation at 30°C for 45 min all tubes are removed from the water bath and exactly 0.5 ml of 0.867 *M* thioacetamide is added to each tube. Immediately after addition of thioacetamide each tube is mixed gently by hand and then allowed to stand at room temperature for 5 to 10 min. The tubes are then mixed individually (orbital mixer) for 30 sec.

3. *Formation of p-Nitrophenylhydrazones.* After all tubes in a given assay are mixed, 0.5 ml of *p*-nitrophenylhydrazine reagent is added to each tube; after mixing again, all tubes are returned to the 30°C water bath.

4. *Extraction of p-Nitrophenylhydrazones from Aqueous Phase.* After incubation for 45 min at 30°C the tubes are removed from the water bath, 5.0 ml of ethyl acetate is added to each tube, followed by mixing (orbital mixer) for 30 sec.[3] As much as possible of the upper ethyl acetate layer, which separates fairly rapidly after mixing is stopped, is removed using a 5.0-ml pipet with the aid of a pipettor, and the ethyl acetate is transferred to a dry 18 × 150 mm Pyrex tube. A pellet, consisting largely of sulfur, usually appears at the water-ethyl acetate interface. Approximately 4.5 ml of ethyl acetate can be removed without contamination by liquid from the aqueous phase. In order to recover most of the carbonyl

[3] In order to avoid possible loss of liquid during mixing, each tube is first covered with an inverted polyethylene thimble (Size No. 2, Cat. No. 9314-F, Arthur H. Thomas Co., Philadelphia), which is kept in position by the index finger of the hand used to hold the tube.

acid derivative still remaining in the small amount of ethyl acetate covering the aqueous phase, 2 ml of ethyl acetate is added to the residual ethyl acetate layer and, without further mixing, this ethyl acetate is removed and placed in the tube containing the bulk of the original ethyl acetate used for extraction. This procedure is repeated once more to make a total of two 2-ml rinses.

5. *Extraction of Carbonyl Acid p-Nitrophenylhydrazones.* To the ethyl acetate extract is added 4.0 ml of 0.5 *M* Tris. The carbonyl acid *p*-nitrophenylhydrazones are extracted into the Tris by mixing (orbital mixer) for 30 sec. After separation of a clear bottom layer of Tris, as much as possible of the upper ethyl acetate layer, which may be somewhat turbid, is removed with a 10-ml pipet (with pipettor) and discarded. The Tris extract is covered with 5 ml of ethyl acetate and washed by mixing (orbital mixer) for 30 sec.

6. *Determination of Concentration of Extracted Carbonyl Acid.* For each tube, approximately 3 ml of Tris extract is removed from beneath the ethyl acetate wash with a 5-ml pipet (with pipettor) and transferred to a 1-cm rectangular absorption cell. The absorbancy of reagent blanks and samples are read at 390 mμ in a Beckman Model DU Spectrophotometer against a water or 0.5 *M* Tris blank. The absorbancies of duplicate tubes, which usually differ by less than 3%, are averaged and subtracted from the average reading for the reagent blanks. As may be seen in Fig. 1, the net absorbancy is a linear function of concentration for compounds that give rise to glyoxylate or pyruvate upon periodate oxidation. Linearity is still maintained using as large a sample as 1.0 μmole of gluconate or 1.0 μmole of pyruvate and diluting the derivative-containing Tris extract ten-fold with 0.5 *M* Tris before determining the absorbancy.

7. *Reduction prior to Periodate Oxidation.* To 1.9 ml of sample in an 18 × 150 mm Pyrex tube is added 0.1 ml of 1.0 *M* sodium borohydride solution. After mixing, all tubes treated with the reducing agent, including reagent blanks, are incubated in a 30°C water bath for 30 min. The tubes are then removed from the water bath and to each is added 1.0 ml of 10 *N* H_2SO_4. After thorough mixing the tubes are incubated at 30°C for 10 min followed by mixing (orbital mixer) for 30 sec. To each tube is then added 1.0 ml of 0.1 *M* paraperiodic acid, or of 0.1 *M* sodium meta periodate. The tubes are mixed thoroughly and incubated at 30°C for 45 min. The remaining procedure is the same as steps 2–6 outlined above.

Destruction of excess borohydride with acid results in lactonization of any sugar acids present. The following procedure is therefore employed, when sugar acids are present, to reduce and delactonize. To a 5-ml sample, containing 0.15–1.5 μmoles of carbonyl acid precursor, is added 0.5 ml of 1.0 *M* sodium borohydride. After mixing, the solution is incubated at

30°C for 30 min. Excess borohydride is then destroyed by addition of 1.0 ml of 1.0 N H_2SO_4. The acidified sample is incubated at 30°C for 10 min followed by mixing (orbital mixer) for 30 sec. Delactonization is accomplished by adding 0.5 ml of 2.5 N NaOH, mixing, and allowing the solution to stand for several minutes. The standard assay (steps 1–6) is made using 1.0 ml of the delactonized solution. The small amount of alkali in this sample does not cause any interference.

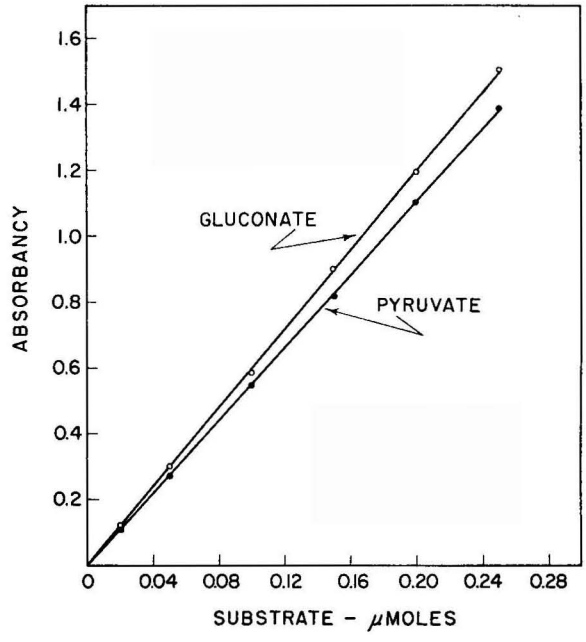

FIG. 1. Absorbancies of carbonyl acid p-nitrophenylhydrazones at 390 mμ as a function of the amount of carbonyl acid, or carbonyl acid precursor, used in the assay. Although no periodate oxidation occurred for the case of pyruvate, the standard assay procedure involving the periodate oxidation step was employed in order to simulate conditions where pyruvate is formed during oxidation of a compound such as α-acetolactate.

8. *Determination of Carbonyl Acids prior to Periodate Oxidation (Modified Friedemann and Haugen Test).* To an 18 × 150 mm Pyrex tube are added 3.0 ml of sample or water (blank) and 1.0 ml of 10 N H_2SO_4, and the contents thoroughly mixed. p-Nitrophenylhydrazones of carbonyl acids are prepared and assayed as outlined in steps 3–6.

STUDY OF VARIABLES IN THE ASSAY PROCEDURE

Acidity of Periodate Oxidation Reaction Mixture. The rates of periodate oxidation of organic compounds and the nature of the resulting

products are profoundly influenced by the pH at which these oxidations take place (10, 11). Periodate oxidations of α-carbonyl acids are known to occur more slowly with decrease in pH of the reaction mixtures (4, 12). Preliminary experiments indicated that oxidation of α-carbonyl acids could virtually be eliminated if the reaction mixtures were acidified with a strong acid such as H_2SO_4. The curves in Fig. 2 show that glyoxylate

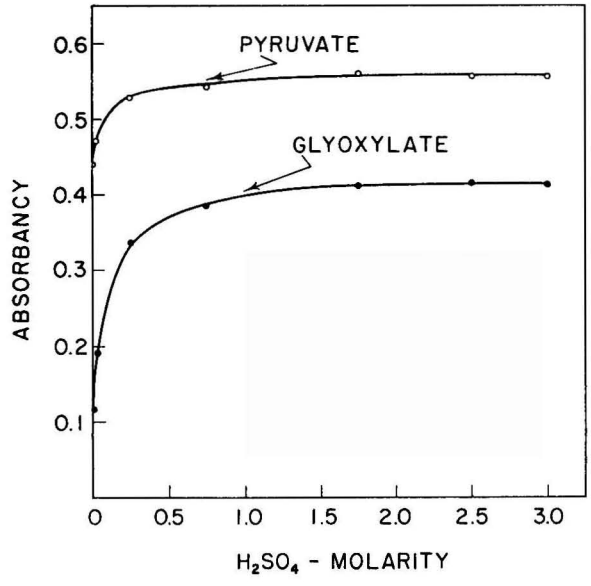

FIG. 2. Recovery of carbonyl acids after incubation for 2 hr in 0.025 *M* paraperiodic acid further acidified with varying amounts of H_2SO_4. The standard assay procedure was altered accordingly using 0.0728 μmole of glyoxylate or 0.1 μmole of pyruvate.

and pyruvate are degraded 72 and 20%, respectively, when incubated in unbuffered 0.025 *M* paraperiodic acid for 2 hr at 30°C. For each of these carbonyl acids, adjusting the acidity range from 1.75 to 3.0 *M* H_2SO_4 resulted in virtually complete recovery of the acid after incubation in 0.025 *M* periodate for 2 hr at 30°C. For glyoxylate the recovery was the same when the H_2SO_4 concentration was varied from 1.75 to 5.0 *M*. In spite of the apparent nonoxidation of glyoxylate at high levels of acidity (Fig. 2) it was found that there was still a small amount of degradation; 2.5% of the glyoxylate was oxidized after 2 hr in acid periodate and 4.8% was degraded when incubation was prolonged for 3 hr. For incubation periods in acid periodate less than 1 hr no oxidation can be detected. By contrast, pyruvate appears to be completely stable in acid periodate for periods as long as 3 hr. The small amount of degradation of glyoxylate

observed after 2 or 3 hr does appear to be due to periodate oxidation. Incubation of glyoxylate with strong acid, in the absence of periodate, for 2- or 3-hr periods does not result in glyoxylate disappearance. An acid concentration of 2.5 *M* H_2SO_4 was chosen for the standard procedure described above. This concentration is sufficiently high to maintain proper acidity even when rather heavily buffered solutions containing carbonyl acid precursors are to be assayed.

pH of Sample to Be Oxidized. Sugar acids form lactones under acid conditions (13, 14). Since certain lactones react differently from the free acids in the assay procedure (8) it is necessary that the sample to be oxidized should first be neutralized or made slightly alkaline in order to hydrolyze any lactone present. Periodate oxidation of sugar acids takes place so rapidly under the conditions of the assay that significant lactonization does not occur when the sample is placed in the acid-periodate solution (8).

Periodate Concentration. The present assay procedure was designed for the determination of approximately 0.05 μmole of carbonyl acid precursor in the presence of 5 μmoles of glucose. Complete periodate oxidation of 5 μmoles of glucose would consume 25 μmoles of periodate, only 25% of the periodate added. The remaining periodate concentration is sufficiently high to oxidize 0.05 to 0.2 μmole of carbonyl acid precursor at very nearly the same rate obtained with the initial periodate concentration (0.025 *M*).

Incubation Temperature. In order to avoid changes in reaction rates due to changes in room temperature, all critical incubations are performed in a water bath at 30°C. This temperature, which is convenient to maintain in a simple water bath, was found to be high enough to give rapid rates of reaction without producing undesirable side effects noted when using higher incubation temperatures during periodate oxidations (10, 11).

Time of Periodate Oxidation. The oxidations of most compounds used in this study were found to be complete in less than 45 min, and this interval was therefore chosen as the standard oxidation time. Exceptions to this rule are discussed for the particular compounds involved (8).

Destruction of Excess Periodate. At the completion of the oxidation period all remaining periodate is removed by reducing it to HI with thioacetamide, a convenient source of H_2S. The reducing agent is oxidized to sulfur, which frequently appears as a pellet made up of many particles. The amount of thioacetamide added is fairly critical. As may be seen in Fig. 3, a slight excess of thioacetamide greater than the 400 μmoles theoretically required to react completely with 100 μmoles of periodate must be added. When less than 425 μmoles of thioacetamide is added there is sufficient residual iodine to interfere with the subsequent reactions.

With more than 460 μmoles of thioacetamide the excess reducing agent reacts with *p*-nitrophenylhydrazine, during the following step of the procedure, to give a product which increases the blank absorbancy at 390 mμ. The blank absorbancy is subtracted from the experimental value obtained to give results which are independent of the particular value of the blank absorbancy. The value of 433 μmoles of thioacetamide used in the standard procedure has been found to give reproducible blanks and also assures that the blank value will remain fairly constant in samples containing large amounts of oxidizable substrates. When larger amounts

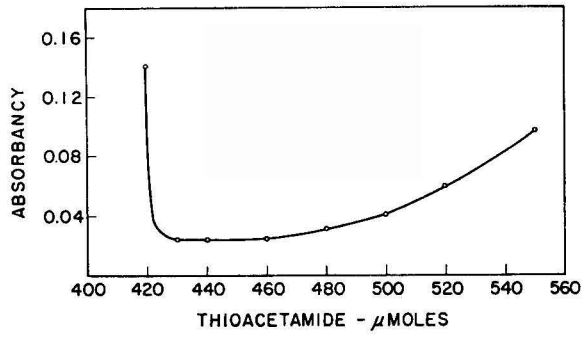

FIG. 3. Effect of the amount of thioacetamide used to destroy excess periodate on the absorbancy of the reagent blank. The standard assay procedure was used except for variation in the amount of thioacetamide.

of substrate are oxidized there remains a greater excess of unreacted thioacetamide due to greater reduction of the initial amount of periodate after oxidation of the substrate. Two commercial brands of thioacetamide have been found to be satisfactory (Distillation Products Industries and California Corp. for Biochemical Research). A third brand tested (Matheson Co.) was found to be yellow instead of white, and gave variable results. Since commercial preparations of thioacetamide are likely to vary in purity, each new bottle should be tested to be certain that low reagent blanks (absorbancy of 0.024 to 0.034) are obtained. High blank absorbancies, usually accompanied by nonmatching duplicates, can be eliminated by increasing slightly the concentration of the particular sample of thioacetamide employed.

When periodate reacts with thioacetamide some of the iodine formed as an intermediate of the reduction combines with sulfur to yield a brown precipitate. This sulfur-iodine complex does not interfere with any of the further steps in the assay procedure. The detailed outline given above for formation of the sulfur pellet has been found to result in fairly uniform and reproducible pellets, although this does not appear to be a crucial factor.

Thioacetamide was selected as the best agent for destruction of excess periodate after fairly extensive testing of existing reducing agents. The chief advantage of thioacetamide for this application appears to be related to the fact that sulfur, the oxidized product of the reaction of thioacetamide with periodate, is insoluble and cannot interfere by oxidizing the *p*-nitrophenylhydrazine reagent used to react with newly formed carbonyl acids.

Choice of Carbonyl Reagent. One of the most widely used methods for the estimation of carbonyl acids involves reaction with 2,4-dinitrophenylhydrazine followed by successive extractions into ethyl acetate and sodium carbonate (6). The density of the color obtained upon addition of strong alkali to part of the carbonate extract is determined colorimetrically. This method was found to be unsatisfactory for the determination of glyoxylate since the color formed upon addition of alkali is unstable and fades rapidly. An alternative procedure, in which the absorbancy of the extracted yellow carbonyl acid 2,4-dinitrophenylhydrazone is determined without addition of alkali (15, 16), seemed more promising. Unfortunately, it was found that 2,4-dinitrophenylhydrazine reacts with formic acid to yield a yellow derivative which is also extracted into sodium carbonate solution. Since the present method was devised to determine carbonyl acid precursors in the presence of sugars, formic acid arising from periodate oxidation of these sugars leads to considerable interference in the assay. The reaction of 2,4-dinitrophenylhydrazine with certain carboxylic acids to form 2,4-dinitrophenylhydrazides has been reported (17). Furthermore, it was found that recovery of carbonyl acids using the 2,4-dinitrophenylhydrazine reagent was considerably inhibited by the presence of the reaction products of periodate and thioacetamide.

A survey of several other carbonyl reagents revealed that *p*-nitrophenylhydrazine was ideally suited for the present purpose. This reagent reacts fairly rapidly with carbonyl acids to form strongly absorbing stable yellow derivatives. The absorption spectra of the *p*-nitrophenylhydrazones of glyoxylate and pyruvate are shown in Fig. 4. Both derivatives have absorption maxima at 390 mμ. The molar extinction coefficients for the derivatives of glyoxylate and pyruvate are 2.46×10^4 and 2.34×10^4, respectively. *p*-Nitrophenylhydrazine does not react with formic acid or acetic acid (8), and the recovery of carbonyl acids is not affected by the presence of the reaction products of periodate and thioacetamide.

The curves in Fig. 5 show the formation with time of the *p*-nitrophenylhydrazone of glyoxylate, derived from gluconate by periodate oxidation, and the *p*-nitrophenylhydrazone of pyruvate in the presence of the reaction products of periodate and thioacetamide. A reaction time of 45 min at 30°C has been chosen as optimal for compounds giving rise to gly-

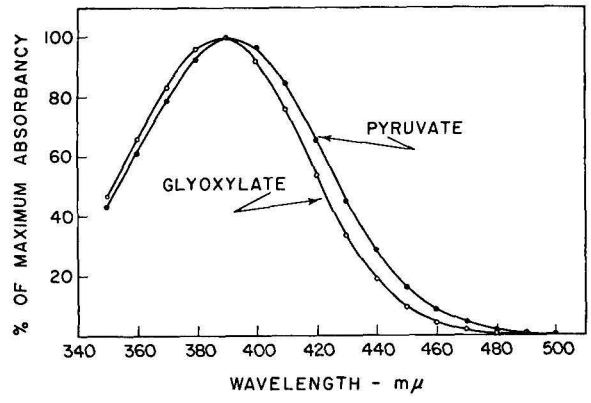

FIG. 4. Absorption spectra of carbonyl acid *p*-nitrophenylhydrazones.

oxylate after periodate oxidation. For compounds giving rise to pyruvate, 30 min at 30°C may be used. Figure 5 also shows reagent blanks obtained using two different concentrations of thioacetamide. It can be seen that the blank absorbancy rises slowly with time of incubation in *p*-nitrophenylhydrazine reagent. For this reason it is essential that reagent blanks

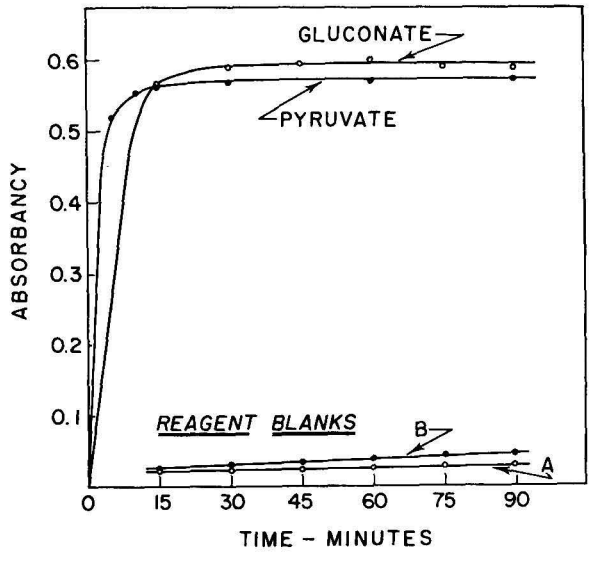

FIG. 5. Formation of carbonyl acid *p*-nitrophenylhydrazones as a function of time of incubation in *p*-nitrophenylhydrazine reagent. Glyoxylate was formed upon periodate oxidation of gluconate (0.1 μmole). Standard assay conditions involving the periodate oxidation step were also used for the case of pyruvate (0.1 μmole). Blanks A and B contained 427 and 450 μmoles of thioacetamide, respectively.

and experimental samples be incubated with the *p*-nitrophenylhydrazine reagent for the same length of time.

Very shortly after preparing an aqueous solution of *p*-nitrophenylhydrazine hydrochloride a red precipitate starts to form. This result, which is probably the formation of an insoluble product of a disproportionation reaction, can be considerably retarded by preparing the acidified reagent described above, in which no precipitate forms for at least a week.

Extraction of p-Nitrophenylhydrazones. Ethyl acetate has been found to be an excellent reagent for extraction of carbonyl acid *p*-nitrophenylhydrazones from the aqueous-acid phase. A single extraction has been shown to result in complete removal of as much as 1.0 μmole of the *p*-nitrophenylhydrazone of glyoxylate or pyruvate. Four different brands of reagent-grade ethyl acetate (Merck, Baker, Mallinckrodt, and Distillation Products) were all shown to be satisfactory.

With a little care, it is possible to remove the bulk of the ethyl acetate extract, as well as the two ethyl acetate rinses, from the aqueous-acid solution without contamination from the aqueous phase. In control experiments it has been shown that accidental removal of 0.05 ml of the aqueous-acid phase along with the ethyl acetate will not affect the final result.

Extraction of *p*-nitrophenylhydrazones into ethyl acetate, as well as subsequent steps in this procedure, have been greatly facilitated by the use of an orbital mixer. It has been shown that a mixing time as short as 15 sec is sufficient for complete extraction of the derivatives into the solvent phase.

The use of two 2-ml ethyl acetate rinses of the aqueous-acid phase, after removal of the bulk of the original ethyl acetate extract, makes it possible to recover approximately 99.6% of the carbonyl acid derivatives formed. Extraction into ethyl acetate has been shown to terminate derivative formation (6).

Selective Extraction of Carbonyl Acid p-Nitrophenylhydrazones. Several mildly alkaline buffers have been tested for ability to extract carbonyl acid *p*-nitrophenylhydrazones from ethyl acetate. For each of the buffers listed in Table 1, a 5-ml sample of ethyl acetate containing 0.1 μmole of glyoxylate *p*-nitrophenylhydrazone was extracted for 30 sec with 4.0 ml of buffer (orbital mixer) and the buffer washed once for 30 sec (orbital mixer) with 5 ml of ethyl acetate. The amount of derivative retained in any buffer was always less than that retained in 0.5 or 1.0 *M* Tris. It has been shown that Tris, in the concentrations employed, completely extracts the *p*-nitrophenylhydrazones of glyoxylate and pyruvate from ethyl acetate. Repeated ethyl acetate washes of each of the buffers

results in a progressive washing out of the derivative as evidenced by a decrease in absorbancy of the aqueous phase. For the case of unbuffered Tris, however, repeated washings with ethyl acetate results in an increase in absorbancy due to concentration of the derivative caused by extraction

TABLE 1

RETENTION OF GLYOXYLATE *p*-NITROPHENYLHYDRAZONE IN VARIOUS BUFFERS AFTER ONE WASH WITH ETHYL ACETATE

Buffer	Per cent of max. absorbancy at 390 mμ[a]
0.5 *M* Tris, unbuffered	100
1.0 *M* Tris, unbuffered	100
1.0 *M* Tris–HCl, pH 9.1	96.5
1.0 *M* Tris–HCl, pH 8.48	93.8
1.0 *M* Tris–HCl, pH 8.15	90.2
1.0 *M* Na_2CO_3	54.0
1.0 *M* Na_2CO_3–$NaHCO_3$, pH 9.78	51.1
1.0 *M* Na_2CO_3–$NaHCO_3$, pH 9.41	42.4
1.0 *M* Na_2CO_3–$NaHCO_3$, pH 9.12	36.8
1.0 *M* Tris: 1.0 *M* Na_2CO_3 (3:1)	86.0
0.5 *M* Na_3PO_4	64.4
1.0 *M* NH_3–NH_4Cl, pH 9.3	67.0

[a] The detailed procedure for determining these values is described in the text.

of small amounts of water by the ethyl acetate washes. When Tris-equilibrated ethyl acetate is used to wash a Tris solution containing glyoxylate *p*-nitrophenylhydrazone, the absorbancy of the Tris solution remains constant after several ethyl acetate washes.

No explanation can be offered at this time to account for this unusual and apparently uniquely efficient behavior of Tris as an extractant of *p*-nitrophenylhydrazones of carbonyl acids. The absorbancy of an aqueous solution of a given concentration of glyoxylate *p*-nitrophenylhydrazone is the same regardless of the nature of the buffer used. The absorbancies of both glyoxylate and pyruvate *p*-nitrophenylhydrazones dissolved in 0.5 or 1.0 *M* Tris remain constant when the solutions are incubated at room temperature for at least 24 hr. Although higher concentrations of Tris are equally effective in extracting carbonyl acid *p*-nitrophenylhydrazones, 0.5 *M* Tris has been chosen for the standard procedure since higher concentrations of Tris result in slightly higher reagent blank absorbancies. Reagent blank absorbancies at 390 mμ using 0.5, 1.0, 2.0, and 3.0 *M* solutions of Tris as extractants in the standard procedure in one experiment were 0.028, 0.030, 0.046, and 0.051, respectively.

When Tris is equilibrated with ethyl acetate some of the solvent dissolves in the aqueous phase to cause a 5–6% increase in volume. As

pointed out above, subsequent washes with ethyl acetate, of ethyl acetate-equilibrated Tris, results in a decrease in volume of the aqueous phase. Since both of these factors come into play during application of the standard assay procedure, it has been found that the absorbancies determined for the derivatives of glyoxylate and pyruvate are approximately 95.5% of the values expected from the molar extinction coefficients for these compounds, assuming that the volume of Tris remains constant during the assay procedure.

Sodium Borohydride Reduction. Samples to be reduced prior to assay are treated with 100 μmoles of $NaBH_4$. Since no more than 5 μmoles of reducing sugar is assumed to be present in any sample, there is always a sufficient excess of reducing agent available. The standard reaction time of 30 min at 30°C has been found to be more than adequate for complete reduction of the carbonyl groups of carbonyl acids and sugars. Reagent blanks containing $NaBH_4$ should also be included in any assay involving prior reduction since the absorbancy of the blank is usually slightly higher when the reducing agent is used. Acidic samples should be neutralized before attempting reduction so that the borohydride is not destroyed.

If sugar acids are present in solutions that have been treated with sodium borohydride, these acids will form lactones during the step involving acidification to destroy excess borohydride. Since various lactones react differently from their corresponding sugar acids in this assay (8), it is necessary to alter the procedure as described in step 7 in order to hydrolyze these lactones before periodate oxidation.

Use of the Procedure as a General Assay for Carbonyl Acids. The application of the assay procedure for analysis of carbonyl acids in the absence of periodate, as described in Step 8, has several advantages over methods involving use of 2,4-dinitrophenylhydrazine as the carbonyl reagent (6). The fact that carboxylic acids such as formate and acetate cause interference in assays using 2,4-dinitrophenylhydrazine, but not when using *p*-nitrophenylhydrazine, has already been discussed. *p*-Nitrophenylhydrazine hydrochloride is considerably more soluble than 2,4-dinitrophenylhydrazine hydrochloride. It is therefore possible to use larger concentrations of the former reagent, a fact that is of considerable advantage when assaying for carbonyl acids in the presence of large excesses of nonacidic carbonyl compounds, which can compete with carbonyl acids for reagent.

Friedemann and Haugen (6) report that ethyl acetate does not completely extract the 2,4-dinitrophenylhydrazone of pyruvate from acid-aqueous solution. In the present procedure, all of the *p*-nitrophenylhydrazones of pyruvate and glyoxylate are extracted into ethyl acetate. The finding reported above, that Tris completely extracts the *p*-nitrophenyl-

hydrazones of pyruvate and glyoxylate, prompted an examination of the possible use of Tris as a final extractant in the Friedemann and Haugen procedure. In marked contrast to the results with sodium carbonate, the use of Tris was found to result in virtually complete extraction of pyruvate 2,4-dinitrophenylhydrazone from ethyl acetate. It has been shown that not all 2,4-dinitrophenylhydrazones of carbonyl acids are extractable with sodium carbonate (18), and also that certain carbonyl acid 2,4-dinitrophenylhydrazones can be easily re-extracted from carbonate solution with organic solvents (19).

It has been customary to determine the concentration of carbonyl acid hydrazones by measuring the absorbancy of the colored solution obtained upon addition of strong alkali (6). Since the color obtained with glyoxylic acid fades whether 2,4-dinitrophenylhydrazine or *p*-nitrophenylhydrazine is used, it has been found more advantageous to determine the absorbancies of solutions of the hydrazones directly without addition of alkali, these derivatives being stable in either carbonate or Tris. This latter procedure has already been employed to estimate the 2,4-dinitrophenylhydrazones of carbonyl acids (15, 16).

As mentioned above, the use of *p*-nitrophenylhydrazine in place of 2,4-dinitrophenylhydrazine also makes it possible to compare directly results of carbonyl acid determinations with and without prior periodate oxidation.

The use of an orbital mixer for extractions between aqueous and organic solvent phases has proved to be extremely efficient and eliminates the need for an aeration mixing apparatus.

Protein Precipitation. Deproteinization of enzyme solutions with trichloroacetic acid, according to Friedemann and Haugen (6), prior to assay was found to cause no interference in the procedure. Proteins do not give rise to carbonyl acids upon periodate oxidation (8) and need not be removed if present in small quantities.

Interfering Compounds. Free carbonyl acids and other compounds that give rise to carbonyl acids upon periodate oxidation will interfere in the assay. Reduction prior to periodate oxidation serves to eliminate interference from free carbonyl acids. A detailed study of other compounds that react in this assay procedure is presented in a second paper (8).

SUMMARY

A method is described for the quantitative analysis of 0.02–0.2 μmole of glyoxylate or pyruvate formed upon periodate oxidation of certain compounds such as gluconic acid and α-acetolactic acid, respectively. Reduction with sodium borohydride, prior to periodate oxidation, eliminates interference from free carbonyl acids and also makes possible the

assay of compounds such as 2-ketogluconate and hydroxypyruvate by converting them to glyoxylate precursors. The procedure developed for the determination of carbonyl acids can be used in the absence of periodate and has several advantages over existing methods for analysis of these acids.

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Determination of Carbonyl Acids Formed upon Periodate Oxidation[1] II. Analysis of Various Carbonyl Acid Precursors

ELLIOT JUNI AND GLORIA A. HEYM

From the Department of Microbiology, Emory University, Atlanta, Georgia

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INTRODUCTION

A method for the quantitative analysis of carbonyl acids produced upon periodate oxidation of certain organic compounds has been reported in a previous paper (1). The present study presents several applications of this assay procedure. Compounds such as sugar acids, sugar acid phosphates, tartronic semialdehyde, glyceric acid, and tartaric acid produce theoretical quantities of glyoxylate when tested by this procedure. Reduction with sodium borohydride, prior to periodate oxidation, makes possible the determination of 2-ketogluconate, hydroxypyruvate and α-acetolactate in the presence of large excesses of carbonyl acids. An unusual action of periodate involves oxidation of active methylene groups (2). Such "overoxidations" take place fairly rapidly under the conditions of the assay employed and lead to the production of glyoxylate. Certain sugar acid lactones yield some glyoxylate due to "overoxidation." For compounds such as 1,4-linked disaccharides "overoxidation" produces exactly one equivalent of glyoxylate. The present procedure can be applied to the determination of these compounds and also provides a new means for the kinetic analysis of various periodate oxidations. The procedure should be of special interest for application to certain compounds of biological significance.

MATERIALS AND METHODS

Chemicals

The suppliers of the various compounds used were: J. T. Baker, sodium formate, lactic acid, formaldehyde; California Corp. for Biochemical Research, D-glucuronolactone, cellobiose, α-D-glucoheptose, 2-deoxy-D-glucose, α-melibiose, α,α-trehalose, 3-*O*-methyl-D-glucopyranose, polygalac-

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turonic acid; Difco Laboratories, D-sorbitol, dextrin; Aldrich Chemical Co., dihydroxyfumaric acid, tartronic acid; Eastern Chemical Corp., sodium α-glycerophosphate; Distillation Products Industries: L(—)-rhamnose, L-malic acid; Mann Research Laboratories, glucono-δ-lactone, α-glucoheptonolactone, glucosaminic acid, D-galactose, D-mannitol, meso-tartaric acid, D,L-threonine; Matheson Coleman and Bell, L(—)-sorbose, D(—)-ribose, sodium alginate; Merck, ascorbic acid, D-glucose, soluble starch, sodium acetate; National Bureau of Standards, D-glucose; Nutritional Biochemicals Corp., monopotassium saccharate, calcium 2-keto-gluconate, calcium cellobionate, sodium isocitrate, pectic acid, chondroitin sulfate, α-ketoglutarate; Pfanstiehl Laboratories, Inc., sodium D-gluconate, D-galactono-γ-lactone, D-gulono-γ-lactone, sodium α-glucoheptonate, calcium D-galactonate, lactose, maltose, D-fructose, dulcitol, L-serine; Schwarz Bioresearch, Inc., glucose-1-P,[2] glucose-6-P, fructose-6-P, ribose-5-P, 3-P-glycerate; Sigma Chemical Co., D-glucuronic acid, 6-P-gluconate, calcium lactobionate, fructose-1,6-P, dextran, grade V, bovine albumin, calcium glycerate, L(+)-tartaric acid, sodium glyoxylate.

Lithium hydroxypyruvate was prepared according to the method of Dickens and Williamson (3) and was crystallized once from water. α-Acetolactate was prepared by hydrolysis of methylacetoxyethylaceto-acetate with sodium hydroxide (4). Lactate was used as the lithium salt (5). The potassium salt of tartronic semialdehyde was prepared according to the procedure of Fukunaga (6). Dihydroxyfumarate was recrystallized before use (7).

Chemical Methods

The assay procedure for determination of carbonyl acids produced as a result of periodate oxidation, before or after reduction with sodium borohydride, is described in a previous paper (1). Solutions of the salts of all sugar acids were neutralized prior to periodate oxidation in order to hydrolyze any possible lactones that might have been present. Lactones of sugar acids were prepared by acidifying with 1 *N* HCl and incubating in a boiling water bath for 5 min. Lactones were converted to the salts of free acids by neutralization with sodium hydroxide to stable end points. α-Acetolactate was determined as acetoin after acid decarboxylation (4). Solutions of cellobionate and lactobionate were standardized by comparison with glucose and galactose standards, respectively, using the

[2] The following abbreviations are used: glucose-1-P, glucose-1-phosphate; glucose-6-P, glucose-6-phosphate; fructose-6-P, fructose-6-phosphate; ribose-5-P, ribose-5-phosphate; 3-P-glycerate, 3-phosphoglycerate; 6-P-gluconate, 6-phosphogluconate; fructose-1,6-P, fructose-1,6-diphosphate; Tris, tris(hydroxymethyl)aminomethane; DPNH, reduced diphosphopyridine nucleotide.

anthrone reagent (8). Hydroxypyruvate was assayed manometrically (9) as well as by spectrophotometric determination of DPNH disappearance in the presence of lactic dehydrogenase (10). Carbonyl acid *p*-nitrophenylhydrazones dissolved in Tris were identified by acidification of the Tris solution in an ice bath, extraction of the hydrazone into ethyl acetate, evaporation of the solvent under a stream of cool air, and paper chromatography of the concentrated derivatives (11).

RESULTS AND DISCUSSION

Periodate Oxidation of Sugar Acids

The appearance of glyoxylate as a function of incubation time in 0.025 *M* periodate has been studied for several aldonic acids. Because of the virtual lack of oxidation of glyoxylate at the pH of these reactions (1) it is to be expected that oxidation of an aldonic acid such as gluconic acid should yield an equimolar quantity of glyoxylate:

$$CH_2OH—CHOH—CHOH—CHOH—CHOH—CO_2H \xrightarrow{+4\ HIO_4} HCHO + 3\ HCO_2H + CHO—CO_2H \quad (a)$$

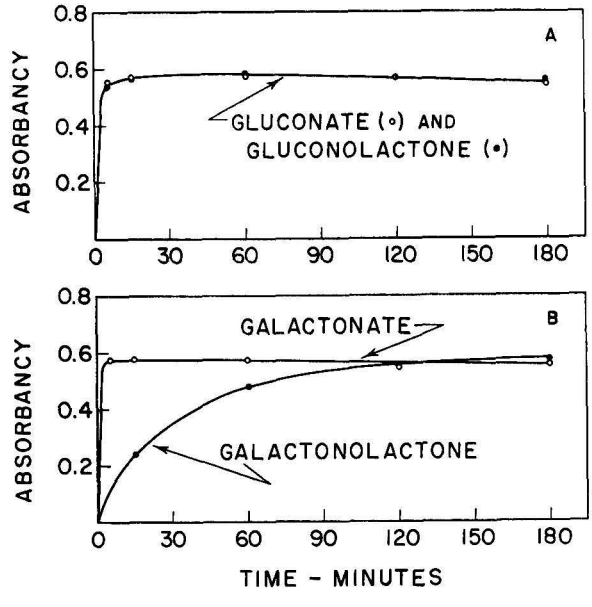


FIG. 1. Appearance of glyoxylate as a function of incubation time of aldonic acids and their lactones in 0.025 *M* periodate. Standard assay conditions were used in all cases except for the variation of time in periodate. (A) Periodate oxidation of 0.1 μmole of sodium gluconate and 0.1 μmole of glucono-δ-lactone. (B) Periodate oxidation of 0.1 μmole of calcium galactonate and 0.1 μmole of γ-galactonolactone.

Time curves depicting glyoxylate formation during periodate oxidation of gluconate and galactonate are shown in Fig. 1. Similar curves for gulonate and glucoheptonate are shown in Fig. 2. For each of these aldonates,

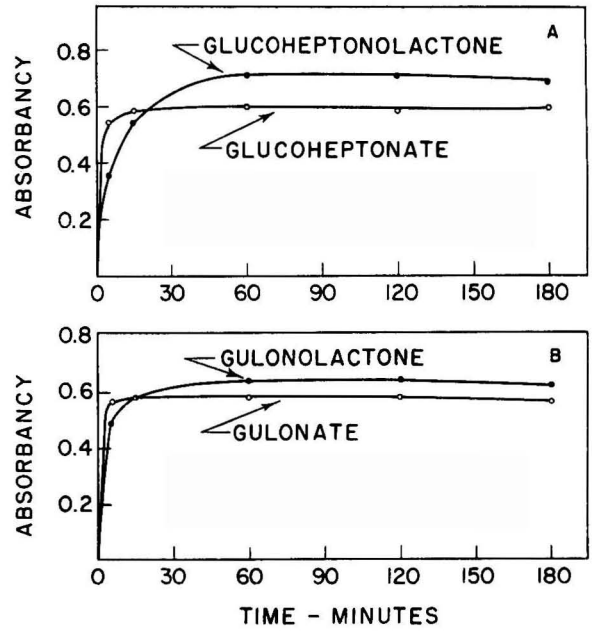
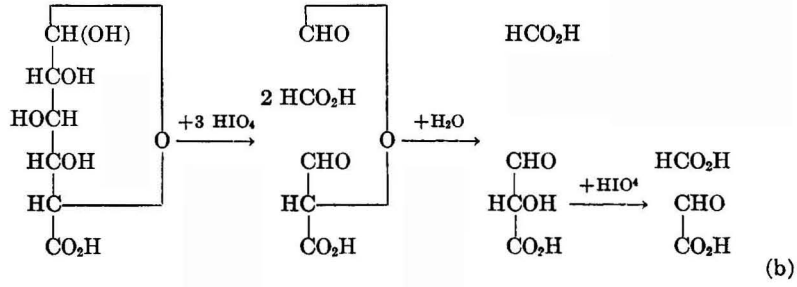


FIG. 2. Appearance of glyoxylate as a function of incubation time of aldonic acids and their lactones in 0.025 *M* periodate. Standard assay conditions were used in all cases except for the variation of time in periodate. (A) Periodate oxidation of 0.1 μmole of sodium glucoheptonate and 0.1 μmole of glucoheptono-γ-lactone. (B) Periodate oxidation of 0.1 μmole of sodium gulonate and 0.1 μmole of γ-gulono-lactone.

oxidation to glyoxylate is complete in 5–15 min. A possible explanation for the slower oxidation of glucuronate to glyoxylate (Fig. 3A) can be given in terms of the rate-limiting step in a sequence of reactions involving oxidative cleavage of the bonds between carbons 1 and 2, carbons



(b)

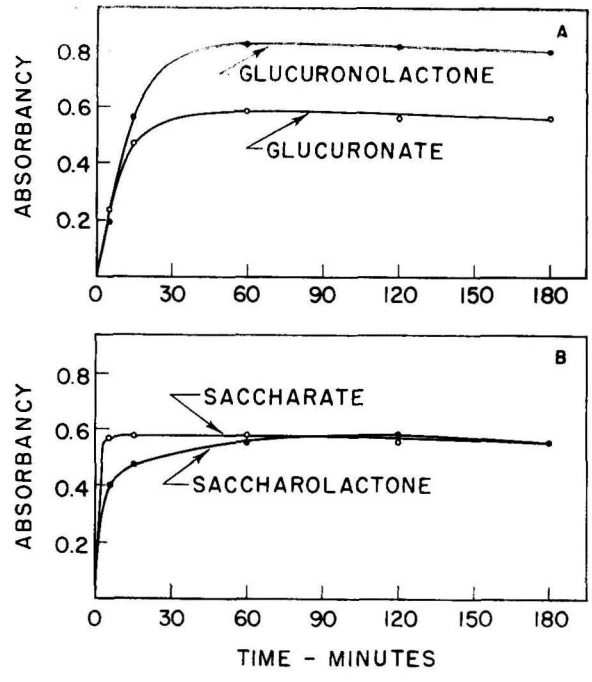


FIG. 3. Appearance of glyoxylate as a function of incubation time of a uronic acid, a dicarboxylic sugar acid, and the lactones of these acids in 0.025 *M* periodate. Standard assay conditions were used in all cases except for the variation of time in periodate. (A) Periodate oxidation of 0.1 μmole of sodium glucuronate and 0.1 μmole of glucuronolactone. (B) Periodate oxidation of 0.05 μmole of sodium potassium saccharate and 0.05 μmole of saccharolactone.

2 and 3, and carbons 3 and 4, hydrolysis of the resultant formate ester, followed by periodate oxidation of the intermediate tartronic semialdehyde (Eq. b).

Periodate oxidation of saccharic acid should yield two moles of glyoxylate per mole of saccharate:

$$HO_2C{-}CHOH{-}CHOH{-}CHOH{-}CHOH{-}CO_2H \xrightarrow{+3\ HIO_4} 2\ CHO{-}CO_2H + 2\ HCO_2H \quad (c)$$

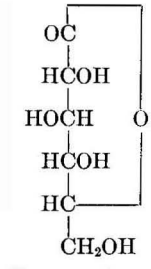
The curve in Fig. 3B shows that saccharate is rapidly oxidized to two moles of glyoxylate.

For all the aldonates studied, the absorbancy of 0.1 μmole of sugar acid, treated by the standard assay procedure, did not differ by more than 2% from a value of 0.585. Application of the method of Hestrin (12) for the assay of aldonic acids has been shown to give different absorbancies for

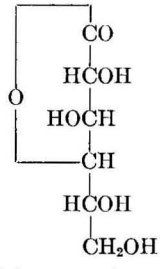
different aldonates (13, 14). The sensitivity of the latter method is considerably lower than that of the present procedure.

Periodate Oxidation of Sugar Acid Lactones

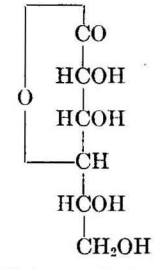
Sugar acids in acid solution form chiefly either δ or γ lactones, depending upon the structure of the particular sugar acid (15). Periodate oxidation of these lactones can be best explained by reference to the reactions with glucono-δ-lactone, galactono-γ-lactone, and gulono-γ-lactone.



Glucono-δ-lactone



Galactono-γ-lactone



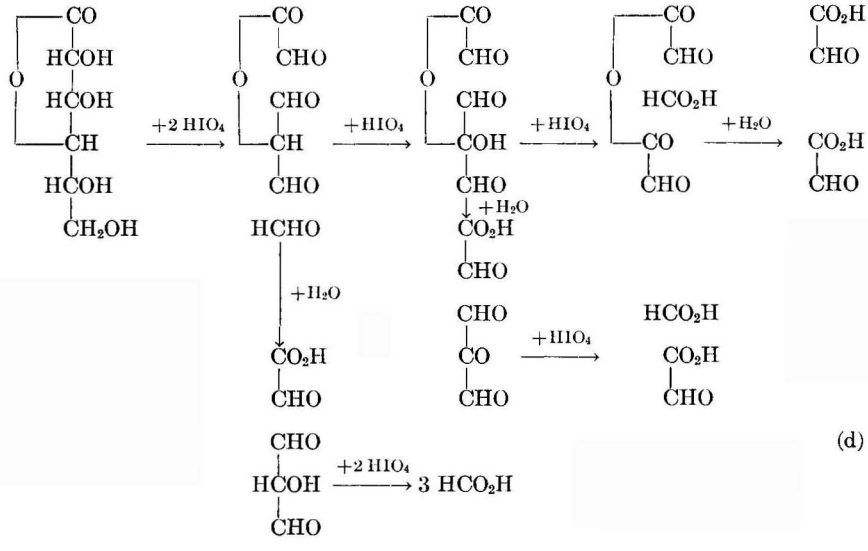
Gulono-γ-lactone

The low rate of oxidation of galactonolactone (Fig. 1B) compared with the rapid rate for gulonolactone (Fig. 2B) can be explained by the fact that formation of the lactone ring results in trans hydroxyl groups at carbons 2 and 3 for galactonolactone in contrast with the corresponding cis hydroxyl groups at carbons 2 and 3 for gulonolactone. It has been shown that for cyclic compounds containing vicinal glycols the structure having *cis* hydroxyl groups is oxidized more rapidly by periodate than is the structure having *trans* hydroxyl groups (16). Under the conditions of acidity employed in the standard assay procedure, in the absence of periodate, half of the galactonate present is lactonized in 3.3 min, all of it being lactonized in 20 min. The fact that galactonate is oxidized quantitatively to glyoxylate in the standard assay serves to emphasize the fact that periodate oxidation to glyoxylate takes place very rapidly.

The presence in gluconolactone of two pairs of vicinal *trans* hydroxyl groups provides a greater opportunity for periodate oxidation of the ring than is possible in the γ-lactones, in which the rings contain only one pair of vicinal hydroxyl groups. As may be seen in Fig. 1A, oxidation of gluconolactone is almost as rapid as oxidation of the free acid.

Oxidation of gulonolactone (Fig. 2B), glucoheptonolactone (Fig. 2A), and glucuronolactone (Fig. 3A) each leads to more than one mole of glyoxylate per mole of lactone. The "excess" obtained with these lactones, over and above the one mole obtained per mole of sugar acid oxidized, can

be explained in terms of an "overoxidation" reaction (2). A scheme for periodate oxidation of gulono-γ-lactone is shown in (d).



(d)

Oxidative cleavage of the bond connecting carbons 2 and 3 and the bond connecting carbons 5 and 6 results in the formation of the glyoxylate ester of malonic dialdehyde. It has been shown (2) that periodate can oxidize certain compounds having an active methylene group. Activation of the methylene hydrogen of malonic dialdehyde ester by the two adjacent aldehyde groups results in oxidation of the central hydrogen to an hydroxyl group. Periodate oxidation of the resultant hydroxymalonic dialdehyde ester gives formate and glyoxylic anhydride, the latter compound hydrolyzing to two moles of glyoxylate.

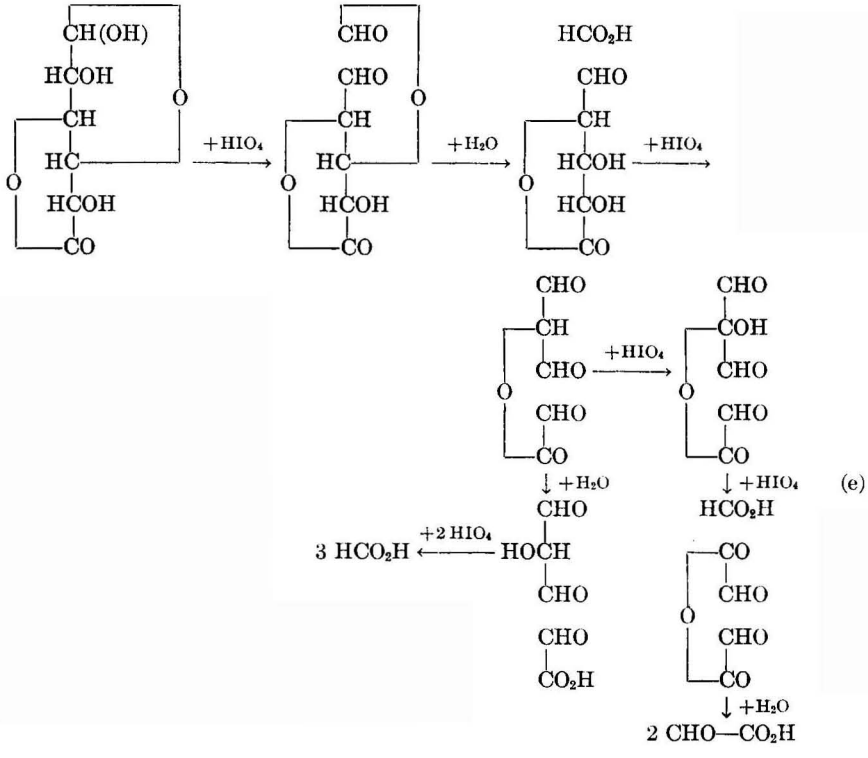
Alternatively, it is possible for hydrolysis to occur prior to the final oxidative step (17, 18) to yield glyoxylate and ketomalonic dialdehyde (mesoxalic dialdehyde), the latter compound being oxidized to formate and glyoxylate. The fact that two moles of glyoxylate are not formed per mole of γ-lactone oxidized provides evidence for the relative instability of the glyoxylate ester of malonic dialdehyde; most of this ester must be hydrolyzed before further oxidation of the methylene group takes place.

When hydrolysis of the glyoxylate ester of malonic dialdehyde occurs before "overoxidation" the resultant tartronic dialdehyde is probably largely oxidized to three moles of formic acid. Since tartronic dialdehyde contains an active hydrogen, it is possible that some "overoxidation" to ketomalonic dialdehyde can take place. Unlike the case for malonic

dialdehyde ester, where oxidation of the active hydrogen must precede further oxidative reactions, tartronic dialdehyde is susceptible to the usual type of periodate oxidation with the formation of three moles of formate. It has been shown, using different conditions of oxidation, that tartronic acid can, in part, be "overoxidized" by periodate (2).

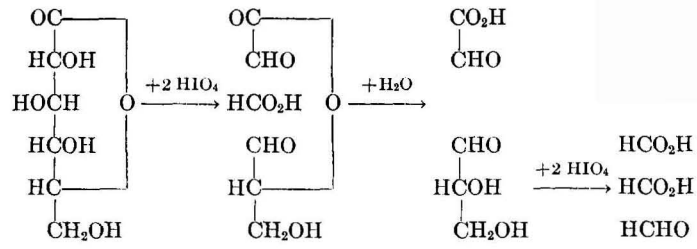
Results from the studies of periodate oxidation of glucuronate, discussed above, have shown that formate esters, as well as glyoxylate esters, are also unstable under the conditions of the present assay. It has been shown that glyoxylate esters (19) and formate esters (20) are stable at pH 3.6 but are rapidly hydrolyzed in alkaline or strongly acid solution.

Periodate oxidation of glucuronolactone (Eq. e) involves initial hydrolysis of a formate ester followed by partial hydrolysis of a glyoxylate



ester. When "overoxidation" takes place before hydrolysis of the glyoxylate ester, further oxidation of the resulting ketomalonic dialdehyde produces glyoxylate, as illustrated in (Eq. d) for gulonolactone.

Since the oxygen in the ring of gluconolactone links carbon 1 with carbon 5, periodate oxidation of this compound does not involve formation of a malonic dialdehyde ester (Eq. f). The curve for appearance of



(f)

glyoxylate from gluconolactone as a function of time in periodate (Fig. 1A) does not show any additional glyoxylate formation over and above the equimolar amount of glyoxylate obtained after periodate oxidation of gluconate. Apparently only one carbonyl group adjacent to a methylene group does not result in sufficient activation to permit "overoxidation" with periodate.

It is theoretically not possible to obtain glyoxylate by "overoxidation" of the lactones of dicarboxylic acids such as saccharic acid. The time curve for oxidation of saccharolactone (Fig. 3B) shows that the final yield of glyoxylate formed during periodate oxidation of this material is the same as for the free acid.

Periodate Oxidation of 6-P-Gluconate, 2-Ketogluconate, and Ascorbate

The data in Table 1 show that 6-P-gluconate reacts in the standard assay in the same manner as gluconate. One of the products of periodate oxidation of 6-P-gluconate should be glycolaldehyde phosphate. Since glycolaldehyde phosphate is a carbonyl acid, it might be expected to cause some interference in the assay. It seems likely, however, that this phosphate ester, which is unstable in acid solution (21), is hydrolyzed before or during derivative formation. α-Glycerophosphate, another precursor of glycolaldehyde phosphate, does not result in derivative formation as determined in the standard assay procedure (Table 1).

Reduction of 2-ketogluconate with sodium borohydride results in a compound which is oxidized by periodate to glyoxylate in a 91.5% yield (Table 1). The apparent failure to obtain complete recovery of reduced 2-ketogluconate as glyoxylate may be due to a lack of purity of the commercial product used. It was shown that varying reduction times from 10 to 30 min did not alter the recovery of glyoxylate from 2-ketogluconate. When 2-ketogluconate was oxidized before reduction there was an approximately 7% yield of glyoxylate (Table 1), possibly due to contaminating gluconate in the preparation. Periodate oxidation of 2-ketogluconate should convert the first two carbons of this compound to oxalate, which does not react in the test.

Substitution of an amino group for the hydroxyl group on carbon 2 of gluconate results in a compound (glucosaminate) which does not yield glyoxylate upon periodate oxidation (Table 1).

TABLE 1
CARBONYL ACIDS FORMED AFTER PERIODATE OXIDATION OF VARIOUS COMPOUNDS

Compound	Amount oxidized (μmoles)	Absorbancy at 390 mμ[a]	Absorbancy at 390 mμ after $NaBH_4$ reduction[a]
Gluconate	0.1	0.585	0.585
6-P-Gluconate	0.1	0.582	
2-Ketogluconate	0.1	0.040	0.534
Glucosaminate	0.1	0	
Hydroxypyruvate	0.1	0.028	0.573
Glycerate	0.1	0.576	
3-P-Glycerate	0.1	0	
α-Glycerophosphate	0.1	0.020	
Pyruvate	0.1	0.564	
Pyruvate	10.0		0.028
Lactate	10.0		0.023
α-Acetolactate	0.1		0.569
Glyoxylate	10.0		0.014
α-Ketoglutarate	10.0		0.045
Serine	1.0	0.002	
Threonine	1.0	0.012	
L-Malate	0.1	0	
Tartronate	0.1	0.019	
Isocitrate	0.1	0	
Dihydroxyfumarate	0.1	0.057	
Formaldehyde	20.0	0	
Formate	50.0	0	
Acetate	50.0	0	

[a] Values determined using standard assay procedure.

The formation of glyoxylate from ascorbate during periodate oxidation occurs relatively slowly (Fig. 4A). The rate-limiting step in this reaction is probably that involving conversion of the enediol to the 3-keto acid, presumably only the latter compound being susceptible to periodate oxidation. An attempt was made to reduce ascorbic acid prior to oxidation. If all the ascorbic acid had been reduced to an aldonic acid it would be expected that periodate oxidation to glyoxylate would be complete in 5–15 min, as was the case for each of the other aldonic acids tested (Figs. 1 and 2). The results with reduced ascorbic acid (Fig. 4A) strongly suggest that the time of incubation with sodium borohydride was not sufficient to

allow complete reduction. The rate-limiting step in reduction, as well as periodate oxidation, of ascorbic acid may be the one concerned with conversion of the enediol to the 3-keto acid. The maximum yield of glyoxylate from ascorbate was approximately 91% after incubation in periodate for 2 hr.

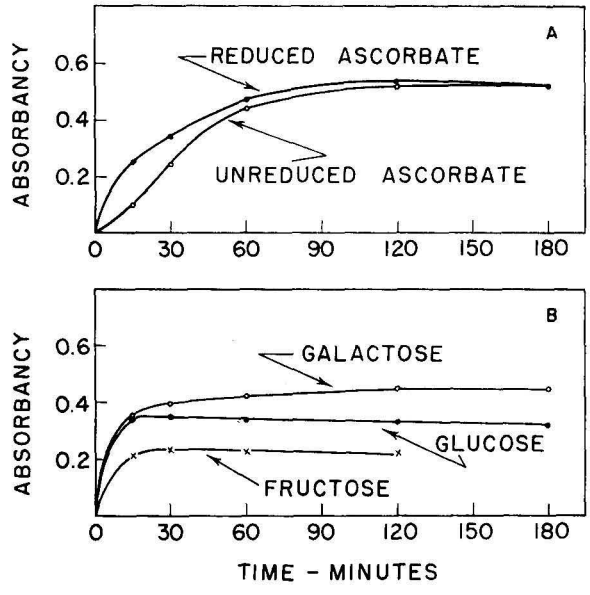
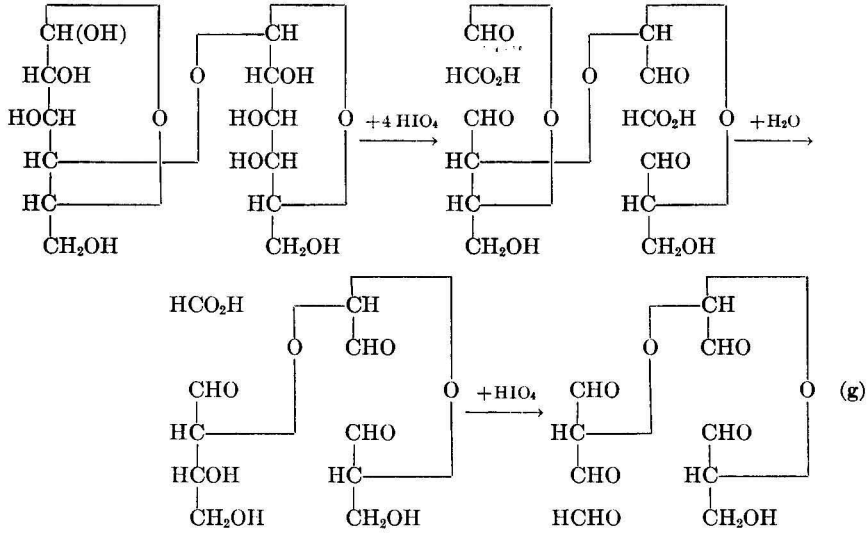


FIG. 4. Appearance of glyoxylate as a function of incubation time of ascorbate and monosaccharides in 0.025 *M* periodate. Standard assay conditions were used except for the variation of time in periodate. (A) Periodate oxidation of 0.1 μmole of ascorbate before and after reduction with sodium borohydride for 30 min. Ascorbic acid was dissolved in equimolar $KHCO_3$ prior to oxidation. (B) Periodate oxidation of 5.0 μmoles of glucose, 5.0 μmoles of galactose, and 0.1 μmole of fructose.

When a sample is reduced with sodium borohydride prior to periodate oxidation, the reaction mixture is acidified following reduction in order to destroy the excess borohydride. Acidification of solutions containing sugar acids results in lactone formation (15). If the sugar acid present during reduction is gluconic acid, no special precautions need be taken to avoid lactonization since gluconolactone will give the same glyoxylate yield as gluconate in the standard assay procedure (Fig. 1A). Variable results will be obtained for the other lactones studied (Figs. 1B, 2, and 3A) and it is therefore necessary to delactonize sugar acid lactones present in solutions that have been acidified. A typical procedure for delactonization is outlined in step 7 of the assay procedure (1).

Periodate Oxidation of Disaccharides and 3-O-Methyl Glucopyranose

It has been shown that complete oxidation of the disaccharide lactose yields nine moles of formate, two moles of formaldehyde, and one mole of CO_2 (22). The appearance of CO_2 in this oxidation indicates probable formation of a malonic dialdehyde intermediate, further oxidation of which should, in the present assay, give rise to one mole of glyoxylate per mole of disaccharide (Eq. g).



Since several oxidative steps, as well as hydrolysis of a formate ester, occur before appearance of glyoxylate, it was expected that a lag would be evident in the curve of glyoxylate formation as a function of incubation time in periodate. The curves obtained for three 1,4-linked disaccharides (Fig. 5A) do indeed exhibit characteristic lags followed by the eventual formation of exactly one mole of glyoxylate per mole of disaccharide. This last result proves that the hemiacetal bond linking the methylene carbon of malonic dialdehyde is fairly stable under the acid conditions employed in this assay procedure. The finding of different characteristic lag periods for glyoxylate appearance from the various disaccharides is especially interesting when the results for maltose and cellobiose are compared. In this case, the only difference between the two sugars is the anomeric character of the glycosidic linkage. Bentley (23) has interpreted the different rates of bromine oxidation of maltose and cellobiose in terms of a conformational analysis.

By contrast, periodate oxidation of 3-*O*-methyl glucopyranose yielded

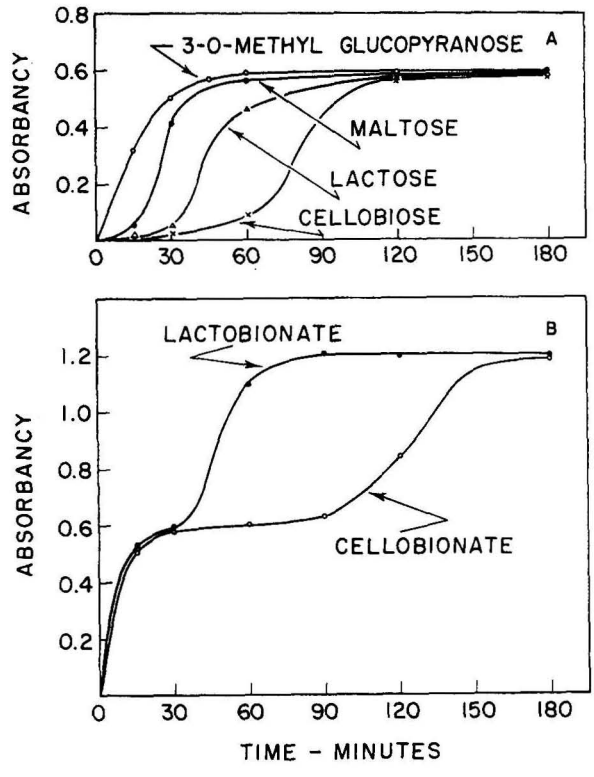
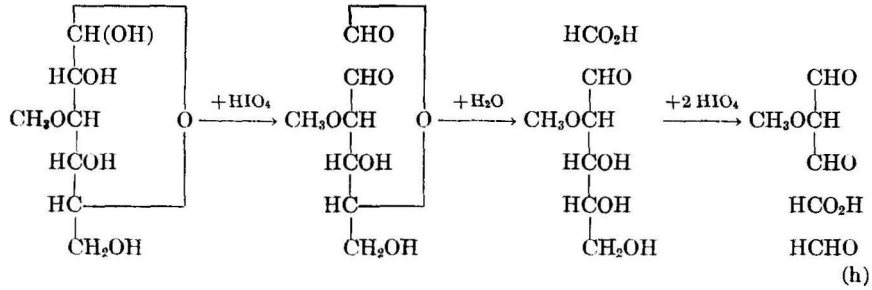


FIG. 5. Appearance of glyoxylate as a function of incubation time of various disaccharides, aldobionic acids, and 3-*O*-methylglucopyranose in 0.025 *M* periodate. Standard assay conditions were used in all cases except for the variation of time in periodate. (A) Periodate oxidation of 0.1 μmole of lactose, maltose, and cellobiose and 0.1 μmole of 3-*O*-methylglucopyranose. (B) Periodate oxidation of 0.1 μmole of cellobionate and 0.1 μmole of lactobionate.

a mole of glyoxylate without a lag (Fig. 5A). The rate-limiting step for this oxidation may be the hydrolysis of a formate ester (Eq. h).



(h)

No glyoxylate could be detected after periodate oxidation of trehalose (1,1-linkage) or melibiose (1,6-linkage) (Table 2). From consideration of the respective structures of various disaccharides, it is expected that

TABLE 2
CARBONYL ACID FORMATION AFTER PERIODATE OXIDATION OF SUGARS AND RELATED COMPOUNDS

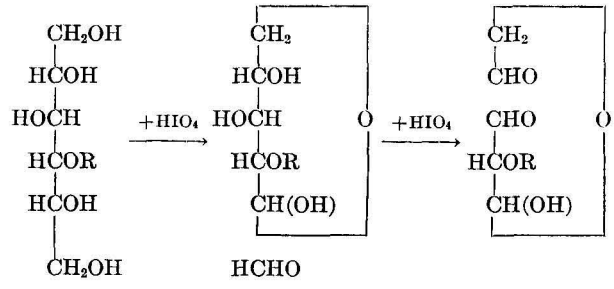
Compound	Amount oxidized (μmoles)	Absorbancy at 390 mμ[a]	Absorbancy at 390 mμ after $NaBH_4$ reduction[a]	Moles carbonyl acid per mole compound tested
Glucose	5.0	0.330		0.0113
Glucose	5.0		0.065	0.0022
Galactose	5.0	0.460		0.0157
Galactose	5.0		0.030	0.0010
Rhamnose	5.0	0.025		0.0009
Ribose	5.0	0.075		0.0026
Ribose	5.0		0.051	0.0017
Fructose	0.1	0.227		0.388
Fructose	0.1		0	0
Fructose	5.0		0.093	0.0032
Sorbose	0.1	0.286		0.489
Lactose	0.1		0.012	0.0205
Maltose	0.1		0.006	0.0102
Cellobiose	0.1		0.005	0.0086
Cellobiose	0.1		0.058[b]	0.0990
Melibiose	0.1	0		0
Trehalose	0.1	0		0
2-Deoxy-D-glucose	0.1	0.024		0.041
Mannitol	5.0	0.063		0.0022
Dulcitol	5.0	0.064		0.0022
Sorbitol	5.0	0.089		0.0030
Glucose-1-P	1.0	0.031		0.0053
Glucose-6-P	1.0	0.107		0.0183
Fructose-6-P	1.0	0.747		0.128
Fructose-1,6-P	1.0	0.186		0.0318
Ribose-5-P	1.0	0.107		0.0183

[a] Values obtained using standard assay procedure except for differences indicated.
[b] Incubation in HIO_4 for 3 hr instead of 45 min.

glyoxylate will be formed upon periodate oxidation of only 1,3- or 1,4-linked disaccharides. It is thus possible to use this assay procedure for linkage studies in disaccharides and oligosaccharides.

When disaccharides are reduced with sodium borohydride prior to periodate oxidation (1), the yield of glyoxylate is considerably reduced (Table 2). This result can be explained if it is assumed that, for most of the reduced disaccharide molecules oxidized by periodate, the first bond ruptured in the reduced hexose unit is the one joining carbons 5 and 6,

and that cyclic hemiacetalization takes place before further oxidation occurs (24) (Eq. i).



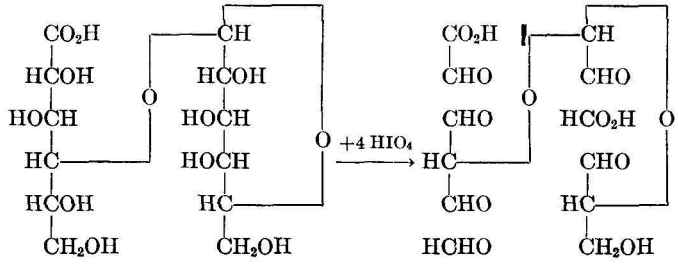
(i)

(R = $C_6H_{11}O_5$)

The stability of the hemiacetal linkage, already demonstrated for the conditions of this assay procedure, make it impossible to obtain the malonic dialdehyde structure required for "overoxidation" to glyoxylate. The small increase in absorbancy for reduced cellobiose upon incubation in periodate for 3 hr (Table 2) may be an indication that the hemiacetal linkage is not completely stable.

Periodate Oxidation of Aldobionic Acids

One mole of a 1,4-linked aldobionic acid should be oxidized to yield two moles of glyoxylate. With lactobionate, for example, it is expected that one mole of glyoxylate, arising from carbons 1 and 2 of the gluconate half of the molecule, will be formed rapidly. Appearance of the second mole of glyoxylate, as a result of "overoxidation" of the malonic dialdehyde intermediate, should be preceded by a time lag for the same reasons a time lag was predicted for the appearance of glyoxylate during periodate oxidation of 1,4-linked disaccharides (Fig. 5A) (Eq. j).



(j)

Time curves for the appearance of glyoxylate during periodate oxidation of lactobionate and cellobionate (Fig. 5B) reveal that one mole of glyoxylate is formed rapidly for each aldobionic acid. The time course for production of the second mole of glyoxylate parallels that found for

the corresponding disaccharides (Fig. 5A). Since the first mole of glyoxylate is completely formed after 30 min in periodate for both aldobionic acids (Fig. 5B), it is evident that the nature of the constituent linked through oxygen to the methylene carbon of malonic dialdehyde must have a profound effect on the further oxidation of the activated hydrogen.

Clancy and Whelan (25) have shown that glyoxylate is stable in the presence of dilute unbuffered sodium metaperiodate. They have taken advantage of this finding to study the nature of the linkages of various aldobionic acids, glyoxylate being expected from the periodate oxidation of 1,4-, 1,5-, or 1,6-linkages but not from 1,2- or 1,3-linked acids. Since recovery of glyoxylate is complete after periodate oxidation of aldobionic acids using the present assay procedure (Fig. 5B), it is possible to differentiate 1,4-linked acids from 1,5- or 1,6-linked samples. The former acids would yield two moles of glyoxylate while the latter acids would be expected to yield only one mole of glyoxylate per mole of aldobionate.

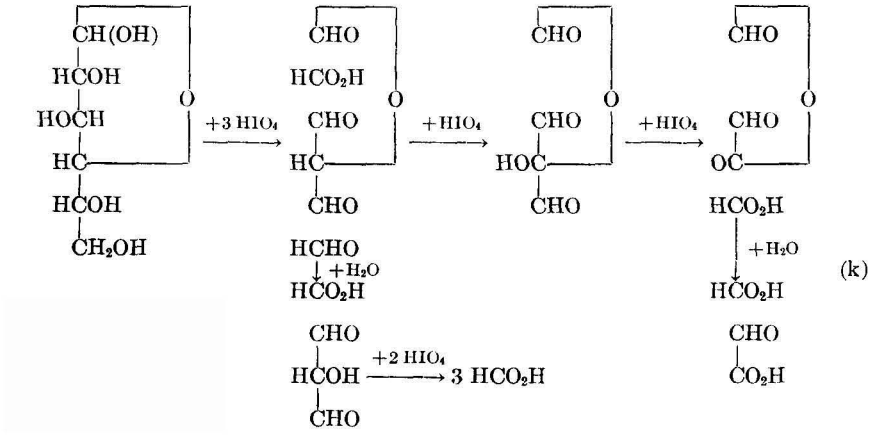
Periodate Oxidation of Sugars and Related Compounds

The data in Table 2 show the amounts of carbonyl acids, calculated as glyoxylate, which are formed after periodate oxidation of various sugars. Although the carbonyl acid yields from sugars are relatively small (1.13% for glucose), these reactions were studied in some detail since the same percentage yield was found for a commercial sample of glucose (Merck, Reagent Grade) as well as for a standard glucose sample prepared by the National Bureau of Standards. Chromatography of the carbonyl acid derivatives derived from both glucose and galactose by periodate oxidation showed these to be *p*-nitrophenylhydrazones of glyoxylate. The small amounts of glyoxylate obtained after periodate oxidation of sugars were shown not to be due to contamination with sugar acids since there was no diminution in glyoxylate yield after several sugars had been neutralized to delactonize and then passed through a Dowex-1 acetate column. This anion-exchange column was shown to remove gluconate.

Appearance of glyoxylate from glucose and galactose as a function of time in periodate is shown in Fig. 4B. It has been reported (26) that reagent-grade glucose contains small quantities of various disaccharides. Since the curves for glyoxylate formation from glucose and galactose as a function of time in periodate (Fig. 4B) do not possess characteristic lags, demonstrated for three 1,4-linked disaccharides (Fig. 5A), it seems likely that the small amounts of glyoxylate formed from these sugars are not due to contaminating disaccharides.

It is possible that glyoxylate may arise from the small proportions of the sugars that exist in furanose forms. Oxidation of glucofuranose, for

example, could yield the formate ester of malonic dialdehyde. Oxidation of the active hydrogen would then give a precursor of glyoxylate (Eq. k).



(k)

Since it has been demonstrated that formate esters are somewhat unstable under the conditions of the assay procedure, it may well be questioned whether hydrolysis of the formate ester takes place before oxidation of the active hydrogen of the malonic dialdehyde ester can occur. It has been shown in (d) that the glyoxylate ester of malonic dialdehyde is largely, but not completely, hydrolyzed before "overoxidation" to glyoxylate can take place. In order to test the relative stability of the formate ester, periodate oxidation of glucoheptose has been studied as a model reaction system. All of the pyranose structure of this seven-carbon sugar should be oxidized by periodate to the formate ester of malonic dialdehyde. It has been shown that approximately 25% of glucoheptose is oxidized rapidly, without a time lag, to glyoxylate. It is therefore possible that the small amount of glyoxylate obtained upon periodate oxidation of hexoses may be due to a partial "overoxidation" reaction involving those sugar molecules in the furanose form.

An alternative explanation should also be considered. During periodate oxidation of hexoses there are three possible ways in which hydroxymalonic dialdehyde (tartronic dialdehyde) may be formed as an intermediate. "Overoxidation" of a small proportion of this intermediate to ketomalonic dialdehyde, similar to the partial "overoxidation" of tartronic acid to ketomalonic acid (mesoxalic acid) (2), would provide a precursor of glyoxylate. On the basis of the data presented, it is not possible to decide the mechanism(s) of glyoxylate formation from sugars. It should be possible to explore this problem further using specifically labeled sugars, since C^{14}-glyoxylate *p*-nitrophenylhydrazone can be iso-

lated and counted. The relatively high glyoxylate yield from 2-deoxyglucose (Table 2) may result from "overoxidation" of malonic dialdehyde formed from the first three carbons by periodate oxidation of the portion of the sugar present in the free aldehyde form.

Periodate oxidation of ketoses is known to produce a mixture of glyoxylate and glycolate, since initially some molecules are cleaved between carbons 1 and 2 while others are cleaved between carbons 2 and 3 (27, 28). The proportions of glyoxylate obtained after periodate oxidation of fructose and sorbose (Table 2) are somewhat smaller than other reported values (27, 28) obtained under different conditions. A curve for the appearance of glyoxylate from fructose as a function of time in periodate is shown in Fig. 4B.

Reduction of hexoses with sodium borohydride (1) results in products which form considerably less glyoxylate upon subsequent periodate oxidation than is the case for the unreduced sugars (Table 2). The amounts of glyoxylate obtained compare with the values determined for several sugar alcohols (Table 2). No definite explanation for these results can be given at the present time. If most of the sugar alcohol molecules are initially cleaved into two three-carbon fragments, the formation of glyoxylate by "overoxidation" is precluded.

The results of periodate oxidation of several sugar phosphate esters are shown in Table 2. The percentage of glucose-1-P converted to glyoxylate is somewhat less than that obtained from glucose. In order to correlate this finding with the proposals made above to account for glyoxylate formation from glucose, it must be assumed that either there is less furanose structure in glucose-1-P than in glucose, or that esterification at position one prevents formation of hydroxymalonic dialdehyde upon periodate oxidation. The glyoxylate yield from glucose-6-P is slightly higher than that from glucose and may be due to contamination with small amounts of ketose. Oxidation of fructose-6-P results in only 33% as much glyoxylate as is obtained by oxidation of fructose (Table 2). Since esterification on the six position of fructose results in a furanose ring having trans hydroxyls, it is expected that oxidation may not be complete after the incubation time in periodate used in the standard assay procedure (45 min). Esterification on the one position, as well as the six position of fructose, should also prevent oxidative cleavage of the bond joining carbons 1 and 2. The very small amount of glyoxylate formed from fructose-1,6-P (Table 2) may be due to some fructose-6-P or free ketose present in the preparation used.

As expected, periodate oxidation of various polysaccharides did not result in very significant glyoxylate yields (Table 3). Only the relatively few free reducing end groups of 1,3- or 1,4-linked polysaccharides would

TABLE 3
CARBONYL ACID FORMATION AFTER PERIODATE OXIDATION OF VARIOUS POLYSACCHARIDES AND A PROTEIN

Material[a]	Absorbancy at 390 mμ[b]	Material[a]	Absorbancy at 390 mμ[b]
Pectic acid	0.043	Dextrin	0.016
Polygalacturonic acid	0.035	Dextran	0.007
Alginic acid	0.002	Chondroitin sulfate	0.012
Soluble starch	0.010	Bovine albumin	0.003

[a] 0.2 mg of each material was oxidized.
[b] Values obtained using standard assay procedure.

be expected to give rise to glyoxylate through "overoxidation." Very little glyoxylate was obtained from the polyuronic acid polymers (Table 3), a result consistent with the structures of these compounds.

Determination of Hydroxypyruvate, Glycerate, α-Acetolactate, and Tartrate

Hydroxypyruvate is readily reduced to glycerate with sodium borohydride. Periodate oxidation of glycerate yields an equimolar amount of glyoxylate (Table 1):

$$CH_2OH—CHOH—COOH \xrightarrow{+HIO_4} HCHO + CHO—CO_2H \quad (1)$$

Reduction of a reaction mixture containing hydroxypyruvate followed by periodate oxidation (1) provides a convenient and fairly specific method for the quantitative estimation of this carbonyl acid in the presence of other carbonyl acids, the latter acids being reduced to hydroxy acids, which react to a negligible extent (Table 1).

The appearance of glyoxylate as a function of time of incubation of reduced hydroxypyruvate in periodate is shown in Fig. 6A. The rate of production of glyoxylate from glycerate is not as fast as the corresponding rates from aldonic acids (Figs. 1 and 2). Under the present assay conditions, small molecules appear to be oxidized at slower rates than larger ones. Complete oxidation of tartrate to glyoxylate is a slow process (Fig. 6B) (28). It is difficult to explain the relatively more rapid rate of oxidation of L(+)-tartrate compared with the rate for mesotartrate.

It has been verified that application of the standard assay procedure preceded by sodium borohydride reduction (1) makes it possible to determine α-acetolactate in the presence of as much as a 100-fold excess of pyruvate, the enzymic precursor of α-acetolactate, as well as a 100-fold excess of acetoin, the decarboxylation product of α-acetolactate. Although α-acetolactate should be oxidized by periodate to pyruvate and acetate, it

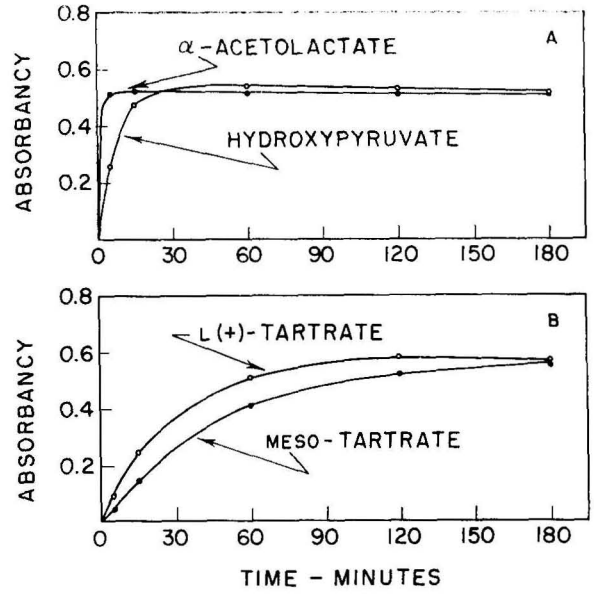
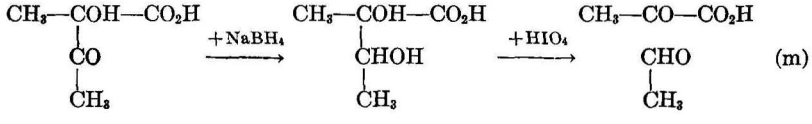


FIG. 6. Appearance of glyoxylate or pyruvate as a function of incubation time of several carbonyl acid precursors in 0.025 *M* periodate. Standard assay conditions were used in all cases except for the variation of time in periodate. (A) Periodate oxidation of reduced hydroxypyruvate (0.0933 μmole) and reduced α-acetolactate (0.0912 μmole). These compounds were reduced prior to periodate oxidation (1). (B) Periodate oxidation of 0.05 μmole of mesotartrate and 0.05 μmole of L(+)-tartrate.

is likely that the acid conditions of the assay procedure would result in decarboxylation of an appreciable amount of α-acetolactate before it could all be oxidized (4). Prior reduction with sodium borohydride (Eq. m) under slightly alkaline conditions (1), where α-acetolactate is stable (4), has the advantage of producing a dihydroxy acid, which should be stable in strong acid, and also serves to reduce any pyruvate present to lactic acid, which reacts to a negligible extent (Table 1). Acetoin is re-



(m)

duced to 2,3-butanediol, which is oxidized by periodate to acetaldehyde and not determined in the assay. Fig. 6A shows the rate of appearance of pyruvate during periodate oxidation of reduced α-acetolactate. Another compound which should give rise to pyruvate upon periodate oxidation is saccharinic acid.

Application of Assay Procedure to Biological Systems

Treatment of various crude microbial cell-free extracts in the standard assay procedure gave small amounts of carbonyl acid derivatives. Such derivatives may be due to the presence of free carbonyl acids, glyceric acid, ketoses, fructose-6-P, aldonic acids, and certain oligosaccharides. Protein does not appear to cause any interference (Table 3). Dialysis of crude extracts should result in removal of all interfering compounds. By using the standard assay procedure it has been possible to study the intermediary accumulation and subsequent utilization of gluconate, formed during oxidation of glucose by certain microorganisms.[3]

The enzyme glyoxylate carboligase (29) catalyzes the conversion of glyoxylate to tartronic semialdehyde (30):

$$2\ \mathrm{CHO{-}CO_2H} \rightarrow \mathrm{CO_2} + \mathrm{CHO{-}CHOH{-}CO_2H} \qquad \text{(n)}$$

It was shown[3] that both chemically and enzymically synthesized samples of tartronic semialdehyde react quantitatively when assayed by the standard procedure. As anticipated, there was no difference in glyoxylate yield when tartronic semialdehyde was reduced with sodium borohydride prior to periodate oxidation. It is thus possible to use this assay procedure to distinguish tartronic semialdehyde from its isomer hydroxypyruvate, since the latter acid will not yield glyoxylate unless it is first reduced to glycerate before periodate oxidation takes place (Table 1).

On the basis of several preliminary tests with biological systems it seems likely that the present assay procedure should have a fairly wide application in this area.

Periodate Oxidation of Other Possible Carbonyl Acid Precursors

Serine and threonine are known to be oxidized by periodate with the intermediary formation of glyoxylate (31, 32). These amino acids are apparently not oxidized under the strongly acidic conditions of this assay (Table 1). Malate and isocitrate, compounds which could possibly be precursors of carbonyl acids, are also not oxidized (Table 1). Since tartronate has been shown to be, in part, "overoxidized" to ketomalonate (2), it was of interest to test for this reaction under the present conditions. The result in Table 1 shows that very little carbonyl acid derivative is formed after incubation in the standard assay. Since ketomalonate forms a *p*-nitrophenylhydrazone which absorbs maximally at 390 mμ, the result obtained for tartronate proves that this compound is neither oxidized nor "overoxidized" under the conditions of the assay procedure.

Dihydroxyfumarate gives very little carbonyl acid when assayed in the

[3] Juni, E. and Heym, G. A., unpublished results.

standard procedure (Table 1). Since dihydroxyfumarate is in equilibrium with oxaloglycolate, it might be expected that oxidation of the latter compound would give glyoxylate:

$$HO_2C{-}COH{=}COH{-}CO_2H \rightleftharpoons HO_2C{-}CO{-}CHOH{-}CO_2H \xrightarrow{+HIO_4} HO_2C{-}CO_2H + CHO{-}CO_2H \quad (o)$$

It has been shown (28, 33), however, that periodate oxidizes dihydroxyfumarate first to diketosuccinate and then to oxalate. When assayed as a carbonyl acid, in the absence of periodate (1), dihydroxyfumarate gives rise to the formation of a *p*-nitrophenylhydrazone that absorbs at 390 mμ, thus verifying the equilibrium between dihydroxyfumarate and oxaloglycolate under the conditions of the assay.

SUMMARY

An analysis has been made of the time course for appearance of carbonyl acids from certain compounds as a function of incubation time in acid periodate. Sugar acids, phosphorylated sugar acids, glycerate, tartronic semialdehyde, and tartrate yield theoretical quantities of glyoxylate upon periodate oxidation by the particular procedure employed. Reduction with sodium borohydride prior to periodate oxidation of 2-ketogluconate, hydroxypyruvate and α-acetolactate makes possible the quantitative determination of these compounds in the presence of large excesses of carbonyl acids. The production of glyoxylate from ketoses has been studied. Disaccharides containing 1,4-linked hexoses are oxidized to one mole of glyoxylate per mole of sugar. Two moles of glyoxylate are obtained per mole of aldobionic acid oxidized. Periodate oxidation of sugar acid lactones has been shown to result in small excesses of glyoxylate formed as a result of an "overoxidation" reaction of periodate. The production of small amounts of glyoxylate during the periodate oxidation of hexoses has been studied. Reduction with sodium borohydride prior to periodate oxidation greatly reduces the glyoxylate yield from sugars. The periodate oxidation of ascorbic acid has been shown to yield an almost theoretical quantity of glyoxylate. Some applications of the assay procedure to biological systems have been discussed.

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ANALYTICAL BIOCHEMISTRY 4, 182–189 (1962)

SHORT COMMUNICATIONS

Fractionation of RNA from Mammalian Cells[1]

Ribonucleic acid (RNA), extracted from mammalian cells with aqueous phenol, contains three major components sedimenting at approximately 34, 18, and 4 *S* in the ultracentrifuge (1). For physical and metabolic studies it is necessary to have a simple means of separating these various components. Recently, Rosenbaum and Brown (1) described a method for rat liver RNA based on precipitation, first with 1 *M* NaCl and then with ethanol, to separate the high molecular weight (h-RNA) components, *S*34 and *S*18, into one fraction, and soluble RNA (s-RNA), *S*4 component, into another. Efficient separation required highly concentrated solutions of RNA. In routinely applying this method to *in vitro* cultures of mammalian cells, and necessarily to limited amounts of material, it became apparent that the s-RNA fraction was frequently contaminated by large amounts of DNA and occasionally by small amounts of h-RNA.

To circumvent these difficulties, a new method of separating h-RNA and s-RNA has been developed based on differential precipitation of RNA components with streptomycin (2, 3). The details of this method and properties of the isolated fractions are described in the present paper. Applications of the method in studies of h-RNA structure (4) and in metabolic studies with isotopically labeled precursors of RNA (5) have been presented elsewhere. Two strains of mammalian cells were used: L strain mouse cells and the L5178-Y strain of mouse ascites tumor cells. Both strains were propagated in suspension and maintained in the logarithmic phase of growth (6, 7). Cells from such cultures were collected by centrifugation at room temperature with the Servall "Szent-Gyorgyi and Blum" continuous-flow centrifuge. Immediately after centrifugation, and without further manipulation, the pellets were frozen in a dry ice-acetone bath to prevent degradation of RNA. This

[1] This investigation was supported by a U. S. P. H. S. research grant E-2454 from the Institute of Allergy and Infectious Diseases, training grant 2G-142, the National Science Foundation grant No. G-6479, American Cancer Society grants No. E-88A and E-149D, and the Samuel S. Fels Fund.

frozen material could be stored for prolonged periods at —70°C without apparent change. Rat liver powder was obtained as previously described (1).

All of the following operations were carried out at 4°C. To a frozen pellet containing 10^9 cells were added 6 ml of water-saturated, redistilled phenol, 4 ml of 0.14 *M* LiCl-0.01 *M* Tris-HCl buffer, at pH 7.6, and 0.1 ml of 1% ethylenediaminetetraacetic acid (EDTA) solution. After shaking vigorously by hand for 2 to 3 min to homogenize the mixture, the phases were separated by centrifugation at $1000 \times g$ for 10 min. The resulting aqueous phase was extracted twice more with phenol in a similar manner, and then extracted three times with ether to remove the residual phenol. Nitrogen gas was then bubbled through the solution to eliminate the ether.

h-RNA was precipitated from this aqueous solution by stepwise addition of a 0.5% solution of streptomycin sulfate. Streptomycin was added until the turbidity, which first appeared, was replaced by a heavy flocculent precipitate; 3 to 5 mg streptomycin/mg h-RNA was required. Any DNA present in the solution would also be precipitated as the DNA-streptomycin complex. This mixture was allowed to stand for 1 to 2 hr and then centrifuged at $6000 \times g$ for 10 min; the supernatant solution was decanted.

The streptomycin-precipitated nucleic acid was resuspended in a volume of 1 *M* NaCl equal to that of the original aqueous phase and vigorously shaken to dissociate the complex (2). By this means, free RNA (Fraction A) was precipitated and any DNA present should have remained in solution. Solid sodium chloride was added to the supernatant solution, resulting from the streptomycin precipitation, to a concentration of 1 *M* to break up any soluble streptomycin-RNA complex. Then exactly 2 vol of 95% ethanol was added to precipitate the RNA (Fraction B). Both fractions were allowed to stand overnight to ensure complete flocculation of RNA, and were centrifuged at $6000 \times g$ for 10 min; the sediments were dissolved in 0.14 *M* LiCl-0.01 *M* Tris-HCl buffer, pH 7.6. The optical densities of these solutions were measured at 260 mμ and converted to RNA concentrations by the factor of 50 μg RNA/ml/optical density unit. Both Fraction A and Fraction B had typical nucleic acid spectra with values of 0.45 to 0.47 for the ratios, optical density at 280 mμ/optical density at 260 mμ.

Two control experiments were done to determine the behavior of cellular DNA during the extraction and fractionation procedure. The DNA of L cells was labeled with C^{14} by the addition of thymidine-2-C^{14} to a growing culture. In the first experiment, RNA was extracted from part of this labeled culture and separated into Fractions A and B as

described in the present paper. Radioactivity was assayed in a Packard Tri-Carb scintillation counter, using the scintillation system described by Bray (8). While there was about 900 μg of DNA in the cells, containing C^{14} equivalent to 10,000 counts per minute (cpm), no measurable C^{14} was found in 440 μg of Fraction A or 190 μg of Fraction B. The 1 *M* NaCl solution obtained from dissociation of the Fraction A-streptomycin complex contained less than 0.1% of the total C^{14} of the cells. Thus, virtually no DNA was extracted from the cells along with the RNA. In the second experiment DNA was extracted from the remainder of the labeled L cell culture by the method of Colter, Brown, and Ellem (9). This DNA, 180 μg (10) containing C^{14} equivalent to 2000 cpm, was added to the RNA extracted from a culture of unlabeled cells. Fraction A (990 μg) and Fraction B (265 μg) were separated and found to contain no C^{14}: all of the radioactivity added with the DNA was recovered in the 1 *M* NaCl solution after dissociation of the Fraction A-streptomycin complex. This experiment showed that, if some DNA were extracted inadvertently from the cells, along with the RNA, it would not appear in either Fraction A or Fraction B.

Sedimentation velocity analyses of the RNA fractions were carried out in the ultracentrifuge as previously described (1). Average sedimentation constants were calculated directly from the densitometer tracings. These analyses showed that, for the various cell types investigated (Table 1),

TABLE 1
FRACTIONATION OF CELLULAR RNA

Source	Sedimentation rates of various RNA components in Svedberg units	
	Fraction A[a]	Fraction B[a]
L cells	34, 18	4
5178-Y ascites tumor cells	32, 23, 18	4
Rat liver	34, 22	4

[a] Generally, the amount of material isolated in Fraction A was about 8 times that of Fraction B.

Fraction A contained all of the RNA sedimenting at rates of 18 *S* or higher (h-RNA) and none of Fraction B. Fraction B contained a single component sedimenting at a rate of 4 *S* (s-RNA), appeared homogeneous, and routinely contained less than 5% of h-RNA. Rat liver s-RNA prepared by this method, and tested with the "pH 5 amino acid activating enzyme system," accepted amino acids with an efficiency comparable to that previously obtained (1).

The use of streptomycin in the described manner, therefore, effects a

clean separation between the h-RNA and s-RNA of mammalian cells. No measurable amounts of DNA are present in either fraction.

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JANET P. HARSHAW
R. A. BROWN
A. F. GRAHAM

The Wistar Institute
Philadelphia, Pennsylvania
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A New Method for the Detection of Thyroxine and Other Iodinated Derivatives on Paper Chromatograms

In connection with the study of metamorphosis of tadpoles it became necessary to develop a method for the detection of thyroxine and other iodinated intermediates in the biosynthesis of thyroxine i.e., iodotyrosines and iodothyronines. Taurog *et al.* (1) have developed a method for detection of some iodinated compounds on chromatograms using sulfanilic acid diazonium chloride as the spraying reagent. Low sensitivity of the diazonium reagent precludes its use in determining stable iodine compounds in small amounts (less than 5 μg) on paper chromatograms. A considerable amount of work has been carried out with the help of paper chromatographic technique and using I^{131}-labeled compounds as tracers (2–5). The short half-life of I^{131} is, however, a great disadvantage. Bowden *et al.* (6) have developed a sensitive method for the detection of thyroxine and related substances by the application of the ceric sulfate-arsenious acid reaction. Modifications of this method have been made by many investigators—Fletcher and Stanley (7), Mandl and Block (8), Bird and Farran (9), Robbins (10), and others. Another extremely sensi-

tive method has been developed by Gmelin and Virtanen (11) using the ferrichloride-ferricyanide-arsenic acid reaction.

Iodine, linked by a covalent bond, does not give a positive test with typical iodine reagents. For example, thyroxine, iodotyrosines, and iodothyronines are negative with starch. A very simple method has been developed by which the iodine of the above-mentioned compounds can be liberated on the paper chromatogram by ultraviolet irradiation and detected by starch and potassium iodate solution. This is a new method for the detection of stable iodine compounds but is not as sensitive as the Ce-As reaction. Solutions of thyroxine, diiodothyronine, triiodothyronine, and diiodotyrosine, procured from L. Light & Co., England, were applied on Whatman No. 1 filter paper (usually 0.01 ml of 0.01 to 0.1% solution). These compounds were not completely soluble at this concentration. Diiodotyrosine went to solution on slight warming and in order to dissolve thyroxine, diiodothyronine, and triiodothyronine the pH had to be adjusted to 8.0 with a few drops of 1 *N* NaOH. The paper chromatograms were then developed with three different solvents in the ascending way for 15 to 18 hr, the composition of the solvents being (*a*) butanol:ethanol:2 *N* ammonia (5:1:2), (*b*) butanol:2 *N* ammonia (5:2), and (*c*) collidine: water:ammonia (cf. ref. 1). After the papers were well dried at room temperature, they were sprayed first with 1% starch solution and then with 1% potassium iodate solution. Potassium iodate can effectively be replaced by potassium bromate. The papers when still wet were irradiated with ultraviolet light from a Chromatolite of Hanovia Ltd., Slough, England, with the filter removed (that is, predominantly with 2537 A). Within 3 to 4 minutes spots with blue color, typical of the starch-iodine reaction, appeared. The positions of the spots were marked immediately, as the color is quite unstable and fades quickly on drying the paper. *Rf* values of the iodinated compounds in different solvents are given in Table I. This table shows that the compounds separated best in butanol:

TABLE I

Rf VALUES OF SOME IODINATED COMPOUNDS

Sample	*Rf* value in			
	Butanol: ethanol: 2 *N* NH_3	Butanol: 2 *N* NH_3	Collidine: water	Collidine: water[a]
Thyroxine	0.51	0.43	0.56	0.58
Diiodothyronine	0.68	0.62	0.68	
Triiodothyronine	0.63	0.58	0.70	
Diiodotyrosine	0.17	0.17	0.19	0.20

Procedure is given in the text.

[a] Figures in this column were calculated from the paper of Taurog *et al.* (3).

ethanol: 2 N ammonia. It may be further noted from the table that there is a good agreement between our R_f values in the collidine: water system with those calculated from the autoradiogram in the publication of Taurog *et al.* (3). This emphasizes that the spots obtained by us are not artifacts and are due to the iodinated compounds applied on the chromatogram.

The method is sensitive enough to detect thyroxine and diiodotyrosine on paper chromatograms in concentrations as low as 0.5 and 0.05 μg, respectively. Preliminary experiments indicate that this method can be used for the detection of thyroxine in the extracts of tadpoles. Further work on the mechanism of this reaction as well as in the detection of different iodinated compounds in tissues and tissue extracts is in progress.

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ASOKE G. DATTA
A. K. MEDDA
J. DUTTA

Department of Chemistry
Bose Institute
Calcutta, India
Received March 7, 1962

Oxygen Flask Combustion in Determination of C^{14} and H^3 in Biological Materials

Recently, Kelly and co-workers (1) introduced a relatively simple and rapid method for the quantitative measurement of radioactive C^{14} and H^3 in biological materials by modification of the Schöniger combustion technique in combination with liquid scintillation counting. The accuracy and reproducibility of this method has been confirmed by us in our pharmacological studies with C^{14}- and H^3-labeled compounds in animals. We now wish to report some modifications we have found to be convenient and economical.

First, the electric filament or induction coil ignition in the oxygen flask was replaced by infrared light ignition using the Thomas-Ogg apparatus.[1] This permits a simple rubber stopper closure of the combustion flask with no electrical connections. The platinum baskets were similar to those described by Kelly *et al.* but construction required only the use of a Bunsen burner, tack hammer, and a pair of hemostats instead of an electric spot welder. A 15-cm length of 18-gage platinum-iridium 10% wire serves as a center support post between the glass rod and the basket for holding the cellulose casing and the sample to be ignited. The perimeter of the basket is made from a 12-cm length of 22-gage platinum-iridium 10% wire shaped into a square. The wire ends are held overlapping by clamping with a pair of hemostatic forceps, heated to a dull red glow in a Bunsen burner flame, and fused with the tap of a tack hammer. The remaining wires for the basket are similarly welded into place. The completed grid is folded across the center horizontal axis to make the basket. We have subjected each basket to more than one hundred ignitions and have observed no deterioration or disconnection of the platinum wires at the "flame" welded spots.

Ordinarily, with the Thomas-Ogg apparatus, rapid ignition of the sample in the closed oxygen flask is initiated by a strip of heat-absorbing black paper. India ink was substituted for the black paper and simultaneously provided a means for numbering the cellulose bags without adding significant weight to the material to be combusted.

Custom glass blowing was avoided by use of ordinary 2-liter heavy-walled filter flasks without side arm reservoirs and stopcocks for delivery of CO_2- and H_2O-absorbing and scintillation-counting solvents.

[1] Arthur H. Thomas Co., Philadelphia, Pa.

Delivery of such solvents was made with a pipet or syringe through a short length of translucent silicone rubber tubing[2] attached to the side arm of the flask (black silicone rubber was unsatisfactory). The tubing is conveniently clamped off with a pair of rubber-sheathed hemostats during combustion and after solvent delivery. The counting efficiencies of our samples have not been lowered by color quenching since the silicone rubber tubing appears to be resistant to leaching by toluene and methanol.

Finally, for CO_2 absorption, instead of the more expensive and less efficient Hyamine hydroxide, 15 ml of phenethylamine (distilled in a flash evaporation apparatus at 10 mm with a water bath at 90°C) solution in the concentration described by Woeller (2) was used. After absorption was complete, 5 ml of stock toluene scintillator solution was added through the side arm as a rinse prior to withdrawing an aliquot for counting C^{14}. The quantitative absorption of $C^{14}O_2$ reported by Woeller was confirmed and our absolute counting efficiency was 64% at 0°C.

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VINCENT T. OLIVERIO
CHARLENE DENHAM
JACK D. DAVIDSON

Clinical Pharmacology and Experimental Therapeutics Section
Medicine Branch
National Cancer Institute
National Institutes of Health
Bethesda, Maryland
Received April 5, 1962

[2] Five millimeter bore × 1.6-mm wall, Esco (Rubber) Ltd., 34–36 Somerford Grove, London, N16, England.

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Separation and Estimation of the Principal Human Urinary 17-Ketosteroids as Trimethylsilyl Ethers

W. J. A. VANDENHEUVEL, B. G. CREECH, AND E. C. HORNING

From the Lipid Research Center, Baylor University College of Medicine, Texas Medical Center, Houston, Texas

Received January 8, 1962

INTRODUCTION

The three principal human urinary 17-ketosteroids may be separated and estimated by gas chromatography through the use of two columns (SE-30 and NGS) (1). Abnormal patterns obtained by this gas chromatographic method have been described (2). This procedure, while superior in speed, resolution, and precision to previous methods, suffers from the disadvantage that two separate chromatographic runs are necessary. The gas chromatographic behavior of the parent steroids and two kinds of derivatives was therefore investigated further with the aim of developing a procedure which would give qualitative and quantitative data in a single run. A method of this kind has the added advantage that abnormal patterns may be compared directly with the normal in terms of each of the three major steroids.

MATERIALS AND METHODS

Relative retention times and quantitative data were obtained with a Barber-Colman Model 10 instrument equipped with a Lovelock argon ionization detection system with a radium foil source. The columns were 6 ft $\times$ 5 mm or 9 ft $\times$ 4 mm glass U-tubes. The preparation of the column packings followed the usual practice of this laboratory. The support was Gas-Chrom P, 100–140 mesh, inactivated with dichlorodimethylsilane. The support (25 gm) was suspended in a 5% solution of dichlorodimethylsilane in toluene (100 ml) in a side-arm filter flask; the pressure was reduced for about one minute through use of a water aspirator, and the flask was swirled gently to dislodge bubbles from the surface of the support. The treated support was removed by filtration and washed with toluene (100 ml), and then with methanol to ensure complete reaction. The support was dried at 80°. Coating was carried out by a filtration technique for phases SE-30 (methyl silicone polymer),

NGS (neopentyl glycol succinate), CNSi (2-cyanoethyl methyl silicone, 65 mole % cyanoethyl) and QF-1-0065 (fluoroalkyl silicone). Phases SE-30, NGS and QF-1 were used at a 1% level; phase CNSi was used at a 2% level. The "flash heater" zone was kept 30–40° above the column temperature, and the detection cell was held at 240°. Gas pressures were 10–20 psi. Samples (1-μl volume) were injected in hexane (trimethylsilyl ethers) or tetrahydrofuran (parent steroids and trifluoracetates). The syringe needle was allowed to remain in the heated tube until the solvent front emerged.

Reference samples of steroids were obtained from commercial sources. Minor impurities were observed in some samples on gas chromatographic examination. Trifluoracetates were prepared as usual (3) by reaction with trifluoracetic anhydride. Trimethylsilyl ethers were prepared by reaction with hexamethyldisilazane (4). The rate of formation of the ethers at room temperature was followed by gas chromatography, and it was found that 2–3 hr was sufficient for complete reaction. [For quantitative work it is essential that conversion to the ether be complete. If incomplete reaction is suspected, or if it is desired to follow the course of the reaction, a gas chromatographic analysis may be made at any time with a CNSi, NGS, or QF-1 phase. Unreacted steroids may be detected at the appropriate point (Table 1) in the analytical separation.]

The sensitivity and linear range of the determination were established by dilution procedures. The precision and accuracy of the method were determined by use of laboratory reference compounds. Weight per cent relationships were obtained by taking the product of the peak height and retention time; this is satisfactory in the present instance (5).

A mixture of reference steroids was converted to trimethylsilyl ether derivatives and subjected to gas chromatographic analysis with a CNSi column under conditions described in Table 1. This experiment was designed to compare the result of a gas chromatographic analytical procedure involving formation of the ethers with an authentic weight relationship of the urinary steroids.

A sample of normal female urine gave the pattern shown in Fig. 2. Sulfate esters were hydrolyzed at pH 1 with continuous ether extraction (6). Glucuronides were hydrolyzed with a commercial glucuronidase preparation (Sigma) of bacterial origin and the free steroids were extracted with chloroform.

For the results described in this paper, a 24-hr urine specimen was collected, and separate 50-ml portions were used in the two hydrolysis procedures. The glucuronide hydrolysis was carried out at pH 6.5 with 80 units of bacterial enzyme for each milliliter of urine. The steroids were extracted with chloroform, and the solvent was removed from the

extract under reduced pressure. The residue was dissolved in ether, and the ether solution was washed with 2 *N* sodium hydroxide solution and with water. The solvent was removed under reduced pressure (acetone was added to remove water). The residue was converted to trimethylsilyl ether derivatives as described previously (4).

A Florosil column may be used for preliminary purification of the steroid mixture (7). This is a rapid and effective procedure, but it is not necessary if the determination is limited to pregnanediol and the major 17-ketosteroids.

RESULTS AND DISCUSSION

The separation of androstane-3α-ol-17-one (I, androsterone), 5β-androstane-3α-ol-17-one (II, etiocholanolone), and 5-androstene-3β-ol-17-one (III, dehydroisoandrosterone) is difficult because of the close

TABLE 1
RELATIVE RETENTION TIMES

Compound	Column			
	CNSi	NGS	QF-1	SE-30
Cholestane	1.00[a]	1.00[b]	1.00[c]	1.00[d]
5β-Androstane-3β-ol-17-one	3.61	3.66	1.70	0.34
Androstane-3α-ol-17-one (I)	3.97	4.02	1.83	0.37
5β-Androstane-3α-ol-17-one (II)	4.19	4.17	1.93	0.34
Androstane-3β-ol-17-one	4.66	4.44	2.12	0.38
5-Androstene-3β-ol-17-one (III)	4.64	4.72	1.84	0.37
Pregnane-3α,20α-diol	5.60	7.25	1.92	0.61
Trimethylsilyl Ethers				
5β-Androstane-3β-ol-17-one	1.07	0.94	1.04	0.37
Androstane-3α-ol-17-one (I)	1.09	0.94	1.03	0.37
5β-Androstane-3α-ol-17-one (II)	1.34	1.22	1.12	0.39
Androstane-3β-ol-17-one	1.62	1.44	1.44	0.48
5-Androstene-3β-ol-17-one (III)	1.57	1.44	1.28	0.46
Pregnane-3α,20α-diol	0.93	0.83	0.96	0.98
Trifluoracetates				
5β-Androstane-3β-ol-17-one	1.74	1.34	1.79	0.25
Androstane-3α-ol-17-one (I)	1.76	1.32	1.78	0.26
5β-Androstane-3α-ol-17-one (II)	1.85	1.52	1.90	0.26
Androstane-3β-ol-17-one	2.15	1.74	2.20	0.31
5-Androstene-3β-ol-17-one (III)	1.97	1.61	1.89	0.29

[a] Cholestane time, 10.1 min; 202°C; 9-ft column.
[b] Cholestane time, 6.3 min; 212°C; 6-ft column.
[c] Cholestane time, 4.1 min; 195°C; 6-ft column.
[d] Cholestane time, 10.6 min; 205°C; 6-ft column.

structural similarity of the three compounds. It is now recognized that retention time relationships found for stereoisomeric hydroxy steroids with selective phases are dependent upon the conformation of the hydroxyl group. The axial isomer is always eluted before the equatorial isomer. It has also been established that 5β-H steroids are always eluted before the corresponding 5α-H steroid with a nonselective phase. The latter relationship may be altered for hydroxy steroids if the separation is carried out with a selective phase. From Table 1 it may be seen that androsterone, I, is eluted after etiocholanolone, II, and after the 5β-H, 3β-ol isomer, when SE-30 is employed; this effect is due to the A/B ring relationship. With selective phases the order of elution is altered. The relative retention time order within this group of compounds is 5β-H,3β-ol (ax.) < 5α-H,3α-ol (ax.) < 5β-H,3α-ol (eq.) < 5α-H,3β-ol (eq.) for all phases with relatively great selective retention properties for hydroxy steroids. The separation problem is more difficult when dehydroisoandrosterone, III, is present. In the absence of any selective effect arising from the presence of the Δ^5 bond, the hydroxyl group retention effect results in a retention time which falls between the extremes observed for the saturated axial and equatorial relatives. When the phase has in addition a selective retention effect for unsaturation, the Δ^5 compound is eluted after the 5α-H,3β-ol (eq.) isomer. This is shown in the data for an NGS column, and it is equally true for a phenyl methyl silicone phase (not shown) (4).

These effects make it difficult to separate I, II, and III with a single column. Two columns may be used as described previously (1). For the present, it is clear that the best opportunity for a separation of all three compounds lies in the use of derivatives, and the table contains data relating to trimethylsilyl ethers (TMSi derivatives) and trifluoracetates (TFA derivatives).

TMSi derivatives were found to be particularly satisfactory. The conversion with hexamethyldisilazane is quantitative, and ketone groups are not affected. The gas chromatographic properties are excellent, the retention times are much shorter than would be expected from the molecular weight, and the response/mass relationship is constant within the group (4, 8). The result of an analysis of reference compounds is shown in Table 2; the gas chromatographic analytical values for a steroid mixture which was converted to trimethylsilyl ether derivatives are compared with the authentic weight relationships.

Figure 1 shows a separation of trimethylsilyl ether derivatives of I, II, and III with a CNSi phase. TFA derivatives were found to be less satisfactory than TMSi derivatives for the separation of I, II, and III.

The excellent separation found with a CNSi column suggested that

TABLE 2
ANALYSIS OF 17-KETOSTEROIDS AS TRIMETHYLSILYL ETHERS

Compound	Weight per cent	Found[a]
Androstane-3α-ol-17-one	29.4	29.0 ± 0.3
5β-Androstane-3α-ol-17-one	31.7	32.1 ± 0.1
5-Androstene-3β-ol-17-one	38.9	38.9 ± 0.2

[a] After conversion to trimethylsilyl ethers. The value given is the mean of five gas chromatographic determinations.

this condition might be quite satisfactory for both research and routine purposes in many studies of urinary 17-ketosteroid patterns. By a dilution procedure it was found that the lower limit of sensitivity for these derivatives was about 0.01 μg, and that the upper limit of the linear range, with a separation period of about 10–12 min for all three compounds, was about 10 μg for each component. This value is likely to vary greatly with different detection systems and with different conditions, and it should not be regarded as a fixed value for the method. The precision was found to be about ±0.5% (Table 2).

The presence of C-21 steroids, particularly pregnanediol, leads to an

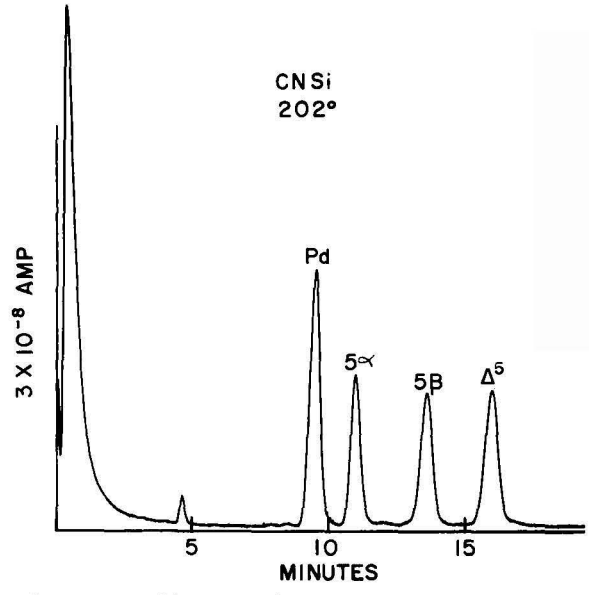

FIG. 1. Gas chromatographic separation of a mixture of pregnanediol di-TMSi (Pd), androsterone TMSi (5α), etiocholanolone TMSi (5β), and dehydroisoandrosterone TMSi (Δ^5). Column conditions same as in Table 1 (2% CNSi column, 9 ft × 4 mm U-tube).

additional separation problem in human studies. The data in the table indicate that a CNSi or NGS phase should be used when pregnanediol is present, and Figs. 1 and 2A show a separation of the TMSi derivatives of

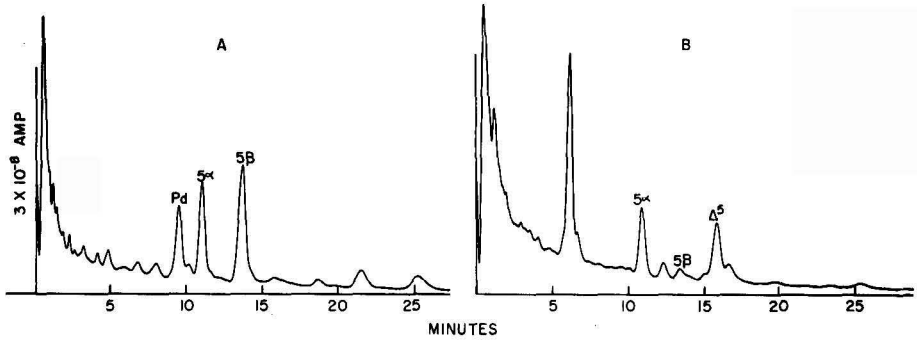

FIG. 2. Gas chromatographic separations of trimethylsilyl ethers of urinary 17-ketosteroids and of pregnanediol from a normal female. Panel A is from a glucuronidase hydrolysis and represents glucuronic acid-conjugated steroids; Panel B is from pH 1 hydrolysis with continuous ether extraction, and represents sulfate-conjugated steroids. The compounds are identified as before. The identity of other components in the mixture has not been established, and compounds other than steroids may be present. The CNSi column used here was the same as that employed for Fig. 1; the conditions were the same as those described in Table 1.

I, II, III, and pregnanediol. This procedure may be used for estimating pregnanediol along with the 17-ketosteroids.

Figure 2 shows an application of the method to a normal female. The steroid glucuronides and sulfates were hydrolyzed separately, and individual patterns are shown for each group. Dehydroisoandrosterone was found only as a sulfate-conjugated steroid, in accordance with previous work (6). Abnormal patterns may easily be compared with the normal for all three steroids.

SUMMARY

A method for the separation and estimation of androsterone, etiocholanolone, and dehydroisoandrosterone by gas chromatographic techniques is described. The separation is carried out through the use of trimethylsilyl ether derivatives. Phases which may be used include cyanoethyl methyl silicone and neopentyl glycol succinate. Pregnanediol may be estimated at the same time.

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We are indebted to Dr. Arthur Martellock of General Electric Co. for a sample of CNSi liquid phase (65 mole % cyanoethyl substituted). We should also like to

thank General Electric Co. for silicone polymer SE-30, and Dow Corning Corp. for polymer QF-1-0065. We are indebted to Dr. Joseph Corse of the Western Regional Research and Utilization Laboratory for a sample of neopentyl glycol succinate.

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Quantitative Determination of 3,7,12-Triketocholanic Acid in Biological Fluids by Gas-Liquid Chromatography[1]

ROBERT I. ELLIN,[2] ALBERT I. MENDELOFF, AND DAVID A. TURNER

From the Biochemistry Research Division, Department of Medicine, Sinai Hospital, Baltimore, Maryland

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INTRODUCTION

Bile acids have been used therapeutically for many years. In addition to those conditions in which they replace deficiencies of bile, they have been used nonspecifically for various digestive malfunctions. The bile acid, 3,7,12-triketocholanic acid, commonly known as Decholin,[3] has been shown to increase the volume of bile flow after injection into the systemic circulation, the portal vein, or the hepatic artery and may be useful in the investigation of bile formation. Hepatic blood flow is known to be increased only slightly after the intravenous injection of Decholin and apparently contributes little to the observed increase in the volume of bile.

There has been no practical method for the determination of microquantities of 3,7,12-triketocholanic acid in biological fluids, and present knowledge of its function is based on indirect evidence. Recent advances in the study of the formation of bile and its constituents by liver cells has reawakened interest in precise quantitative determination of the processes involved. A reliable method for the separation and analysis of 3,7,12-triketocholanic acid in biological material would make possible better understanding of the biological function of this material.

Methods have been devised in this laboratory for the analysis of cholanic acid derivatives, using gas-liquid phase chromatography (1). The present paper is concerned with the application of these techniques

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[2] Trainee, N. H. I. Training Program HTS-5399. Permanent address: United States Research and Development Laboratories, Directorate of Medical Research, Physiological Chemistry Branch, Army Chemical Center, Maryland.

[3] Ames Company, Inc., Elkhart, Indiana.

to a study of the changes in concentration of free triketocholanic acid in blood and bile following the intravenous injection of the sodium salt of triketocholanic acid in humans.

METHODS

A simple quantitative extraction technique was a necessary first step in the development of a useful analytical method employing gas-liquid chromatography (GLC). The GLC analysis of bile acids in biological material is much more difficult than the analysis of pure compounds, due to other interfering substances. Thus the removal of interfering material with similar retention time was essential. With the techniques described here, the quantitative extraction and measurement of 3,7,12-triketocholanic acid in blood and bile has been achieved.

3,7,12-Triketocholanic Acid in Plasma

One ml of plasma was diluted with 1 ml of distilled water and the pH adjusted to 1 with 8 *N* HCl. Three successive extractions of the sample were performed using 8-ml volumes of ethyl ether saturated with water. An ethereal solution of diazomethane was added in excess to the ether extract until the solution appeared yellow. Methylation of the free bile acid was usually assured in 30 min.

The solution containing the methyl 3,7,12-triketocholanate was evaporated to dryness under nitrogen. The remaining residue was extracted with three 0.5-ml portions of acetone. This was transferred to a graduated test tube, and evaporated to a convenient volume. Aliquots were then taken for injection into the GLC column, using a Hamilton microsyringe (10-μl capacity). The concentration of solution was usually such that 1–2 μl injections were made.

TABLE 1

QUANTITATIVE RECOVERY OF STANDARD 3,7,12-TRIKETOCHOLANIC ACID SOLUTIONS ALONE OR WHEN ADDED TO HUMAN BLOOD PLASMA

Sample	3,7,12-Triketocholanic acid (mg/ml)	Vol. of extract injected (μl)	Area of peak (mm^2)
Standards	3.75	2	61
	7.50	1	65
	7.50	2	123
Plasma	3.75	2	63

Table 1 shows the quantitative recovery of 3,7,12-triketocholanic acid added to plasma and extracted by the procedure described. Triangulation of the peak areas in successive analyses using either aliquots of stand-

ards or replicate samples of plasma showed the quantitative error to be in the range of ±5%.

3,7,12-Triketocholanic Acid in Bile

Equal volumes (10 ml) of bile and water were mixed in a separatory funnel. The solution was acidified to approximately pH 1 with 8 *N* HCl. Fifteen ml of water-saturated ethyl ether was then added to the separatory funnel, which was shaken. After the acidification, a precipitate occurred which formed a layer at the ether-water interface. The aqueous layer was removed and twice reextracted with 15 ml volumes of ethyl ether. The ether extracts were pooled and shaken in the original separatory funnel containing the precipitate, in order to extract any residual bile acid from the precipitate.

The free bile acid was methylated using an ethereal solution of diazomethane as previously described. After methylation, the solution was first dried under nitrogen, then redissolved in 2 ml acetone, from which samples were taken for analysis.

Gas-Liquid Phase Chromatography

The analyses were performed with a commercial chromatograph using a 6-ft, 4-mm i.d. U-shaped glass column. The support was Gas-Chrom P, 100–120 mesh, coated with 0.5% of a new nitrile polysiloxane liquid phase (No. 287-108-949)[4] consisting of 50 mole % methyl-β-cyanoethylsiloxane and 50 mole % dimethyl-β-siloxane. A Lovelock argon ionization cell equipped with a 20-mc strontium-90 foil was used for detection.

RESULTS

The technique was applied to the analysis of free 3,7,12-triketocholanic acid in human blood following the administration of 1 gm of the sodium salt (as 5 ml of a 20% solution) by intravenous injection into the antecubital vein. An indwelling needle, placed in the vein of the opposite arm, was used for obtaining the blood samples at various time intervals after injection. Figure 1 shows the rate of disappearance of 3,7,12-triketocholanic acid from the plasma in three subjects.

Each of the three curves demonstrates the decrease in concentration of 3,7,12-triketocholanic acid over a period of 20 min. The fall in the concentration of the bile acid was rapid between 0 and 8 min, after which the rate gradually decreased. The serial values shown between 2 and 8 min demonstrate the characteristics of a pseudo first-order disap-

[4] The liquid phase was made by Dr. A. C. Martellock of Silicone Products Department, General Electric Company, Waterford, New York.

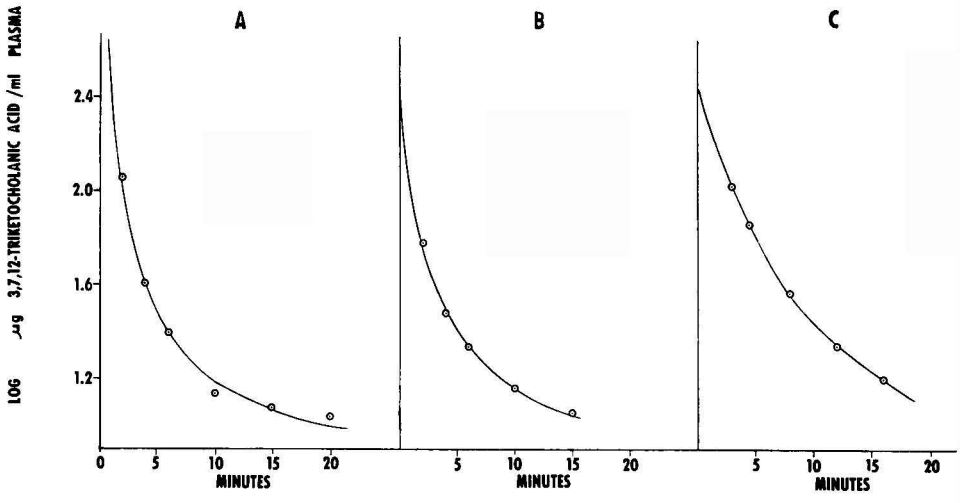

FIG. 1. Disappearance of 3,7,12-triketocholanic acid from blood plasma in three patients.

pearance rate, i.e., 0.35, 0.30, and 0.23 min^{-1}, respectively. The average value for the disappearance rate of 3,7,12-triketocholanic acid under these circumstances is 0.29 min^{-1}.

DISCUSSION

The lack of quantitatively reliable and convenient analytical methods has been a major obstacle in the elucidation of the chemistry and physiology of the bile acids in man. Many details in the metabolism of the bile acids in various species have been carefully studied by Bergström (2) and his colleagues in Sweden. The analytical techniques used by most workers were recently critically discussed by Levin *et al.* (3). The present method provides an accurate technique for the rapid qualitative and quantitative determinations of 3,7,12-triketocholanic acid in amounts as little as 0.075 μg in plasma. However, this is a relative figure which may vary with the conditions of sensitivity of the gas-liquid chromatography instrument.

The direct extraction of bile acids from tissue preparatory to GLC analysis presented two major obstacles. First, both bile acids and other steroid compounds which normally occur in greater concentration are extracted together with solvents commonly employed. If the latter group have similar retention values to those of the bile acids, the analysis of either group of compounds is difficult. Second, because of the highly polar nature of certain bile acids, the retention times using liquid phases

such as Apiezon and the common silicones are too long to be practical. Under these conditions, severe tailing and asymmetry of peaks is commonly observed and prevents accurate triangulation of the peak area.

The particular liquid phase and conditions employed with these columns allowed for the rapid elution of methyl 3,7,12-triketocholanate. Most interfering steroids which might have been extracted, with the exception of cholesterol, were eluted along with the solvent and did not interfere with the 3,7,12-triketocholanate peak, which was usually sharp and symmetrical. No evidence of decomposition of 3,7,12-triketocholanate was observed during these experiments.

Ethyl ether was the solvent of choice in the extraction procedure because it was shown that, using the methods described, the 3,7,12-triketocholanic acid was quantitatively extracted along with a minimum concentration of other contaminating plasma and bile constituents.

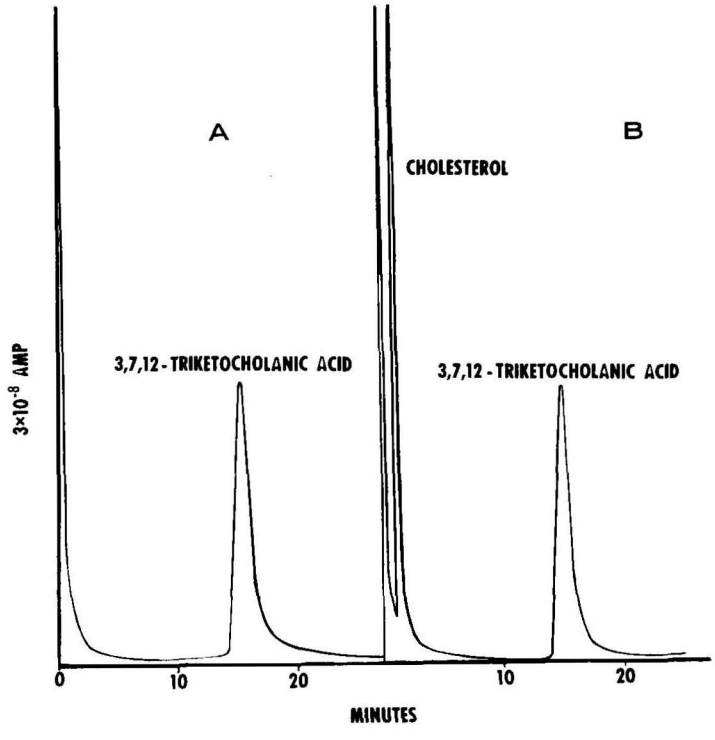

FIG. 2. Gas-liquid chromatography of (A) standard methyl 3,7,12-triketocholanate and (B) human fistula bile extract, collected for 12 hr after intravenous injection of 3,7,12-triketocholanic acid. Conditions: 6-ft column, 4 mm i.d., 100–120 mesh Gas-Chrom P, coated with 0.5% nitrile polysiloxane. Inlet pressure, 25 psi; flow rate 140 ml/min; column temperature, 228°C.

Preliminary extraction with petroleum ether only resulted in the extraction of larger quantities of biological impurities.

The high recovery of unconjugated 3,7,12-triketocholanic acid in human fistula bile following its intravenous administration was surprising. Approximately 40% recovery was obtained in a 12-hr collection. The finding of free 3,7,12-triketocholanic acid in human fistula bile (Fig. 2B) has prompted further study since this was not expected in the absence of the intestinal flora. Bacterial contamination in the T-tube and collecting vessel may have resulted in hydrolysis of a 3,7,12-triketocholanic acid conjugate. The analysis of bile and plasma from patients not receiving 3,7,12-triketocholanic acid failed to reveal the presence of this material.

SUMMARY

The quantitative measurement of 3,7,12-triketocholanic acid in plasma and bile has been achieved using gas-liquid chromatography. A new polysiloxane liquid phase permitted rapid elution of the bile acid with minimal tailing. The peak areas were linear in all concentrations employed.

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ANALYTICAL BIOCHEMISTRY 4, 204–212 (1962)

The Quantitative Analysis of Fecal Neutral Sterols by Gas-Liquid Chromatography[1]

W. W. WELLS[2] AND M. MAKITA

From the Biochemistry Department, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania

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Physiological and nutritional studies of sterol metabolism often require the accurate analysis of fecal neutral sterols (1–4). Most of the commonly employed methods of sterol analysis have been found to be inadequate when applied to the complicated mixture of isomeric sterols of fecal origin (5). For example, difficulties arise from the fact that these sterols respond unequally to most reagents of colorimetric or spectrophotometric methods (6, 7), and that not all sterols (chiefly coprostanol) are completely precipitated by digitonin (8).

The successful application of gas-liquid chromatography to the steroid field (9–11) provided the opportunity for the precise measurement of individual sterols with a minimum of error from contaminants. Several attempts have been made to analyze sterols of biological interest by gas-liquid chromatography (12, 13). In the case of fecal sterols (13) the best separation factor between cholesterol and coprostanol has been approximately 0.9. Improved separation of closely similar sterols has been achieved by the use of nonpolar derivatives and polar supports (14–16). Recently Luukkainen *et al.* (17) reported the applicability of trimethylsilyl ethers (originally introduced by Langer *et al.* (18), for the gas-liquid chromatography of phenols and alcohols) for sterol separations. These workers found quantitative formation of corresponding phenolic and hydroxy steroid ethers under mild conditions. The data presented indicated the usefulness of this technique for the separation of certain epimeric steroids and estrogens on silicone SE-30, fluoralkyl silicone, and neopentenyl glycol succinate polyester columns. In our laboratory, the trimethylsilyl ethers of the common fecal sterols were

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[2] This work was carried out during the tenure of an Established Investigatorship of the American Heart Association.

first chromatographed on polyester phases at various temperatures, pressures, and column dimensions. Separation of the trimethylsilyl ethers of coprostanol and cholesterol was excellent, but some overlapping occurred between the corresponding derivatives of methostenol and lanosterol. Further disadvantage of the polyester phase was the temperature limitation (excessive bleeding of the phase resulted at temperatures required for reasonable retention times). Currently, our best results have been obtained by the use of a new polar silicone gum containing 50 mole % of β-cyanoethylmethylsiloxy units and 50 mole % of dimethylsiloxy units. Utilization of this phase at the 5% level on washed Gas-Chrom P afforded excellent separations of the trimethylsilyl ethers of a number of biologically important sterols. This report deals with the adaptation of this column for the quantitative analysis of fecal neutral sterols.

METHODS AND MATERIALS

Pure samples of four major fecal sterols were obtained as follows: coprostanol, m.p. 99–100°C, from rat feces; cholesterol, m.p. 146–7°C, from Armour and Company, then purified through the dibromide (19); Δ^7-cholestenol, m.p. 122–3°C, and methostenol (4α-methyl-Δ^7-cholestenol), m.p. 146–7°C, synthesized in this laboratory (20, 21). For the determination of the retention times of the trimethylsilyl ethers of several minor fecal sterols or those of biological interest, samples were obtained as follows: 7-dehydrocholesterol and β-sitosterol from Calbiochem, Inc.; cholestanol from Nutritional Biochemical Co.; lanosterol isolated in this laboratory (22) from isocholesterol, a generous gift from The Hekman-Meeter Co. We are indebted to Dr. C. C. Sweeley for a sample of 24-dehydrocholesterol, and to Dr. A. A. Kandutsch for a sample of 4α-methyl-Δ^8-cholestenol.

Gas-Liquid Chromatography

Gas-Chrom P,[3] 80–100 mesh, was used to support the stationary phase essentially as described by Horning *et al.* (23) and Sjövall *et al.*, (24). Ten grams of the acid-washed dimethyldichlorosilane-treated Gas-Chrom P was added to a 250-ml round-bottom flask containing 50 ml of a 5% solution of a nitryl silicone gum[4] in acetone. Air bubbles were removed at all stages in the coating and previous washing process by applying an intermittent water pump vacuum. After standing for 30 min, the

[3] Applied Science Laboratories, Inc., State College, Pennsylvania.

[4] We are indebted to Dr. A. C. Martellock, Silicone Products Department, General Electric Company, Waterford, New York, for a generous gift of the nitryl silicone gum, 149-348.

excess solution was filtered off on a Büchner funnel and the damp support was spread out on two large (18¼ × 22½ inch) Whatman No. 1 filter paper sheets to air-dry for approximately 2 hr. The sheets with support were then transferred to an oven at 80°C for 2–4 hr. The column (9 ft × 3 mm) was packed by applying a water pump vacuum at the exit end and tapping lightly with a file as support was added through a funnel. The glass wool used to pack the flash heater area of the column was previously silanized with dimethyldichlorosilane and washed to neutrality with methanol. A Barber-Colman instrument equipped with an argon ionization detector (A 4183) was employed. Flash heater temperature was 285°C, column temperature 235°C, detector temperature 265°C, argon pressure 15 psi. The electrometer gain was 30 and detector voltage was 600. The number of theoretical plates[5] for the coprostanyl ether was found to be 4600. Suitable solutions of trimethylsilyl sterol ethers in anhydrous tetrahydrofuran were prepared and injections of 0.2–4.0 μl were made with a 10-μl Hamilton syringe (No. 701, Hamilton Co., Whittier, California). For quantitative work, the areas under each peak were determined by a Keuffel and Esser Co. compensating polar planimeter and the area[6] plotted against the mass based on the original weight of the sterol.

EXPERIMENTAL

Preparation of Sterol Trimethylsilyl Ethers

Using cholesterol as a representative member of the sterols to be analyzed, a study was conducted to determine the optimum quantity of the reagents, hexamethyldisilazane[7] and trimethylchlorosilane,[8] necessary for quantitative ether formation. A series of tetrahydrofuran solutions containing 10.0 mg of cholesterol in stoppered centrifuge tubes were treated with between 0.003–0.5 ml of hexamethyldisilazane and 2.5 μl of trimethylchlorosilane. The final volume was adjusted to 5.0 ml and the tightly stoppered tubes were kept for 18 hr at room temperature. Aliquots of the reaction mixtures were injected directly into the chromatograph and the extent of the ether formation was determined. The retention time of the trimethylsilyl ether of cholesterol was 29.7 min while the retention time of cholesterol was 51.9 min. The results of this experiment (Table 1) indicated that 0.003 ml of hexamethyldisilazane

[5] Number of theoretical plates $= (x/y)^2$ (16), where $x =$ distance from injection to peak and $y =$ width of peak base.

[6] One unit on vernier = 0.01 sq in.

[7] Peninsular Chemresearch, Inc., Gainesville, Florida.

[8] General Electric Company, Silicone Products Department, Waterford, New York.

TABLE 1

FORMATION OF CHOLESTEROL TRIMETHYLSILYL ETHER UNDER VARIOUS CONDITIONS[a] OF REAGENTS AND REACTION TIME

Sample[b] containing 10 mg cholesterol	Reaction time (hr)	Hexamethyl-disilazane added (ml)	Peak area[c]	
			Ether	Free sterol
1	18	0.003	89	7
2	18	0.010	104	Absent
3	18	0.050	102	Absent
4	18	0.100	104	Absent
5	18	0.500	103	Absent
6	2	0.100	95	5
7	5	0.100	101	Absent

[a] Column conditions: 5% of 50 mole % β-cyanoethylmethylsiloxy unit and 50 mole % dimethylsiloxy unit silicone gum polymer (General Electric Co.) on 80–100 mesh Gas-Chrom P; 9 ft × 3 mm, 235°C, 15 psi.

[b] The final volume of the reaction mixture in each case was 5 ml (each sample contained 2.5 μl of trimethylchlorosilane as catalyst).

[c] Vernier units described in text; 3 μl of each reaction was injected.

was nearly adequate to complete the formation of ether from 10.0 mg of cholesterol, but that 0.01 ml afforded complete ether formation from the same amount of sterol.

An ancillary experiment was conducted to determine the minimum time required to effect complete ether formation at room temperature conditions. Samples of cholesterol (10.0 mg) were treated with 0.1 ml of hexamethyldisilazane and 2.5 μl of trimethylchlorosilane dissolved in tetrahydrofuran to a final volume of 5.0 ml. After standing for periods of 2, 5, and 18 hr, appropriate injections of the reaction mixtures were made and the products analyzed as described above. From the results shown in Table 1, it can be seen that 2 hr is not quite sufficient for complete ether formation, but that 5 hr was satisfactory.

Quantitative Response

Standard solutions of the trimethylsilyl ethers of coprostanol, cholesterol, Δ^7-cholestenol, and methostenol in tetrahydrofuran were prepared according to the method previously described. Detector response was found to be satisfactory over a range of 1–6 μg (Fig. 1). It was noted that a slight decrease in detector sensitivity occurred which seemed to be related to the relative retention times of the three C-27 sterol ethers but, in the case of methostenol (C-28), the decrease in relative area response was more striking (incomplete ether formation was ruled out since no free methostenol peak was detectable on the recording). This behavior of

ionization detectors has been thoroughly investigated by Sweeley and Chang (25).

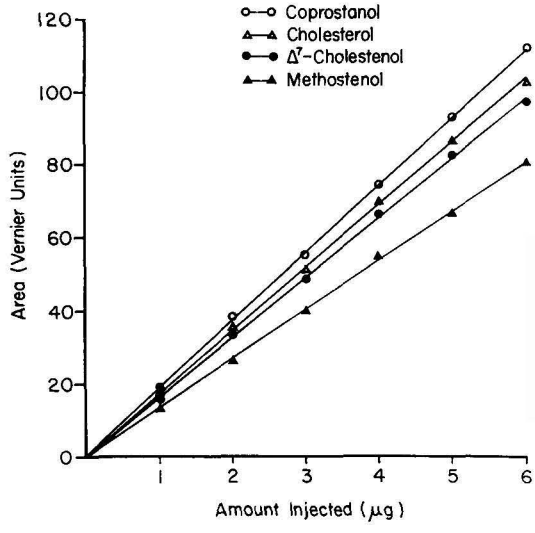

FIG. 1. Relationship of varying amounts of the trimethylsilyl ether of coprostanol, cholesterol, Δ^7-cholestenol, and methostenol to area recorded. Column and conditions are the same as for Table 1.

Relative Retention Times of Various Common Sterol Trimethylsilyl Ethers

The absolute and relative (to cholestane) retention times for a number of common sterol ethers can be seen in Table 2. There was overlapping in some critical cases, e.g., cholestanol vs. cholesterol and Δ^7-cholestenol vs. 7-dehydrocholesterol but resolution was excellent for most of the other samples studied (also see Fig. 2).

Fecal Sterols

The feces from various species were obtained in the following way: for small animals such as the rat, wire screens were placed on top of the refuse trays. No attempt was made to trap the fecal pellets in organic solvents nor to prevent coprophagy during a typical 24- or 48-hr collection period. Human stool collection was facilitated by the use of 18-in. wide sheets of heavy strength Saran Wrap,[9] and the individual 24-hr samples were frozen immediately.

In a typical preparation, 14 male rats of mixed ages (6–12 weeks)

[9] Dow Chemical Company, Midland, Michigan.

TABLE 2
RETENTION TIMES OF VARIOUS STEROL TRIMETHYLSILYL ETHERS

Trimethylsilyl ether[a]	Absolute[b] (min)	Relative[c]
Coprostanyl-	22.0	1.57
Cholesteryl-	29.7	2.12
Cholestanyl-	30.4	2.17
24-Dehydrocholesteryl-	34.7	2.48
Δ^7-Cholestenyl-	35.3	2.52
7-Dehydrocholesteryl-	35.8	2.56
4α-Methyl-Δ^8-cholestenyl-	38.0	2.71
4α-Methyl-Δ^7-cholestenyl-[d]	42.6	3.04
β-Sitosteryl-	46.8	3.34
Lanosteryl-	47.2	3.37

[a] Sterol ethers are prepared as described in text.
[b] Column and conditions are the same as for Table 1.
[c] Relative to cholestane (14.0 min).
[d] 4α-Methyl-Δ^7-cholestenol is known as methostenol.

and mixed strains (Long-Evans and Holtzman) were placed in group cages and fed a semisynthetic diet consisting of sucrose 62.8%, casein 18%, cottonseed oil 15%, Wesson salt mixture 4%, choline chloride

TABLE 3
ANALYSIS AND RECOVERY OF VARIOUS STEROLS FROM RAT FECES BY GAS-LIQUID CHROMATOGRAPHY OF THEIR TRIMETHYLSILYL ETHERS[a]

Material analyzed	Coprostanol found (mg)	Cholesterol found (mg)	Δ^7-Cholestenol found (mg)	Methostenol found (mg)
1 ml NS[b]	1.03 ± 0.06[c]	0.83 ± 0.06[c]	0.28 ± 0.04[c]	0.08 ± 0.02[c]
0.5 ml NS[d] + 1.01 mg coprostanol	1.46	0.43	0.15	Trace
0.5 ml NS + 1.00 mg cholesterol	0.52	1.39	0.14	Trace
0.4 ml NS + 1.04 mg Δ^7-cholestenol	0.42	0.37	1.11	Trace
0.4 ml NS + 0.60 mg methostenol	0.39	0.33	0.12	0.61
Average recovery, %	94	98	96	96

[a] The values represent the weight of free sterol calculated from their trimethylsilyl ethers.
[b] NS signifies standard rat feces nonsaponifiable fraction. See text for description.
[c] Standard deviation for five separate determinations,

$$\sigma = \sqrt{\Sigma X^2 - \frac{(\Sigma X)^2}{N}} \Big/ N - 1.$$

[d] All recovery data are the average of duplicate determinations.

0.1%, vitamin mix 0.1%, and ample supplements of α-tocopheryl acetate in cod-liver oil for a period of 4 days. Feces collected for the next 48 hr, were air-dried, and extracted with alcohol/acetone (1:1) for 24-hr in a Soxhlet apparatus. The lipid (4.4 gm) was saponified by refluxing for 2 hr in 30 ml of 10% KOH in 85% ethanol under a stream of N_2. The flask was cooled and 2 vol of water added. The mixture was then extracted with 3 equal vol of ether and the combined ether extracts were washed thoroughly with water. The ether extract was dried over anhydrous sodium sulfate for a minimum of 1 hr, filtered through Whatman No. 42 filter paper, and the ether removed *in vacuo*. The nonsaponifiable matter (360 mg) was dissolved in 50 ml of anhydrous tetrahydrofuran and was designated the rat feces nonsaponifiable fraction in later analytical experiments. For studies of reproducibility, aliquots of 1 ml of the rat feces nonsaponifiable fractions, 0.1 ml of hexamethyldisilazane,

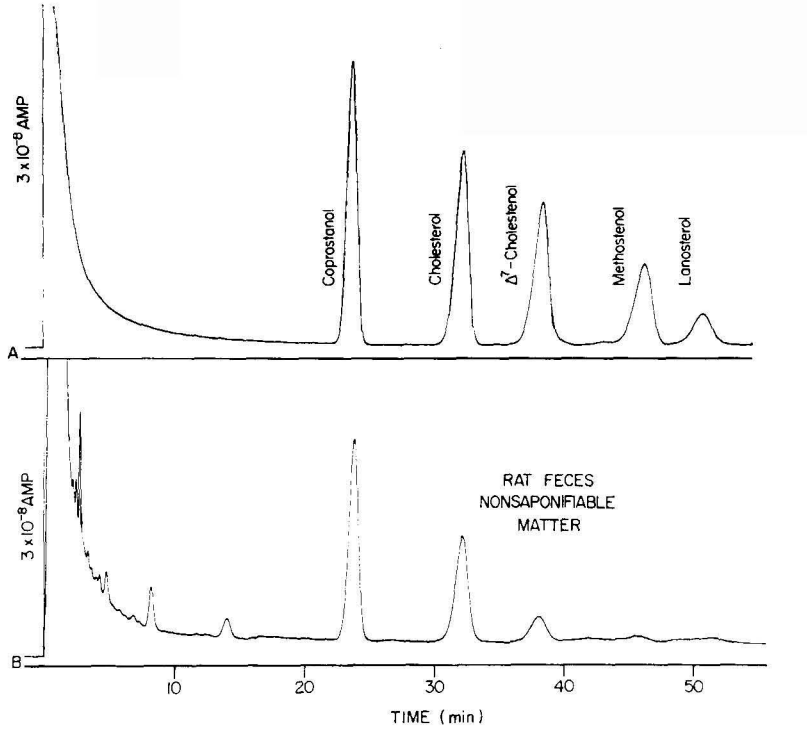

FIG. 2. Analytical record obtained in the gas-liquid chromatography of: A, 2 μg each of trimethylsilyl ethers of authentic coprostanol, cholesterol, Δ^7-cholestenol, methostenol, and lanosterol; B, rat feces nonsaponifiable matter treated with hexamethyldisilazane and trimethylcholorsilane in tetrahydrofuran. Column and conditions are the same as for Table 1.

and 2.5 μl of trimethylchlorosilane were allowed to stand for at least 5 hr. The averages and standard deviations (Table 3) of the amounts of coprostanol, cholesterol, Δ^7-cholestenol, and methostenol were 1.03 ± 0.06, 0.83 ± 0.06, 0.28 ± 0.04, and 0.08 ± 0.02 mg, respectively. The values obtained are in good agreement with those previously reported in the literature (5). The recovery of added quantities of each of the above sterols varied from 94–98%. A comparison of the chromatographic record of some known fecal sterol trimethylsilyl ethers with that of a treated aliquot of rat feces nonsaponifiable matter (Fig. 2) demonstrates the agreement of retention time found for authentic samples and those of sterols derived from the natural material.

DISCUSSION

The column described in this report was used continuously for approximately 6 weeks without deterioration. Certain precautions must be observed, however, for the successful use of this procedure. The nonsaponifiable matter must be thoroughly dry (we codistil residual water with anhydrous benzene) before addition of tetrahydrofuran and reagents. The strongly hydroscopic tetrahydrofuran must be distilled first from KOH pellets and again from $LiAlH_4$ and kept in a double seal bottle. We have observed that tetrahydrofuran and excess hexamethyldisilazane and trimethylchlorosilane cannot be evaporated from the mixture and the derivatives redissolved in a new volume of solvent without resulting in the appearance of interfering peaks in the sterol ether elution area. These new peaks have not been identified but appear only in the case of a fecal nonsaponifiable preparation (pure sterol samples do not develop this difficulty when similarly treated). Since the ionization detector response may vary from day to day, all analytical work should be preceded by and concluded with injections of a standard solution of at least one of the sterol trimethylsilyl ethers under investigation. One possible explanation for the changing detector sensitivity is deposition of the silicone polymer on the insulator and inner chamber of the detector by the constant bleeding of the phase from the column. We have found that, in the case of the model A 4183 detector of Barber-Colman Co., the Teflon seal can be cleaned satisfactorily by rubbing the seal with an optical abrasive moistened with acetone.

The trace quantities of fecal 7-dehydrocholesterol and cholestanol (5), which are poorly resolved from Δ^7-cholestenol and cholesterol, respectively, by this column would be a major concern only if large dietary quantities of these sterols were known to be administered to experimental animals. The method does allow for the separation of dietary plant sterols which will be found in fecal nonsaponifiable matter of animals fed lipids of plant origin.

Note added in proof. R. B. Clayton [*Biochemistry* **1,** 357 (1962)] has recently reported the gas-liquid chromatography of sterol methyl ethers on polydiethylene glycol succinate columns.

SUMMARY

1. A method has been developed for the quantitative determination of fecal sterols by the gas-liquid chromatography of their trimethylsilyl ethers on a 5% nitryl silicone gum phase supported on Gas-Chrom P.

2. The formation of the trimethylsilyl ethers of coprostanol, cholesterol, Δ^7-cholestenol, and methostenol were shown to be complete and recoveries of added amounts of pure sterols were shown to vary from 94–98%.

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A Simplified Procedure for Automatic Amino Acid Analysis

HYMAN ROSEN, COSTAN W. BERARD, AND STANLEY M. LEVENSON[1]

From the Department of Surgical Metabolism and Pathology, Division of Basic Surgical Research, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D. C.

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Since the introduction of the reduced ninhydrin reagent in a method for the routine estimation of amino acids by Stein and Moore (1, 2), particularly in connection with fractionation devices, a number of improvements have been introduced in order to facilitate pH adjustments and to improve color yield and stability. The original method required adjustment of each fraction with acid or base to bring the pH to the required value of 5. This requirement was eliminated by Stein and Moore by the introduction of concentrated buffers in the reagent solution (3). Yemm and Cocking (4) showed that small amounts of cyanide eliminated the need for reduced ninhydrin, a fundamentally unstable substance, and Rosen (5) adapted this reagent for use with fraction collectors.

The present report deals with an extension of this method which makes possible the use of a similar reagent in automatic amino acid analyzers. The reagent automatically adjusts pH, does not involve reduced ninhydrin, is stable for weeks in air, and has the additional advantage of not clogging the instrument lines. Indeed, it was for the last purpose that the reagent was developed. It has been used in this laboratory for approximately one year with the procedure of Piez and Morris (6), some details of which we have modified.

The important features are replacement of sodium acetate by sodium propionate to prevent clogging and introduction of cyanide into column buffers rather than into the reagent itself. In this way, the cyanide and the ninhydrin are brought together only minutes before the reaction begins. The cyanide has no detectable effect on any parameters of function of the column.

[1] Present Address: Department of Surgery, Albert Einstein College of Medicine, New York 61, New York.

MATERIALS

The method and equipment used are the same as that reported by Piez and Morris (6) with the following modifications. For the ninhydrin reservoir it is unnecessary to use an amber bottle, oil, or nitrogen atmosphere; we employ a simple 1- or 2-liter glass bottle with a 2-hole stopper, through one hole of which the line to the ninhydrin pump is inserted to the bottom of the bottle. The other hole is left open to room air. Since sediment does not appear in the reagent after even 3–4 weeks' storage and since dissolved air has never in our experience caused bubbles, it is unnecessary to interpose a trap between the ninhydrin reservoir and the pump. The lack of sediment formation eliminates the need for particular attention to cleaning and maintenance of the ninhydrin reservoir and the feeding line. Clogging and erratic pump operation are not observed, the irregularity of baseline (particularly on the 440 mμ trace) does not appear, and blank values are essentially constant from day to day.

The gradient elution device (Varigrad) of Peterson and Sober (7) and buffer reservoir are used essentially as described by Piez and Morris (6) with the exception that these are connected and disconnected to the buffer pump manually. We eliminated the automatic switching device for this step because we found mechanical failure common and manual switching was reliable and not inconvenient. The ion-exchange column, its water bath, and the manifold have not been altered.

The reaction coil is likewise unaltered but a standard Precision Instrument Co. water bath has been substituted for the 1-liter resin reaction flask and heating mantle. The 130-ft coil, wound spoolwise on two stainless-steel columns, is immersed in the bath, with lines of entry and exit passing through small holes in the covered top section. Originally operating this bath at 100°C, we employed a stainless-steel condenser, through the jacket of which cold water was circulated, to prevent rapid loss of water by evaporation. After discovering essentially no decrease in color yield when operating at 95°C, we have eliminated the condenser and effectively prevented evaporation by layering the surface with ¼ to ½″ of mineral oil. Photometer, recorder, and resin are as described (6) except that the automatic timer to regulate the 3-way stopcock has been eliminated.

The ninhydrin reagent employed in this procedure has been altered as follows: $SnCl_2$ is not used, the proportions of methyl Cellosolve and water have been changed, and propionate buffer has been substituted for acetate buffer. Since the reagent is stable indefinitely, it may be prepared in any quantity desired in the following proportions: sodium

propionate, 201.8 gm; propionic acid, 93 ml; methyl Cellosolve, 500 ml; ninhydrin, 20 gm; H_2O, up to 1 liter (about 250 ml). The reagent can be prepared on a simple magnetic stirring device without protection from light or air. It is itself a canary yellow color but gives a water clear blank when mixed and heated at 95°C with an equal quantity of buffer. Except for refrigerator storage between runs, the reagent is not protected in any way. We have never detected a change in the appearance of the reagent, the blank values, or the color yield with reagent stored up to 6 weeks.

The proportions of water and Cellosolve in this reagent can be altered by choice since we have found that ninhydrin is soluble in these reagents without any Cellosolve at all, i.e., using 750 ml of H_2O per liter of reagent. This is undesirable, however, since the use of the reagent without Cellosolve decreases the color yield at least 50%. Use of Cellosolve: H_2O in the 2:1 proportion suggested above gives maximal color yields and yet effectively prevents clogging of the lines by either salts or ninhydrin reagent.

The buffering capacity of this reagent is sufficient to adjust the eluent at pH 2.91 to pH 4.75 and the 0.8 *M* Na citrate to pH 5.45. These limits are narrow enough to ensure optimal and reproducible color development with this procedure. An additional important advantage of this reagent with its effective buffering capacity is the almost total elimination of the tendency of the baseline to rise, especially during the second half of the chromatogram when the pH of the eluent is changing rapidly. By increasing the concentrations of sodium propionate and propionic acid by 50% (i.e., 302.7 gm of Na propionate and 139.5 ml of propionic acid per liter of reagent), one can maintain the baseline at absolute flatness throughout the chromatogram, but we have elected to use the less concentrated reagent because it provides a very acceptable chromatogram with no risk of clogging.

The pH 2.91 buffer and 0.8 *M* sodium citrate solution are prepared as described by Piez and Morris (6); the pH 2.91 buffer must be deammoniated before use to prevent a sharp rise in blank just before the ammonia in the sample.

The additional reagent employed in this procedure is 0.01 *N* NaCN. This reagent is added to the 9 chambers of the gradient elution device (after filling) and to the pH 2.91 reservoir in the proportion of 0.2 ml cyanide solution to 75 ml of buffer. This gives a final cyanide concentration of 0.000026 *M* in each chamber. The concentration of cyanide employed is rather critical since reduction decreases color yield while increase causes a pink color which raises the blank values. The stock NaCN solution is kept stoppered and refrigerated except when removing

aliquots from it. In 8 months we have seen no change in this solution so that it, like the ninhydrin reagent, is stable for long periods under the simple conditions employed. The buffer containing the cyanide is thus pumped through the columns and is mixed with the ninhydrin reagent at the manifold just prior to entering the reaction coil. In months of pumping such cyanide-containing buffer through the column, we have detected no change in parameters of function of the column.

At the completion of a run, after the elution of arginine, we continue to pump pH 2.91 buffer through the column and coil for 1–2 hr. At the same time, we pump a 1:1 mixture of Cellosolve: H_2O through the coil with the ninhydrin pump. This wash step prevents clogging in the reaction coil even if the machine is turned off and the reaction coil is allowed to remain at room temperature for several days. If, of course, successive runs are to be done, this wash step can be shortened by replacing the wash solution with ninhydrin reagent as soon as the next sample is on the column. In this case the water bath would not be turned off, and flow through the coil would be essentially continuous.

Procedure for Performance of Analysis

1. Preparation of sample and its adsorption onto column is as described by Spackman, Stein, and Moore (10).

2. While sample is being adsorbed, the gradient elution device is filled as described by Piez and Morris (6) and to each of the 9 chambers is added 0.2 ml of 0.01 *N* NaCN. The stirring device is started.

3. Ninhydrin reagent, fresh or refrigerator-stored, is connected to ninhydrin pump by inserting Teflon tube to pump into one of the holes of the 2-hole stopper.

4. When sample has been adsorbed, the column space above the resin is filled with 2.91 buffer and the exit line from buffer pump is connected to the column. The gradient elution device is connected to the buffer pump feeding line and the valves connecting the chambers are opened manually.

5. Buffer pump, ninhydrin pump, and recorder are started. (If the machine has not been in use and the photometer lamps have been off, these should be turned on 3–4 hr prior to a run to allow warmup.)

6. After approximately 45 min, when ninhydrin has reached the absorption cells, the baselines will have risen and leveled and should be reset.

7. After approximately 21 hr, when the gradient elution device is empty, disconnect it from the buffer pump and connect in its place the 2.91 buffer reservoir.

8. After elution of arginine, replace the ninhydrin solution with a

mixture of 1:1 Cellosolve: H_2O and pump for 1–2 hr to wash the reaction coil and absorption cell thoroughly. The machine may now be turned off or started on another analysis.

Figure 1 demonstrates a typical chromatogram of a standard amino acid mixture[2] containing 0.5 μmole of each amino acid except for 0.25 μmole of cystine. Figure 2 shows a chromatogram of a hydrolyzed ultrafiltrate of the first fraction of acetic acid solubilized rat tail tendon chromatographed on carboxymethylcellulose. This represents a continuation of the work initially reported by Kessler, Rosen, and Levenson (8), showing that at least four components of rat tail tendon collagen are separable by gradient elution chromatography and that the first component is composed of low molecular weight peptides. In a subsequent publication (9), the same authors established the metabolic inhomogeneity of rat tail tendon collagen by studying the distribution dynamics of methyl-labeled glycine-C^{14} in the four chromatographic components. In work as yet unpublished we have succeeded in isolating a number of subfractions of the first component, probably peptide in nature, and are determining the amino acid composition of each of these.

DISCUSSION

Since the introduction of an automatic analytical procedure for amino acid analysis by Spackman, Stein, and Moore (10), the number of laboratories doing routine, highly precise work of this type has increased steadily. The modification proposed by Piez and Morris (6), with a single column and a continuous gradient, permitted a complete analysis of a protein hydrolyzate in 24 hr using one sample and less equipment.

The modifications in this report make simpler and less troublesome the method proposed above (6). The use of a stable ninhydrin reagent eliminates the need for special precautions in preparation, storage, and use of the reagent. The more favorable solubility characteristics of this reagent ensures against the clogging (due to crystallization) that has been a continuous hazard in instruments using capillary tubing.

The wash step, adding no further time to an analytical procedure, permits the investigator whose use of the instrument is not continuous to shut down the equipment with assurance that it will be immediately functional when used again. There has been essentially no "maintenance time" involved since the development of this procedure, except for the ½ to 1 hr used in starting a new run. The cyanide reagent, added to buffer in catalytic quantity, makes possible optimal color yield from a reagent which is insensitive to light and air.

[2] Purchased from Phoenix Precision Instrument Company, Philadelphia 40, Pa.

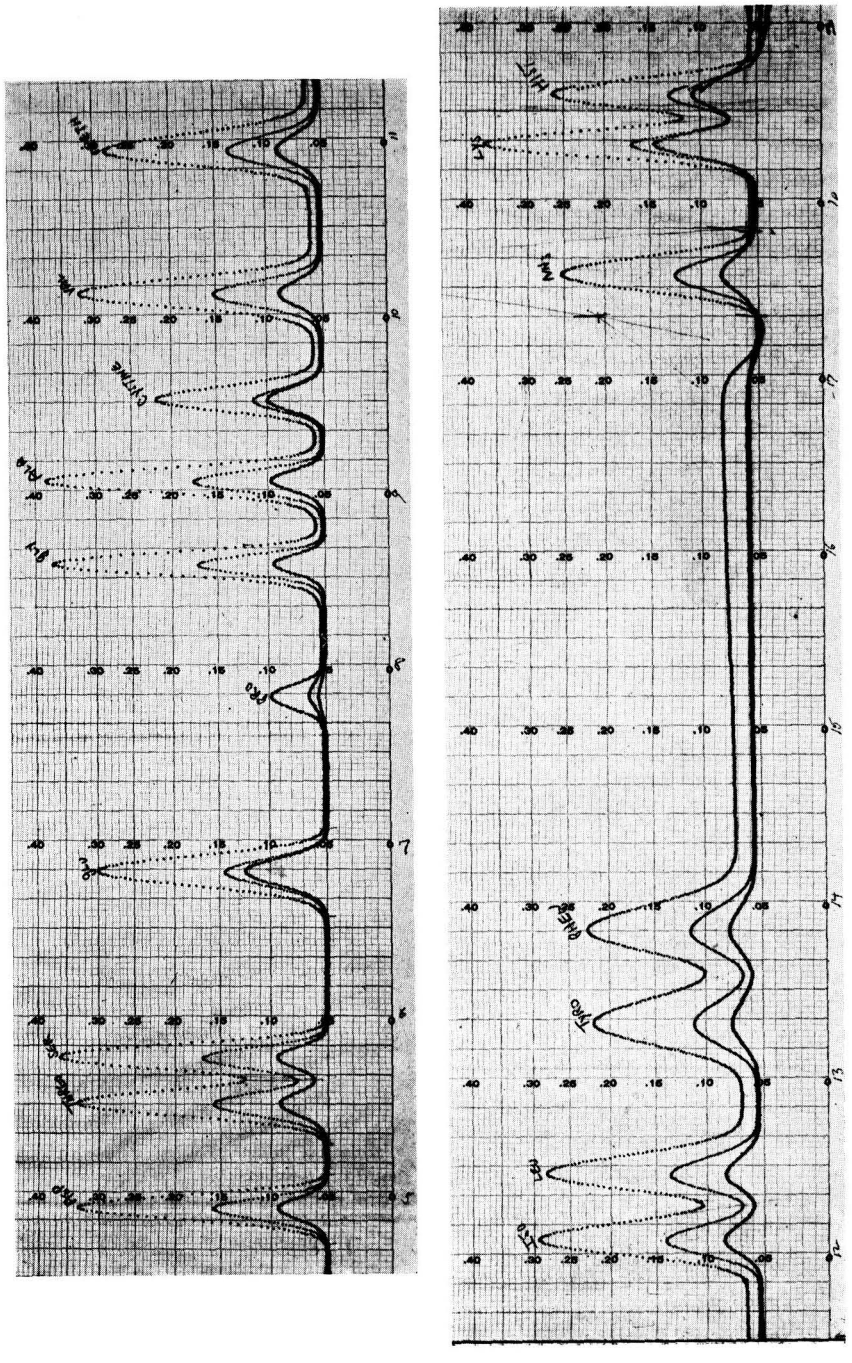

FIG. 1. Typical chromatogram of standard amino acid mixture containing 0.5 μmole of each amino acid except for 0.25 μmole of cystine. Arginine was not determined on this chromatogram.

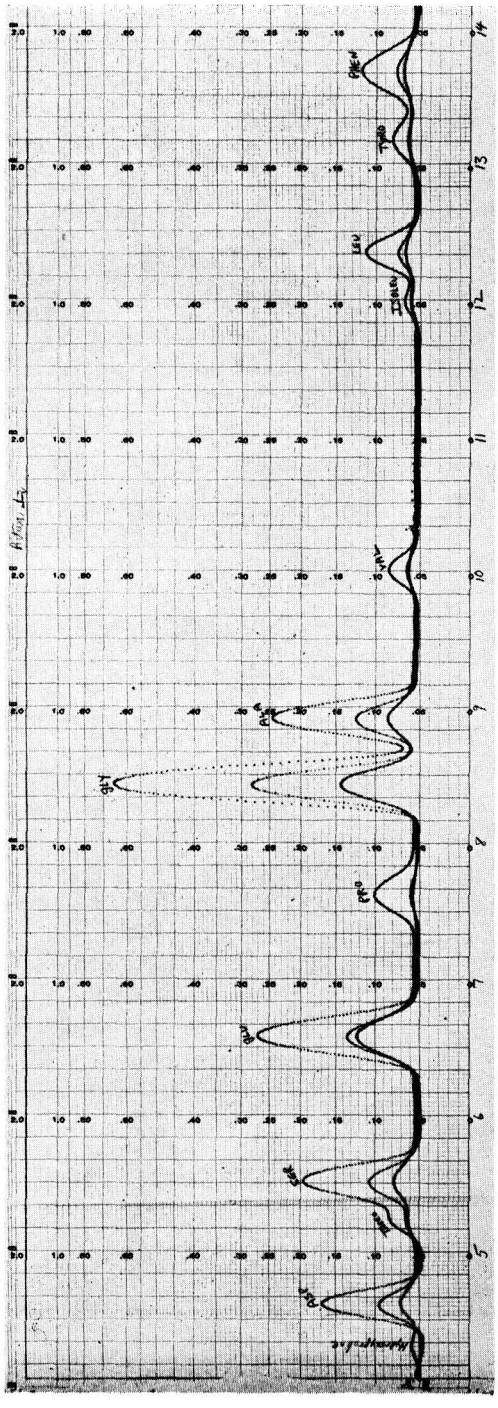

FIG. 2. Chromatogram of hydrolyzed ultrafiltrate of first fraction of acetic acid solubilized rat tail tendon chromatographed on carboxymethylcellulose (*9*, *10*).

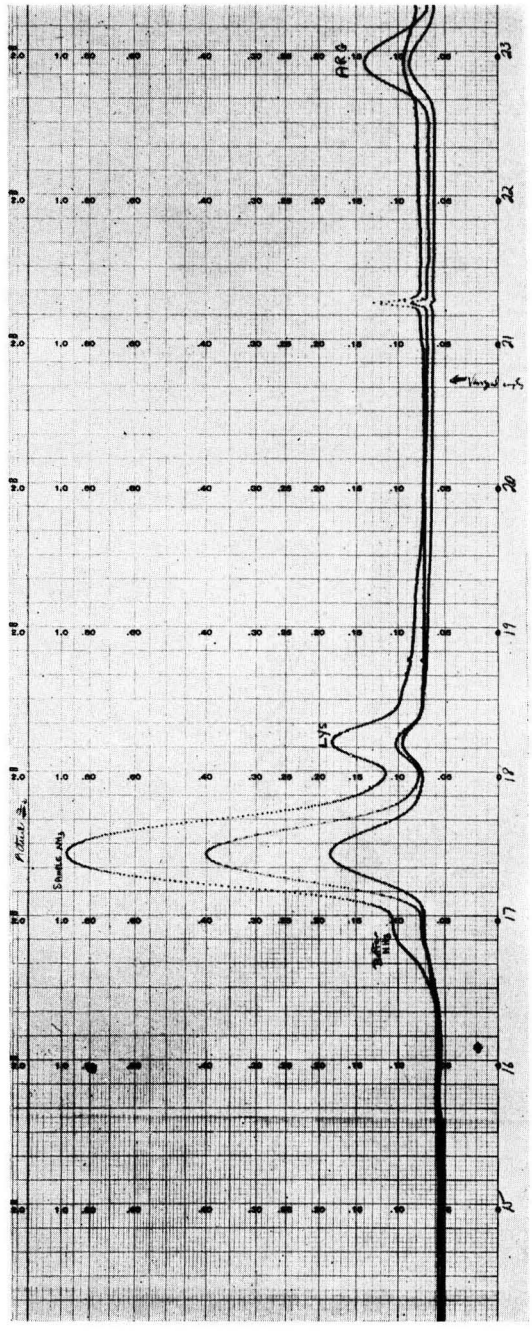

FIG. 2 (*continued*)

The color values obtained with mixtures of known amino acids are comparable to those reported by Spackman *et al.* (10) and by Piez and Morris (6). These values vary, however, with the instrumentation and procedures used and should be determined separately for each laboratory.

Amino acid analyzers of other types can be adapted to these modifications with no changes in instrumentation. It is merely necessary to alter the composition of the ninhydrin reagent and add NaCN to the buffers in the proportions indicated.

SUMMARY

Further simplifications of the procedures for the automatic analysis of amino acids are presented. The basic modifications are alterations in the ninhydrin reagent and addition of cyanide to the buffer system. These developments render the reagent stable indefinitely in light and air and eliminate the risk of clogging experienced in the past.

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An Ultramicro Method for Determination of Carbonic Anhydrase Activity[1]

H. MATTENHEIMER AND HENDRINA DEBRUIN

From the Departments of Biochemistry and Medicine, Presbyterian-St. Luke's Hospital, and Department of Biological Chemistry, University of Illinois College of Medicine, Chicago, Illinois

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In the course of investigations into the activity of enzymes in the various anatomical units of the nephron (1), it was necessary to have available an ultramicro method for serial determinations of carbonic anhydrase activity in tissue fragments. These fragments are dissected from frozen dried 16-μ thick kidney slices, and they weigh between 10 and 200 mμg.

When CO_2 is dissolved in water, it is hydrated to H_2CO_3 according to the equation:

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$$

Carbonic anhydrase accelerates this reaction equally in both directions (2, 3). The difference between the spontaneous hydration of CO_2 and the hydration in the presence of carbonic anhydrase is used as a parameter for carbonic anhydrase activity, which has been measured: (*a*) manometrically (4–7), (*b*) colorimetrically (3, 7–10, 12), and (*c*) electrometrically (11–13).

The manometric "boat" technique of Meldrum and Roughton (4) was later modified, using Warburg manometers (6), and was adapted to the ultramicro scale, using the Cartesian diver technique (18). However, the diver technique is far too time consuming for the serial measurements required in the studies mentioned above. The stop flow technique (10) is the most accurate method to measure the very fast reaction, but the incubation volume cannot be reduced to ultramicro quantities. Of the colorimetric methods, the CO_2-Veronal-Indicator method of Roughton and Booth (3) seemed to be the most suitable for an ultramicro modification although the carbonic anhydrase activity is somewhat effected by the buffer and by the indicator used (2, 3, 12). In the Roughton-

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Booth procedure the incubation volume is 10.5 ml. Pilot experiments in our laboratory with frozen dried kidney sections revealed that, to measure the carbonic anhydrase activity in 10–200 mμg of tissue, the final incubation volume should be about 17 μl.

Accordingly, the following technique has been developed.

METHOD

Principle

CO_2-saturated water is added to a weak Veronal buffer of pH 8.0 containing bromothymol blue and the enzyme. The time is measured for the pH to drop to 6.3 (color change from blue to yellowish green). The enzyme activity is calculated from the time difference between the zero reaction (without carbonic anhydrase) and the enzymic reaction.

Equipment

1. *Test tubes* 50 mm long and 2 mm internal diameter. The test tubes have to be siliconed on the inside (Dow Corning stopcock grease). The freshly siliconed tubes are heated at 150°C for at least 2 hr.

2. *Constriction pipets* (λ pipets): 300 μl, 230 μl, 12 μl, 8 μl, and a double constriction pipet of 9 μl. Polyethylene constriction pipets (14) are used in our laboratory. Glass constriction pipets may be used except for the 8-μl pipet, with which the CO_2-saturated water is pipetted, and the double constriction pipet, which is much easier to make from polyethylene than from glass. A double constriction pipet is shown in Fig. 1. The volume to be delivered is measured between the two constrictions. The constrictions are made as previously described (14).

3. *Incubation unit,* consisting of an ice bath, vibrator (a simple massage vibrator connected to a power stat is satisfactory) with an attached holder for 2 test tubes, a 100-ml polyethylene bottle, a stirrer, and a CO_2 (100%) tank.

4. *Ice bath* with rack for the micro test tubes.

5. Stop watch.

Reagents

(A) Veronal, 0.022 *M*.

(B) Veronal-sodium, 0.22 *M*.

(C) Veronal/Veronal-Na buffer, 0.02 *M*, pH 8.0: 10 ml A + 1.1 ml B (adjust pH by measuring with pH meter).

(D) Bromothymol blue, 0.2% in 50% ethanol.

(E) Distilled water with 11.8 mg peptone per 100 ml (Bacto-Peptone, Difco Laboratories).

(F) Distilled water saturated with CO_2 at 0°C (= 0.071 *M* (3)).

(G) Phosphate buffer, 0.02 *M*, pH 6.3.

(H) Incubation medium: 300 μl buffer (C), 230 μl peptone water (E), 12 μl indicator (D).

(I) Color standard: made up as (H) but using phosphate buffer (G) instead of (C).

Procedure

In the upper third of each of the siliconed test tubes a "seal" is formed with incubation medium (H), using the 9-μl double constriction pipet, which is filled with incubation medium up to constricton 2 (Fig. 1). The tip of the pipet is set on the wall of the test tube at the place where the "seal" is to be formed, and the fluid is delivered slowly by applying slight pressure through the mouth tubing, while the test tube is slowly rotated around its axis. A "seal" will form easily and is brought to a volume of 9 μl by emptying the pipet to constriction 1 (Fig. 1). If more than 9 μl is needed to form the "seal," excess fluid can be delivered from the pipet but must be sucked back to constriction 1 after the "seal" has formed. A "seal" formed with incubation medium is preferable to placing the medium in the bottom of a micro test tube because (*a*) the color change is easier to see if, as in the "seal," the diameter is uniform, and (*b*) the mixing by vibration is faster in a "seal." One test tube is filled completely with color standard solution (I). The filled test tubes are then placed in a rack in an ice bath, in order to equilibrate the seal to 0°C. The tissue specimens—weighed on a glass fiber balance (15, 16)—are inserted into the cold seal under a stereoscopic microscope with a glass needle. If enzyme solutions are being used, an aliquot which should not exceed 0.4 μl is added to the seal with a constriction pipet.

In order to measure the carbonic anhydrase activity, a test tube containing the seal with the specimen added (or for the zero reaction, containing the seal without a specimen) is placed in the holder in the ice bath beside the test tube with the color standard (Fig. 2). The seal must be well below the water level. CO_2-saturated water is then added to the seal with the 8-μl pipet. This pipet must be kept in the ice-cold CO_2 water because, if the pipet is allowed to warm up and is then used, gas bubbles would appear in the pipet, not only blocking the pipet but also leading to a loss of CO_2. The tip of the pipet containing CO_2-saturated water is placed on the glass wall approximately 1 mm above the seal and then emptied quickly by applying pressure through the mouth tubing. As soon as the pipet is emptied, the stop watch is pressed, and the vibrator is switched on for not more than 1–2 sec. The solutions are mixed by the vibration. It is important not to continue the vibration

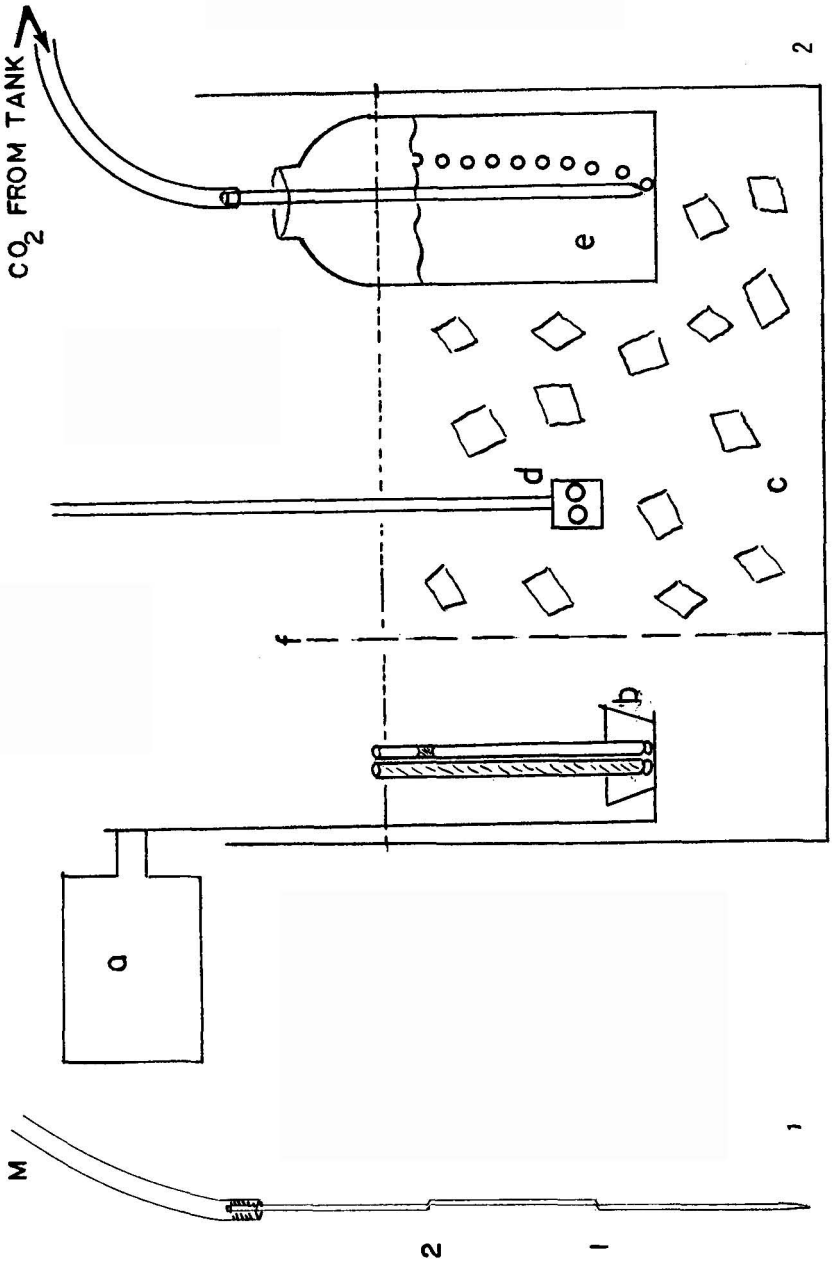

FIG. 1. Polyethylene double constriction pipet made according to (14): (1, 2) constrictions; (M) mouth tubing. Details for operation are given in the text.

FIG. 2. Incubation unit: (a) vibrator connected to a power state; (b) holder for 2 micro test tubes (made from a rubber stopper); (c) ice bath; (d) stirrer; (e) 100-ml polyethylene bottle for saturated CO_2 water; (f) wire gauze. Not shown in the sketch is a bright light source, preferably placed in front of the ice bath.

more than 1–2 sec because it would lead to a loss of CO_2. The strength of the vibration has to be adjusted with the power stat so that thorough mixing is achieved. It is usually necessary to repeat the mixing for 1 sec when the color change becomes visible because the color change seems to be somewhat slower at the surface. The time is measured for the color to change to yellowish green, which can be observed with little difficulty by comparison with the color standard. It is advisable to have a good light source in front of the incubation unit. The time for at least 5 zero reactions (spontaneous hydration of CO_2) must be measured, in addition to the enzyme reaction.

Calculations [According to Roughton and Booth (3)]

The rate of enzymic hydration of CO_2 (R_E) is equal to the difference between the rate of the over-all catalyzed reaction ($R = 1/(t - 1)$), and the uncatalyzed spontaneous reaction ($R_0 = 1/(t_0 - 1)$), where $t - 1$ is the reaction time in seconds, 1 sec allowed for mixing.

$$R_E = R - R_0 \tag{1}$$

The ratio of the intrinsic enzymic reaction to the zero rate is therefore:

$$\frac{R_E}{R_0} = \frac{R - R_0}{R_0} = \frac{t_0 - t}{t - 1} \tag{2}$$

This ratio can be used to express the enzyme activity in enzyme units:

$$\text{EU} = \frac{t_0 - t}{t - 1} \tag{2a}$$

(e.g., $t_0 = 100$, $t = 61$; EU $= (100 - 61)/(61 - 1) = 0.65$). To obtain R_E in moles/liter/sec, the velocity constant k_u of the uncatalyzed reaction has to be known:

$$k_u = \frac{[\text{acid formed}]}{[\text{average } CO_2] \times (t_0 - 1)} \text{ moles/liter/sec} \tag{3}$$

for which [acid formed] is calculated, using the Henderson-Hasselbach equation (pH range 8.0–6.3, pk = 7.96). Average CO_2 can be approximately calculated as shown below.

The amount of acid formed during the pH shift can also be determined simply by titrating the "seal" with acid to the color of the standard phosphate buffer (as suggested by Dr. Bonting, personal communication). However in standardizing the procedure a 10-fold volume of the "seal" (170 μl) was titrated with 0.1 N H_2SO_4 from an ultramicroburet (Beckman/Spinco 153 Microtitrator). We found at 0°C, 62.0×10^{-4} moles/liter $\pm$ 1.3% (coefficient of variation, $n = 10$), which agrees well with 61.6×10^{-4} moles/liter as calculated below.

For the volumes and concentrations given above

$$\text{at pH 8.0:} \quad [\text{veronate}^-] = 64 \times 10^{-4}\,\text{M}\left(0.22 \times \frac{1.1}{11.1} \times \frac{300}{542} \times \frac{9}{17}\right)$$

$$[HCO_3^-] = \text{zero}$$

$$[CO_2] = 33.4 \times 10^{-3}\,M\left(0.071 \times \frac{8}{17}\right)$$

Since

$$[HCO_3^-] \text{ at pH } 6.3 - [HCO_3^-] \text{ at pH } 8.0 = [\text{veronate}^-] \text{ at pH } 8.0 - [\text{veronate}^-] \text{ at pH } 6.3 = [CO_2] \text{ at pH } 8.0 - [CO_2] \text{ at pH } 6.3$$

it follows that

$$\text{at pH 6.3:} \quad [\text{veronate}^-] = 2.4 \times 10^{-4}\,M$$

$$[HCO_3^-] = 61.6 \times 10^{-4}\,M$$

$$[CO_2] = 27.2 \times 10^{-3}\,M$$

The average CO_2 equals approximately $30.3 \times 10^{-3}\,M$ $(33.4 + 27.2)/2)$. Hence (Eq. 3):

$$k_u = \frac{61.6 \times 10^{-4}}{30.3 \times 10^{-3} \times (t_0 - 1)} \text{ moles/liter/sec}$$

For $t_0 = 100$, $k_u = 20.5 \times 10^{-4}$.

From Eq. (2) the enzymic rate of hydration of CO_2

$$R_E = \frac{t_0 - t}{t - 1} \times R_0 \tag{4}$$

and since $R_0 = k_u$ [average CO_2]

$$R_E = \frac{t_0 - t}{t - 1} \times k_u \text{ [average } CO_2\text{] moles } CO_2\text{/liter/sec} \tag{6}$$

For $t_0 = 100$:

$$R_E = \frac{t_0 - t}{t - 1} \times 6.21 \times 10^{-5} \text{ moles } CO_2\text{/liter/sec}$$

To express the enzyme activity in MKH units (moles CO_2 hydrated per kilogram tissue dry weight per hour) the following equation must be used (weight measured in mμg and incubation volume 17 μl):

$$R_E = \left(\frac{t_0 - t}{t - 1} \Big/ \text{ weight}\right) \times k_u \text{ [average } CO_2\text{]} \times 6.1 \times 10^{10} \text{ MKH} \tag{7}$$

For $t_0 = 100$:

$$R_E = \left(\frac{t_0 - t}{t - 1} \Big/ \text{ weight}\right) \times 3.79 \times 10^6 \text{ MKH}$$

RESULTS AND DISCUSSION

The time for the uncatalyzed hydration of CO_2 averaged 100 ± 3 sec in all our experiments. From this, the velocity constant $k_u = 20.5 \times 10^{-4}$ moles/liter/sec was calculated (Eq. 3). This value is in close agreement with $k_u = 22.5 \times 10^{-4}$ found by Roughton and Booth (3) using the colorimetric macromethod, and $k_u = 21.0 \times 10^{-4}$ found manometrically by the same authors. With a commercial carbonic anhydrase preparation (Fig. 3) and with tissue homogenates (not shown in Fig. 3) a direct relationship exists between enzyme concentration and enzyme activity

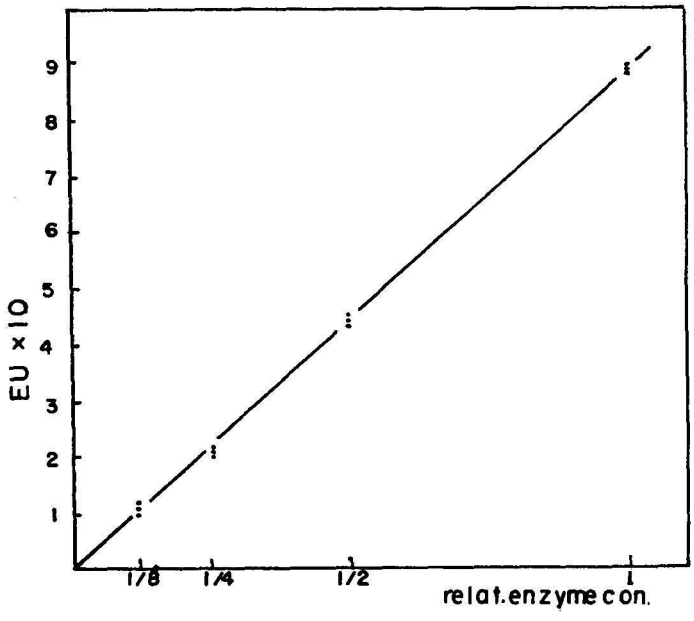

FIG. 3. CA activity versus enzyme concentration. A commercially available CA preparation has been used (Nutritional Biochemical Corporation, Cleveland, Ohio; prepared according to Keilin and Mann (17)). 1.0 mg CA preparation was dissolved in 2.5 ml water with 0.05% peptone added. From this stock solution 1:100, 1:200, 1:400, and 1:800 dilutions were prepared using peptone water as diluent. From the dilutions 0.24 μl was added to the seal. Relative enzyme concentration 1 = 0.97 mμg CA preparation.

at least in the range of 0.1–1.0 EU (Fig. 3). The commercial carbonic anhydrase preparation maintained its full activity when dissolved in water (0.05% peptone added) and stored frozen. This solution was frequently used as a standard to check the assay system.

The coefficient of variation (standard deviation in per cent) of the

data in Fig. 3 is 12.6%, while it was less than 5% in experiments in which only one enzyme concentration (0.48 mμg/assay) was used. With 8 different homogenates from dog kidney—each run in triplicate—coefficients of variation from 4.0 to 8.4% (average 5.7%) were found. On the other hand, the coefficient of variation was 16.9% ($n = 20$) in an experiment with pieces from frozen dried hemolyzate of various weights (Table 1). Since the coefficient of variation for weighing is in

TABLE 1

CARBONIC ANHYDRASE ACTIVITY OF HEMOLYZATE FROM HUMAN ERYTHROCYTES

Hemolyzate from washed human erythrocytes was lyophilized. From the dry material, pieces of varying weights (quarts fiber balance) were cut under a stereoscopic microscope and inserted into the seal with a fine glass needle.

No.	Weight (mμg)	t	$t_0 - t$	EU[a]	EU/mμg
1	158.0	48	52	1.11	7.0×10^{-3}
2	112.0	54	46	0.87	7.8
3	103.0	60	40	0.68	6.6
4	113.0	57	43	0.77	6.8
5	80.4	65	35	0.55	6.8
6	39.1	81	19	0.24	6.1
7	40.8	78	22	0.29	7.1
8	19.0	90	10	0.11	5.8
9	71.5	64	36	0.57	8.0
10	41.0	78	22	0.29	7.1
11	13.4	91	9	0.10	7.5
12	14.6	90	10	0.11	7.5
13	11.1	90	10	0.11	9.9
14	14.2	89	11	0.13	9.1
15	30.6	84	16	0.19	6.2
16	49.7	72	28	0.39	7.8
17	20.3	83	17	0.20	9.8
18	21.5	84	16	0.19	8.8
19	10.7	93	7	0.08	7.5
20	12.2	89	11	0.13	10.6

Mean = 7.7×10^{-3}
S.D. = $\pm 1.3 \times 10^{-3}$
Coeff. of variation = 16.9%

[a] EU = $(t_0 - t)/(t - 1)$.
Determination of t_0: 101, 100, 99, 99, 101, average t_0 = 100.

the order of 1% (16), this implies that carbonic anhydrase is not homogeneously distributed in lyophilyzed hemolyzate. As can be seen in Table 1 the variation does not depend on the weight of the sample. In general the accuracy of the presented ultramicro modification is in

good agreement with the accuracy of the original CO_2-Veronal-Indicator method for which the authors (3) claim an "experimental uncertainty" of $\pm 10\%$.

It has to be assumed that the values for carbonic anhydrase activity are about 10% below the actual value, due to an inhibitory effect of the indicator, as found by several authors (2, 3, 12).

SUMMARY

Based on the CO_2-Veronal-Indicator method of Roughton and Booth, an ultramicro method for the determination of carbonic anhydrase was developed. Carbonic anhydrase activity can be determined in tissue fragments with dry weights between 10 and 200 mμg or in 0.2–0.4 μl tissue homogenates.

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Fluorometric Measurement of Reduced Pyridine Nucleotide in Cellular and Subcellular Particles

RONALD W. ESTABROOK[1]

From the Department of Biophysics and Physical Biochemistry, Johnson Foundation, University of Pennsylvania, Philadelphia, Pennsylvania

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The central role of pyridine nucleotide in oxidative reactions, and its intimate relationship to the processes of oxidative phosphorylation, have served as an impetus to detailed studies of the interactions of dehydrogenases, pyridine nucleotides, and the respiratory chain in cells and subcellular particles. Three general approaches to this problem have been applied in recent years. The first is the application of sensitive spectrophotometric methods as first demonstrated by Chance (1) and more recently by Buecher and Klingenberg (2). This has provided a direct evaluation of the kinetics of reduction and oxidation of cytochromes and pyridine nucleotide during oxidative metabolism. The second approach employs the application of chemical analytical techniques, as exemplified by the studies of Purvis (3) and Kaufman and Kaplan (4), as well as Hunter and co-workers (5). A third technique involves the measurement of the fluorescence of reduced pyridine nucleotide, as described by Duysens and Amesz (6), Chance *et al.* (7), and Avidor *et al.* (8). This last method permits the application of a spectrofluorometer to evaluate the fluorescence excitation and emission spectra of reduced pyridine nucleotide [cf. Avidor *et al.* (8) and Chance and Baltscheffsky (9)] or the measurement of the change in fluorescence as a function of time [cf. Acidor and Olson (10)]. These latter studies have complimented the spectrophotometric determinations and opened new facets to the problem, such as the relationship of pyridine nucleotide to mitochondrial structure or the postulated role of pyridine nucleotide as an "active" intermediate in oxidative phosphorylation.

A previous paper (11) has described the application of a commercial fluorometer for the microdetermination of adenine and pyridine nucleo-

[1] This study was carried out during the tenure of a U. S. Public Health Service Senior Fellowship (SF 206).

tides. The present paper describes the use of the same instrument for the measurement of the kinetics of concentration changes of intracellular or intramitochondrial pyridine nucleotides during various metabolic conditions. In addition, the present study describes the correlation of the fluorescence change observed with turbid suspensions with the spectrophotometric measurement of pyridine nucleotide reduction as well as direct chemical analyses for changes in pyridine nucleotide concentration. Such a comparison permits the evaluation of the degree of reduced pyridine nucleotide binding in such systems and illustrates the pronounced fluorescence enhancement of reduced pyridine nucleotide associated with liver mitochondria. In agreement with the recent report by Avidor *et al.* (8) the present study presents data to support the observation that reduced diphosphopyridine nucleotide of isolated liver mitochondria displays a fluorescence enhancement of 10- to 12-fold when compared to the fluorescence of DPNH in solution. This is in contrast to a fluorescence enhancement of 2- to 4-fold observed with TPNH in the same system. The pronounced fluorescence enhancement observed with mitochondria is discussed in terms of a ternary complex for reduced pyridine nucleotide possibly related to a hypothetical intermediate of oxidative phosphorylation.

METHODS AND MATERIALS

Apparatus. An Eppendorf photometer with fluorometer attachment was modified for recording as described in the previous paper (11). The optical geometry of the excitation light beam and the fluorescence intensity detector is optimized in the design of this instrument by placing the measuring photomultiplier in a position so that fluorescence from the irradiated surface of the reaction vessel is monitored. This principle was also employed in the specially constructed instruments described by Duysens and Amesz (6) and Chance *et al.* (7) and minimizes artifacts arising from small changes in the light-scattering properties of the reactant suspensions during the course of a reaction.

When oxygen utilization was measured simultaneously with the change in fluorescence, a vibrating platinum electrode of the type described by Chance (12) was mounted in the cell compartment, employing a KCl bridge and calomel electrode as the reference half-cell. The fluorescence increase obtained by adding a standard solution of DPNH to the reaction mixture was used routinely for calibration of the instrument.

Spectrophotometric measurements of pyridine nucleotide reduction were made with a developmental model of the Aminco dual wavelength spectrophotometer in a manner analogous to that described by Chance

(12). The change in absorbancy of pyridine nucleotide was measured at 340 mμ employing 375 mμ as reference wavelength. Calibration of the instrument was obtained by adding a standardized solution of DPNH to a turbid suspension and measuring the change in absorbancy.

Analysis of Pyridine Nucleotides. The concentration of pyridine nucleotides was determined in neutralized samples employing the fluorometric procedure described previously (11).

Chemicals. Adenine and pyridine nucleotides were obtained from Sigma Chemical Company, St. Louis, Mo., or from Boehringer & Soehne, Mannheim, Germany.

Preparations. Rat liver mitochondria were prepared in 0.25 *M* sucrose following the procedure described by Schneider (13). Mitochondria were diluted in an isotonic buffer of pH 7.0 containing 80 m*M* KCl, 10 m*M* potassium phosphate, 10 m*M* triethanolamine hydrochloride buffer, and 5 m*M* $MgCl_2$. Starved yeast cells were obtained by aeration of commercial bakers' yeast washed in 0.02 *M* KH_2PO_4 and suspended to a concentration of 30% (w/v) in 0.02 *M* KH_2PO_4. *Escherichia coli* was grown on the synthetic medium described by Cohen (14) and harvested during the log phase of growth [cf. Estabrook *et al.* (15)].

RESULTS

Comparison of Spectrophotometric and Fluorometric Measurement of Pyridine Nucleotide Reduction. The reduction of pyridine nucleotides (DPN and TPN) is characterized by an increase in light absorption at 340 mμ as well as the appearance of an associated fluorescence emission band in the 440–460 mμ region. Mitochondria, isolated from rat liver, show characteristic changes (16) in the extent of pyridine nucleotide reduction depending on the presence of substrate, phosphate acceptor (ADP), and oxygen. These changes are illustrated in Fig. 1, where the extent of pyridine nucleotide reduction was measured spectrophotometrically and fluorometrically. The pattern of transitions of pyridine nucleotide reduction obtained at various metabolic states is observable by either method. The addition of an aliquot of a standardized solution of DPNH to calibrate the absorbancy or fluorescence changes observed permits the determination of the apparent change in reduced pyridine nucleotide concentration and serves as a means of relating the two methods of measuring pyridine nucleotide reduction. For the experiment illustrated in Fig. 1, the calculated changes in pyridine nucleotide concentrations are summarized in Table 1. These data show that the observed increase in fluorescence associated with pyridine nucleotide reduction of mitochondria is 4 to 6 times greater than expected on the

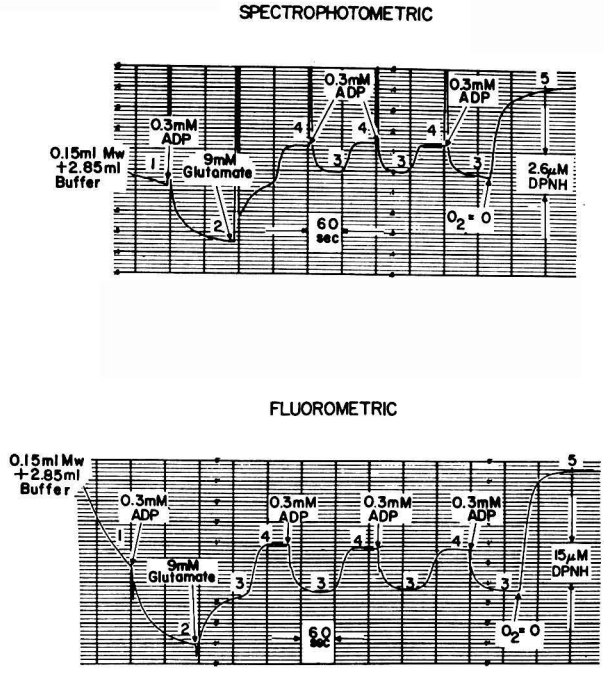

FIG. 1. Comparison of fluorometric and spectrophotometric measurement of pyridine nucleotide reduction of rat liver mitochondria. A 0.15-ml sample of rat liver mitochondria (6.3 mg protein) was diluted to 3.0 ml with isotonic buffer in a reaction cuvette. Aliquots of ADP and sodium glutamate were added as indicated. The upper recording is the change observed spectrophotometrically by measuring the absorbancy change at 340 mμ relative to 375 mμ as reference wavelength. A comparable sample was assayed in the same manner fluorometrically (lower recording) using the Eppendorf fluorometer with 366 mμ excitation light and a filter transmitting 400–3000 mμ. The various metabolic states are indicated using the terminology suggested by Chance and Williams (16). A solution of standardized DPNH was employed for calibration of the fluorometer and spectrophotometer. The calculated changes in pyridine nucleotide concentration are summarized in Table 1. Experiment 860.

basis of the absorbancy change measured spectrophotometrically. This fluorescence enhancement may reflect the extent of binding of the reduced pyridine nucleotide of mitochondria.

Analysis of Pyridine Nucleotide. Neither the spectrophotometric nor the fluorometric measurement of reduced pyridine nucleotide of cellular material differentiates between diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) but merely gives the sum of the changes of either component. Such a distinction can be obtained, however, by employing the microanalytical techniques described in a

TABLE 1

COMPARISON OF SPECTROPHOTOMETRIC AND FLUOROMETRIC MEASUREMENT OF PYRIDINE NUCLEOTIDE REDUCTION

Metabolic states	Apparent change in reduced pyridine nucleotide concn. (mole/ml)		Ratio fl./sp.
	Fluorometric	Spectrophotometric	
3–2	18×10^{-9}	3.8×10^{-9}	4.6
4–2	37	6.1	6.1
3–2	19	4.6	4.2
4–2	36	6.3	5.7
3–2	20	4.6	4.3
4–2	36	6.2	5.8
3–2	20	4.3	4.6
5–2	64	9.9	6.5

The changes in pyridine nucleotide concentrations were determined from the data presented in Fig. 1 by relating the extent of change of fluorescence or absorbancy to the change observed upon addition of an aliquot of a standardized solution of DPNH. The terminology employed to designate the various metabolic states is that suggested by Chance and Williams (16). The concentration of mitochondrial protein employed in the reaction cuvette was 2.1 mg/ml (Expt. 860).

previous paper (11). A series of such experiments were therefore carried out to relate the change in pyridine nucleotide fluorescence, as described above, with the change in concentration of DPN and TPN. These experiments would also serve as a second means of assessing the extent of fluorescence enhancement and would support the calculations relating the observed changes determined spectrophotometrically and fluorometrically.

Figure 2 demonstrates the changes in pyridine nucleotide fluorescence observed with liver mitochondria during the various metabolic states associated with β-hydroxybutyrate oxidation. The pattern of steady state changes of pyridine nucleotide oxidation and reduction initiated by ADP or substrate are similar to those described for Fig. 1. A parallel experiment was carried out with the same preparation of mitochondria and with additions of reagents at times correlated with those employed in the experiment presented in Fig. 2. At the times indicated by the arrows in Fig. 2, samples of the diluted mitochondrial mixture were removed and the reaction terminated by addition to perchloric acid. The results of assays for DPN and TPN in the neutralized supernatant fluid are presented in Table 2. The concentrations of pyridine nucleotide determined in this manner are similar to the results reported by Jacobson and Kaplan (17) and to the values obtained by Klingenberg *et al.* (18) but slightly lower than those reported by Birt and Bartley (19).

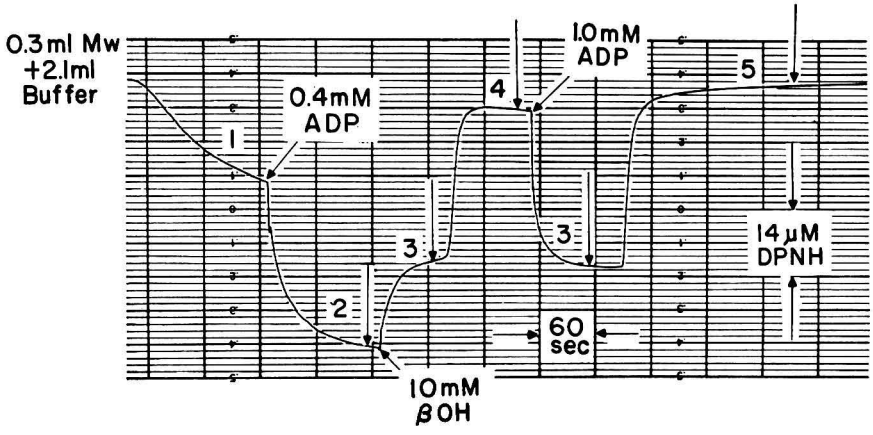

FIG. 2. Fluorometric measurement of pyridine nucleotide reduction of rat liver mitochondria during β-hydroxybutyrate oxidation. A 0.3-ml aliquot of mitochondrial suspension (25.5 mg protein/ml) was diluted to 2.4 ml with isotonic buffer and the change in fluorescence measured with the Eppendorf fluorometer. Additions of ADP and β-hydroxybutyrate are as indicated. The various metabolic states are designated by the numbers associated with each transition of pyridine nucleotide reduction. The establishment of anaerobiosis (state 5) was confirmed by the parallel polarographic measurement of oxygen utilization. The arrows indicate times when samples were removed in the parallel experiment described in Table 2. An upward deflection of the recording indicates an increase in fluorescence.

TABLE 2

ANALYSIS FOR PYRIDINE NUCLEOTIDE OF RAT LIVER MITOCHONDRIA DURING β-HYDROXYBUTYRATE OXIDATION

Metabolic state	Pyridine nucleotide (mole/mg protein)	
	DPN	TPN
2	2.1×10^{-9}	2.4×10^{-9}
3	1.9	1.1
4	1.1	0.1
3	1.8	1.1
5	1.0	0.05

A 6-ml sample of mitochondrial suspension (25.5 mg protein/ml) was diluted to 42 ml with isotonic buffer. The mixture was stirred and additions of ADP and β-hydroxybutyrate were made at the times indicated in Fig. 2. Establishment of the metabolic states was ascertained from the parallel experiment illustrated in Fig. 2 (i.e., arrows) at which times 2-ml samples were removed and added to centrifuge tubes containing 1 ml of 15% perchloric acid. The samples were neutralized, centrifuged, and the concentration of DPN and TPN determined as described previously (11). When the mitochondria were in state 2 (i.e., in the presence of ADP without added substrate) duplicate 2-ml samples were removed and the reaction terminated by addition to 1 ml of 3 *N* KOH. The alkaline samples were neutralized and assayed for DPNH and TPNH. These analyses showed absence of any detectable concentration of DPNH and 0.5×10^{-9} mole/mg protein TPNH (Expt. 854).

Of considerable interest are the changes in TPN reduction during β-hydroxybutyrate oxidation. This result is in agreement with Klingenberg and Slenczka (27), who also studied liver mitochondria, as well as Estabrook *et al.* (28), who observed similar transitions with digitonin particles. The significance of these changes will be described in detail in a future communication.

The extent of pyridine nucleotide reduction as calculated from the analytical data presented in Table 2 is summarized in Table 3 where it

TABLE 3

COMPARISON OF FLUOROMETRIC MEASUREMENT OF PYRIDINE NUCLEOTIDE REDUCTION AND CHANGE IN ANALYZABLE PYRIDINE NUCLEOTIDE CONCENTRATION

Metabolic states	Apparent change in reduced pyridine nucleotide concentration (mole × 10^{-9}/mg)				Ratio fl./anal.
	Fluorescence	Analysis			
		DPNH	TPNH	Total	
3–2	7.0	0.2	1.3	1.5	4.6
4–2	18.6	1.0	2.3	3.3	5.7
3–2	6.4	0.3	1.3	1.6	4.0
5–2	20.0	1.0	2.4	3.4	5.9

The change in pyridine nucleotide concentration was determined from the fluorometric recording presented in Fig. 2. The changes in DPN and TPN concentrations were determined from the data presented in Table 2. The same preparation of rat liver mitochondria was employed for both studies (Expt. 854).

is related to the fluorometric changes illustrated in Fig. 2. Such a comparison confirms the spectrophotometric data presented in Table 1 and illustrates the 4- to 6-fold fluorescence enhancement for the sum of reduced pyridine nucleotides of mitochondria.

Avidor *et al.* (8) recently reported a similar fluorescence enhancement for the reduced pyridine nucleotides of mitochondria. Applying their method of calculation of the simultaneous equations derived from data of the type presented in Table 3, the fluorescence enhancement for TPNH is 2 to 4 while that for DPNH is 8 to 12. As will be discussed in a subsequent paper, the extent of fluorescence enhancement for DPNH varies depending upon the metabolic state of the mitochondria and therefore may be related to one of the proposed intermediates of oxidative phosphorylation [cf. Chance and Williams (16) or Slater (20)].

From these studies comparing the spectrophotometric, analytical, and fluorometric measure of pyridine nucleotide changes of mitochondria it is apparent that the qualitative picture of steady-state kinetics can be measured fluorometrically although the differences in fluorescent enhancement under various metabolic conditions detracts from the quanti-

tative validity of this method. Aware of this limitation, we investigated other parameters affecting the usefulness of the fluorometric method.

Influence of Mitochondrial Concentration. In order to ascertain the useful range of the fluorometric technique for the measurement of pyridine nucleotide kinetics a series of experiments were carried out to determine the linearity of change in fluorescence as a function of mitochondrial concentration. Various dilutions of suspensions of rat liver mitochondria were treated with ADP to oxidize pyridine nucleotide as described in Figs. 1 and 2. A mixture of glutamate and malate was then added as substrate followed by additions of ADP in order to determine the magnitude of cyclic oxidation and reduction of pyridine nucleotide. The per cent fluorescence observed during the steady-state reduction of pyridine nucleotide in the metabolic states 2, 3, and 4 are plotted as a function of mitochondrial protein concentration in Fig. 3. The change in fluorescence is linear to a concentration of mitochondrial protein of about 2.0 mg/ml. At higher concentrations the curve departs from linearity with an error of about 10% at a concentration of 2.5 mg/ml. This loss

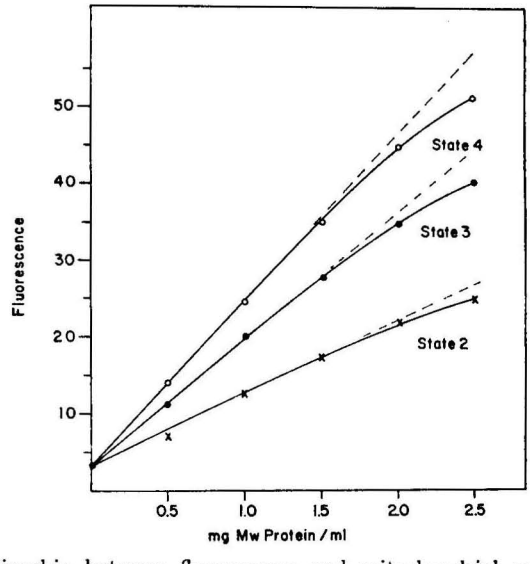

FIG. 3. Relationship between fluorescence and mitochondrial protein concentration. A series of experiments similar to those described in Figs. 1 and 2 were carried out with various dilutions of rat liver mitochondria employing a mixture of malate-glutamate-malonate as substrate. The mitochondrial suspension was treated with ADP to oxidized pyridine nucleotide (state 2) followed by the addition of substrate causing a reduction of pyridine nucleotide to the actively metabolizing state (state 3) or to the ADP limited quiescent state (state 4). Protein concentration was determined with biuret reagent standardized with bovine serum albumin.

of linearity of fluorescence observed with higher concentrations of mitochondria is comparable to that observed with a solution of DPNH, i.e., a quenching of fluorescence is observed at concentrations greater than 4×10^{-5} *M* DPNH.

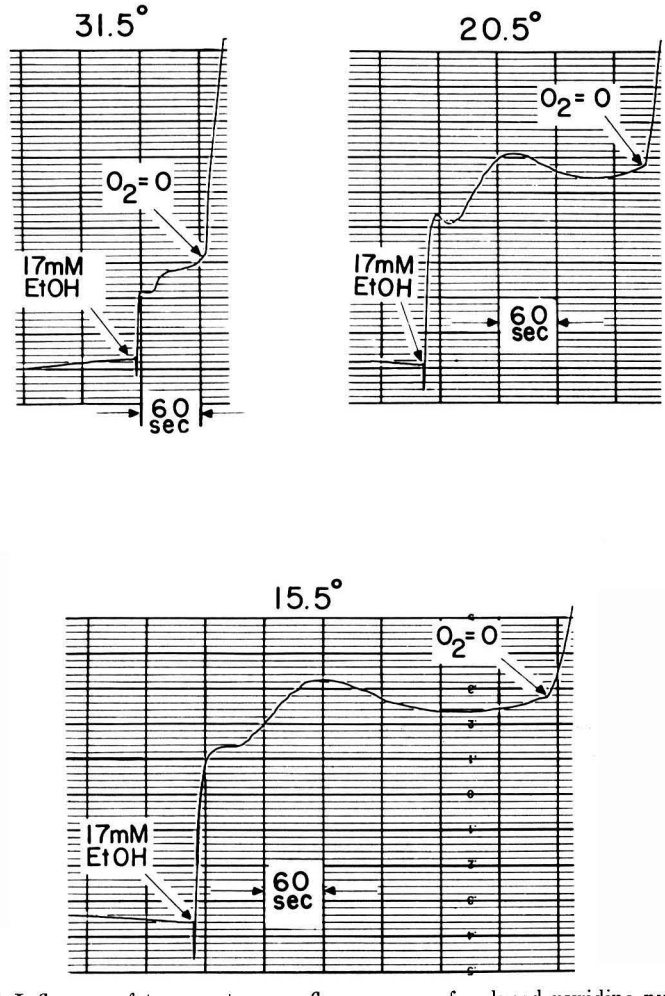

FIG. 4. Influence of temperature on fluorescence of reduced pyridine nucleotide of yeast during alcohol oxidation. A 0.4-ml sample of a 30% suspension of yeast cells aerated 48 hr to deplete endogenous substrates was diluted to 2.5 ml with 0.02 *M* triethanolamine buffer of pH 7.4 of the temperature indicated. The mixture was placed in a water-jacketed cuvette holder to maintain temperature and the reaction initiated by the addition of 0.025 ml of a 10% solution of ethanol as indicated. An upward deflection of the tracing represents an increase in fluorescence. Experiment 850.

The data presented in Fig. 3 also illustrates the magnitude of the background fluorescence associated with mitochondria (i.e., the fluorescence observed is state 2). This may be due in part to TPNH, which is not completely oxidized on addition of ADP (cf. Table 2) as well as other fluorescent compounds, such as flavins, possibly present in mitochondria. The introduction of the bias voltage circuit with the Eppendorf

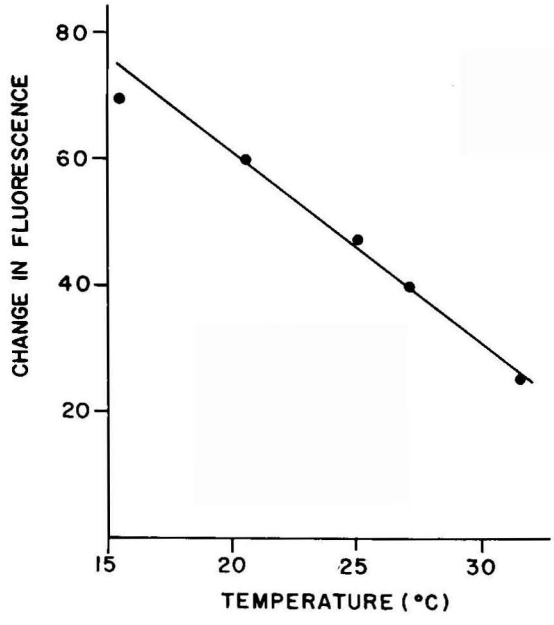

FIG. 5. Relationship of temperature to pyridine nucleotide fluorescence during the aerobic steady state of alcohol oxidation by yeast. A series of experiments comparable to those illustrated in Fig. 4 were carried out at various temperatures. The magnitude of increase in fluorescence upon addition of ethanol is plotted as a function of temperature. Change in fluorescence is expressed in arbitrary units.

(11) permits one to compensate for this background fluorescence and amplify the changes observed during the various metabolic states.

Effect of Temperature. Another parameter which alters pyridine nucleotide fluorescence is the temperature of the reaction medium. This was tested by applying the techniques described above for the measurement of mitochondrial pyridine nucleotide reduction to a study of reduced pyridine nucleotide fluorescence of yeast during glucose or alcohol oxidation [cf. Duysens and Amesz (6)]. The magnitude of fluorescence increase associated with pyridine nucleotide reduction of yeast during alcohol oxidation was therefore measured at various tem-

peratures by incorporating a water-jacketed constant-temperature reaction cuvette holder into the fluorometer attachment of the Eppendorf. The recordings presented in Fig. 4 show that the fluorescence of reduced pyridine nucleotide is inversely related to the temperature of the reaction medium, i.e., reduced pyridine nucleotide fluorescence has a negative temperature coefficient. The magnitude of change in fluorescence is plotted as a function of temperature in Fig. 5. This is in agreement with the observations of Lowry *et al.* (21), who determined a negative temperature coefficient of 1.6% per degree for the fluorescence of solutions of DPNH as a function of temperature.

Studies with Bacterial Systems. In addition to the applicability of the technique to the study of pyridine nucleotide fluorescence of mitochondria and yeast, studies have been carried out to measure the change in pyridine nucleotide reduction of bacteria during various types of metabolism (15). Such studies should give a further insight into the metabolism of microorganisms and the role of pyridine nucleotide in coupled enzymic reactions—either the oxidation and reduction initiated by dehydrogenase and the respiratory chain or between two or more dehydrogenases. An example of the type of recording obtainable is illustrated in Fig. 6, where the reduction of pyridine nucleotide during

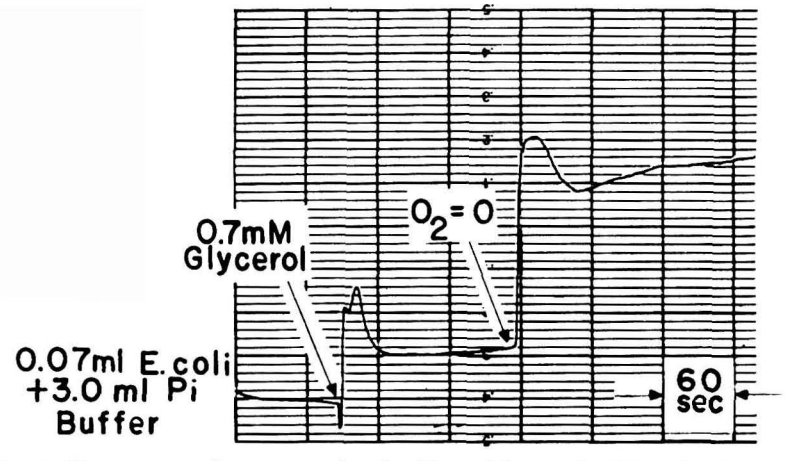

FIG. 6. Fluorescence changes associated with pyridine nucleotide reduction during glycerol metabolism by *E. coli*. *E. coli* were adapted to glycerol by growth on Cohen's medium (14) and harvested during the log phase of growth. A 0.07-ml sample of the washed suspension of *E. coli* was diluted to 3.0 ml with 0.1 *M* phosphate buffer of pH 7.0 containing 6 m*M* NH_4Cl. At the point indicated glycerol was added to initiate respiration and pyridine nucleotide reduction. An increase in fluorescence is indicated by an upward deflection of the tracing. Experiment 856.

glycerol metabolism by *E. coli* has been measured. This shows the rapid reduction of pyridine nucleotide on initiation of respiration, presumably due to the activation of α-glycerol phosphate dehydrogenase. A second aerobic cycle of reduction is also observed which may represent the contribution of triose phosphate dehydrogenase prior to the establishment of the aerobic steady state of glycerol metabolism. Studies of this type, combined with measurements on the influence of inhibitors as well as determinations of changes in concentration of metabolic intermediates (glyceraldehyde 3-phosphate, pyruvate, etc.), will extend our understanding of the integrated metabolic reactions functional in bacterial cells.

Correlated with these studies of pyridine nucleotide reduction in whole cell systems, samples of yeast and bacteria have been assayed for the change in pyridine nucleotide concentration to determine the extent of fluorescence enhancement. These results are summarized in Table 4

TABLE 4
EXTENT OF FLUORESCENCE ENHANCEMENT OF REDUCED PYRIDINE NUCLEOTIDE

System	Fluorescence enhancement
Liver mitochondria	
Sum of DPNH + TPNH	
State 4	6
State 3	4
Calcd. from anal. data	
DPNH	8–12
TPNH	2–4
Yeast (alcohol oxidation)[a]	3–4
E. coli (glucose oxidation)[a]	1
ADH·DPNH complex	2–3
GDH·DPNH complex	1.7
LDH·DPNH complex	3.5
GDP·DPNH complex	Quenching

Fluorescence enhancement is expressed as the ratio of fluorescence observed to the fluorescence of a comparable sample of DPNH solution. The ratio for alcohol dehydrogenase (ADH) is from the data of Duysens and Kronenberg (23). The ratio for glutamic dehydrogenase (GDH) is from the data of Frieden (25), while the ratio for lactic dehydrogenase (LDH) and glyceraldehyde 3-phosphate dehydrogenase (GPD) were obtained from the data of Velick (26).

[a] Studies carried out in collaboration with Dr. P. K. Maitra.

together with results obtained by others with purified enzyme systems. These studies show that with bacteria (*E. coli*) no fluorescence enhancement is observed while with liver mitochondria a 4- to 6-fold enhancement is obtained. This comparison of the extent of fluorescence enhance-

ment with various systems shows no consistent pattern but does point out that reduced pyridine nucleotide of mitochondria must be bound in a unique manner resulting in a fluorescence enhancement far exceeding any other system yet tested.

DISCUSSION

The simplicity of the fluorometric method for the measurement of reduced pyridine nucleotide offers a convenient technique for monitoring changes in the extent of steady-state reduction during various metabolic conditions. However, one uncertainty is associated with such measurements, particularly with turbid protein systems. This resides in the extent of fluorescence enhancement resulting from the binding of reduced pyridine nucleotides and the difference in enhancement observed with DPNH and TPNH.

The property of fluorescence enhancement of reduced pyridine nucleotide has been observed with a variety of purified enzyme systems. Boyer and Theorell (22) as well as Duysens and Kronenberg (23) have reported on the increase in fluorescence intensity of reduced pyridine nucleotide when bound to alcohol dehydrogenase. In addition, a shift in the maximum of the emission spectrum occurs on binding (8). This is a property which Chance and Baltscheffsky (9) have employed to determine that reduced pyridine nucleotide in mitochondria is in the bound form. The previous studies (22, 23) on the increase in fluorescence yield on binding DPNH to alcohol dehydrogenase showed only a 2- to 3-fold increase in fluorescent intensity. Recently, however, Winer and Theorell (24) have shown that the addition of acetamide or imidazole to form a ternary complex with DPNH and alcohol dehydrogenase further enhances the fluorescent intensity.

Frieden (25) recently investigated the applicability of fluorescent enhancement measurements to determine coenzyme binding to enzymes (glutamic dehydrogenase) and concluded that the observed enhancement may not represent, however, the binding of reduced pyridine nucleotide to the necessary catalytic site on the protein. In addition, Velick (26) has discussed the factors influencing the enhancement or quenching observed during the formation of complexes of pyridine nucleotide and enzyme. He (26) attributes the enhancement of fluorescence to a change in the conformation of the bound coenzyme and the "rigid orientation of the reduced pyridine ring on the enzyme by bonds which raise the energy level of the first electronic excited state."

The high fluorescent yield observed with reduced pyridine nucleotide associated with liver mitochondria has also been observed by Avidor *et al.* (8) and confirmed by the present study. This enhancement may

represent evidence for a ternary complex and may be related to the presence of a "high energy intermediate" form of reduced pyridine nucleotide in mitochondria. Studies to be presented in detail in a subsequent paper indicate a direct correlation between the extent of fluorescence enhancement of DPNH associated with mitochondria and the presence of phosphate, ADP, and substrate.

SUMMARY

1. The application of a commercial fluorometer for the measurement of pyridine nucleotide reduction and oxidation of biological particulate preparations, such as mitochondria, yeast, and bacteria, has been described and demonstrated.

2. The changes of concentration of reduced pyridine nucleotide as measured fluorometrically with rat liver mitochondria have been compared to the spectrophotometric measurement of pyridine nucleotide kinetics and to the changes in pyridine nucleotide concentration determined by chemical analyses.

3. These studies show a fluorescence enhancement for reduced pyridine nucleotide of rat liver mitochondria of 4- to 6-fold suggesting the presence of a ternary complexing of pyridine nucleotide in mitochondria. The greatest fluorescence enhancement is observed with the DPNH fraction of mitochondria with increases as high as 8 to 12 obtainable under various metabolic conditions.

4. The extent of fluorescence enhancement observed with mitochondria, bakers' yeast, and *E. coli* is compared to previous reports on the DPNH complexing with purified enzyme preparations.

5. The influences of mitochondrial concentration and temperature on the measurement of pyridine nucleotide fluorescence are described.

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ANALYTICAL BIOCHEMISTRY 4, 246–251 (1962)

Determination of H^3 and C^{14} in Biological Materials Using Oxygen Bomb Combustion[1]

HERBERT SHEPPARD AND WALDTRAUT RODEGKER

From the Research Department, CIBA Pharmaceutical Company, Division of CIBA Corporation, Summit, New Jersey

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The determination of low levels of H^3 and C^{14} in biological materials presents the problem of combusting relatively large samples. Recently, two methods appeared which attempted to solve this problem. Kelly *et al.* (1) utilized the Schöniger oxygen flask technique for samples below 300 mg dry weight, while Peets *et al.* (2) used an oxygen furnace of special design for samples up to 2 gm dry weight. At the same time we had investigated the possibility of using the Parr oxygen bomb for samples of 1.0 to 1.5 gm of dry biological material.

The use of the metallic Parr oxygen bomb suggested certain advantages derived from simplicity of design, economy of space, and absence of memory effect. The feasibility of this approach was suggested by the use of the bomb in calorimetry, where completeness of combustion is essential. In addition, Payne (3) used a hand-made bomb for the determination of tritium in somewhat smaller samples. The following sections describe the methods developed primarily for the determination of H^3 in animal tisues but which have also been applied to the determination of C^{14}.

METHODS

Apparatus. Double-valve, self-sealing, 360-ml capacity oxygen combustion bomb No. 1101 plus accessories (Parr Instrument Co., Moline, Illinois). Tri-Carb liquid scintillation spectrometer (Packard Instrument Corp., La Grange, Illinois).

Reagent. PPO (2,5-diphenyloxazole), POPOP (1,4-bis-2-(5-phenyloxazole)benzene), and thixotropic gel powder (Cab-O-Sil) were all obtained from Packard Instrument Corp., La Grange, Illinois. Gold Shield absolute ethanol was used while all other chemicals were reagent

[1] The contents of this paper were presented at the Fifth Annual Symposium on Advances in Tracer Methodology, October 20, 1961, Washington, D. C.

grade. The toluene-phosphor solution was prepared by dissolving 3 gm of PPO and 0.1 gm of POPOP in a liter of toluene. The dioxane-naphthalene-phosphor solution was prepared by dissolving 7.0 gm of PPO, 0.05 gm of POPOP, and 100 gm of naphthalene in 1 liter of 1,4-dioxane. The thixotropic gel-phosphor was prepared by adding 25 gm of thixotropic gel powder to 1 liter of the toluene-phosphor solution. The $Ba(Cl)_2$–NH_4Cl solution contained 0.44 N NH_4Cl in 5 N *aqueous* $Ba(Cl)_2$.

Combustion Procedure. The sample was placed in the ignition cup and the electrodes were connected with a Nicrome wire according to procedures described in the operation booklet. The suggested addition of 1 ml of distilled water was omitted in order to keep the water volume to a minimum. The electrode unit was then placed into the body of the bomb and the screw cap tightened by hand. The inlet valve was attached to an oxygen tank and charged to 25 atm. The bomb was then submerged in water in the 2-liter metal container purchased with the bomb. The ignition unit was attached and the button was pressed to initiate the combustion. The combustion was complete in a matter of seconds, but the bomb was left in the water for about 10 min to allow the water vapor cloud to condense along the inside walls of the bomb. The bomb was then removed from the water, wiped dry with a towel, and connected to the water and carbon dioxide collection train as shown in Fig. 1.

The first two small traps were immersed in a Dewar flask containing

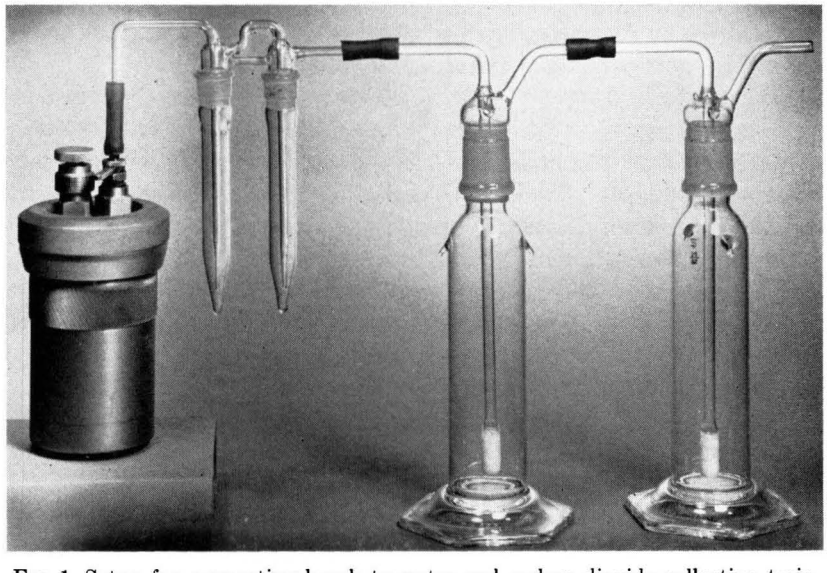

FIG. 1. Setup for connecting bomb to water and carbon dioxide collection train.

a dry ice-acetone mixture for the collection of any water vapor escaping from the bomb after the release valve was opened. From there the escaping gases pass through two gas-collecting columns, the first containing 200 ml and the second 100 ml of 1 *N* NaOH. The bomb was recharged with 5 atm of oxygen after all of the original gases had been released. This new charge with any remaining water or CO_2 was exhausted through the same solutions used for the original collections.

If no C^{14} was present in the sample, the absorbing solutions of alkali as well as the recharging of the bomb were omitted.

Collection of $H_2{}^3O$ *for Counting*

The water collection tubes were removed from the Dewar flask. After the ice had melted, the delivery and collection tubes were rinsed with 1 ml of ethanol delivered with a glass syringe. This ethanol wash was transferred to a counting vial and the process was repeated with another 1 ml of absolute ethanol. These two ethanol washes were combined and counted to determine the water lost from the bomb. The bomb was then opened and the inner surfaces rinsed with a total of 12 ml of ethanol. Care must be taken to collect all of the ethanol rinses. By first rinsing off the inner surface of the head which includes the electrodes and cup, the drippings were collected in the bottom part. This rinse of absolute ethanol was transferred to a 16-dram counting vial containing 40 ml of toluene-POPOP-PPO solution and counted in the liquid scintillation spectrometer. Absolute counting rates were calculated from counting efficiencies determined on each sample by the addition of 0.1 ml of toluene-H^3 after the initial count was obtained.

Preparation of $C^{14}O_2$ *for Counting*

From each collection flask, 20-ml aliquots were removed and mixed with 80 ml of a $Ba(Cl)_2$-NH_4Cl solution. The precipitate of $BaCO_3$ which formed was collected by centrifugation and washed successively with water, ether-ethanol (1:1), and acetone. The $BaCO_3$ was then dried, weighed, and placed in a 16-dram vial. To this was added 40 ml of a thixotropic gel-phosphor solution and the vial was shaken vigorously to suspend the $BaCO_3$. This suspension was then counted in the liquid scintillation spectrometer. Absolute counts rates were calculated from counting efficiencies determined through the addition of 0.1 ml of toluene-C^{14}.

Preparation of Samples

All biological samples were lyophilized in a pharmaceutical research size lyophilizer. Most of the tissues could be combusted simply by

putting the dried piece in the ignition cup of the bomb. Some tissue lyophilates such as lung, along with the powdery lyophilates of blood, urine, bile, and feces, were placed into gelatin capsules (No. 00) and combusted. All samples were weighed before and after lyophilization in order to determine the original water content.

RESULTS AND DISCUSSION

After complete combustion of the sample had occurred, it was necessary to exhaust the gases. The speed with which the gases left the bomb could be regulated by the exhaust valve. It was recognized that some $H_2{}^3O$ might be lost as water vapor along with the escaping gases and that the amount lost would depend to some extent on the rate at which the gases left the bomb. The effect of the time used to exhaust the gases on the amount of $H_2{}^3O$ found in the water traps may be found in Table 1.

TABLE 1
EFFECT OF EXHAUST TIME ON ESCAPE OF $H_2{}^3O$

Exhaust time (min)	% found in trap	% recovered
2.5	4.6	101
6.0	3.7	102
9.5	4.1	101
10.0	3.4	96
15.0	1.2	96
30.0	0.7	96

It is seen that slightly less than 5% of the total $H_2{}^3O$ was recovered in the traps even if the exhaust valve was opened all the way (2.5 min exhaust time). The loss of $H_2{}^3O$ to the traps did not begin to fall appreciably until the exhaust time was greater than 10 min. This reduction in loss of $H_2{}^3O$ to the traps did not seem to warrant the greater time needed for exhaustion of the gases. It was decided, therefore, that when $H_2{}^3O$ alone was being collected, the exhaust valve could be opened fully. As may be seen in Table 1 the total recoveries of H^3 ranged from 96 to 102%. A typical series of analyses with tissues is found in Table 2.

When C^{14} was being determined, the exhaust time had to be extended to 10 min in order to prevent excess foaming and frothing of the sodium hydroxide solutions as well as the blowing off of the tops of the collecting columns. Table 3 shows that the recoveries obtained with C^{14} ranged from 93 to 104%. With 200 ml of 1 *N* NaOH in the first flask only 1 to 3% of the $C^{14}O_2$ actually gets into the second collection flask. In some experiments, when 100 ml of 2 *N* NaOH was used in the first collection

TABLE 2
ANALYSIS OF TISSUES OF TURKEYS RECEIVING H^3-LABELED DRUG

Tissue	Sample		Water loss (%)	H^3 found (mμc)
	Wet wt. (gm)	Dry wt. (gm)		
Heart	3.10	0.753	75.7	0.36
Spleen	2.60	0.610	76.6	0.54
Lung	3.05	0.701	77.0	0.98
	3.25	0.787	75.7	0.93
Liver	3.60	1.175	67.4	4.38
	3.05	0.976	68.0	4.60
Kidney	3.05	0.655	78.7	0.43
Breast	3.45	1.034	70.0	N.D.[a]
Muscle	3.15	0.923	70.8	N.D.[a]
Plasma	4.7 ml.	0.377	92.8	0.72

[a] Not detectable.

TABLE 3
RECOVERY OF C^{14} ADDED TO 700 MG OF CHICKEN FEED

DPM C^{14} added	% recovered
30,000	98
30,000	97
3,145	100
3,145	102
3,145	104
3,145	93

flask, as much as 12% of the $C^{14}O_2$ was carried over into the second one.

The use of the 16-dram vial was disadvantageous, and attempts were made to use a 5-dram vial. If less than 500 mg of tissue was being combusted, 4 ml of ethanol was sufficient, and therefore, the 5-dram vial could be used. Thus, one could combust more tissue but count the equivalent of only 500 mg. However, to maximize the sensitivity of the method, it would be desirable to count all of the water from 1 to 1.5 gm of lyophilized tissue in a 5-dram vial. For this purpose the dioxane-naphthalene-phosphor system seemed ideal. Tests with pure water indicated that up to 1.0 ml of water could be counted with a loss of efficiency of only 30%. When, however, this system was applied to bomb combustion water several problems became apparent. Naphthalene and the oxazole phosphors were found to precipitate along the walls and valves of the bomb when the dioxane solution was used to collect the water. This could be corrected by using pure dioxane to wash the bomb and placing weighed amounts of naphthalene and phosphor in the counting

vial. At this point, however, it was found that the counting efficiency had fallen to 1 to 1.5% as compared with 3 to 4% efficiencies with the ethanol-toluene-phosphor system. The reason for this soon became apparent when it was observed that the fluorescence of the solutions containing combustion water was not as intense and appeared to be less purple in color. Remembering that water of combustion is acidic due to the formation of nitric and sulfuric acids from the nitrogen and sulfur contained in the samples, it was thought that these could be the cause of the trouble. Brief experimentation with pure acids demonstrated that the same change in fluorescence could be effected, with nitric acid being more potent in this regard. Apparently, in the ethanol-toluene system, these acids have less effect. The problem of the effects of acids on counting efficiencies for H^3 was described recently by Belchor (4). Because of the complexities of the dioxane-naphthalene system, the ethanol-toluene system appeared more satisfactory.

The system as initially outlined has been used for hundreds of combustions with excellent results. Only one bomb has been used—with no accidents, failure of function, or serious corrosion. It has been possible to determine approximately 4.0×10^{-4} μc of H^3 and 1.0×10^{-4} μc of C^{14} per 3 gm of fresh tissue. The setup is compact and the operation is relatively rapid. Where sensitivity is not a great problem, one may combust the fresh tissue without lyophilization by placing a weighed amount in a gelatin capsule.

SUMMARY

A method has been presented which makes possible the determination of 0.4 and 0.1 mμc of H^3 and C^{14}, respectively, in 1–1.5 gm of lyophilized tissue. The sample is combusted in a Parr oxygen bomb under 25 atm pressure of oxygen and the resulting water and carbon dioxide collected and counted in a liquid scintillation spectrometer.

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ANALYTICAL BIOCHEMISTRY 4, 252–256 (1962)

Microdetermination of Cysteinesulfinic Acid[1]

FRANZ-J. LEINWEBER AND KENNETH J. MONTY

From the McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland

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Although cysteinesulfinic acid (CSA) has been widely studied as a metabolite in biological systems, convenient methods for the microdetermination of this compound are notably lacking in the literature. The conventional analytical approach has been first to separate the compound from interfering substances by chromatography on filter paper or columns, and then to determine the amount of CSA recovered by virtue of the amino group or the reducing power of the sulfinic acid moiety (1–3).

A convenient and precise microanalysis of CSA can be achieved through the use of glutamic-oxalacetic transaminase. CSA is reacted with α-ketoglutarate in the presence of the enzyme, yielding glutamic and, presumably, β-sulfinylpyruvic acids as products. The latter compound does not accumulate, but gives rise to inorganic sulfite, which may be determined colorimetrically. The determination consists, then, of the analysis for sulfite which has been released specifically and stoichiometrically from CSA. The method is applicable to as little as 50 mμmoles of the sulfinic acid.

MATERIALS

Pig heart glutamic-oxalacetic transaminase, A grade, was obtained from California Corporation for Biochemical Research.[2] Before use, the enzyme was diluted with 0.04 *M* phosphate buffer, pH 6.8, to a concentration of approximately 2 mg/ml, and dialyzed overnight against the same buffer. Solutions of the enzyme prepared in this fashion were found to be moderately stable, retaining 85% of their activity after storage for 8 days at 0°C. Lower storage temperatures did not offer any advantage

[1] This work was supported in part by Contract AT(30-1)-1816 between the Atomic Energy Commission and The Johns Hopkins University. This is contribution No. 366 from the McCollum-Pratt Institute.

[2] The supplier reports that this enzyme is prepared by the method of Jenkins *et al.* (9).

in preserving enzymic activity. A unit of enzyme activity is that amount of enzyme which produces an initial rate of change of 0.185 optical density unit per minute at 280 mμ (1 cm, light path) when incubated at 37°C in 3 ml of solution containing 360 μmoles of L-monosodium-glutamate, 7.5 μmoles of oxalacetic acid, and 250 μmoles of phosphate buffer, pH 8.0. This rate is equivalent to the uptake of 1 μmole of oxalacetate per minute.

The L-cysteinesulfinic acid cited in Table 1 was obtained from California Corporation for Biochemical Research. The supplier cited the following analytical data for the sample: carbon, 23.55%; hydrogen, 4.65%; $[\alpha]_D = +27.5°$, $c = 1\%$ in 1 N HCl, 19°C.

Hypotaurine was prepared by the oxidation of cystamine according to the method of Cavallini *et al.* (4). This product had the following physical and chemical properties: m.p. (corr.), 171–173°C, in agreement with that cited by Bricas *et al.* (5); equivalent weight as determined by permanganate titration, 62.8 (theoretical for hypotaurine monohydrate, 63.5); free of inorganic sulfite, as determined by the method of Grant (6); descending chromatography on filter paper with phenol/water (4/1) revealed a single ninhydrin-positive spot with an R_f of 0.62, in agreement with that cited by Bricas *et al.* (5) for hypotaurine; oxidation of the synthetic material with H_2O_2 yielded complete conversion to a second ninhydrin-positive material which was chromatographically indistinguishable from taurine.

METHODS

Appropriate amounts of a neutral solution containing cysteinesulfinic acid (CSA) were combined with 15 μmoles of α-ketoglutarate and 210 μmoles of phosphate buffer, pH 8.0, in a total volume of 2.4 ml. The reaction was initiated by the addition of 25 units of glutamatic-oxalacetic transaminase in a volume of 0.1 ml. After incubation at 37°C for 15 min, the reaction was terminated by the addition, in quick succession, of 0.5 ml of 1% alcoholic KOH and 1 ml of a saturated aqueous solution of $HgCl_2$. After mixing thoroughly, a clear supernatant was obtained by short-term centrifugation. One ml of the supernatant fluid was added to 4 ml of the fuchsin reagent of Grant (6).[3] Precisely 15 min later, the optical density of the solution was determined at 580 mμ, using a Beckman Model DU spectrophotometer, slit width of 0.03 mm, and a light path of 1 cm.

Since inorganic sulfite may be present in the sample to be analyzed or in the reagents utilized, a control, wherein the KOH and $HgCl_2$ were

[3] It is desirable to decolorize this reagent after preparation by shaking with 2 mg of activated carbon per milliliter for 15 min and filtering.

added immediately before the transaminase, was carried in parallel with each analysis. The controls are not incubated at 37°C.

The amount of CSA in the original sample was calculated as follows:

$$\text{m}\mu\text{moles of CSA} = (\text{OD}_{\text{incubated sample}} - \text{OD}_{\text{control}}) \times 517$$

The proportionality constant was determined using a solution of inorganic sulfite standardized by iodimetric titration (7).

RESULTS

Sensitivity and Precision. The precision and the useful range of the method are indicated in Table 1. The commercial sample of CSA ana-

TABLE 1
ANALYSIS OF A COMMERCIAL SAMPLE OF CYSTEINESULFINIC ACID

CSA added (μg)	Sulfite (μmoles)	
	Theoretical	Found
7.66	0.050	0.050
15.3	0.100	0.104
23.0	0.150	0.152
30.6	0.200	0.191
46.0	0.300	0.294
61.3	0.400	0.391
76.6	0.500	0.497

lyzed yielded 99.4% of the theoretical amount of sulfite, with a standard deviation of 2.7% for the seven determinations.

By contrast, a second commercial sample from a different source, also claiming to be L-CSA of high purity, yielded 0.0% of sulfite by this method. Elemental analysis of this latter sample suggests that it is a hydrated form of cysteic acid.

Specificity. Since the method involves the colorimetric determination of inorganic sufite released enzymically from CSA, the specificity of both the enzymic release of sulfite and the determination of sulfite are of concern to the analyst. Two organic compounds, hypotaurine and homocysteinesulfinic acid, were considered as potential sources of error by virtue of their sulfinic acid moieties. Application of the analytical method to synthetic hypotaurine indicated clearly that this compound neither gives rise to sulfite in the presence of the transaminase nor interferes in any way with the determination of CSA.

Homocysteinesulfinic acid could not be obtained for direct examination. However, it may be anticipated that this compound will be accepted by the enzyme as a substrate for transamination, but that the product

of deamination, β-sulfinyl-α-ketobutyric acid, will not readily hydrolyze to release inorganic sulfite.

Grant (6) has indicated that a high degree of specificity can be expected from the colorimetric determination of sulfite under the conditions employed above. The method has proved highly satisfactory for the measurement of sulfite in bacterial systems in this laboratory.

Interfering Compounds. The glutamic-oxalacetic transaminase employed in the method will accept as substrates for transamination glutamic, aspartic, cysteic, and homocysteic acids. (Homocysteinesulfinic acid probably should be included in the list, since it is a close structural analog of these compounds.) Under the conditions employed in the method, each of these compounds will act as a competitive inhibitor of the release of sulfite from CSA. This relationship has been studied in detail with a similar enzyme present in extracts of *Escherichia coli* (8).[4] Evidence from a number of laboratories (see ref. 9, for example) suggests, in fact, that any dicarboxylic acid with a 2- or 3-carbon chain separating the carboxyl groups will associate with the pig heart enzyme, and must be suspect as a potential competitive inhibitor for the release of sulfite from CSA. In analyzing samples of unknown composition for the presence of CSA, possible interference by such divalent organic anions may be detected and overcome by increasing the amount of transaminase used until a maximum amount of sulfite is formed.

Inorganic sulfite is not stable at pH 8.0, although the presence of α-ketoglutarate significantly retards the rate of oxidation of this compound. Inorganic sulfite may be carried through the analytical procedure described with a recovery of better than 99%. However, lengthening the duration of the incubation at 37°C to 30 min lowers the recovery to 95%. Consequently, the recommended incubation time of 15 min should not be exceeded. The completing of the enzymic decomposition of CSA should be established instead by using larger amounts of the transaminase.

Grant (6) has discussed adequately the possible sources of interference in the colorimetric determination of sulfite as performed in the present procedure. One complication must be borne in mind. Organic disulfides react rapidly with inorganic sulfite, and, consequently, when present together with CSA, can lead to erroneously low estimates of CSA. When the presence of cystine, oxidized glutathione, or other disulfide compounds is suspected, care should be taken to remove these before attempting the enzymic decomposition of CSA. A convenient method for

[4] A detailed report of these kinetic interrelationships is forthcoming (*Biochim. et Biophys. Acta,* in press).

the removal of thiols and disulfides from CSA with alumina has been described by Fromageot *et al.* (10).

DISCUSSION

The present method for the determination of CSA offers substantial gains in sensitivity, precision, speed and convenience over methods previously described in the literature. It has been used in this laboratory primarily for the purpose of establishing the degree of purity and the degree of hydration of preparations of CSA, but the attributes of the method suggest a more general applicability.

SUMMARY

A highly purified commercial preparation of glutamic-oxalacetic transaminase is used to promote the selective release of inorganic sulfite from cysteinesulfinic acid. The sulfite is then determined colorimetrically with fuchsin. The method is applicable to as little as 50 mμmoles of the sulfinic acid. Advantages in sensitivity, precision, and convenience are offered over earlier methods.

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ANALYTICAL BIOCHEMISTRY 4, 257–267 (1962)

Determination of Tritium in Whole Cells and Cellular Fractions of *Bacillus megaterium* Using Liquid Scintillation Techniques

JOHN H. HASH

From the Biochemical Research Section, Lederle Laboratories, American Cyanamid Company, Pearl River, New York

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The ready availability of relatively inexpensive tritium-labeled compounds has made them attractive for metabolic investigations. However, their soft beta emissions make their detection difficult, and liquid scintillation counting, because of its sensitivity and precision, is the method of choice (1). Unfortunately, the use of liquid scintillation techniques has been hampered by the lack of suitable methods for incorporating various biological materials into organic phosphor solutions and by the requirement for expensive counting equipment. The former has been largely overcome by combustion (2), Hyamine hydroxide (3), and the use of heterogeneous systems (4–6); the latter is being relieved as liquid scintillation equipment becomes more available.

Liquid scintillation counting of tritium in bacteria poses certain problems not encountered in mammalian tissues, namely, bacteria contain a fibrous cell wall which is insoluble in Hyamine hydroxide. Therefore, bacteria must be combusted and the tritiated water collected, or they must be counted in a heterogeneous system with the aid of a suspending agent. Neither method appears attractive for routine use.

The use of a windowless gas-flow counter for counting tritium in bacteria has been described (7), but this method suffers from the inherent lack of sensitivity of such counters to weak beta radiation, and the inability to determine total radioactivity in samples.

With *Bacillus megaterium* KM, these difficulties have been overcome by dissolving the cell wall of the intact bacterium with lysozyme and incorporating the cellular debris into a phosphor solution with the aid of Hyamine hydroxide.

EXPERIMENTAL

Materials

A culture of *Bacillus megaterium* KM was obtained from Dr. S. Spiegelman, University of Illinois, Urbana, Illinois, and H^3-diamino-

pimelic acid was a gift of Dr. J. L. Strominger, Washington University, St. Louis, Mo. H^3-Thymidine (3.0 curies/mmole) was purchased from Schwarz BioResearch Inc., Orangeburg, New York, and 4,5-H^3-DL-leucine (3.5 curies/mmole) and H^3-toluene as internal standard, from New England Nuclear Corp., Boston, Mass. Hyamine hydroxide (benzyldimethyl {2-[2-(*p*-1,1,3,3-tetramethylbutylphenoxy)ethoxy]ethyl} ammonium hydroxide) was obtained in a 1 *M* methanol solution from Packard Instrument Company, LaGrange, Ill. Crystalline trypsin and lysozyme were obtained from Worthington Biochemical Corp., Freehold, N. J., and membrane filters, from Millipore Filter Corp., Bedford, Mass.

All radioactivity measurements were made in a Packard Tri-Carb Scintillation Spectrometer, and the phosphor solution was the polyether 611 system of Davidson and Feigelson (1), which consisted of *p*-dioxane, anisole, and 1,2-dimethoxyethane (6:1:1; v/v/v) and contained 15 gm 2,5-diphenyloxazole (PPO) and 0.5 gm 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene (Dimethyl-POPOP) per liter of solution.

Methods

Synthetic growth medium. The synthetic medium was a modification of the one used by Hancock (8) for the growth of *Staphylococcus aureus*. It contained the following L-amino acids in mmoles/liter: arginine, cystine, histidine, leucine, isoleucine, methionine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, 0.5; alanine, aspartic acid, glutamic acid, 1.25; and glycine, 1.25. In addition, it contained the following components in gm/liter: glucose, 10; NH_4Cl, 2.0; NaCl, 1.0; K_2HPO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.05; and citric acid, 0.25. All the components were dissolved together in distilled water, with the exception of cystine, which was dissolved in 1 *N* HCl prior to adding it to the medium. The pH was adjusted to 7.2 and the medium was sterilized by pressure filtration through a membrane filter (0.45 μ pore size). This medium supported excellent growth of *Bacillus megaterium* KM, which was measured at 610 mμ in a Coleman, Jr. Spectrophotometer in matched 18 × 150 mm test tubes. A typical growth curve of the organism is shown in Fig. 1.

Measurement of radioactivity in whole cells. Where only quantitative information was sought as to the amount or rate at which label was removed from the medium by the organism, radioactivity in the whole cell was measured. Ten ml of synthetic medium containing 0.1–0.3 μc of radioactivity/ml was inoculated with a 16-hr culture of *B. megaterium* KM grown in the synthetic medium. Tubes, inclined at a 30° angle on a reciprocating shaker, were incubated at 28°. At intervals during the exponential phase of growth (Fig. 1), 1.0-ml samples were withdrawn

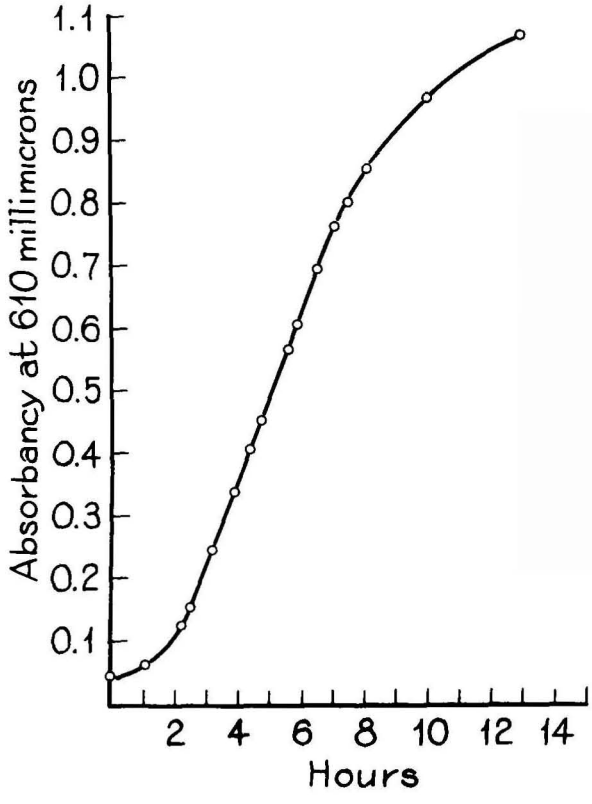

FIG. 1. Typical growth curve of *Bacillus megaterium* KM in synthetic medium.

aseptically for analysis. Samples were chilled rapidly to 0°, immediately centrifuged at 5000 × *g* for 5 min, and washed once with 1 ml of water. The wash water was combined with the culture supernatant for residual radioactivity determinations. The cells were resuspended in 0.1 ml of 0.1 *M* phosphate buffer, pH 7.0, containing 500 μg of lysozyme. After a lapse of at least 1 hr at 37°, 50 μl of 1% trypsin solution was added and incubation was continued for an additional hour. Addition of 0.4 ml of Hyamine hydroxide dissolved completely the cellular debris, which was then carefully washed into a counting vial with 10 ml of scintillation liquid. Trypsin was used to degrade high molecular weight proteins to increase their solubility in the 611 phosphor solution, but this treatment was not absolutely necessary. A slightly greater volume of Hyamine hydroxide may be necessary to dissolve the debris, but there is little sacrifice in counting efficiency.

Samples were then counted to a statistical accuracy of 5% and were

recounted after the addition of H^3-toluene as internal standard. From the calculated counting efficiencies, the sample counts/min (cpm) were converted to disintegrations/min (dpm).

Measurement of radioactivity in cell fractions. Where qualitative as well as quantitative information was sought as to the fate of an incorporated metabolite, cells were fractionated and radioactivity was determined in each fraction. The procedure of Park and Hancock (9) for fractionating *S. aureus* was extended to *B. megaterium* KM. In the Park and Hancock procedure cells are extracted successively with cold 5% trichloroacetic acid (TCA), 75% ethanol, and hot 5% TCA, and then digested with trypsin. The remaining residue consists mainly of the cell wall mucopeptide. The procedure adopted for *B. megaterium* KM was very similar, with the important addition that the residue was fractionated further with lysozyme and *n*-octanol and the entire bacterium was brought into solution. The fractionation scheme is given in Table 1.

TABLE 1
FRACTIONATION SCHEME FOR *Bacillus megaterium*

Fraction	Treatment[a]	Contents of fraction
1. Cold TCA	Suspend 2–4 mg washed cells in 1 ml cold water, add 1 ml cold 10% TCA, centrifuge after 10 min at 0°	All low MW compds. sol. in cold 5% TCA
2. 75% Ethanol	Suspend residue in 2 ml 75% ethanol, centrifuge after 10 min at room temp.	Aq. ethanol-sol. materials
3. Hot TCA	Suspend residue in 2 ml 5% TCA, centrifuge after 6 min at 90°	Degraded nucleic and teichoic acid
4. Trypsin	Suspend residue in 1.5 ml of 0.05 *M* NH_4HCO_3 containing 0.005 *N* NH_4OH, add 0.5 ml of 1 mg/ml trypsin soln., centrifuge after 2 hr at 37°	Trypsin-degraded proteins
5. Lysozyme	Suspend residue in 1.5 ml of 0.1 *M* phosphate buffer, pH 7.0, add 0.5 ml of 1 mg/ml lysozyme solution, centrifuge after 60 min at 37°	Lysozyme-degraded mucopeptide of cell wall
6. *n*-Octanol	Dry residue *in vacuo* at 60° for 60 min, add 1 ml *n*-octanol, warm to 60°; if complete soln. does not occur, add 0.2 ml Hyamine hydroxide, warm till soln. is complete	Degraded poly-β-hydroxybutyrate and perhaps some capsular material

[a] After each centrifugation, the debris was washed with 0.5 ml of the particular reagent and recentrifuged, and the wash water combined with the supernatant in question.

The first four steps are identical with the Park and Hancock procedure. The hydrolysis of the *N*-acetylglucosaminyl-*N*-acetylmuraminyl(pentapeptide) polymer of the bacterial cell wall by lysozyme is well estab-

lished (10). The residue, after lysozyme treatment, has not been analyzed completely but probably consists largely of the polymeric β-hydroxybutyrate found in the lipid granules of the genus *Bacillus* (11, 12), and perhaps some capsular material. These conclusions are based on the following considerations. The residue was completely soluble in 1 *M* Hyamine hydroxide and therefore seems most likely to be lipid or protein rather than carbohydrate. Attempts to dissolve the wet residue with water-immiscible solvents usually gave emulsions, but when dry the residue was soluble in *n*-octanol and chloroform; these properties are characteristic of poly-β-hydroxybutyrate (11). On hydrolysis with 6 *N* HCl, this fraction gave an oily residue that could be extracted with chloroform.

In practice, as 2–4 mg (dry weight) of cells was needed, cells from 5 ml of medium were harvested. Tubes containing 5 ml of medium and 0.1–0.3 μc/ml of label were inoculated and incubated as described in the previous section. When the desired absorbancy was reached, the whole culture was chilled and quickly centrifuged at $5000 \times g$ in the cold. Cells in the late log phase did not form tight pellets on centrifugation; this difficulty was overcome by adding cold TCA to 0.2% concentration in the chilled culture and immediately centrifuging. The cells were washed once, and the combined wash water and supernatant were saved for residual radioactivity determination. Cells were fractionated as described in Table 1. In each case the residue was sedimented at $5000 \times g$ in an angle-head centrifuge, and the supernatant was carefully siphoned off with a Pasteur capillary pipet whose tip had been drawn out to a fine point.

With this procedure the entire bacterium could be brought into solution in a series of fractions, all of which are suitable for incorporation into the 611 scintillation liquid. After recording the total volume of each fraction, 0.5 ml of each was added to 10 ml of the 611 phosphor and counted as described in the previous section. The culture supernatant, ethanol, and TCA fractions dissolved readily in the 611 fluid with no precipitation, but occasionally the trypsin and lysozyme fractions produced slight turbidity which could be dispelled by adding 0.2 ml of Hyamine hydroxide. Occasionally, the photoluminescent effect (13) of Hyamine hydroxide on proteins was observed and this was eliminated by the stoichiometric neutralization of Hyamine hydroxide with glacial acetic acid.

RESULTS

Incorporation of H^3-leucine into whole cells. The results of an experiment incorporating 4,5-H^3-DL-leucine into whole cells are shown in

Fig. 2. The abscissa is given as Δ absorbancy to correct for initial absorbancy of the culture. The incorporation of H^3-leucine is directly proportional to the increase in absorbancy throughout the major part of the exponential phase of growth; that is, the rate of incorporation of label is directly related to the increase in bacterial cell mass.

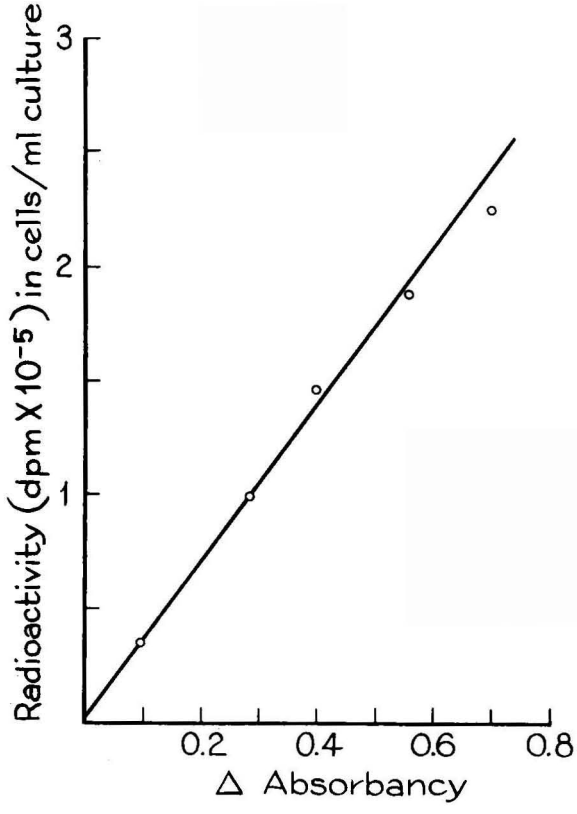

FIG. 2. Incorporation of 4,5-H^3-DL-leucine into *B. megaterium* KM as a function of bacterial growth; 1-ml aliquots were removed at the indicated intervals from 10 ml synthetic medium containing 6,410,000 dpm.

Incorporation of H^3-thymidine, H^3-leucine, and H^3-diaminopimelic acid followed by fractionation. To test the fractionation scheme, metabolites were selected that were known to be incorporated principally into a specific fraction: H^3-thymidine for nucleic acid, H^3-leucine for protein, and H^3-diaminopimelic acid for the cell wall. The results of experiments in which these metabolites were used are given in Tables 2, 3, and 4.

With H^3-thymidine, about 10% of the label had been incorporated

TABLE 2

FRACTIONATION OF *Bacillus megaterium* AFTER INCORPORATION OF H^3-THYMIDINE

	Δ Absorbancy			
	0.165	0.355	0.545	0.735
Fraction	Total radioactivity (dpm)			
Cold TCA	1,000	2,000	11,000	21,000
75% Ethanol	1,000	2,000	2,000	3,000
Hot TCA	320,000	308,000	304,000	304,000
Trypsin	21,000	34,000	31,000	22,000
Lysozyme	9,000	9,000	13,000	16,000
n-Octanol	17,000	25,000	32,000	70,000
Dpm incorporated	369,000	380,000	393,000	436,000
Percentage incorporation	11.5	11.8	12.2	13.6
Culture supernatant	2,771,000	2,712,000	2,693,000	2,655,000
Dpm recovered	3,140,000	3,092,000	3,086,000	3,091,000
Percentage recovery	98	96	96	96

The cells were from 5 ml of synthetic medium containing 3,217,000 dpm and were harvested at the Δ absorbancy indicated.

TABLE 3

FRACTIONATION OF *Bacillus megaterium* AFTER INCORPORATION OF 4,5-H^3-DL-LEUCINE

	Δ Absorbancy			
	0.185	0.375	0.570	0.750
Fraction	Total radioactivity (dpm)			
Cold TCA	8,000	2,000	9,000	9,000
75% Ethanol	3,000	6,000	7,000	10,000
Hot TCA	2,000	3,000	3,000	3,000
Trypsin	130,000	251,000	390,000	436,000
Lysozyme	3,000	6,000	8,000	9,000
n-Octanol	2,000	9,000	13,000	20,000
Dpm incorporated	148,000	277,000	430,000	487,000
Percentage incorporation	12	23	36	41
Culture supernatant	1,004,000	885,000	780,000	700,000
Dpm recovered	1,152,000	1,162,000	1,210,000	1,187,000
Percentage recovery	96	97	100	99

The cells were from 5 ml of synthetic medium containing 1,200,000 dpm and were harvested at the Δ absorbancy indicated.

TABLE 4
FRACTIONATION OF *Bacillus megaterium* AFTER INCORPORATION OF H^3-DIAMINOPIMELIC ACID

	Δ Absorbancy			
	0.195	0.397	0.602	0.775
Fraction	Total radioactivity (dpm)			
Cold TCA	36,000	8,000	32,000	48,000
75% Ethanol	3,000	7,000	8,000	9,000
Hot TCA	10,000	19,000	35,000	36,000
Trypsin	107,000	115,000	130,000	131,000
Lysozyme	799,000	807,000	770,000	752,000
n-Octanol	63,000	78,000	119,000	91,000
Dpm incorporated	1,018,000	1,034,000	1,094,000	1,067,000
Percentage incorporation	45	46	49	47
Culture supernatant	1,115,000	1,100,000	1,151,000	1,151,000
Dpm recovered	2,133,000	2,134,000	2,245,000	2,218,000
Percentage recovery	95	95	100	99

The cells were from 5 ml of synthetic medium containing 2,248,000 dpm and were harvested at the Δ absorbancy indicated.

into the nucleic acid by the time the culture entered the log phase of growth (Fig. 1), and there was no further incorporation of label into nucleic acid. Neither was there any loss of label already incorporated. The bulk of the radioactivity remained in the culture supernatant, and all of it could be adsorbed on charcoal and eluted with 50% ethanol containing 0.3% ammonia. Chromatography of this eluate in a system that separates thymidine and thymine (ethyl acetate: water: formic acid; 60:35:5; upper phase) (14) revealed that all the radioactivity coincided with thymine. Therefore, the *B. megaterium* culture converted thymidine to thymine; this appears to be common for bacteria (7) as well as mammalian cells (15). This conversion would explain the failure of the label to be further incorporated into nucleic acid, as the free base is not in the sequence for DNA synthesis. The slow but steady increase in radioactivity in the *n*-octanol fraction most likely represents catabolic products of thymine which are incorporated into lipid. Hydrolysis of this fraction with perchloric acid (16) did not liberate radioactivity in a form adsorbable by charcoal, indicating that the label is no longer present as a pyrimidine.

With H^3-leucine, the results in Fig. 2 were confirmed. Over the major part of the exponential phase of growth, the incorporation of label was

directly proportional to growth. As the preponderant part of the label was incorporated into the protein fraction, the incorporation is therefore proportional to protein synthesis. The synthetic medium contained unlabeled L-leucine, which diluted the H^3-leucine and allowed it to be incorporated throughout the entire growth period of the culture. The unincorporated label that remained in the supernatant was assumed to be 4,5-H^3-D-leucine, but this was not investigated. Hydrolysis (sealed tubes, 6 *N* HCl, 105°, 16 hr) and chromatography (*n*-butanol: acetic acid: water; 4:1:5; upper phase) of the protein fraction showed that the label was present as leucine. There were no spurious spots.

With H^3-diaminopimelic acid, about 45% of the radioactivity had been incorporated into the cells by the time the culture entered the exponential phase of growth, and there was little further incorporation of label during the remainder of the growth period. About half of the label remained in the supernatant. While the bulk of the radioactivity appeared in the cell wall fraction as expected, appreciable quantities were in the protein and *n*-octanol fractions. Hydrolysis, as above, and chromatography in the solvent system of Hoare and Work (17) revealed that all radioactivity in the protein fraction was in the form of lysine and all radioactivity in the cell wall fraction was in the form of diaminopimelic acid. There was no cross-contamination of these two fractions. The radioactive compounds in the *n*-octanol fraction could not be identified, but only a small fraction appeared to be either lysine or diaminopimelic acid.

As supplied, the H^3-diaminopimelic acid consisted of meso and LL isomers. The sample also contained a small amount of lysine, and it was assumed that it was this lysine that was incorporated into the protein fraction. The possibility that *B. megaterium* KM possesses a diaminopimelic acid decarboxylase is not excluded, however. The unincorporated label was assumed to be the LL isomer, as it is the meso isomer that is known to exist in the cell wall (18). In this experiment there was no unlabeled diaminopimelic acid in the medium to dilute the H^3-diaminopimelic acid, and the small amount of label was immediately incorporated. In all probability, the label could be diluted with unlabeled *meso*-diaminopimelic acid so that incorporation would be directly proportional to cell wall synthesis in the same way that H^3-leucine incorporation was proportional to protein synthesis.

DISCUSSION

The methods described in this paper should, at present, be applicable to any other lysozyme-sensitive organism, not only for the determination of tritium, but also for other radioisotopes. As new cell wall depolymer-

izing enzymes become available, the methods should be of general applicability.

The measurement of label incorporated into whole cells was rapid and the method is suitable for routine application to many samples. The fractionation scheme was validated by incorporation of the preponderant part of selected metabolites into predicted fractions. The present fractionation scheme for *B. megaterium* allows the estimation of four major polymers in this organism, namely, nucleic acid, protein, cell wall mucopeptide, and poly-β-hydroxybutyric acid. Selection of a suitably labeled metabolite should allow estimation of any of these polymers. The hot TCA fraction contains both degraded nucleic and teichoic acid, and the estimation of teichoic acid might be difficult. The use of C^{14}-orotic acid should provide a reasonably accurate measure of nucleic acid, though it would not distinguish between RNA and DNA. The use of P^{32}-phosphate would be of limited value here since both nucleic and teichoic acids are polyphosphate compounds. As teichoic acids are ribitol phosphate polymers, perhaps C^{14}-ribitol or a suitable precursor would allow estimation of this compound in the presence of nucleic acid.

For protein synthesis studies, a wide range of amino acids and labels are available from which to choose, but this choice is more restricted for cell wall synthesis studies. Diaminopimelic acid is the only amino acid in the cell wall which is not contained in protein, and diaminopimelic acid is not a constituent of all bacteria. For those bacteria which contain lysine in the cell wall instead of diaminopimelic acid, the methods in this paper may still be used. The results of incorporating diaminopimelic acid and leucine into *B. megaterium* clearly show that the protein and cell wall fractions are well separated, so that glycine, alanine, glutamic acid, and lysine may all be used for cell wall synthesis studies even though these amino acids will also be incorporated into protein. In fact, these amino acids may be preferable even when diaminopimelic acid could be used, because incorporation of a single amino acid would serve as a reliable estimate of both protein and cell wall. As the teichoic acid also contains ester-linked D-alanine, it seems probable that the use of C^{14}-alanine would allow the simultaneous estimation of teichoic acid, protein, and cell wall mucopeptide in a single sample. In addition, the cold TCA fraction provides an estimate of the pool constituents, regardless of the label.

Counting efficiencies of tritium were considered good. They averaged about 8% for the radioactivity in intact cells. In cellular fractions they ranged from 9% for the TCA fractions to 14% for the ethanol and octanol fractions.

SUMMARY

Noncombustive methods for the determination of tritium in *Bacillus megaterium* using liquid scintillation techniques have been developed. The key step involves the dissolution of the cell wall with lysozyme.

A fractionation scheme that separates *B. megaterium* into six soluble fractions, all of which are suitable for incorporation into liquid scintillation solvents, has been developed. Tritiated leucine incorporation was directly proportional to bacterial growth and incorporation was principally into the protein fraction. Tritiated thymidine was not a stable precursor, being converted to thymine, but a portion was incorporated into the nucleic acid fraction. Tritiated diaminopimelic acid was incorporated into the cell wall fraction.

It is suggested that these methods have equal applicability for other lysozyme-sensitive organisms, not only for the determination of tritium, but also for other radioisotopes. It is further suggested that, as new cell wall depolymerizing enzymes become available, the methods may be of general applicability.

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Erratum

Volume 4, Number 1, pages 1–9 in the article entitled "pH Gradient Elution in Column Chromatography," by John M. Reiner and Buena Reiner:

Footnote 1 should read "This investigation was supported in part by grants from the United States Public Health Service, the National Science Foundation, and the American Cancer Society."

Biochemical Applications of Gas Chromatography

By H. P. BURCHFIELD
Southwest Research Institute, San Antonio, Texas
and ELEANOR E. STORRS
Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York

March 1962, 680 pp., $22.00

This book fulfills a need for a text that supplies both theoretical background and details of the applications of gas chromatography to biochemical problems. Specific procedures are supplied together with enough background information to enable the experimentalist to improvise adaptations without an extensive study of the literature. Although the work is organized along biochemical lines, information is included that will be of value to workers in other fields, such as air pollution and petroleum chemistry.

Gas Chromatography is the technique described most extensively in this book, but it is not the only one. Detailed methods for sample collection are given for each group of compounds, and prefractionation of samples by liquid-solid chromatography and ion exchange techniques is described. Methods are provided for the preparation of stationary liquids, subtraction of unwanted compounds from the gas stream, and synthesis of volatile derivatives of non-volatile compounds.

A special feature is the adoption of a standard form for tabulating conditions for chromatography. Most of the methods were checked for accuracy and updated by the original authors; therefore, many of the methods are more complete than in the original literature. Extensive tables of retention values have been summarized from a variety of sources, and indexes are included to provide easy access to the contents.

This book is the only complete experimental text presently available in the field.

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ANALYTICAL BIOCHEMISTRY 4, 269–283 (1962)

Analytical Techniques for Cell Fractions. II. A Spectrophotometric Column Monitoring System

N. G. ANDERSON

From the Biology Division, Oak Ridge National Laboratory,[1] Oak Ridge, Tennessee

Received November 9, 1961

INTRODUCTION

To follow changes in the composition of cells and tissues occurring as responses to experimental variables, it is necessary to have available rapid automated analytical systems for the major cell constituents. Initially it is prudent to develop those systems that have the widest application. Continuous recording of absorbance of column effluents at one or several wavelengths in the ultraviolet range allows the quantitation not only of proteins, nucleotides, and nucleotide derivatives (4), but also of peptides (with or without aromatic groups) (1–3, 5), and, as will be shown in a subsequent paper, lower fatty acids. In addition, enzymic activities producing changes in ultraviolet-absorbing substrates or cofactors may be monitored.

In this paper the details of two ultraviolet absorbance recording systems are reported. Subsequent papers will deal with methods using them for analyzing specific mixtures.

ANALYTICAL SYSTEMS

Of the four ultraviolet-monitoring systems that we have investigated, two have been found useful for routine analysis.

Single-Beam, Two-Wavelength Systems

The system we have used for more than 2 years (Fig. 1) includes either two Beckman DU spectrophotometers, with spectral energy adapters[2] and power supplies,[3] or one DU and one DUR spectrophotometer.[4] While the latter is not useful below 216 mμ, its electronic stability

[1] Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.

[2] Spectral energy adapter, Catalogue No. 5800, Beckman Instruments, Inc., Fullerton, Calif.

[3] Power supply, Beckman Catalogue No. 23700.

[4] Available only on special order from Beckman.

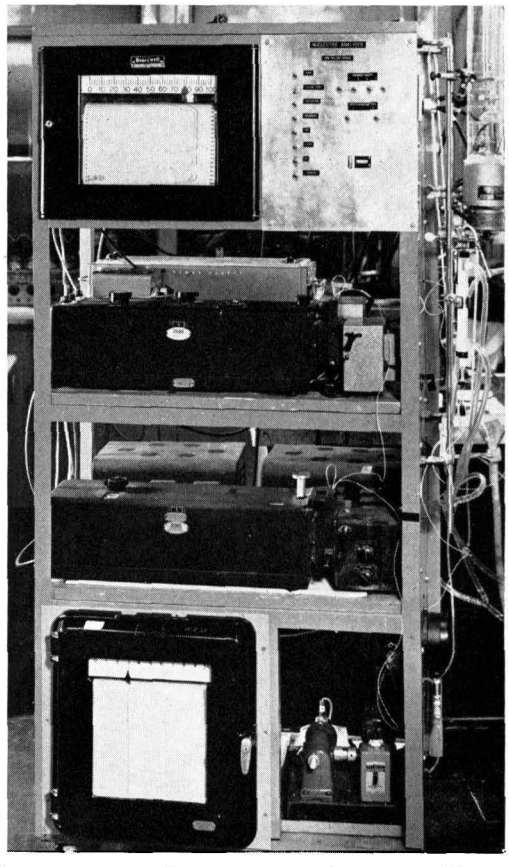

FIG. 1. Single-beam, two-wavelength system. Beckman DU and DUR spectrophotometers (center) are arranged to record absorbance at two wavelengths on the multipoint recorder (top). A recording pH meter with a small-volume electrode assembly is shown in the lower left, while the pump is mounted in the lower right. Columns and gradient-producing devices are mounted on the bars along the right side of the instrument. Control panel (upper right) allows control of each component separately.

is of considerable advantage. The spectrophotometers were attached to a 10-mv Honeywell Multipoint Recorder[5] having a chart speed of 2 in./hr, a 2.5-sec balancing motor, and a 5-sec printing time. The voltages to the spectrophotometers and the recorder were stabilized by a constant-

[5] Model Y 153X67-V12H-II-III-(H16)-A8A9K 12-point cyclic balance strip-chart recorder with a dual nonshorting 12-point selector switch, a separate chart drive motor to obtain a chart speed of 2 in./hr, and a 4× amplifier. Available from Minneapolis Honeywell Inc., Minneapolis, Minn.

voltage transformer.[6] The flow cells (0.2 and 1.0-cm light path)[7] and methods for connecting them to fine plastic tubing have been described (2).

By careful tube selection and maintenance it is possible to obtain very stable baselines. Since considerable drift is seen when the instruments are first turned on, it is our practice not to turn them off unless they are to be idle for more than three days.

The great advantage of these simple single-beam systems is that any two wavelengths from 205 mμ to the near-infrared may be monitored. However, for very prolonged runs (approximately 25 hr) they show a small drift in the dark-current (infinite absorbancy) settings. For this reason a new and relatively inexpensive double-beam spectrophotometer has been modified for flow monitoring.

Double-Beam Monitoring System

The completed system is shown in Fig. 2. A Beckman DB spectrophotometer was modified by the addition of the pneumatic wavelength-shifting mechanism shown in Figs. 3 and 4. A piston, attached through a linkage to the wavelength dial, moves the dial alternately from 260 to 280 mμ (other wavelengths 20 mμ apart may also be selected) in synchrony with the printing cycle of the recorder (Fig. 5). An additional 12-point nonshorting switch[8] is mounted in the recorder and is driven by the same mechanism moving the 12-point circuit selector switch. The second switch actuates alternately two power relays[9] that energize the solenoid valves,[10] which, in turn, drive the pneumatic wavelength-shifting mechanism. The baselines for two wavelengths may be independently adjusted using the arrangement schematically presented in Fig. 5.

If absorbancy at only two wavelengths is to be recorded, the second 12-point switch may be omitted and the signal fed directly into the recorder, in which instance the baseline adjusting circuit cannot be used. The 12-point switch normally in the recorder may then be used for

[6] Harmonic-neutralized, constant-voltage transformer Model CVH-1, Sola Electric Co., Chicago 50, Ill.

[7] Available as Oak Ridge flow cells from Pyrocell Mfg. Co., 207–11 E. 84th St., New York 28, N. Y., or Quaracell Products Co., 401 Broadway, New York 13, N. Y.

[8] Additional 12-point thermocouple switch available with mounting bracket from Minneapolis Honeywell, Inc.

[9] Relay, 115-volt, 60-cycle, 20-amp contact SPDT, Ward Leonard Electric Co., 45 South Street, Mount Vernon, N. Y., No. 105-6512.

[10] Solenoid valve No. V5D18360-V-10, 115-volt, 60-cycle, ⅛-inch NPT, 3-way, bleed-type, Skinner Electric Valve Division of Skinner Chuck Co., 100 Edgewood Ave., New Britain, Conn.

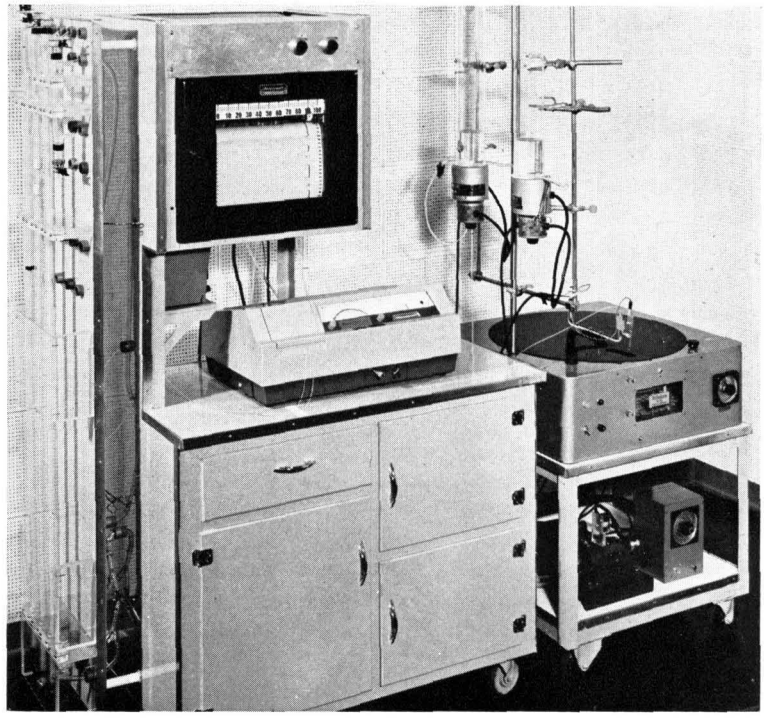

FIG. 2. Complete analytical system for nucleotides, proteins, or other ultraviolet-absorbing materials. Circulating water bath is mounted inside the cabinet in lower left compartment; pump (if not mounted on fraction collector) is in lower right space; hydrogen lamp power supply is mounted in upper right section. Baseline adjusting potentiometers are mounted above the multipoint recorder. The controlling multimeter is mounted between the recorder and the spectrophotometer table. Ion-exchange columns are mounted on a panel projecting from the main cabinet (left) a sufficient distance to allow the column jacket water lines to be easily reached.

wavelength shifting. However, in the present instrument, provision is made for recording pH, conductivity, and radioactivity, in addition to absorbancy, on the same chart. These signals must also pass through a selector switch.

We have used Milton Roy chromatographic pumps.[11] Connections to the pump are made with 3/8-in. Tygon tubing through swagelock connections.[12] In early work, salt rings occurred around these connections

[11] Minipump, CHMMI-B-29R, Milton Roy Co., 1300 E. Mermaid Lane, Philadelphia 18, Pa.

[12] Swagelock fitting No. 600-1-2-316, Crawford Fitting Co., 884 E. 140th St., Cleveland 10, Ohio.

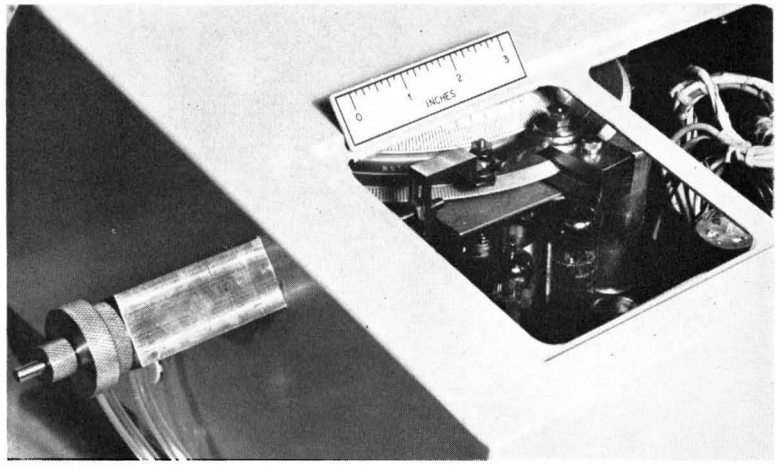

FIG. 3. Air-operated, wavelength-shifting mechanism. The air piston mounted outside the spectrophotometer housing moves a shaft linked to the wavelength dial. Stops are provided in the piston housing to adjust the travel of the piston and to position accurately the wavelengths to be read, in this case 260 and 280 mμ.

because sealing compounds were avoided. However, no leakage is observed when the male threaded parts are wrapped in Teflon tape.[13]

In a specific system, which will be described in a subsequent paper, a change in pumping rate occurs during chromatography. This is accomplished by lifting a flat stainless-steel spacer from between the cross head and set screw with a small, continuous-duty solenoid,[14] as shown in Fig. 6. The solenoid is actuated by a timer that energizes it at the end of a preset time.

Connection from the pump to the top of the chromatographic column is through a polyvinyl chloride ball connector fitted with a silicone rubber "O" ring.[15] All columns are jacketed, with 18/9 top ball connections and 12/2 effluent ball connections. Both glass frit columns[16] and Teflon frit columns[17] of 0.9-cm i.d. were used. Column temperatures were controlled with a circulating constant-temperature bath[18] equipped with

[13] Thread-tape, Crane Packing Co., Morton Grove, Ill.

[14] No. 1R-11-Cont-115-Ac, 60-cycle solenoid, Guardian Electric Mfg. Co., 1621 W. Walnut St., Chicago 12, Ill.

[15] Connector assembly PVC ball 18/1, tubing to column, part No. 120-9100 with "O" ring No. 120-9036, Spinco Division of Beckman Instruments, Inc., Palo Alto, Calif.

[16] Available from Scientific Glass Apparatus Co., Bloomfield, N. J.

[17] Available from Spinco Division of Beckman Instruments, Inc., Palo Alto, Calif.

[18] Constant-temperature circulating bath, Catalogue No. 3050, Labline Inc., 3070-82 W. Grand Ave., Chicago 22, Ill.

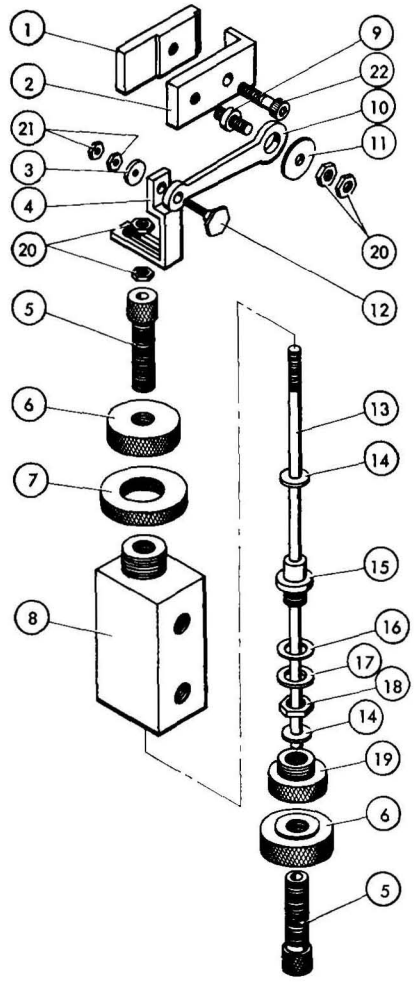

FIG. 4. Assembly of wavelength-shifting mechanism. (1) Lower jaw of wavelength dial clamp. (2) Upper jaw of wavelength dial clamp. (3) Washer. (4) Bracket connecting piston shaft to driving arm. (5) Adjustment for upper wavelength to be read. (6) Lock nut for 5. (7) Nut for holding piston to spectrophotometer housing. (8) Air piston cylinder. (9) Bearing connector between wavelength dial-clamp and driving arm. (10) Driving arm. (11) Washer. (12) Bolt with bearing surface for connecting parts 4 and 10. (13) Air piston shaft. (14) Teflon washers. (15) Piston. (16) Leather washer. (17) Brass washer. (18) Brass piston nut. (19) Piston cylinder end closure. (20, 21) Brass nuts. *Note:* Number corresponds to parts on engineering drawings available from author.

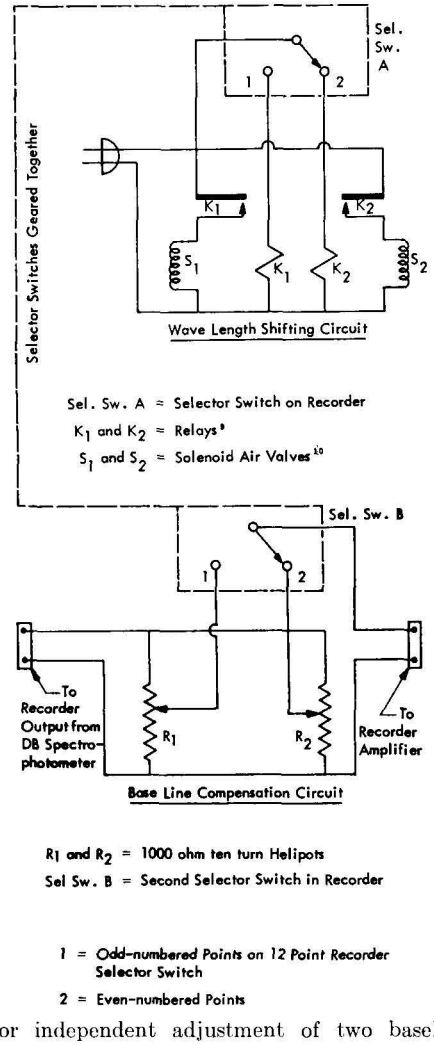

FIG. 5. Circuit for independent adjustment of two baselines and circuit for wavelength switching.

coils for rapid cooling of the bath with tap water. All fluid-handling lines beyond the columns are of Teflon[19] joined as previously described (2), or by heat-shrinking expanded Teflon tubing.[20]

[19] No. PF 22 Teflon tubing, Pennsylvania Fluorocarbon Co., Inc., 1115 N. 38 St., Philadelphia, Pa.

[20] Expanded Teflon AWG# 19 STD Natural tubing available from Pennsylvania Fluorocarbon Co., Inc. When heated, tubing will shrink to form a tight bond with No. PF 22 Teflon tubing.

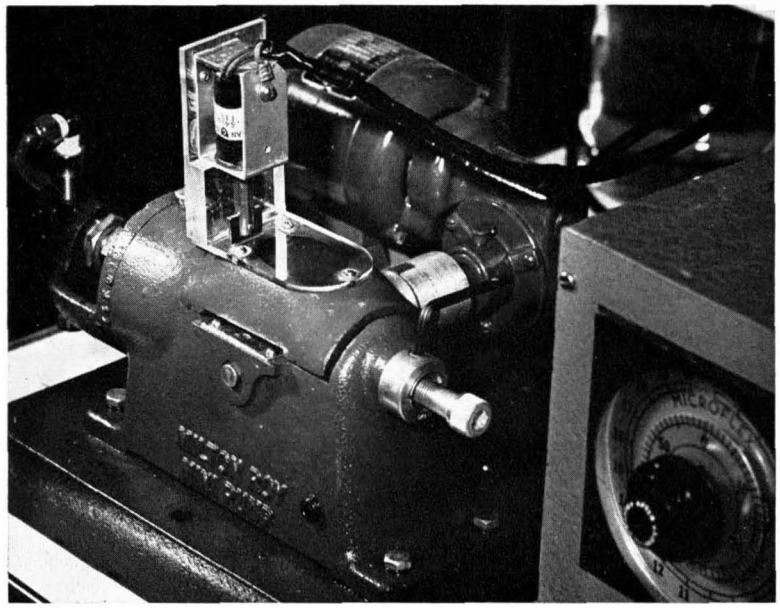

FIG. 6. Timer-operated mechanism for changing pumping rate. At preset time, the timer energizes the solenoid, which raises the stainless-steel plate from between the cross head and set screw, increasing the pump stroke. The blade is shown in down position.

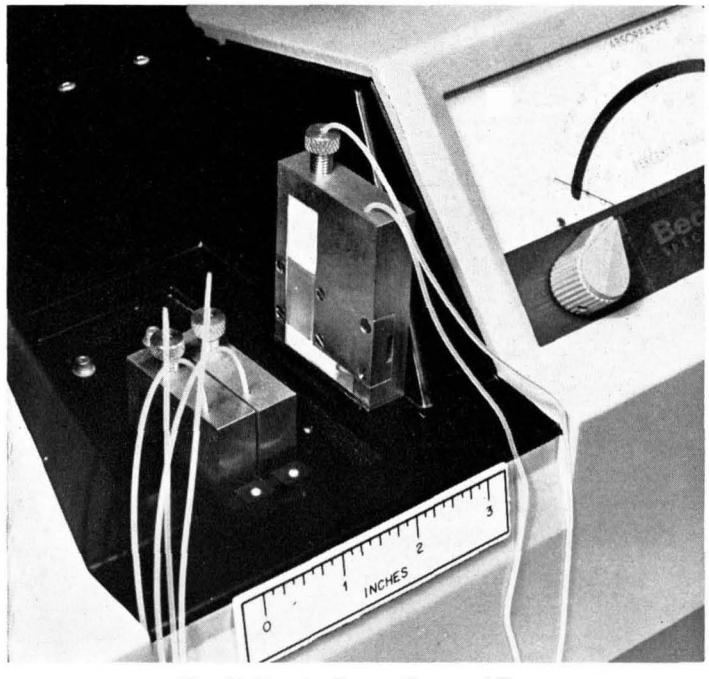

FIG. 7. Quartz flow cell assemblies.

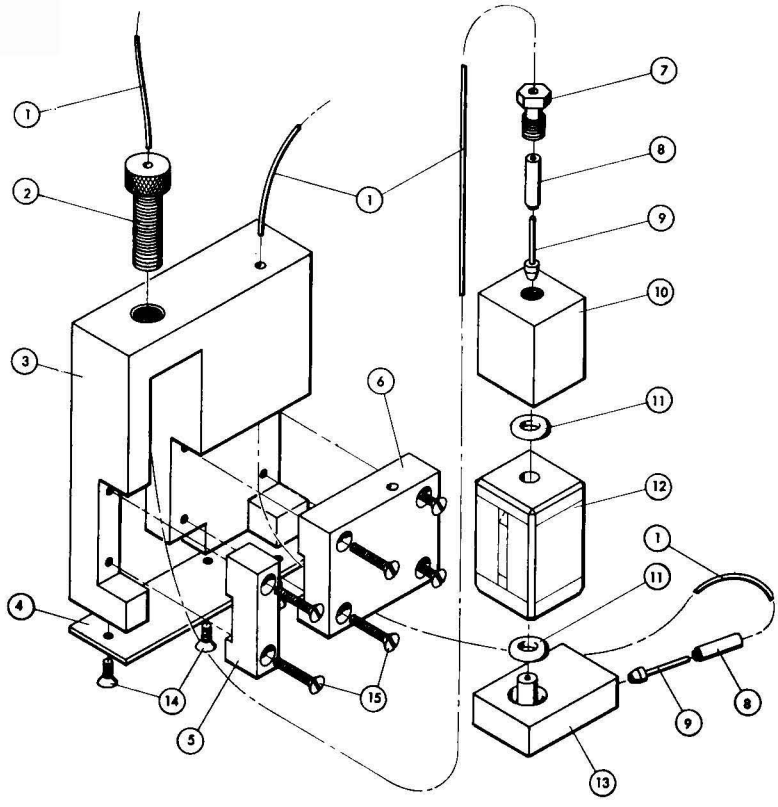

FIG. 8. Assembly of quartz flow cells (see *Note,* Fig. 4). (1) Teflon tubing. (2) Upper pressure plate adjustment screw. (3) Cuvette housing block. (4) Bottom plate of cuvette housing. (5) Front beam guide. (6) Rear beam guide. (7) Swivel fitting pressure screw. (8) Swivel fitting sleeve. (9) Swivel fitting. (10) Upper pressure plate. (11) "O" ring. (12) Quartz cuvette. (13) Lower pressure plate. (14, 15) Screws.

The quartz flow cells (Figs. 7 and 8) are modifications of those previously described (2) with optical paths of 0.2 and 1 cm and volumes of 0.056 and 0.25 ml, respectively.[7] Connections are made through Teflon end blocks and swivel connections.[21] The free area for the light beam is 0.2 by 1 cm. The cell holder completely fills the cell space of the Beckman DB spectrophotometer. For baseline correction the elution fluid may flow through the reference cell before being pumped through the column.

Flow Rate. Quantitative chromatography requires that the flow through

[21] Swivel fitting assembly for standard wall Teflon tubing A.W.G. 22 0.012-in. wall, part 120-311, Spinco Division of Beckman Instruments, Inc., Palo Alto, Calif.

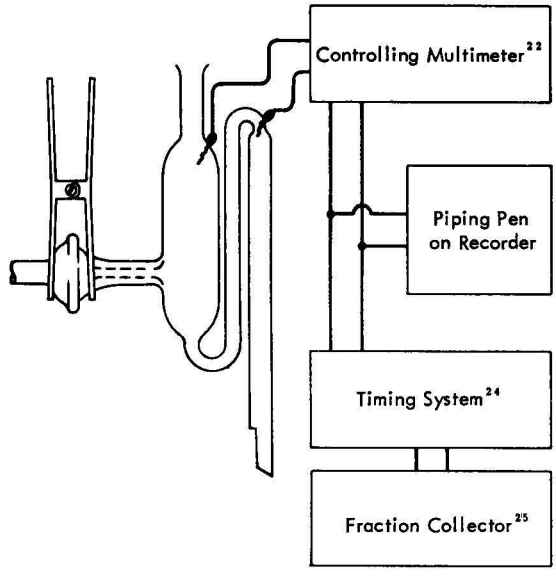

FIG. 9. Flow rate measuring system. Connection at left is to line leading from cuvette. The top is open. The siphon at bottom drains into the fraction collector tube.

the column be precisely known. When a reagent stream is added to the column effluent, as in amino acid analysis, the flow rate both from the column and of the reagent stream must be known, must be constant, and must have a constant ratio. In ultraviolet absorption analysis, however, the only requirement is that the flow rate during the elution of a given peak be known. The flow rate need not be constant from run to run, nor even be constant during the course of a run. Great operational flexibility is therefore obtained by constantly recording the flow rate.

Flow rates may be measured by continuously weighing the effluent on a recording balance or by using a number of commercially available flowmeters. A method based on a calibrated siphon was chosen here, however, because the device is nearly independent of rate and solution density, and because it may be used to actuate a fraction collector. We devised a simple, standard-component method for producing a pulse when the siphon emptied by attaching a controlling multimeter[22] to two platinum wires attached to the siphon,[23] visible above the fraction col-

[22] Controlling Multimeter, Assembly Products, Inc., Chesterland, Ohio, adjusted to measure resistance.

[23] Siphons were obtained from Technicon, Inc., Chauncey, N. Y., and are available in a range of sizes. Platinum wires are fused into the glass in the drain arm and

lector in Fig. 2. Since the actual resistance may be observed constantly, a setting can be obtained that allows actuation with a variety of salt concentrations, but leaves the circuit open when the siphon is not draining.

The multimeter actuates a right-hand piping pen on the recorder, and a collector timer (Fig. 9). The collector timing system is started by a pulse from the controlling multimeter, which, in turn is actuated by the siphon. The timing system runs 20 sec (to allow the siphon to drain fully) and then delivers an impulse to the fraction collector, which has been switched to Count.[26] In this way, constant-volume collections may be made and the flow rate recorded during the entire chromatographic run. The multimeter may also actuate a printing timer, which may record the actual time the siphon drains.

A mounting rack (Fig. 2) holds the constant-temperature bath (lower left), pump (lower right, or under fraction collector), and hydrogen lamp power supply (upper right). Space is provided for four columns, which may be long (150 cm), medium (50 cm), short (15 cm), in any combination. No space or facilities for storing eluting solutions is provided, since requirements differ.

It is a prime rule in the design of automated analytical apparatus that all electrical lines be above and separate from fluid-handling lines. Tubing to the columns exit from the main part of the cabinet a foot or two from floor level and runs up in the readily accessible space between the cabinet and the column mounting board. All lines and connections are within sight and reach. More elaborate flow manifolds for connecting any one of four columns to the spectrophotometer and the rest to drain have been constructed using three precision microvalves.[27] For most applications this is not necessary, however.

Performance of Flow Cells

Since all flow cells contain a finite volume of fluid, it is not possible to avoid completely measurement artifacts when large differences in density, viscosity, or absorbancy occur over a very small volume inter-

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in the body of the siphon so that a current can flow between the two wires only during the period when the siphon is draining.

[24] Series RC timing system, for operating two circuits, for single-cycle, nonrepeating operation with cycle time of 30 sec, Meylan Stopwatch Corp., 264 W. 40 St., New York 18, N. Y.

[25] Technicon Fraction Collector, Technicon, Inc., Chauncey, N. Y.

[26] Detailed circuitry available from the author.

[27] Circle Seal Precision Valve, Model P4 418T, Four Way 3Q61 B obtained from Circle Seal Products Co., Inc., 2181 East Foothill Blvd., Pasadena, Calif.

val. Measurement of the rate of equilibration of the cell contents with the incoming column effluent may be made, but these are of little value in instances in which a small volume of dense fluid (generally the original sample volume containing excess salts and sugars) remains in the cell and convects back through the incoming stream. In the present work the volume of the cells has been reduced as far as is consistent with a good optical cross section. Flow from the bottom of the cell to the top ensures that bubbles are swept out, and results in minimal convective disturbance when gradients of increasing physical density are used for elution.

The correct test with a given system is to observe the performance with the sharpest (contained in the least volume) peaks expected. In our experience these occur at the beginning of the nucleoside-base-nucleotide separation method (4) worked out with the DU-DUR system described

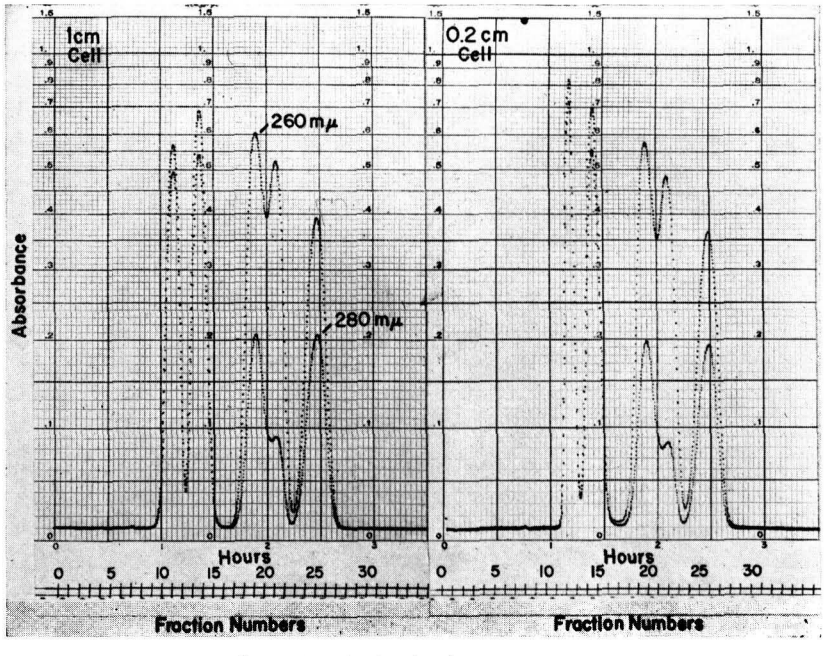

FIG. 10. Comparison of results obtained with 1.0-cm and 0.2-cm light path flow cells. The samples used are described in the text. The elution order is (from left to right) cytosine, cytidine, uridine, uracil, and thymine. These compounds were eluted from a 150 × 0.9 cm Dowex-I X8 column with 0.15 *M* sodium acetate at pH 4.4 at 40°C as described in reference (4). Each mark below the baseline is equivalent to 4.033 ml of column effluent. Absorbance is recorded at 260 and 280 mμ.

here. With a 1-ml sample of a mixture containing 2 μmoles each of cytosine, cytidine, uridine, uracil, and thymine per milliliter with the 0.2-cm cell (0.056-ml cell volume), and 0.2 ml of the same sample with the 1.0-cm (0.25-ml) cell, the recordings shown in Fig. 10 indicate negligible differences between the two cells. The volume width of the first (cytosine) peak is approximately 3 ml. The rest of the peaks are wider and would not be expected to differ. To obtain comparable resolution by reading manually fraction collector samples, a very large number of very small fractions must be collected.

Some difficulty is experienced with the 1-cm cell at the very start of the base-nucleoside-nucleotide analysis when the sample contains a large amount of sucrose. Inverting the cell for about two minutes just after one column volume has passed through eliminates this difficulty. Alternatively, the 0.20- and 1.0-cm cells may be connected in series and the 0.20-cm cell placed in the spectrophotometer until the column volume plus 4–5 ml have passed, during which time the 1.0-cm cell is kept inverted outside the spectrophotometer. The 1.0-cm cell may then be reinserted. If the 0.20-cm cell is to be used for the entire run, no difficulty is experienced. With this single exception no difficulties attributable to the flow cells has been experienced in several hundred runs. The fluid volume below the sintered glass plate in most ion-exchange columns is larger than the volume of the flow cells used here, and is probably a greater source of error than is anomalous flow or back-mixing in the flow cells.

Quantitation of Results

If all of the column effluent containing one ultraviolet-absorbing compound is collected in a single vessel, the amount of that substance may be obtained from the familiar relation

$$\text{mg solute} = \text{MW} \cdot As \cdot \text{ml}/\epsilon \qquad (1)$$

where As is the observed absorbance at a given wavelength in a cell of 1-cm light path, and ϵ is the Molar Absorptivity at the same wavelength.

When the data are obtained in the form of recorded Gaussian curves, the following relations may be used to calculate the amount of a given substance present. In the normal density function the relation between the true area under the curve and the area of a triangle obtained by multiplying the height of the curve by the width at half-height is given by:

$$C = G/H \cdot W \qquad (2)$$

where G is the true area under the curve, and H and W the height and

width at half height, respectively. Since the area $G = 1$ in the normal density function, and the height is $(2\pi)^{-1/2}$, the width at half-height is given by setting $\Phi(X)$ equal to $(2\pi)^{-1/2}/2$ in the equation:

$$\Phi(X) = \frac{1}{(2\pi)^{1/2}} \exp \{-\tfrac{1}{2}X^2\} \tag{3}$$

which gives $X = 1.177$, and $W = 2.354$. By substitution in Eq. (2), C is found to be 1.064.

In the graphic results, however, the height of the curve is given in absorbancy units (A) and the width at half-height in minutes. The curve may be considered comparable to a volume of fluid V having an absorbance A_V since:

$$A_V \cdot V = H_A \cdot W_{\min} \cdot C \cdot \text{ml/min} \tag{4}$$

and:
$$\text{mg solute} = (\text{MW}/\epsilon) \cdot H_A \cdot W_{\min} \cdot \text{ml/min} \cdot C \tag{5}$$

or:
$$\text{mmoles} = (H_A \cdot W_{\min}/\epsilon) \cdot \text{ml/min} \cdot C \tag{6}$$

DISCUSSION

While the system described here was intended primarily for work with nucleic acid-derivatives, it is applicable to analysis of a wide variety of other absorbing substances including peptides by far-ultraviolet absorption, or substances absorbing in the visible range. Its usefulness in spectrophotometric methods for enzyme analysis will be shown in subsequent papers.

One of the greatest advantages of stable automatic analytical systems is the confidence given to evidence of minor components. A "peak" 0.005 absorbancy unit high stands out, whereas one tube in a manually read series that is increased by this amount is generally disregarded. Also very sharp peaks, which would be blurred in plots of discrete fractions, may be followed.

It is often advantageous to use a ratio recorder to distinguish between the light transmitted through the reference and the experimental cells. A recording potentiometer has been used in place of a ratio recorder in the present application because signals generated by a pH meter, conductivity bridge, and a count ratemeter or thermocouple may be conveniently reduced to a millivolt signal and recorded on the same chart.

The analytical system described was developed as a part of the Cell Fractionation Project of the Oak Ridge National Laboratory. Many of the principles are derived from the amino acid analyzer of Spackman, Stein, and Moore (5).

SUMMARY

Two systems for recording absorbancy of chromatographic effluent streams are described. The first utilizes two separate single-beam spectrophotometers and can record at any two wavelengths between 205 and 1100 mμ. The second utilizes a double-beam spectrophotometer and a pneumatic wavelength-shifting device. The latter is specifically designed for the analysis of nucleotides and nucleotide derivatives, and other compounds absorbing at 260 or 280 mμ.

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Synthesis and Chromatography of Dinitrophenyl-Valyl Peptides

HERBERT S. RHINESMITH, RAYMOND RIORDAN, LEONA REICHERT, ESTHER COLDREN, RUTH R. LEVITT, SAMUEL T. MORNEWECK, AND LEONARD PERSINSKI

From the Carnegie Laboratory of Chemistry, Allegheny College, Meadville, Pennsylvania

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INTRODUCTION

Recent quantitative experiments have led to the conclusion that normal adult human hemoglobin contains the *N*-terminal peptides, val-leu and val-his-leu (1a, 2), and that horse hemoglobin contains the *N*-terminal peptides, val-leu and val-glu-leu (3). In these investigations the peptides cited were isolated as the dinitrophenyl derivatives by partial hydrolysis of the appropriate DNP-globin[1] and were characterized by column chromatography.

Kinetic studies of the hydrolysis of DNP-val-leu[1] indicated a first-order reaction (2), but similar studies with DNP-val-his and the tripeptides, DNP-val-his-leu (2) and DNP-val-glu-leu (3), produced irregularities which were difficult to correlate with the known rates of hydrolysis of peptide bonds. Unexpected results were also obtained chromatographically when it was found that two peptides, DNP-val-his and DNP-val-glu, were more strongly adsorbed than the corresponding tripeptides, DNP-val-his-leu and DNP-val-glu-leu (3).

Because of the limited number of valyl peptides available[2] for chromatographic and hydrolytic studies, the object of the present investigation was the synthesis of a series of DNP-valyl peptides. This paper describes a direct synthesis of ten DNP-valyl peptides in which the dinitrophenyl group was employed as the blocking group. Each of

[1] Abbreviations: DNP, dinitrophenyl; DNFB, dinitrofluorobenzene. The abbreviations for solvents and developers follow Green and Kay (6), that is, AA for acetic acid, A for acetone, L for ligroin, F for formic acid, and E for ethyl acetate; thus 3F-8E-L is 3 vol % F and 8 vol % E in L. r.b.s.n., round bottom short neck.

[2] Two valyl peptides, L-val-gly and L-val-L-leu can be purchased from Mann Research Laboratories.

the peptides was purified by chromatography and identified by hydrolysis and subsequent identification of the parts.

EXPERIMENTAL

*Preparation of DNP-*D,L*-Valine.* D,L-Valine was dinitrophenylated in a thermostat at 40°C by the methods of Sanger (4) and Levy and Li (5), modified as follows to permit quantity preparations. Thus, 1.0 gm of D,L-valine was dissolved in a mixture of 2.0 gm of sodium carbonate and 150 ml of water and stirred vigorously for three hours with 2.1 ml of dinitrofluorobenzene (DNFB).[1] The alkaline solution was extracted with ether to remove excess DNFB and the aqueous solution carefully acidified with 6 *N* hydrochloric acid; then re-extracted with 6 × 25 ml of ether. The final extracts were washed with acidulated water, the ether was evaporated, and the product was recrystallized from acetone-ligroin. The yield was about 90% of theoretical.

*Preparation of DNP-*D,L*-Valyl Chloride.* About 50 mg of DNP-D,L-valine was transferred to a dry 100-ml r.b.s.n.[1] flask containing a 24/40 stopper; 5.0 ml of freshly distilled thionyl chloride was added by pipet and the stopper immediately inserted and secured by a copper wire. The flask was then immersed for exactly 10 min[3] in the water bath of a flash evaporator set at 60°C. The flask was then connected directly to the flash evaporator, where evaporation at 60° under a water aspirator and with the condenser in ice removed all but traces of thionyl chloride in 15 min. Upon standing overnight in a vacuum desiccator over solid potassium hydroxide, the DNP-D,L-valyl chloride was free of thionyl chloride and solidified on the walls of the flask. Samples prepared as directed could be stored indefinitely either for use in peptide synthesis or for chloride analysis.

Analysis for Chloride. Since the samples to be analyzed contained only DNP-D,L-valyl chloride and unchanged DNP-D,L-valine, analysis for chloride was rapidly and conveniently accomplished by titrating the samples electrometrically with 0.01 *N* silver nitrate in a Fisher Titrimeter using silver, silver-chloride electrodes, and 50:50 acetone:water as the solvent.

In order to transfer the sample quantitatively to the titrimeter beaker, the reaction flask was rinsed alternately with portions of water and acetone until 100 ml of the combined solution was obtained. This solution was acidified with 1 ml of concentrated nitric acid and titrated with 0.01 *N* silver nitrate until the end point voltage of 0.615 volt (standardized) was reached. The per cent of chloride obtained is a measure of the

[3] Time is counted from time of immersion of the flask.

TABLE 1

SYNTHESIS OF DNP-AMINO ACID CHLORIDES:
A STUDY OF THE EFFECT OF EXPERIMENTAL CONDITION ON PERCENTAGE YIELD

Run No.	DNP-D,L-val (mg)	$SOCl_2$ (ml)	Reaction temp. (°C)	Reaction time (min)	Evap.[a] time (min)	DNP-D,L-val Found (mg)	Chloride Theory (mg)	Total[b] time heat	% yield of acid chloride
1	50.9	5	77[c]	15	15	33.8	54.2	30	62.4
2	50.0	5	77	30	15	25.9	53.3	45	48.7
3	49.8	5	77	60	23	30.1	53.1	83	56.8
4	49.1	5	77	5	103	18.5	52.2	108	35.5
5	51.3	5	77	180	26	2.5	54.7	206	4.6
6	36.9	2	60[d]	3	15	35.6	39.3	18	90.7
7	50.0	2	60	10	15	52.6	53.3	25	98.7
8	50.5	2	60	20	15	51.8	53.8	35	96.3
9	50.8	2	60	30	15	52.8	54.1	45	97.6
10	50.9	5	60	30	15	53.0	54.4	45	97.6
11	50.0	5	60	60	15	51.0	53.3	85	95.7
12	50.7	5	60	90	15	50.3	54.0	105	93.0
13	50.4	5	60	120	15	50.5	53.7	135	94.1
14	49.5	5[e]	60	10	15	50.6	52.7	25	96.4

	DNP-D,L-ala					DNP-D,L-ala chloride			
15	50.0	5	60	10	15	37.4	53.8	25	69.6
16	50.2	5	60	40	15	16.6	53.9	55	30.0
17	50.7	5[e]	60	10	15	49.0	54.5	25	89.9
	DNP-gly					DNP-gly chloride			
18	53.4	5	60	10	15	17.5	57.4	25	30.4
19	50.8	5	60	40	15	33.1	54.6	55	50.6
20	49.7	5[e]	60	20	15	2.0	53.3	35	3.7
21	50.0	5[e]	40	40	15	21.3	53.7	55	39.7
22	51.6	5[e]	60	60	15	44.8	55.4	75	80.8
	DNP-D,L-ser					DNP-D,L-ser chloride			
23	50.0	5	60	10	15	10.0	53.3	25	18.7
24	50.5	5	60	40	15	13.8	53.8	55	25.7
25	51.0	5[e]	60	10	15	13.0	54.4	25	23.9
26	50.0	5[e]	60	40	15	43.4	53.3	55	81.4

[a] All evaporations were carried out at 60°C by rotating the sample in the water bath of a flash evaporator.
[b] Reaction time plus time of evaporation.
[c] 77° is the refluxing temperature of thionyl chloride.
[d] The reaction flask was stoppered and immersed in 60° water for the time indicated.
[e] The thionyl chloride contained 0.007 gm PCl_5/ml.

extent of the conversion of DNP-D,L-valine into DNP-D,L-valyl chloride since the only chloride present is that from the acid chloride. For DNP-D,L-valyl chloride the yield varied from 93 to 99% of theoretical.

Synthesis of DNP-Valyl Peptides. In each run about 50 mg. of DNP-D,L-valine (177 μmoles) was converted to DNP-D,L-valyl chloride as described above. If we assume an average conversion of 95% (Table 1), the reaction flask should contain about 50 mg (166 μmoles) of DNP-D,L-valyl chloride. To this flask was added a solution containing 20 ml of water, 200 mg of sodium carbonate, and a fourfold excess (about 700 μmoles) of the free amino acid to be reacted with the DNP-D,L-valyl chloride to form the peptide. The reaction flask was attached to a flash evaporator and rotated at 60° for 1.5 hr at room pressure. At the end of this time a clear yellow solution was obtained.

Extraction Procedure. The reaction mixture contained the sodium salts of the following: DNP-D,L-valyl peptide, DNP-D,L-valine from hydrolysis of the acid chloride, and excess of the free amino acid used in forming the peptide. This solution was transferred by dropper to a separatory funnel using 5 × 2 ml of water for rinsing. Acidification and ether extraction yielded a yellow solid which was dissolved and re-evaporated with 3 to 5 ml of acetone. The solid was reserved for purification by chromatography, while the aqueous solution containing the excess amino acid was discarded.

Chromatographic Procedure. The residue from the peptide synthesis was dissolved in 50 ml of acetone and a 1-ml aliquot removed by pipet. The 1/50 aliquot and the 49/50 residue were evaporated to dryness under vacuum at 40°C. The latter was reserved for further purification by chromatography; the former was used to obtain movements by chromatography, quantities by spectrum, and peptide analysis by hydrolysis and subsequent identification of the parts.

For the chromatography the method of Green and Kay (6) was used with slight modifications as described by Levitt and Rhinesmith (3). The 1/50 aliquot was dissolved in 2 ml of 5AA-10A-L[1] and chromatographed with 9AA-4A-L. In each chromatogram two main zones were observed. The smaller and faster moving zone corresponded to Group IV in the scheme of Green and Kay (6) and was identified as DNP-D,L-valine by chromatographing with 5AA-2A-L. The larger and slower moving zone contained the DNP-D-L-valyl peptide. The relative positions of the synthetic DNP-D,L-valyl peptides after the addition of 5 and 6 V's[4] of developer are recorded in Fig. 1.

[4] V is the number of milliliters of developer needed to wet the column of adsorbent.

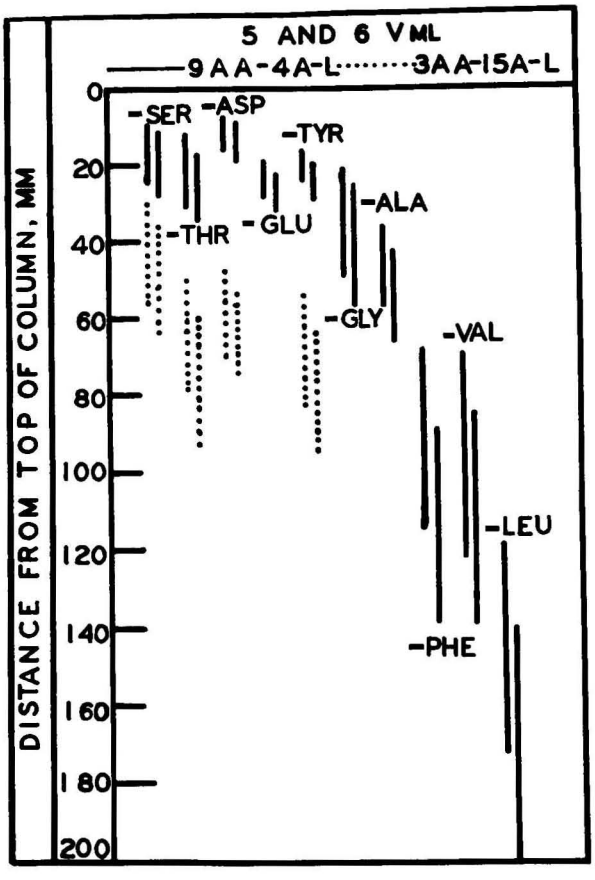

FIG. 1. Positions of zones of ten DNP-valyl peptides on columns of silicic acid-Celite after development with the type and quantity of developer listed.

The concentration of each zone was determined with the Beckman DU spectrophotometer against glacial acetic acid.

The identity of the DNP-D,L-valyl peptides was established by hydrolyzing a chromatographically purified sample for 22 hr in refluxing 6 *N* hydrochloric acid. The *N*-terminal DNP-D,L-valine was extracted with ether and determined quantitatively by chromatography and spectrum; the *C*-terminal amino acid was first dinitrophenylated and then identified by chromatography and spectrum.

The bulk of the DNP-D,L-valyl peptide was readily obtained from the 49/50 residue by single large-scale chromatogram with 9AA-4A-L, using 50 ml of 5AA-10A-L as the sample solvent. The column employed

TABLE 2

QUANTITATIVE DATA FOR SYNTHESIS OF DNP-VALYL PEPTIDES[a]

1 Run No.	2 DNP-D,L-val used[b] (μmoles)	3 Amino acid used (μmoles)	4 DNP-D,L-val recovered[c] (μmoles)	5 DNP-peptide synthesized (μmoles)	6 DNP-D,L-val recovered[d] (%)	7 % yield DNP peptide[e]	8 % yield (corr.)[f]
1[g]	177.5	660 gly	14.5[h]	145.0[i] D,L-val-gly	90.0	81.7	89.0
2	177.0	670 D,L-phe	41.0	131.0 D,L-val-D,L-phe	97.2	74.0	96.0
3	178.0	664 D,L-leu	92.6	71.8 D,L-val-D,L-leu	93.4	40.3	84.2
4	177.0	435[j] D,L-val	58.5	100.0 D,L-val-D,L-val	93.5	56.5	85.0
5	177.4	686 D,L-ala	52.3	111.5 D,L-val-D,L-ala	92.0	63.0	89.0
6	177.0	350 L-glu	119.0	37.4 D,L-val-L-glu	94.8	21.2	65.0
7	177.0	677 D,L-asp	84.0	59.0 D,L-val-D,L-asp	81.0	33.3	63.0
8	177.0	1680[k] D,L-thr	39.0	123.0 D,L-val-D,L-thr	91.5	70.0	89.0
9	177.0	729 D,L-ser	47.4	105.7 D,L-val-D,L-ser	87.0	58.7	80.0
10	189.0	720 D,L-tyr	162.0	21.1 D,L-val-D,L-tyr	97.0	11.2	68.0

[a] The best yields were obtained when 1 part of DNP-D,L-valyl chloride, 4 parts of amino acid and 4 parts of sodium carbonate in 20 ml of water were heated for 90 min at 60°C.

[b] Pure DNP-D,L-valine was weighed and converted to DNP-D,L-valyl chloride. A 95% conversion was assumed (Table 1) and the crude reaction product was used without further purification.

[c] This amount includes about 5% of DNP-D,L-valine not converted to the chloride as well as the DNP-D,L-valine produced by hydrolysis of the chloride during the 90-min period of the reaction.

[d] This material balance was made by comparing μmoles of DNP-D,L-valine used as starting material with the sum of DNP-D,L-valine recovered and DNP-D,L-valyl peptide synthesized.

[e] This over-all theoretical yield is based on μmoles of DNP-D,L-valine used to prepare the chloride.

[f] The μmoles of DNP-D,L-valine recovered were subtracted from the starting material in making this correction.

[g] This run was heated for 60 instead of 90 min.

[h] The μmoles of recovered DNP-D,L-valine were corrected for 2.5% loss on chromatography as determined by Rhinesmith, Schroeder, and Pauling (1b).

[i] Corrected for the average loss for DNP-D,L-valyl peptides of about 4% per chromatogram.

[j] In this run only 2.5 parts of amino acid were used, and the reaction was heated for 160 min.

[k] This large excess of amino acid (9.5 parts) increased the yield of DNP-D,L-val-D,L-thr over DNP-D,L-val-D,L-ser by about 11%.

was 35 × 225 mm. The V was 75 ml; the prewash (6) contained 100 ml of 50:50 ether:acetone and 50 ml of developer. A good separation was obtained in 2 to 3 V's and the column could either be extruded and the zones separated mechanically or more developer could be added and the DNP-valine collected in the receiver. The peptide was eluted from the column with 100 ml of 1:4 ethyl alcohol:ether and usually crystallized on evaporation of the solvent. The quantitative data are given in Table 2.

RESULTS AND DISCUSSION

The synthesis of peptides has been thoroughly covered in two excellent reviews (7, 8). In most cases the Schotten-Baumann reaction was employed to unite the two amino acid residues, and the amino group was protected by the use of a blocking group to prevent side reactions. Among the various blocking groups employed, the carbobenzoxy (9), phthaloyl (10), tosyl (11), and trityl (12) groups not only are typical, but are widely used because the protective group can readily be removed, either by mild hydrolysis or by catalytic hydrogenation.

In the present investigation the dinitrophenyl (DNP) group was employed as the blocking group for the first time. Normally, it would not be used, since it cannot be removed without simultaneously destroying the peptide linkage. However, the separation and identification of amino acids by Sanger's DNP-method (4), adapted for column chromatography on silicic acid by Green and Kay (6) and extended to peptides by Schroeder and Honnen (13), requires the DNP-peptide for chromatographic identification and analysis. A direct synthesis of DNP-peptides would therefore provide reference compounds for chromatographic identification of the many peptides now obtainable from protein hydrolyzates. Our results show that the use of the dinitrophenyl group as a blocking group permits a simple, direct synthesis of DNP-peptides. Experimentally, 25 to 150 μmoles of chromatographically pure material can be prepared in good yield in a relatively short time. The synthesis starts with the appropriate DNP-amino acid, which is converted to the acid chloride with $SOCl_2$ or a mixture of $SOCl_2$ and PCl_5. To produce the peptide the Schotten-Baumann reaction in aqueous sodium carbonate is employed. In Table 1 experimental data for the synthesis of DNP-D,L-amino acid chlorides are tabulated. Runs 1 through 5 indicate that at 77°C, in refluxing thionyl chloride, the maximum yield is about 62% and that the yield decreases rapidly with increasing reaction time. Runs 6 through 14, however, show that at 60°C the reaction is essentially complete in 25 min with an average yield of about 96%. Heating for less than 25 min or more than 100 min decreases the yield slightly.

The use of DNP-D,L-amino acids other than valine, however, indicates that the reaction is not a general one. Thus, under the conditions worked out for DNP-D,L-valine, DNP-D,L-alanine gives a 70% yield, DNP-glycine a 30% yield, and DNP-D,L-serine a 19% yield. An increase in reaction time sometimes increases the yield (Runs 19 and 24), but may also decrease the yield (Run 16). On the other hand, the addition of one equivalent of PCl_5 to the $SOCl_2$ together with a fourfold increase in reaction time gives yields of 90% (Run 17), 81% (Run 22), and 81% (Run 26) for the three DNP-D,L-amino acids previously cited. The 96% yield in Run 14 further indicates that the PCl_5 has no adverse effect on the preparation of DNP-D,L-valyl chloride.

The quantitative data for the peptide synthesis are presented in Table 2. The material balance for DNP-D,L-valine (Column 6) shows an average recovery of 92%, with a probable experimental error in Run 7. Also some destruction of DNP-D,L-valine is likely during the 90-min heating period in alkaline solution at 60°C.

The quantity of DNP-peptide produced varied from 10 to 50 mg. This is an ample amount for chromatographic and hydrolytic studies and is also sufficient for isolation in crystalline form since no further purification is necessary.

The percentage yields based on DNP-D,L-valine are low, indicating that hydrolysis of the acid chloride by hydroxyl ion is favored over the Schotten-Baumann condensation with the amine. This is particularly true in cases such as tyr, glu, and asp, in which the amino acid is complicated by the side chain functional group. Use of the appropriate ester would no doubt increase the amine function, but it would also involve additional steps in the synthesis. We are concerned with a simple, direct synthesis and the production of pure DNP-peptides in a minimum of time. Thus the acid chloride is readily prepared in one hour, and after drying overnight can be converted into the peptide in one day.

The percentage yields based on the chloride reacting are excellent, and it should be noted that the DNP-D,L-valine recovered is chromatographically pure and can hence be reused without further purification.

The chromatographic behavior of the DNP-D,L-valyl peptides is indicated by the bar graphs in Fig. 1. Each peptide was chromatographed with 9AA-4A-L[1] and the position of the zones recorded at the end of 5 and 6 V ml, respectively. For several of the more strongly adsorbed peptides their movements with 3AA-15A-L are also included. In general, the movements of the DNP-D,L-valyl peptides follow the predictions of Schroeder and Honnen (13) for a series of peptides containing a second amino acid whose adsorption affinity, in turn, decreases from Group I through Group IV in the order listed by Green and Kay (6).

Isomerism. The combination of DNP-D,L-valyl chloride with D,L-amino acids would yield a mixture of four diastereoisomeric forms while reaction with an optically active amino acid, such as L-glutamic acid, would produce two diastereomers.

In general, chromatographic procedures do not separate D and L isomers either as the free amino acids on ion-exchange resins or as the DNP derivatives on silicic acid-Celite. Likewise, the extinction coefficients for the DNP enantiomorphs or their racemic mxitures are identical, while the melting point of the racemate, as would be expected, usually differs from that of the optically active forms. On the other hand, the situation with diastereomers is less clear. On ion-exchange resins such pairs as isoleucine and alloisoleucine (14), threonine and allothreonine (15), as well as hydroxylysine and allohydroxylysine (16), have been separated. However, most of the DNP-peptides used in column chromatograms are obtained from natural sources and represent pure L *forms.* Thus little information is available in the literature concerning the chromatography of DNP diastereomers on silicic acid-Celite.

To resolve this problem we prepared, by the method described in this paper, seven of the nine DNP-peptides or mixtures theoretically possible on combining the optical isomers of valine and leucine. The compounds or mixtures prepared were: (a) DNP-D,L-val- D,L-leu, (b) DNP-L-val-L-leu, (c) DNP-D-val-D-leu, (d) DNP-L-val-D-leu, (e) DNP-D-val-L-leu, (f) DNP-D,L-val-L-leu, and (g) DNP-L-val-D,L-leu.

(b), (c), (d), and (e) represent the four pure diastereoisomeric forms present as a mixture in (a). (f) would be a mixture of (b) and (e) while (g) would be a mixture of (b) and (d). DNP-D,L-val-D-leu and DNP-D-val-D,L-leu were not prepared. These would be structural opposites of (f) and (g), respectively, and were not needed to solve the problem.

Each of the seven peptides or mixtures was chromatographed on silicic acid-Celite, first with 3F-8E-L to separate them from the indicated form of DNP-valine and then with 9AA-4A-L on an extended column to obtain their movements with 6V ml of developer. For each developer the seven chromatograms were identical within the experimental limits of the procedure.

In a previous paper (1), we reported that the first-order rate constant for the acid hydrolysis of DNP-val-leu obtained from human hemoglobin was 0.143 hr^{-1}, the reaction being pseudo unimolecular. Since peptides isolated from nature would be of the L variety, it seemed important to determine whether the diastereomers and diastereoisomeric mixtures of DNP-val-leu prepared in this manner would have hydrolysis rates which differed from the naturally occurring isomer. To this end we hydrolyzed representative samples, i.e., (a), (b), (c), (f), and (g), above.

The times selected were 1, 4, 8, 12, 16, and 22 hr, respectively. By plotting the quantity $-\ln x_t/x_0$ (where x_0 is the initial quantity of DNP-val-leu and x_t the amount after t) against time t, in hours, we found that the rate of hydrolysis of the diastereoisomeric forms was identical with that of the naturally occurring L variety.

We thus conclude that our rapid method for the synthesis of DNP-peptides is independent of the optical nature of the amino acids employed, since diastereoisomeric forms may be compared with naturally occurring peptides with respect to chromatographic identification and hydrolytic studies.

SUMMARY

A rapid analytical method for the direct synthesis of DNP-valyl peptides is described in which the dinitrophenyl group is employed as the blocking group. The quantitative data for the synthesis and the chromatographic behavior of ten DNP-valyl peptides are presented and application of the method to amino acids other than DNP-valine is considered. Seven of the nine DNP-peptides or mixtures thereof, theoretically possible by combining the isomers of valine and leucine, were also prepared. Neither the chromatographic behavior on silicic acid-Celite nor the rate of hydrolysis in boiling 6 *N* hydrochloric acid was affected by using these diastereoisomeric mixtures in place of pure optically active forms.

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Milligram-Scale Preparative Gas Chromatography of Steroids and Alkaloids

HENRY M. FALES,[1] EERO O. A. HAAHTI,[2] TAPANI LUUKKAINEN,[3] W. J. A. VANDENHEUVEL,[4] AND E. C. HORNING[4]

From the Laboratory of Chemistry of Natural Products, National Heart Institute, Bethesda, Maryland, and the Lipid Research Center, Baylor University College of Medicine, Houston, Texas

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INTRODUCTION

The separation of a few milligrams of a pure substance from a mixture of biological origin is often a matter of paramount importance in studies of steroids, alkaloids, and many other compounds. The use of relatively thin-film column packings prepared with highly thermostable liquid phases for the analytical separation of compounds of this kind is now well established, and the present investigation was carried out to determine if these column packings could be used effectively in milligram-scale preparative gas chromatography. Problems involving sample injection, sample collection, and column behavior under near-overload conditions were studied.

Much prior work has been done on the development of apparatus and techniques for preparative gas chromatography. However, the emphasis has usually been on attaining a high load capacity, and thick-film columns containing 15–50% of liquid phase have usually been used. A number of high heat capacity vaporizing chambers have been developed to provide for very rapid conversion of the sample to the gas phase, and a variety of condensation or collection devices have been described. These advances are extremely valuable for much work, but the techniques have comparatively little relevance to the problems involved in steroid separations. Thick-film column packings are not useful because they would require the use of very high temperatures, or long retention times. The use of a high temperature-high heat capacity vaporizer for

[1] National Heart Institute, Bethesda, Md.
[2] University of Turku, Finland.
[3] University of Helsinki, Finland.
[4] Baylor University College of Medicine, Houston, Texas.

the "instantaneous" vaporization of milligram amounts of steroid mixtures is undesirable, and the collection of individual steroid fractions is particularly difficult because of aerosol formation. For these reasons the present work is not directly comparable to much published information on preparative methods, and, conversely, the techniques described may have limited application to problems involving smaller molecules. The specific applications studied in this work include the separation of the components of a steroid and an alkaloid mixture.

MATERIALS AND METHODS

A Barber-Colman Model 15 gas chromatograph was modified through the construction of a special low mass column compartment designed to operate as an air bath according to a method developed earlier in this laboratory by Haahti (1) for smaller compartments. A Model 40 F & M temperature programing controller was used to provide heating rates up to 11° per minute. Satisfactory temperature control was obtained by taping the thermocouple element in a central position on the glass column, and by using a slow current of air as the heating fluid. The column was a 9 ft $\times$ 12.4 mm I.D. glass U-tube with a narrower (8 mm O.D.) "flash heating" zone. The column exit was connected by means of short sections of stainless steel and Teflon tubing through a T-joint to an external glass delivery tube. The delivery tube was equipped at the exit end with a 10/30 ST joint, and the entire exit section was maintained at 250–260°. The collecting device was a glass U-tube; one side was of 6-mm tubing and was equipped with a 10/30 ST joint to fit the delivery tube, and the other was of 20-mm tubing. This U-tube was immersed in liquid nitrogen during collection of the sample. A short (4-in.) section of stainless steel capillary tubing (0.01 in. I.D.) was used as a connection to a second T-tube; this T-tube was used as the point of entry of a scavenging flow of argon, and the tube was also connected to an argon ionization detection cell.

The columns were prepared according to methods developed earlier for analytical work with steroids (review, 2). The support was 100–140 mesh Gas-Chrom P, acid washed and siliconized with a 5% solution of dichlorodimethylsilane in toluene. A coating of methyl silicone polymer SE-30 was applied by the filtration technique. Packings containing 0.75% and 4% SE-30 were used in studies of overload behavior. Conditioning was carried out by overnight heating at 300°. The column was treated with hexamethyldisilazane (50 μl by injection) after it was conditioned but before it was used.

The mixture of steroids used in the separation shown in Fig. 5 was a synthetic mixture of reference compounds. The alkaloid mixture used in

the separation shown in Fig. 6 was the entire mixture of chloroform-soluble hydrochlorides obtained from an extract of *Buphane guttata*. The salts were converted to free bases before chromatography.

RESULTS AND DISCUSSION

Column Size and Column Behavior with Varying Load. The effect of increasing the column diameter for a thin-film steroid column is shown in Fig. 1. A 9 ft $\times$ 4 mm I.D. glass U-tube with a column packing of

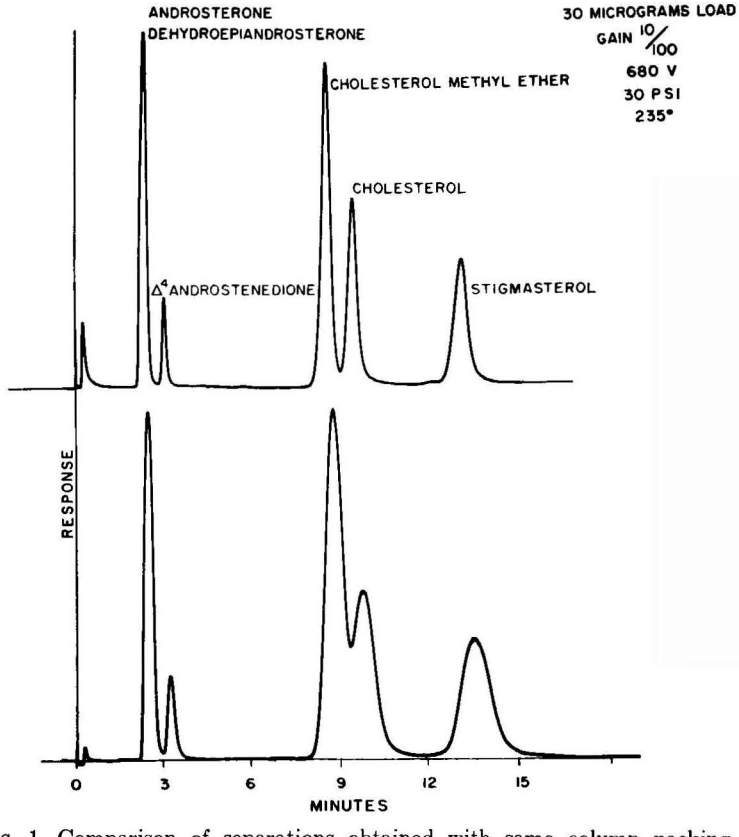

FIG. 1. Comparison of separations obtained with same column packing (0.75% SE-30 on 100–140 mesh Gas-Chrom P) with 9 ft $\times$ 4 mm I.D. and 9 ft $\times$ 12.4 mm I.D. columns. The conditions were chosen to give maximum efficiency for the larger diameter column.

0.75% silicone polymer SE-30 on 100–140 mesh Gas-Chrom P was tested for column efficiency under the conditions indicated. A tube of the same length and containing the same packing, but with an internal diameter

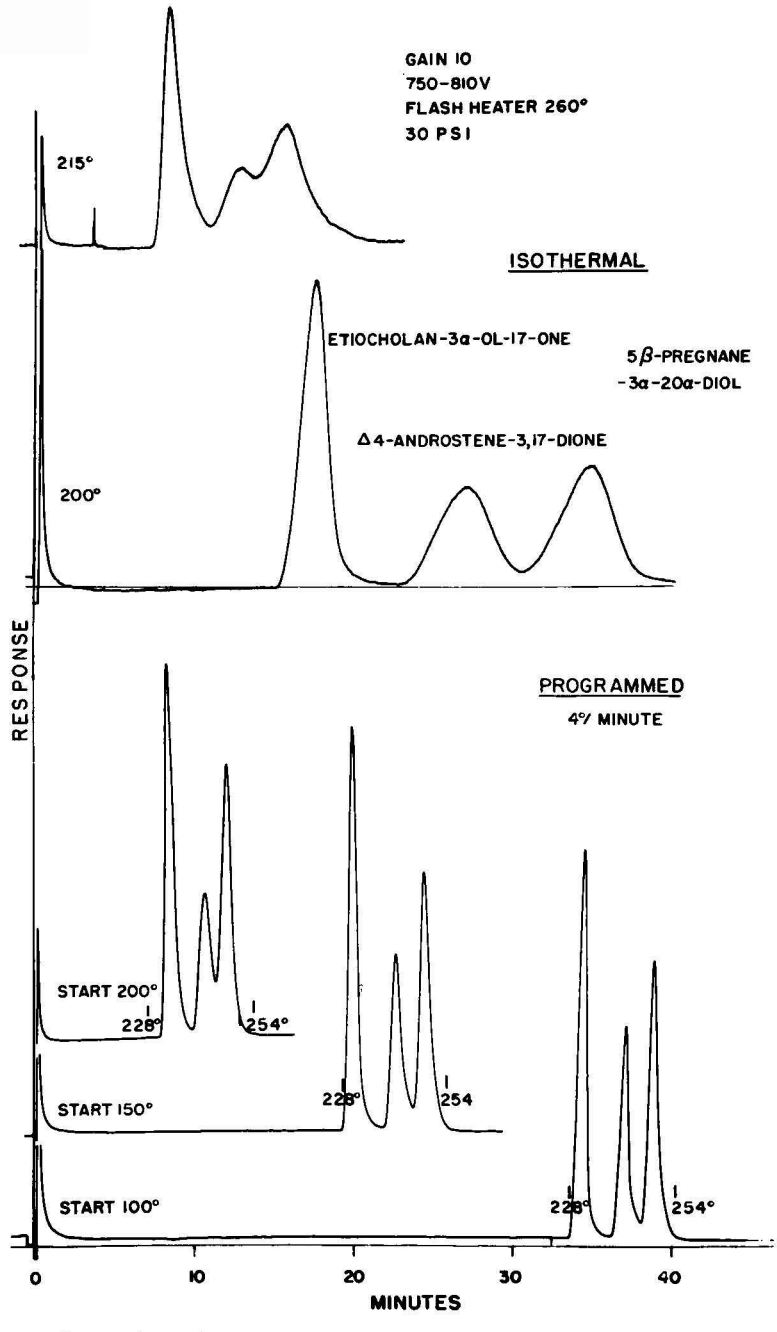

FIG. 2. Comparison of column behavior showing effect of variation in temperature in isothermal separations, and effect of variation in retention time in temperature programed separations. Sample size was 1 mg in each instance.

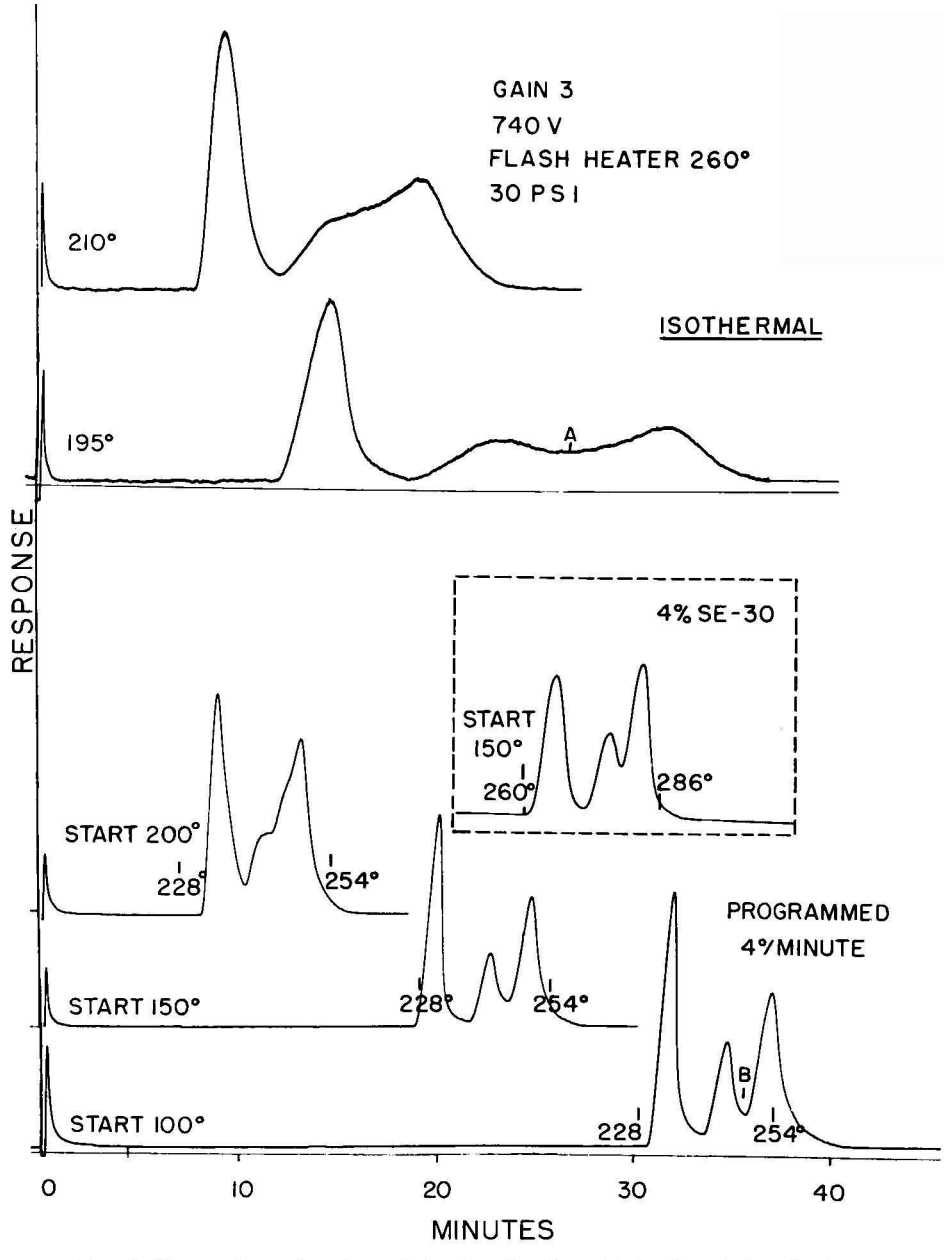

FIG. 3. Comparison of column behavior showing effect of variation in temperature in isothermal separations, and effect of variation in retention time in temperature programed separations. Sample size was 2 mg in each instance. The insert shows the result of an experiment with a 4% SE-30 column packing with a sample size of 4 mg; resolution was about the same as that observed in the other experiments.

of 12.4 mm, was compared for column behavior with the same steroid mixture. A total load of 30 μg was used for both separations, and the entire sample was channeled through the detector system of the preparative column. The theoretical plates observed under these conditions fell from about 4200 to 900 (stigmasterol). A different mixture (etiocholanolone, 4-androstene-3,17-dione, 5β-pregnane-3α,20α-diol) was chosen for studies of load effects and for a comparison of column behavior with the 12.4-mm column under isothermal and temperature programed conditions. The results are in Figs. 2, 3, and 4. Under isothermal conditions a

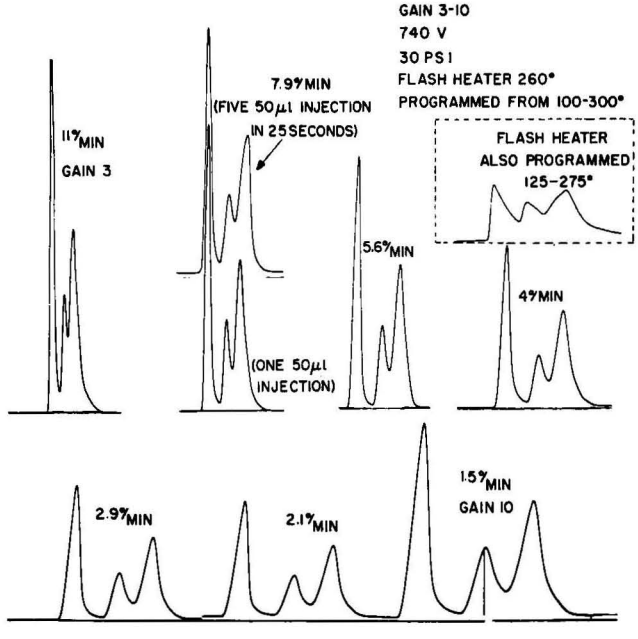

FIG. 4. Effect of heating rate in a temperature programed separation. Sample size was 2 mg. Effect of a multiple injection procedure for the sample is shown in the comparisons with a 7.9°/min heating rate. Effect of a temperature programed procedure for vaporizing the sample is shown in the insert.

complete separation occurred at 200° for a total sample of 1 mg, but a considerable loss of column efficiency resulted when the total sample was increased to 2 mg, and the separations were less satisfactory. When temperature programing was used, several effects were apparent. The extent of separation of each component was as good or better than that obtained under isothermal conditions. The rate of heating had very little effect on the separation at rates between 1.5° and 7.9°/min (Fig.

4). A short residence time was undesirable, but very little difference was observed for a separation starting at 100° and a comparable run starting at 150° (Figs. 2 and 3). The effectiveness of the separation decreased with an increased load, but the relatively sharp changes in concentration found during the temperature programed runs (compare region B with region A, Fig. 3) made it easy to determine the appropriate time for fraction cutting.

An increase in the amount of phase to 4% approximately doubled the capacity of the column for equivalent resolution (insert, Fig. 3; the sample was 4 mg) but an undesirable increase in the column temperature was needed for elution of the steroids.

The effect of variations in flow rate was not studied in detail. Maximum efficiency for the preparative column was obtained at about 30 psi inlet pressure, and this condition was used in studies of load effects.

It is undesirable to draw broad conclusions from these limited data, but several effects are apparent. A loss in column efficiency when the column diameter is increased has been observed in many laboratories and for many types of packings. There is reason to believe that the loss in efficiency is not sharply dependent on the preparative column diameter for values near the size used here. Clifford (3) found very nearly the same efficiency for columns in the range 5–20 mm in diameter. Other investigators [Bayer, Hupe, and Witsch (4); Bayer, Wahl, and Witsch (5); review, Mechelijnck-David (6)] have suggested that preparative columns may have efficiencies approaching those of ordinary packed columns used in analytical work, and columns with diameters of 1.5–2 in. have been used for preparative work (7, 8). The 12.4-mm I.D. tube used in this work is close to that used in preparative studies by Lukes and Herout (16–17 mm) (9), Catalette, Beaufils, Gras, and Germain (16 mm) (10), and Mechelijnck-David (15 mm) (6). It should be added that many investigators have collected samples from analytical columns simply by using a little larger load than usual. This is an effective process with columns containing thick-film packings but it is less successful with thin-film columns since overloading occurs relatively quickly.

Sample Introduction. Perhaps the most striking advantage of a temperature programed separation is shown in Fig. 4. A separation observed after introduction of the sample in five 50-μl portions of dilute solution over a 25-sec period was compared with the result obtained when a single injection of 50 μl of solution was used for the same total amount of steroids. Very little difference was found. This suggests that there is no particular need for "instantaneous" application of the sample

to the column (instantaneous vaporization of a large sample is undesirable in any case because of pressure surges, but it is generally considered that for isothermal separations the process of sample application should require only a few seconds). When temperature programing is used, the sample in an appropriate solvent may be added in several portions over about 15–20 sec to an ordinary "flash heater" chamber which may be part of the chromatographic tube. However, it is not desirable to use a temperature programed vaporization of the sample. The insert in Fig. 4 shows the effect resulting from an experiment in which the sample was injected as usual but vaporized by a programed procedure, with the same heating rate for the"flash heater" zone and the column.

It may be desirable to provide a larger vaporizing chamber than the 8-mm diameter tube used in this work, but the present results suggest that syringe introduction of the sample in solution is an entirely satisfactory method of sample injection for steroid samples of a few milligrams when temperature programing is used. This has the practical advantage that a special vaporizing chamber is not needed as part of the preparative chromatography system.

Sample Collection. At the start of this work it was found that relatively high melting solids formed smoke-like aerosols as soon as the effluent gas stream was cooled. "Fog" or "smoke" problems in condensation have been described by Atkinson and Tuey (11), who developed an electrostatic fog precipitator; Thompson (12) has also described an electrostatic device. In the present work it was found that use of a glass U-tube chilled in liquid nitrogen gave excellent results (usually quantitative recovery). The reason for the effectiveness of this method may lie in the "rain" effect caused by the condensation of the argon carrier gas. The collection tubes were found to contain liquid argon, which was permitted to evaporate in order to obtain each fraction as a crystalline residue.

The simplicity and effectiveness of this procedure with regard to the trapping and isolation of the eluted material suggests that it may be useful in other instances.

Applications. A synthetic mixture of reference standards of steroids was used for the experiment described in Fig. 5. Temperature programing was used (150° starting temperature) and fractions were collected as indicated on the chart. The infrared spectrum was taken for each crystalline solid collected as shown, and the spectra were identical in each case with those of the appropriate reference compounds. The melting points of each compound were within a few degrees of the literature

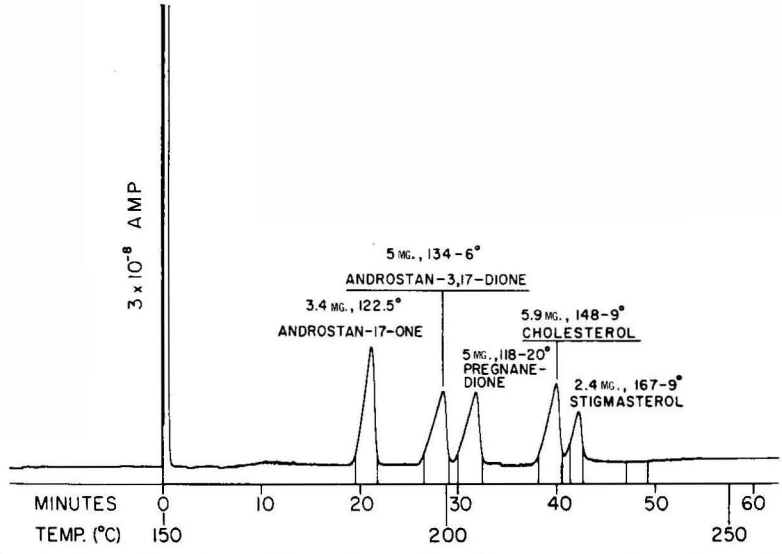

FIG. 5. Separation of a mixture of steroids with a preparative column. Times and temperatures for the temperature programed (2.1°/min, 30 psi) procedure are indicated. Vertical lines indicate the "fraction cutting" operation. Amounts and melting points for each major fraction are shown. Identification of each component was completed by infrared spectra comparisons. The shape of the peaks indicates column overloading, but nevertheless the separation was satisfactory. Recovery of individual components was nearly quantitative, except for the last compound (stigmasterol, recovery about 50%).

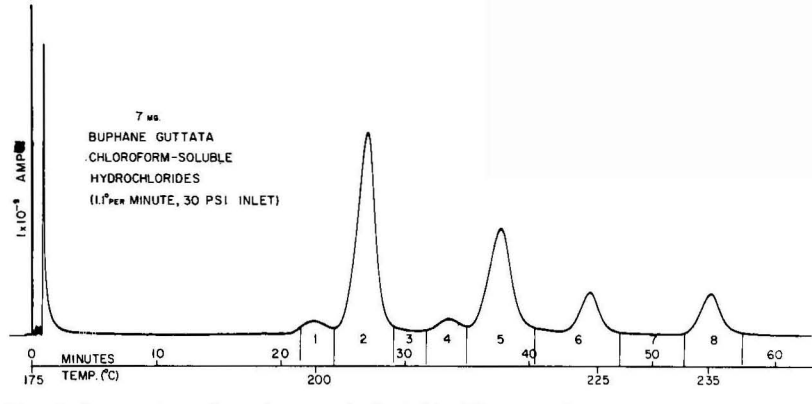

FIG. 6. Separation of a mixture of alkaloids. The sample was the mixed free bases from the chloroform-soluble hydrochlorides of a *Buphane guttata* alkaloid extract. Times and temperatures for the procedure are indicated. Vertical lines indicate the "fraction cutting" operation. All components were found to be above 98% pure by analytical gas chromatography. Compounds of known structure were identified as buphanisine (fraction 2), buphanidrine (fraction 5), and undulatine (fraction 6). Compounds 1, 4, and 8 are new alkaloids, isolated for the first time by this procedure.

values. Analytical gas chromatography of the collected fractions indicated that the separation was complete (above 99%) in each instance. The separation required about 45 min, as indicated in the figure.

The alkaloid mixture used in the experiment described in Fig. 6 was derived from a plant of the Amaryllidaceae. The column behavior with larger samples indicated an overload, but with a total load of 7 mg of mixture the result shown in Fig. 6 was obtained. The fraction number and vertical lines indicate the regions of fraction collection. All fractions were examined by analytical gas chromatography, by infrared spectroscopy, and by other physical methods, and the compounds in fractions 2, 5, and 6 were identified as known alkaloids of the Amaryllidaceae family. Compounds 1, 4, and 8 were previously unknown and their first isolation was by this technique.

SUMMARY

It has been found that milligram-scale preparative chromatography may be carried out for steroid and alkaloid mixtures through the use of thin-film columns with analytical-type packings. The use of temperature programing is helpful. Two examples of experimental separations are given.

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ANALYTICAL BIOCHEMISTRY 4, 306–309 (1962)

Separation of Plasma Protein Fractions by Continuous-Flow Paper Electrophoresis Using a Volatile Buffer[1]

RUTH F. ARONSON, GLENN D. LUBASH,[2] AND ALBERT L. RUBIN[3]

From the Cardiorenal Laboratory, Second (Cornell) Medical Division, Bellevue Hospital, and the Department of Medicine, Cornell University Medical College, New York, New York

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INTRODUCTION

Fractions with differing electrical charges may be separated out of large quantities of a protein mixture, such as plasma, by continuous-flow paper electrophoresis (1). Separation may also be chemically accomplished by precipitation techniques (2), and choice of procedure depends on the aims of isolation. The electrophoretic technique has several practical advantages for certain studies. First, it is particularly suitable for study of plasma proteins as carriers of other substances, including steroids, fatty acids, and peptides, since these bound materials are less likely to be liberated than by the method of chemical separation at the isoelectric point (3). Second, the subsequent identification of these bound materials by chromatography is facilitated in the proper salt environment, a state sometimes difficult to achieve after chemical manipulation. Finally, the isolation of protein fractions in a buffer at pH above the isoelectric point reduces the possibility of altering natural states (4).

We describe herein a method of separating plasma proteins by continuous-flow electrophoresis using a volatile buffer with pH above the isoelectric point and in which plasma proteins are soluble with markedly reduced salt concentration.

[1] This work was supported in part by grants from the American Heart Association, the Health Research Council of the City of New York (contract U-1022), and the U. S. Public Health Service (H-4148).

[2] Advanced Research Fellow, American Heart Association.

[3] Established Investigator, American Heart Association.

METHOD

Three hundred to 500 ml of blood from human subjects was collected by gravity under ice in a plastic bag containing 50 ml of the anticoagulant, disodium ethylenediaminetetraacetate. It was centrifuged immediately at 2700 rpm for 30 min at 4°C. Plasma was separated with a Fenwal Extractor and dialyzed[4] with constant agitation against recirculated buffer (V 50:1) at 4°C for 17 hr. The dialysis removed almost all measurable plasma electrolytes and obviated any salt problem.

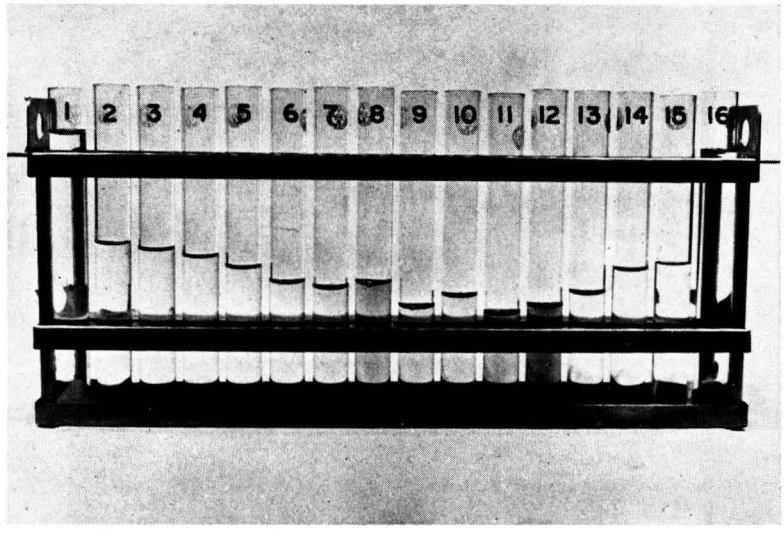

FIG. 1. Separation of plasma protein fractions in test tubes. Albumin has the deepest color and other protein fractions are lighter. These fractions are easily identifiable by electrophoresis (see Fig. 2). Uncontaminated buffer is collected in several test tubes at either end.

The buffer employed was a triethylamine (TEA)-acetic acid solution in water. TEA [5] is a clear, colorless liquid with boiling point at 88–90°C. At 20°C, 1.5 gm is soluble in 100 ml water. In this study, 7.5 ml of TEA was combined with 2.5 ml of glacial acetic acid (3:1 ratio) and diluted to 1 liter with distilled water (pH 9.0–9.3).

Continuous-flow electrophoresis was performed using a Spinco Model CP and a Constat regulated power supply.[6]

[4] Visking $^{20}/_{32}$ cellulose tubing, pretreated by boiling in 1% sodium bicarbonate, rinsing with distilled water and then TEA, and storing in distilled water.

[5] Matheson Coleman & Bell, East Rutherford, N. J.

[6] Beckman Instruments, Spinco Division, Palo Alto, Calif.

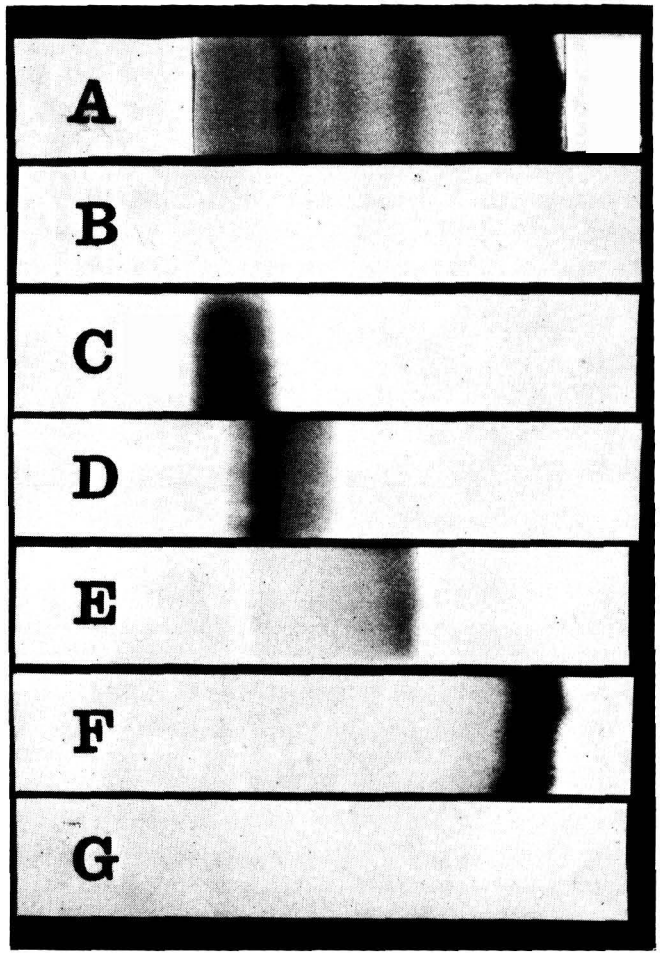

FIG. 2. Electrophoretic patterns[8] of plasma protein fractions obtained by continuous-flow electrophoresis. Samples from each of the test tubes of Fig. 1 were studied and representative strips are shown. (A) Plasma prior to separation. (B) Tubes 1–4, buffer. (C) Tubes 5–6, γ-globulin. (D) Tubes 7–8, β-globulin. (E) Tubes 9–10, α-2-globulin. (F) Tubes 11–12, albumin. (G) Tubes 13–16, buffer.

The electrophoresis paper[7] was correctly placed in the apparatus and washed for 4 days with 4 liters of fresh TEA-acetic acid buffer, changed daily, to reduce contamination with α-amino-ninhydrin reacting substances. The flow rate was kept maximal for the first 24 hr and then reduced. During the last 48 hr of the washing period, 580–620 volts at 95 mamp were applied. The flow rate, voltage, and current approximated

[7] Schleicher and Schuell 470: Spinco No. 400-235, lower curtain paper.

those used in the actual run. The electrophoresis apparatus was mounted in a refrigerator at 4°C.

Plasma was fed on to the curtain at a rate of 1.5 ml/hr. When using 130–200 ml of plasma, a single run took 3–6 days. Fractions of 15 cc each were collected in sets of test tubes which changed automatically at 12-hr intervals. Following collection and identification, separate fractions were pooled.

RESULTS AND DISCUSSION

Ten specimens have been satisfactorily separated by continuous-flow electrophoresis using the volatile buffer. Figure 1 illustrates the collection of fractions by this process. The excellence of separation is demonstrated by paper electrophoresis of samples from different test tubes[8] (Fig. 2).

Test tubes containing different protein fractions may be pooled for additional study. The TEA-acetic acid buffer is volatile and therefore well suited for lyophilization. Pooled protein fractions may be concentrated under vacuum and then reduced to a powder by lyophilization. In the process, the TEA vaporizes before water, and pH falls. Pooled albumin fractions of about 100 ml have yielded 1.5–2.0 gm of dry albumin. Other fractions which are reconstituted in the TEA-acetic acid buffer after this procedure are also unchanged electrophoretically.

The use of the TEA-acetic acid buffer during continuous-flow paper electrophoresis of human plasma protein has, therefore, provided reliable results. Fractions are obtained with a high degree of purity and are unchanged electrophoretically. Their separation in the volatile buffer, as described, facilitates reduction to the solid state.

SUMMARY

A method is described for use of a volatile triethylamine-acetic acid buffer for continuous-flow paper electrophoresis of human plasma. Separation is excellent and contamination is minimal. The properties of the buffer are well suited for additional analytical studies.

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[8] Electrophoresis done with Schleicher and Schuell paper 2043A gl: Spinco 300-846; Spinco Model R apparatus run for 16 hr in a barbital buffer, pH 8.6, ionic strength 0.75, at 60 volts, 2.5 mamp.

ANALYTICAL BIOCHEMISTRY 4, 310–315 (1962)

A Procedure for *in Vivo* $C^{14}O_2$ Collection and Subsequent Scintillation Counting

PAUL GODFREY[1] AND FRED SNYDER

From the Medical Division, Oak Ridge Institute of Nuclear Studies, Oak Ridge, Tennessee[2]

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INTRODUCTION

As a part of a larger research project involving the cerium fatty liver problem (1, 2) it was found desirable to compare the rate of oxidation of various metabolites. Several investigators, e.g., McCalla and associates (3), have used bubble towers containing aqueous alkali for collecting C^{14}-labeled carbon dioxide from intact animals, with subsequent conversion of the soluble carbonate to barium carbonate for counting the radioactivity. Our wish to simplify the collection of the radioactivity for liquid scintillation counting and our previous success (4) in using Hyamine[3] (5) as a carbon dioxide absorbent led to the procedure described in this paper. The method involves no transfer steps and the radioactivity can be counted immediately after the collection period.

EXPERIMENTAL

To study the rate of oxidation of a C^{14}-labeled compound by an intact rat, the animal was placed in a cylinder of galvanized wire screen (0.5″ mesh, 7.5″ × 6″ circumference), which was closed at one end with a hinged door of the same material and fastened with an alligator clip. A nail across the open end restrained the rat. An anesthetized rat was intravenously injected with 0.5 ml of a solution containing 1 μc of radioactivity. A diagram of the apparatus used to collect the $C^{14}O_2$ is shown in Fig. 1. The rat and restrainer were immediately placed in the

[1] Summer Research Participant, University Relations Division, Oak Ridge Institute of Nuclear Studies. Present address: Department of Chemistry, Louisiana College, Pineville, Louisiana.

[2] Under contract with U. S. Atomic Energy Commission.

[3] Trade name for *p*-(diisobutyl-cresoxyethoxyethyl) dimethylbenzylammonium hydroxide, 1 *M* in methanol, obtained from Packard Instrument Company, Inc., La Grange, Illinois.

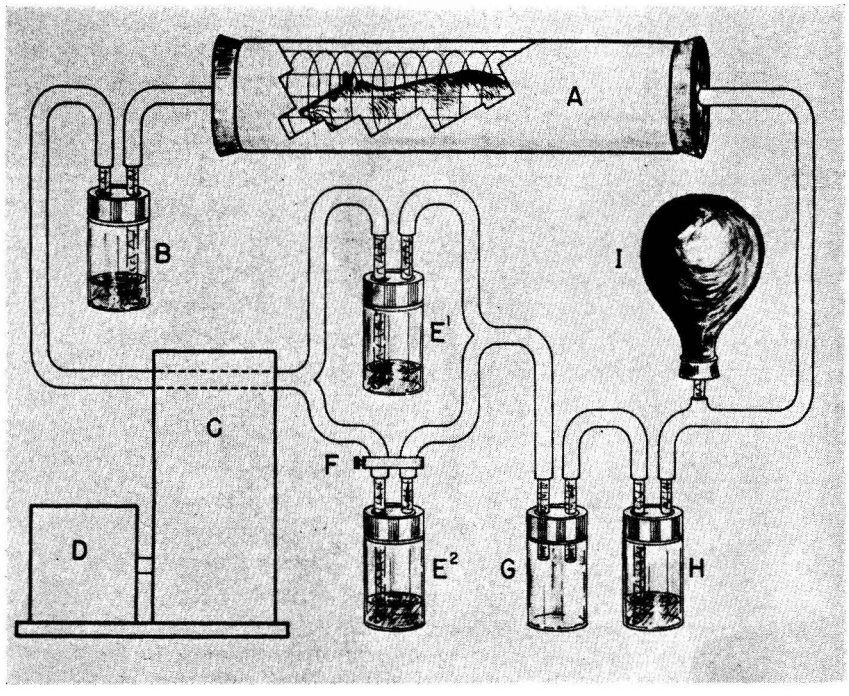

FIG. 1. Apparatus for collecting $C^{14}O_2$ (*in vivo*) for direct scintillation counting.

metabolism chamber, A, with the head at the exit end of the chamber, and the time of entry was recorded. The metabolism chamber consisted of a piece of hard acrylic tubing ($2^{11}/_{16}$" i.d., 10" long), stoppered at both ends, and fitted with glass tubing. The exit tube was connected by rubber tubing to a scrubber, B, containing 5 ml concentrated sulfuric acid for removal of exhaled water. The exhaled gases were circulated through the system by the peristaltic action of a Sigmamotor pump,[4] CD. The speed of the pump was adjusted to give a flow rate of about 185 ml/min through plastic tubing (¼" i.d., Fisher Cat. No. 14-169-5) attached to a Y tube. This Y tube was connected by means of rubber tubing to two caps, which at the time of each experiment received 20-ml counting vials, E^1 and E^2, containing 4 ml Hyamine 10-X. Holes (¾") drilled in the caps received specially made Teflon plugs (Fig. 2) seated on thin rubber washers, which gave gastight seals with the counting vials when the caps were screwed securely. The washers were cut with size 10 and 12 cork borers. The intakes of the vials were fitted with a piece of 8-mm

[4] Available from Travenol Laboratories, Inc., Middleport, New York; pump for the Travenol coil kidney tank unit, Model No. TL.

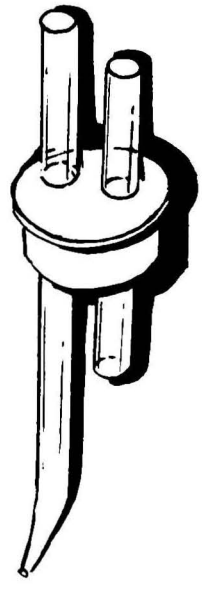

FIG. 2. Sketch of Teflon plug designed for cap that receives the glass collecting vials. The plug was machined in our instrument shop according to the following dimensions: diameter, top, $1\frac{4}{16}$"; diameter, bottom, $1\frac{1}{16}$"; distance, top to bottom of plug, $\frac{13}{16}$"; and thickness of flanged overlap on top, $\frac{1}{16}$".

glass tubing drawn to a small opening and bent at a 45° angle near the tip (which was immersed in the Hyamine) to reduce spattering of Hyamine. A disk of filter paper was slipped on each intake tube well above the level of the Hyamine to prevent spattering on the underside of the Teflon plugs. These two vials were connected to a second Y tube, which in turn was connected to a trap, G. Attached to this trap was a vial, H, containing sulfuric acid (5 ml, fresh each run) to absorb any methyl alcohol that evaporated from the Hyamine. Vial H was connected by means of a T-tube to a balloon, I (containing about 1 liter of oxygen), and to the metabolic chamber, A. The arm of the T-tube carrying the balloon was fitted with a notched rubber plug as a safety feature to minimize diffusion of carbon dioxide into the balloon. The purpose of the balloon was to permit a continual replacement of oxygen to the system: the amount of oxygen released by the balloon was proportional to the pressure changes that resulted from the metabolic activity of the rat (a liter of oxygen would last about 4 hr). Connections to the Hyamine vials were tested for leaks by immersing them in a

beaker of methanol. Other connections were tested with a few drops of detergent solution. The tubing was rinsed with methanol after each experiment to prevent possible contamination. Residual alcohol was quickly evaporated by forcing air through the rubber tubing.

The circulating gas was allowed to pass through only one vial of Hyamine at a time by use of the screw clamp, F. Although the gas usually was allowed to flow through a given vial for 15 min, shorter time intervals were sometimes used. The 4-ml sample of Hyamine used in our experiments was estimated to be able to absorb carbon dioxide from a 250-gm rat for about 20 min. A vial was removed for analysis 10 min after switching the flow to the other vial. The filter paper disk was kept in the counting vial and 15 ml of scintillator solution (4 gm PPO,[5] 0.1 gm POPOP,[5] and 1 liter of toluene) was added. After the contents of the vial were mixed, the radioactivity was measured in an automatic Tri-Carb[5] liquid scintillation spectrometer.

For testing the rate of recovery of carbon dioxide from the system, a Warburg flask containing $Na_2C^{14}O_3$ and sulfuric acid was inserted into the circuit (in some experiments at the entrance of the metabolism chamber, and in the other experiments at its exit).

RESULTS AND DISCUSSION

Release of a known quantity of $C^{14}O_2$ in this closed collecting system showed that 97.8% of the radioactivity was recovered; the results were in agreement with those obtained when the same quantity of radioactivity was released and collected in a Warburg flask (4). The time required for quantitative collection was determined by releasing $C^{14}O_2$ in the system near the exit (between A and B) of the metabolism chamber containing a rat; successive 15-min samplings gave 1030, 107, 30, 15, and 8 counts/min. More than 95% of the radioactivity collected was obtained by the end of the first 30 min. Because all the radioactivity is not collected during the first passage through the Hyamine, an experiment was designed whereby a known quantity of $C^{14}O_2$ was released at the entrance (between I and A) of the metabolism chamber in which the passage of the radioactive gas over the rat was imperative. Five successive 15-min samplings under this condition gave 669, 221, 131, 65, and 36 counts/min; this time 79% of the total radioactivity collected was obtained during the first 30 min. Diffusion and perhaps a small exchange of the carbon dioxide on moist surfaces of the rat

[5] Available from Packard Instrument Company, Inc. PPO = 2,5 diphenyloxazole. POPOP = 1,4-bis-2-(5-phenyloxazolyl)benzene.

could account for the longer time required for quantitative collection when the radioactivity was released behind the rat. However, the total radioactivity collected over the 75-min period in both cases was essentially the same.

Figure 3 shows typical curves obtained with 1-μc quantities of sodium

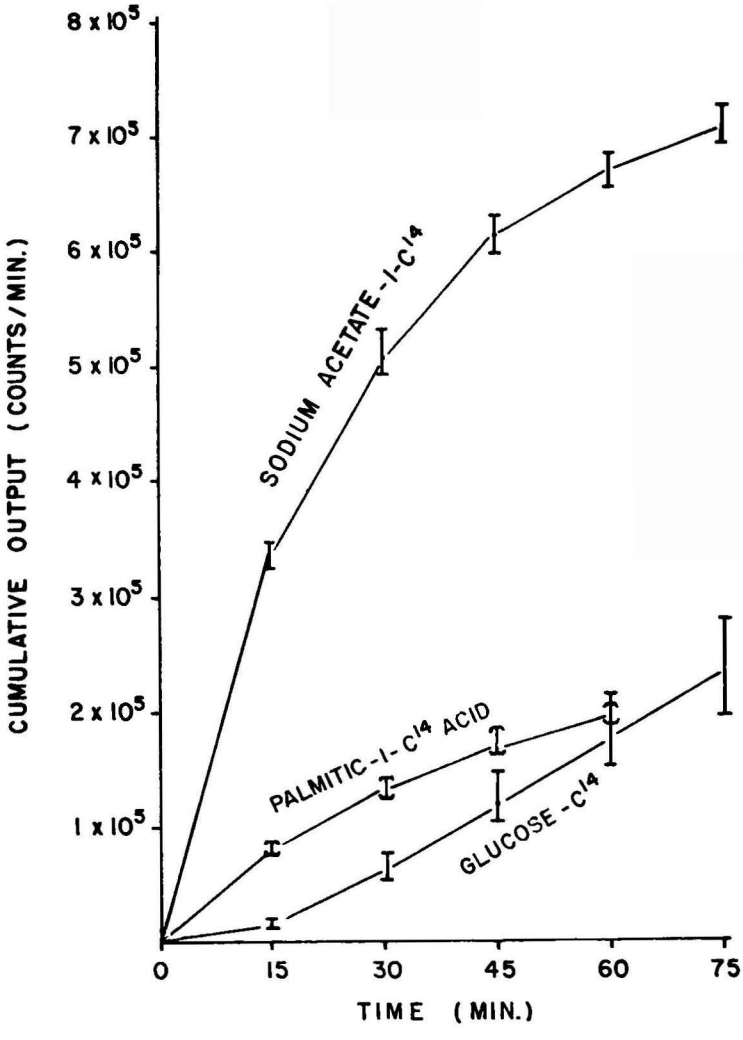

FIG. 3. Oxidation of sodium acetate-1-C^{14}, palmitic acid-1-C^{14} (albumin complex), and glucose-C^{14} (uniformly labeled) measured according to the procedure described in the text. The bar at each time interval represents the range observed for 3 rats.

acetate-1-C^{14}, uniformly labeled D-glucose-C^{14}, and an albumin complex of palmitic acid-1-C^{14} injected into 200-gm rats which had been fasted 24 hr.

SUMMARY

A convenient method for the *in vivo* collection and assay of $C^{14}O_2$ uses Hyamine as the carbon dioxide absorbent with no transfer steps required. The method permits an efficient monitoring of the oxidation of C^{14}-labeled materials administered to small animals.

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ANALYTICAL BIOCHEMISTRY 4, 316–321 (1962)

Conditions for the Hydrolysis of DNP-Gelatin by Dowex 50 Catalysis

F. S. STEVEN

From the Department of Physiology and Biochemistry, University of St. Andrews, Scotland

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INTRODUCTION

The complete hydrolysis of dinitrophenyl (DNP) proteins is usually performed by heating in the presence of strong mineral acids as originally described by Sanger (1). Certain DNP-amino acids are rapidly destroyed under these conditions in the presence of carbohydrates and free amino acids liberated during the course of hydrolysis. The greatest losses are usually found in the DNP-derivatives of glycine, proline, hydroxyproline, serine, and threonine (2–4).

Commercial gelatins, by the definition of Ward (5), have been extensively examined by Courts (6–8) employing a modification of the DNP technique of Sanger (1). The gelatins had a number of different *N*-terminal residues of which the major component was always found to be glycine.

In a recent study of the recovery values obtained when DNP-amino acids were hydrolyzed for 18-hr in the presence of collagen with boiling 5.7 *N* HCl it was found that only 16% of the added DNP-glycine was recovered after chromatographic isolation (4). This recovery value for DNP-glycine is in excellent agreement with the figure quoted by Sanger (1).

Acid hydrolysis has been attempted on DNP-gelatin with only limited success in this laboratory. This was mainly due to the high content of glycine present in the *N*-terminal position coupled with the low recovery of DNP-glycine after acid hydrolysis, which resulted in the inaccurate determination of the major *N*-terminal residue. Reproducible results for other DNP-amino acids could not always be obtained. An attempt has been made to develop a form of hydrolysis which yields a high recovery of the DNP derivatives of glycine, serine, threonine, proline, and hydroxyproline from DNP-gelatin. This improved hydrolysis would be of value since glycine, serine, and threonine, are the most common

N-terminal residues in commercial gelatin (6–8). Proline and hydroxyproline account for nearly a third of the total amino acid composition of gelatin (9). The possibility of these imino acids appearing in *N*-terminal positions cannot be ignored as is the usual practice when conditions of strong acid hydrolysis are employed. Hydrolysis catalyzed by sulfonated polystyrene resins such as Dowex 50 seemed to offer the best possibility since very mild acid conditions are used throughout and the liberated amino acids are bound to the resin, which might be expected to afford some protection to the DNP-amino acids during hydrolysis. The DNP-amino acids are not bound to the resin and can be readily removed by washing with water. Paulson and Deatherage (10) demonstrated the selective rupture of peptide bonds during Dowex 50 hydrolysis of proteins; gelatin was completely hydrolyzed in 10–12 hr at 100° as demonstrated by a negative biuret reaction (11). DNP-serum albumin was found to be more resistent to hydrolysis than the original protein (10).

The present work describes the conditions for Dowex 50 hydrolysis of DNP-gelatin which have been found to yield consistently reproducible results.

MATERIALS

A standard preparation of commercial gelatin has been used throughout this work. The gelatin was a lime-processed calf hide gelatin kindly provided by the British Gelatine and Glue Research Association, London, and classified by this institute as "gelatine X-127." The preparation and properties of this material have been described (5, 12, 13). The total nitrogen contents of all solutions of "gelatine X-127" were determined prior to reaction with DNFB[1] in order to find the weight equivalent of the original protein in the DNP reaction product. "Gelatine X-127" had an ash and moisture-free total nitrogen content of 17.0%.

Dowex 50H (200–400 mesh) was regenerated before use by the method described by Hirs, Moore, and Stein (14).

METHODS

DNP-gelatin was prepared according to Sanger's method (1). The gelatin was dissolved in 5% w/v $NaHCO_3$ to yield a final protein concentration of approximately 1.5% w/v; 20 ml of this solution was then added to 30 ml of ethanol containing 0.5 ml DNFB. The mixture was shaken for 24 hr before the reaction was stopped by acidification with 1 *N* HCl and the entire reaction mixture concentrated to dryness

[1] Dinitrofluorobenzene.

by vacuum distillation. An excess of Dowex 50H (approximately 20–30 times weight of protein) was added to the dry DNP reaction mixture in a 50-ml Quick-fit round-bottomed hydrolysis flask. A few porous chips and 30 ml of water were added and the resultant suspension was found to have a pH of 3–4, which did not alter during hydrolysis. The flasks were heated under reflux in the dark for a given time; the resin was then washed fifteen times with 50-ml portions of boiling water to extract the DNP-amino acids. The hot aqueous extract was filtered through Whatman No. 41 paper. The ether-soluble DNP derivatives were then extracted and estimated as described in an earlier paper (15).

The optimal time for Dowex 50 hydrolysis of DNP-gelatin was determined as follows. Four samples of DNP-gelatin were prepared and subjected to different times of hydrolysis and the liberated DNP-amino acids estimated. The results of this analysis are given in Table 1. It was concluded that 48 hr of hydrolysis provided the greatest yields of DNP-amino acid and this was chosen as the optimal hydrolysis time. Shorter periods did not liberate all the DNP derivatives while longer periods of hydrolysis resulted in reduced yields that were probably caused by hydrolytic destruction. In order to ensure that all the ether-soluble DNP-material had been extracted in the water washing, the following examination of the resin was carried out. Two short columns of the washed resin were prepared: one was eluted with 1 *N* HCl and the other with 1 *N* NH_4OH. The alkaline eluate was then acidified and both eluates were extracted with ether; the ether-soluble fractions were examined for DNP-amino acids, which were found to be absent in each case. This evidence was taken to indicate that all the ether-soluble DNP-amino acids were extracted at the water washing stage.

Control hydrolyses to determine the losses of DNP-amino acids after a 48-hr hydrolysis were performed as follows. A standard solution of mixed DNP-amino acids was prepared and its composition determined by chromatographic analysis. Known amounts of this solution were then hydrolyzed in the presence of gelatin and the recovery values of the individual DNP-amino acids determined after isolation by chromatography. These values are presented in Table 1. The products of a 48-hr resin hydrolysis of gelatin were shown to be completely hydrolyzed, as demonstrated by a negative biuret test.

Substitution of free α-NH_2 groups took place when excessive quantities of commercial gelatin (e.g., 3 gm) were hydrolyzed with a minimal amount of Dowex 50 (e.g., 0.1 gm) in the presence of excess FDNB. This resulted in the appearance of measurable quantities of the DNP derivatives of glycine, glutamic and aspartic acids, serine, threonine, and

alanine, as well as trace quantities of valine, leucine, phenylalanine, and the prolines. The unexpected reactivity of FDNB in acid solution may be accounted for by a partial dissociation of protons at 100° from acidified free amino acids, with subsequent substitution of the free amino groups with dinitrophenyl radicals. When excess resin was used under identical hydrolysis conditions no measurable DNP substitution took place. It is therefore essential to use a large excess of resin.

TABLE 1

RESIN HYDROLYSIS OF DNP-GELATINE (X-127)

N-Terminal residues presented as moles per 10^6 gm gelatin isolated after the given period of hydrolysis.

DNP-amino acid	Time (hr) 18	48	80	170	% recovery of synthetic mixt. of DNP-amino acids after 48 hr	Corr. for recovery after 48 hr	Courts (6)
Glycine	1.55	6.20	5.30	3.71	63.5	10.30	8.3
Glutamic acid	0.12	0.78	0.61	0.58	75.1	1.04	1.0
Aspartic acid	0.11	0.54	0.42	0.35	76.8	0.70	1.1
Serine	0.45	1.35	0.83	0.90	61.5	2.19	1.9
Threonine	0.11	0.76	0.70	0.63	74.5	1.02	1.1
Alanine	0.12	0.76	0.74	0.67	80.0	1.05	1.1
Valine		0.20			87.0	0.23	
Leucine Isoleucine	0.10	0.28	0.19	0.21	42.5	0.66	
Phenylalanine	0.07	0.23	0.29	0.21	68.0	0.34	"Others" 1.2
Lysine	Trace	Trace	Trace	Trace		Trace	
Tyrosine	Trace	Trace	Trace	Trace		Trace	
Pyroline	—	0.16	—	—	55.5	0.24	
Hydroxyproline	—	0.20	0.10	0.02	35.0	0.57	

RESULTS

In Table 1 the actual molar values of the *N*-terminal amino acids, per 10^6 gm "gelatine X-127," isolated after the given hydrolysis times are given. The recovery values of these DNP-amino acids are given for the optimal 48-hr period of resin hydrolysis. The *N*-terminal analysis of DNP-"gelatine X-127" hydrolyzed under these conditions and corrected for hydrolytic loss is calculated on the assumption that the recovery values for free DNP-amino acids are an approximation for recovery values of the same DNP-amino acids when present as *N*-terminal residues in the intact DNP-protein. The values for the 48-hr hydrolyzate were taken from the mean of four estimations, each value being within ± 0.02 mole/10^6/gm of the quoted value. Trace amounts

of di-DNP-lysine and DNP-tyrosine were often found as a single overlapping spot on the paper chromatograms. The estimation of the components of this spot was not possible since the extinction coefficients of these two DNP derivatives have very different values. However it can be stated that less than 0.5 mole of the combined derivatives was present.

DISCUSSION

The recovery values of DNP-amino acids obtained in the 48-hr resin hydrolysis were very much higher than those previously reported for acid hydrolysis (4). It follows that the actual molar quantities of the isolated *N*-terminal derivatives will more closely approach those present in the intact DNP-protein than are found in the case of the corresponding strong acid hydrolysis technique. This statement assumes in both cases that the terminal peptide bonds linked to the DNP-amino acids are quantitatively hydrolyzed.

In this laboratory it has never been found possible to obtain constantly reproducible results for the strong acid hydrolysis of DNP-"gelatine X-127." Courts presented results for the acid hydrolysis of DNP-lime processed gelatins; and "calf skin gelatine (C)" in his paper (6) refers to "gelatine X-127" (personal communication). These values are included in Table 1 for comparison with those obtained in the present work. His value for glycine is lower than the value found after resin hydrolysis and it is probable that the stability of DNP-glycine during the mild conditions of resin hydrolysis accounts for this difference. The values given by Courts for the DNP derivatives of serine, threonine, alanine, glutamic acid, and aspartic acid were in good agreement with those given above. The sum of those described by Courts as "Others" is in good agreement with the sum of the values found after resin hydrolysis of valine, leucines, and phenylalanine. Resin hydrolysis has the additional advantage that it allows the quantitative estimation of *N*-terminal proline and hydroxyproline to be achieved as in the present case of a DNP-gelatin. This technique should also be of value in the *N*-terminal analysis of proteins rich in carbohydrate since these are well known to take part in the destruction of DNP-amino acids during strong acid hydrolysis.

SUMMARY

A method has been described for the hydrolysis of DNP-proteins by Dowex 50 catalysis which is considered to be more reliable than the strong acid hydrolysis usually employed for the estimation of *N*-terminal residues. The new technique has been specially developed for the estimation of DNP-amino acids that are unstable during acid hydrolysis.

ACKNOWLEDGMENTS

I wish to thank Dr. A. Courts of Unilever Research Laboratories, Sharnbrook, for the gift of several of the DNP derivatives used in this work as standards for chromatography and also in recovery analysis. I am indebted to Imperial Chemical Industries for a Research Fellowship which enabled this work to be carried out.

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ANALYTICAL BIOCHEMISTRY 4, 322–329 (1962)

Chromatography of Bovine Pancreatic Ribonuclease on Carboxymethylcellulose Columns[1]

RAYMOND SHAPIRA

From the Department of Biochemistry, Emory University, Atlanta, Georgia

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Aqvist and Anfinsen (1) first chromatographed crystalline bovine pancreatic ribonuclease on carboxymethylcellulose (CMC) columns and reported the presence of four active peaks and one inactive peak in the effluent volume. The inactive material was removed from the column with 0.01 *M* sodium phosphate buffer, and the active peaks were removed by gradient elution performed by adding 0.1 *M* phosphate buffer, pH 7.5, to a constant mixing volume of 250 ml of the 0.01 *M* buffer. The sequence of elution of peaks was I, B (II), A (III), and IV—the major components A and B corresponding to the two bovine ribonucleases separated by Hirs *et al.* (2) by column chromatography on IRC-50 (XE-64).

Taborsky (3) chromatographed ribonuclease in CMC columns by gradient elution in 0.005 *M* Tris-HCl buffer, pH 8.00, and a sodium chloride gradient obtained by adding 0.15 *M* NaCl in buffer to a constant mixing volume of 500 ml of the Tris buffer. Under these conditions, the enzyme was successfully separated into five components designated A–E, A being the initial inactive peak, and B–E corresponding to the four active components of Aqvist and Anfinsen.

In attempting to chromatograph ribonuclease under similar conditions, we tested the effects of effluent flow rate and column temperature. An airless, constant-temperature column fed by a variable-speed pump was constructed for this study. Optimum conditions for the chromatography of ribonuclease have been determined. The reproducibility of the results and the reliability of the equipment employed have prompted this report.

EXPERIMENTAL PROCEDURE

Methods

A 250-mm jacketed Kimax modern Liebig condenser with a 450-mm condenser tube (Kimble 18003) was converted into a chromatographic

[1] This investigation was supported by grant funds from the National Institutes of Health of the Public Health Service.

column (Fig. 1) in the following manner. A 3-cm piece of 7–8 mm Pyrex rod was attached to the condenser tube just above and perpendicular to the straight tube. The condenser jacket was modified by removing the inlet and outlet tubes and replacing them with male 12/5 ball joints. The drip tip of the condenser tube was removed and a 60-cm piece of 7–8 mm capillary tubing (1¼ to 1¾ mm bore) was attached. The capillary tubing was bent, as shown in Fig. 1, and was attached to the rod

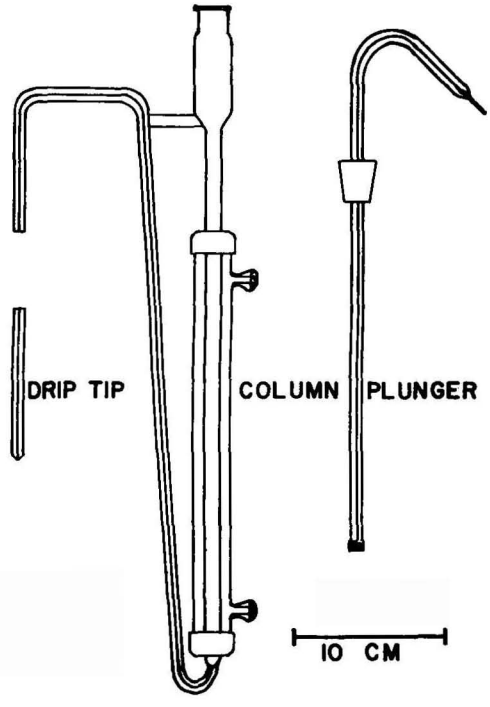

FIG. 1. A chromatographic column constructed for ribonuclease studies.

at the top of the condenser tube. The end of the capillary was evenly cut with a glass saw or cutter to the desired length. A drip tip was prepared from 10 cm of capillary tubing which had been sawed evenly on one end and polished in a 45° angle to form a tip at the other end. The drip tip was attached to the column by use of a short piece of rubber tubing in such a way that the sawed ends of the capillary tubing met. Other types of tips were substituted easily and breakage was minimized with this design. The column was constructed so that there was no constriction smaller than the bore of the capillary tubing. This facilitated loading of the column with resin and controlled the packing of the resin.

A plunger was constructed from another 40 cm of capillary tubing by sawing or cutting one straight end and by blowing a 3–4 mm bulb near the other end. The bulb was heated and pulled to form a tip which was approximately the same bore as the capillary tubing, but now the outside diameter measured about 2 mm. A No. 3 rubber stopper and a $\frac{3}{8}$" piece of seamless black latex rubber tubing, $\frac{3}{16}$" inner diameter, $\frac{1}{16}$" wall thickness, was added to the plunger, as shown in Fig. 1. The plunger tube was bent to an angle which allowed easy attachment of Polypenco Teflon spaghetti tubing, size AWG-15, approximately $1\frac{1}{2}$ mm inner diameter (The Polymer Corporation of Pennsylvania, Reading, Pa.).

A simple linear gradient mixing device, shown in Fig. 2, was constructed from two size-matched 500-ml 29/42 Erlenmeyer flasks (Corning 92550). The siphon arrangement was made from 6-mm outside diameter tubing and 29/42 joints, joined in the middle with rubber tubing or a 12/5 ball joint. The ends of the siphon tubes were cut to set within 1 mm of the bottoms of the flasks. Soda lime tubes to remove CO_2 were attached with rubber tubing or 12/5 ball joints. A straight-bore $1\frac{1}{2}$ mm capillary stopcock (Corning 7300) was attached to the mixing flask level with the bottom, and the other end of the capillary was enlarged and pulled to 2 mm outside diameter in the same manner as the plunger. The gradient apparatus was placed upon a $\frac{1}{2}$" thick wooden box or shelf which could be leveled with the aid of leveling bulbs. A magnetic stirrer was placed in the box or under the shelf, leaving at least a few millimeters of air space.

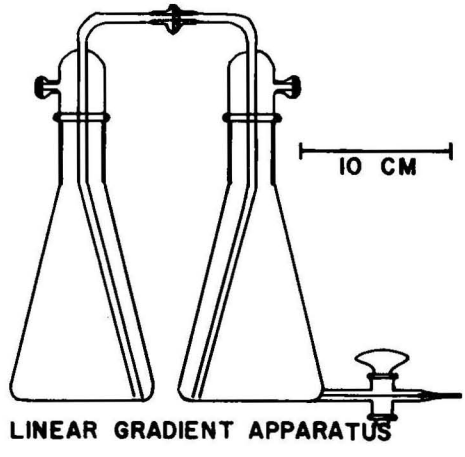

FIG. 2. A simple linear gradient mixing device.

Carboxymethylcellulose (Brown Company reagent lot 1051, 0.93 meq/gm) was cleaned by mixing thoroughly 0.5-gm portions with 25 ml of a mixture of 0.5 *M* NaOH and 0.5 *M* NaCl in a 40-ml centrifuge tube. After centrifugation, the alkali was removed with a bulbed pipet and the resin was washed with distilled water approximately five times until the solution was pH 8 or below as determined by pHydrion 8.0–9.5 paper or thymol blue indicator solution. The resin was then washed twice with 0.01 *M* sodium borate buffer, pH 8.00, hereafter referred to as borate buffer.

Borate buffer was added to the column and a ¾″ length of cotton roll plug, ⅜″ diameter (half of cotton roll, dental, Johnson & Johnson No. 2, 1½″) was added to the column and pushed gently to the bottom of the column. Resin from three centrifuge tubes was added to the column followed by rinsing with buffer. After all of the resin had settled, 10 lb of nitrogen pressure were pressed upon the column for 5 min.

The column was placed next to an LKB Radirac fraction collector with a 3406A turntable disk and a timer. The condenser jacket of the column was connected to an Aminco Type RHC constant-temperature bath or an equivalent 5 to 70° circulating bath. A Sigmamotor T-8 low-capacity pump with 3/32″ diameter holes, a zero-max 14 speed changer, and a 3–1 or a 10–1 gear reduction was placed as close to the column as possible, and the gradient mixing apparatus was placed near the pump. Two glass connectors 1.5 cm long and 1.5–2.0 mm outside diameter were cut from Kimax melting point tubes and were used to connect an 8-cm piece of Tygon tubing (R-2603), 1/32″ inside diameter, 1/32″ wall thickness, to two pieces of Teflon spaghetti tubing, 1½ mm inner diameter, cut long enough to connect the Tygon tubing to the gradient apparatus and to the column, 20 cm and 50 cm in our setup. The Teflon tubing was connected to the plunger and to a length of melting point tubing, and all connections were secured with cellophane tape.

The column was filled with buffer and the plunger was inserted until it was a few millimeters from the resin. The other end of the tubing was placed in buffer during this process, and additional buffer from the column was forced through the tubing when the plunger was inserted. The rubber stopper which served to center the plunger was adjusted to fit the top of the column loosely. If this operation was carried out properly, no air remained in the entire system. If a small amount of air entered the system during a chromatographic procedure, it was trapped above the resin and generally could be ignored.

The Tygon tubing was inserted into the Sigmamotor pump and the tubing was anchored with tape and rubber bands to the outside of the pump to prevent the pump from pulling it through. Because the holes of

the pump were cut close to the bottom adjustment screws of the pressure plate, the pressure regulation of the pump on the tubing was obtained by completely releasing the bottom screws and adjusting the top screws of the pressure plate so that the moving levers almost touched the plate. The pump was normally adjusted to pump 1.5 ml in 16 min. Borate buffer was pumped through the column for about 1 hr.

The linear gradient apparatus was prepared by adding 400 ml of 0.01 *M* borate buffer, pH 8.00, to the mixing flask. Another 400 ml of 0.01 *M* borate buffer, 0.2 *M* in NaCl, adjusted to pH 8.00, was placed in the other flask. A 1″ magnetic stirring bar was added to the mixing flask and the stopcock was opened to allow the buffer to just fill the delivery tube. If exact results were desired, it was necessary to compensate for the volume of this delivery tube (½ ml), the stirring bar (2 ml), and the siphon (5 ml) by using 407 ml of buffer with NaCl solution. After the soda lime tubes were attached, the siphon was filled by blowing into the flask containing the salt solution until all of the air of the siphon tube was eliminated in the mixing flask.

In a 10-ml graduated centrifuge tube, 20 to 200 mg (usually 100 mg) of 5X bovine pancreatic ribonuclease (Mann Biochemicals lot C-2185) was dissolved to 6.0 ml with borate buffer; this solution was pumped onto the column. As soon as all of the enzyme solution was pumped up the short glass tube to the Teflon tubing, the glass tube was removed and the gradient apparatus was attached in a manner so that no air entered the system.

The temperature of the column was regulated by circulating constant-temperature water ($\pm 0.1°$) through the column jacket. Fractions were collected in 16 × 100 mm culture tubes (Kimax 45048) which had been converted into square-top tubes by melting the top ½″ of the tube and inserting a 4-bladed shaper (Scientific Products G-7060) to 2½ cm, and removing it after brief cooling. These tubes, and other sized tubes treated in the same way, fitted the turntable disk and this shape top also facilitated transfer of fractions to cuvettes for assay.

Fractions were collected and assayed immediately or frozen until assays were performed. Fractions (1.5 ml) were transferred into 1-cm² Pyrocell US Silica 4-ml cells and assayed at 278 mμ in a Beckman DU spectrophotometer equipped with a Pyrocell micro attachment. By using the compartment of the cell carrier nearest the operator for the assay cuvette, and the next compartment for the blank, it was found that the assay cuvette could be easily removed from the cell carrier, without removing the cell carrier from the machine. This method made it possible to assay rapidly fractions of 1 ml or greater in the standard cuvettes and smaller volumes in semimicro cells.

RESULTS

Bovine pancreatic ribonuclease was chromatographed at 4, 12, 16, 18, 22, and 25° by the procedure described in the previous section. The absorbancy of each 1.5-ml fraction was determined at 278 mμ, and the results obtained at 4, 22, and 25° are shown in Fig. 3. At 4° an initial enzymically inactive major peak, followed by one and occasionally two minor inactive peaks, appeared. As the temperature was increased to 22° these minor peaks migrated closer to the initial major peak, and at 25° they failed to separate from the major peak.

The first active peak at 4° appeared near 150-ml effluent volume. At 22° this peak separated into two equal peaks and at 25°, into several peaks. Only the latter peak was enzymically active. At 4° the minor active peaks which just preceded the large major active fraction were not well separated. This was the best example of several separations performed at this temperature. At 22° these minor peaks were well separated, and only a slight improvement occurred at 25°. Separations at 12, 16, and 18° were inferior to those performed at higher temperatures.

The flow rate of the column was normally adjusted to 5.625 ml/hr. No effect on the elution pattern was detected when the flow rate was varied from 3.0–6.0 ml/hr. Higher flow rates of 9.0 and 12.0 ml/hr created skew-shaped peaks and poor separation.

DISCUSSION

Column chromatography of bovine pancreatic ribonuclease in an airless system and constant flow rate and temperature yielded superior results to gravity-flow column fractionation. Temperatures of 22° or higher were used to obtain satisfactory separation on carboxymethylcellulose ion-exchange resin. At lower temperatures only the initial enzymically inactive peaks were resolved better than at higher temperatures. This report demonstrates the need for accurate temperature control during analytical column chromatography.

On the other hand, column flow rates could be varied over a relatively wide range without affecting the elution pattern.

The principle of an airless column has also been used on larger diameter columns by inserting a short section of pressure tubing into the column filled with resin and buffer, and then adding a piece of capillary tubing and rubber stopper to the pressure tubing center to form a seal over the resin.

SUMMARY

A convenient method for the chromatographic separation of bovine pancreatic ribonuclease on carboxymethylcellulose in an airless, thermo-

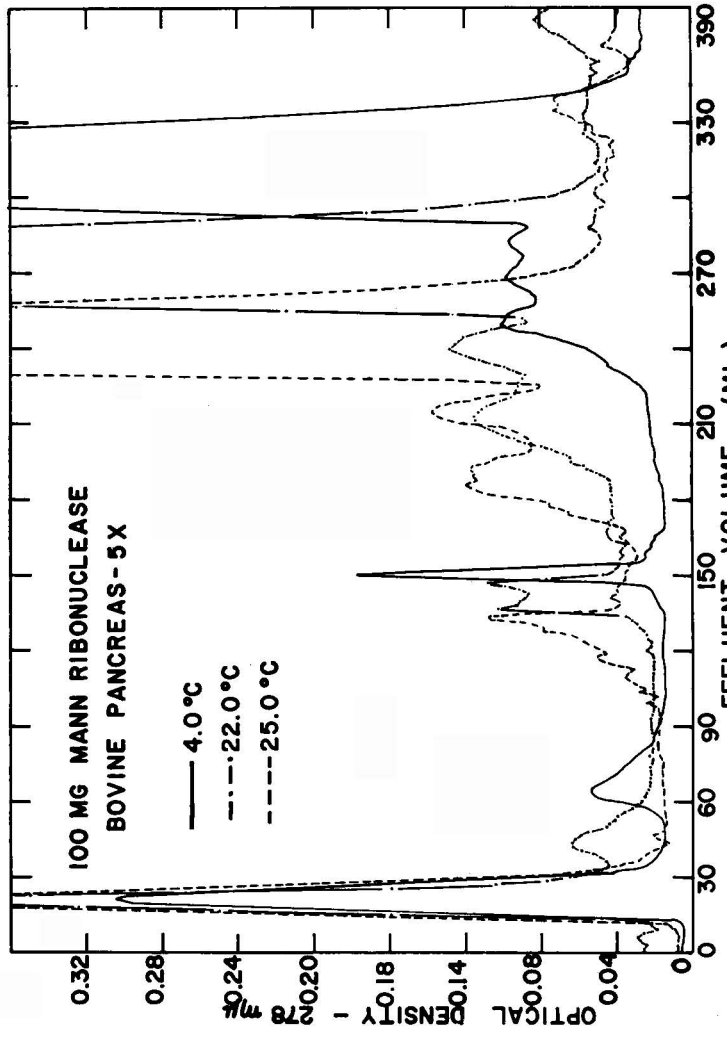

FIG. 3. Chromatography of bovine pancreatic ribonuclease; absorbancy of fractions.

stated, constant-flow column is described. The marked effects of temperature on the elution pattern are discussed.

ACKNOWLEDGMENTS

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ANALYTICAL BIOCHEMISTRY 4, 330–334 (1962)

A Modified Uronic Acid Carbazole Reaction

T. BITTER AND H. M. MUIR[1]

From the Medical Unit, St. Mary's Hospital, London, England

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The reaction of uronic acids with carbazole (1–2) is the most satisfactory method of estimating uronic acids in chromatographic fractions but requires 2 hr for the full development of color and, with certain compounds, the color is partially suppressed by salts (3–5). The color is unstable and sensitive to overheating or dilution by water (9), and impurities in the reagents or the sample interfere with it (6–8).

In keeping with the finding of Aminoff, Morgan, and Watkins (10) for the determination of hexosamines it was established by Gregory (11) that borate ion similarly increases the color yield of the uronic acid-carbazole reaction while in the orcinol-sulfuric acid reaction for hexoses it produces an immediate appearance of color (12).

The present modification using borate in concentrated sulfuric acid is both rapid and quantitative, and results in the following advantages: (*a*) further increase of sensitivity beyond that recorded by Gregory (11), (*b*) immediate appearance of color, (*c*) marked increase of stability of the color, (*d*) greater reproducibility, and (*e*) reduction of interference by chlorides and oxidants. A preliminary report of some of these results has been given (13).

EXPERIMENTAL

Reagents

(*a*) 0.025 *M* Sodium tetraborate·$10H_2O$ (analytical grade) in sulfuric acid, sp.gr. 1.84 (analytical grade).

(*b*) 0.125% Carbazole[2] in absolute ethanol or methanol (analytical grade). Stable for 12 weeks at 4°C in the dark.

(*c*) Glucuronolactone standards of 4–40 μg/ml were prepared by dilution with either glass distilled or deionized water saturated with benzoic

[1] Supported by grants from the Empire Rheumatism Council and the Pearl Insurance Company.

[2] The carbazole obtained from B. D. H. (Poole, Ds., England) did not need recrystallizing as recommended by Dische, and can be stored in the dark at 4°C for 6 months.

acid from a stock standard in water saturated with benzoic acid. Stable for 6 months at 4°C.

Procedure

5 ml of sulfuric acid reagent is placed in tubes fixed in a rack and cooled to 4°C; 1 ml of the sample or standard is carefully layered on to the acid. The tubes are closed with ground glass or Teflon stoppers and the rack shaken at first gently, then vigorously with constant cooling. At no time should the temperature of the mixture exceed room temperature. If these precautions are omitted the temperature can rise to 135°C at the interface.

The tubes are then heated for 10 min in a vigorously boiling distilled water bath and cooled to room temperature. (For extreme accuracy it is recommended that the tubes be cooled to —70°C before the sample is layered and then allowed to warm up to room temperature while being shaken.)

Carbazole reagent (0.2 ml) is then added; the tubes are shaken again, heated in the boiling bath for a further 15 min, and cooled to room temperature. The optical density (OD) is then read at 530 mμ in a 1-cm cell. The OD of the blank against sulfuric acid should be below 0.025.

For economy of sample or reagent when assaying chromatographic fractions, the following proportions can be used: 0.5 ml sample, 3.0 ml sulfuric acid reagent, and 0.1 ml carbazole reagent.

RESULTS AND DISCUSSION

The color is stable for at least 16 hr. The sensitivity of the reaction is approximately double that of the original procedure of Dische (2) for glucuronolactone and most connective tissue heteropolysaccharides tested except heparin. The low color yield of dermatan sulfate (chondroitin sulfate B) due to iduronic acid is 41% of that of chondroitin 4- and 6-sulfates in the Dische method (14). In the present modification the color yield increased to 83% of that of chondroitin 4-sulfate (see Table 1).

Solutions containing 1 μg/ml uronic acid will give a definite color but reproducibility below 4 μg/ml is poor. The optical density is a linear function of concentration between 4 and 40 μg/ml uronic acid. Using glucuronolactone ($\alpha_D^{23} + 19.4°$), the final solution containing all reagents had $E\,{}^{1\%}_{1cm}$ 530 mμ = 1160 ± 3%.

No difference was observed if the acid reagent was added to the sample or vice versa. Borate ion concentration can be varied between 0.025 and 0.1 M and carbazole concentration between 0.125 and 0.5%. Pentoses and tryptophan gave no color at concentrations of 100 μg/ml,

TABLE 1
COMPARATIVE COLOR YIELDS

Polyuronides	Amt. (μg/ml)	OD (Dische)	OD (borate)	Borate/Dische ratio
Hyaluronate (L. Light & Co.)	100	0.274	0.554	2.02
Chondroitin 4-sulfate	100	0.237	0.476	2.04
Dermatan sulfate	100	0.106	0.384	3.65
Heparan sulfate	100	0.352	0.614	1.74
Aortic heparan sulfate (15)	100	0.370	0.612	1.65
Heparin (Hoffmann-La Roche)	100	0.335	0.533	1.59
Pectin	50	0.316	0.632	2.00
Alginate (L. Light & Co.)	100	0.178	0.869	4.88

but 60 μg/ml of glucose give an intensity equivalent to 8 μg of uronic acid using 0.125% carbazole. Heating for 10 min prior to the addition of carbazole is necessary only for the polymers, as glucuronolactone gives similar color yields by the above procedure and after a single heating of 25 min in the presence of carbazole.

Contamination of chromatographic samples by dust or chlorinated tap water gave a green color found to be due to oxidants. Nitrite (5 μM) or hydrogen peroxide (0.01 μM) gives a green color with OD 0.030 at 530 mμ. In Dische's procedure the OD for these concentrations was 0.15 at 530 mμ. It is possible to account for oxidant interference by subtracting the absorption of the contaminant at 920 mμ from the absorption at 530 mμ, at which points the optical densities are almost equivalent (see spectra, Fig. 1). The present modification also suffered

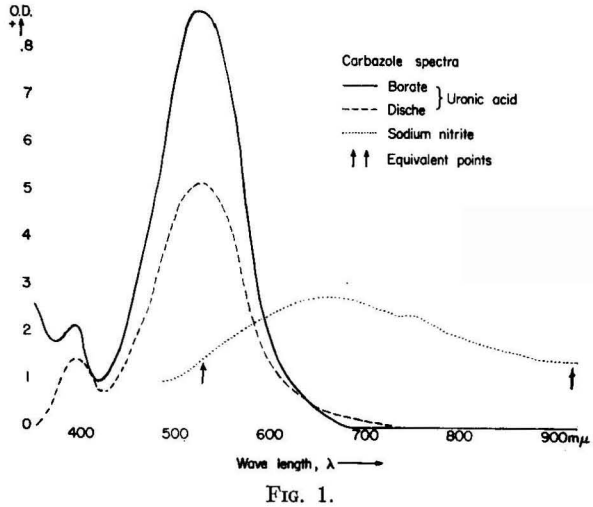

FIG. 1.

less than the original procedure from interference by impurities in reagents. At concentrations above 0.4 N, potassium, sodium, and ammonium chloride lower the color yield of heparin and its derivatives by approximately 33% in the original procedure. In the present modification, only the color yield of heparin itself is depressed. Sodium and potassium carbonate, acetate, or phosphate have no effect. It is thus possible using less than 300 μg of polysaccharide to distinguish between heparin, heparin derivatives, and the other connective tissue polyuronides by adding chloride ion above 0.4 N and comparing the color yields in both procedures (see Table 2).

TABLE 2
SALT DEPRESSION OF CARBAZOLE COLOR YIELDS

Polysaccharides	Dische	Borate
Chondroitin 4-sulfate	Nil	Nil
Chondroitin 6-sulfate	Nil	Nil
Dermatan sulfate	Nil	Nil
Hyaluronate	Nil	Nil
Heparan sulfate	33%	Nil
Aortic heparan sulfate (15)	33%	Nil
Heparin	33%	33%

SUMMARY

A modification of Dische's carbazole reaction for uronic acid in the presence of borate is described. The advantages of the procedure are:

(1) There is an approximately twofold increase of sensitivity. The OD is a linear function of concentration between 4 and 40 μg/ml.

(2) Maximum color develops immediately.

(3) The color is stable for at least 16 hr.

(4) There is greater reproducibility and reduction of interference by chloride ion and oxidants.

It has been found possible to distinguish between heparin, heparin derivatives, and other polyuronides of connective tissue by comparing the effect of chlorides on the color yield in both procedures.

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ANALYTICAL BIOCHEMISTRY 4, 335–340 (1962)

Enzymic Preparation of Labeled Unsaturated Fatty Acid Esters of Cholesterol[1]

LEON SWELL AND C. R. TREADWELL

From the Veterans Administration Center, Martinsburg, West Virginia, and the Department of Biochemistry, School of Medicine, The George Washington University, Washington, D. C.

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In the course of studies on the metabolism of cholesteryl esters it became necessary to prepare small amounts (1 mg or less) of highly labeled unsaturated fatty acid esters of cholesterol. There are several procedures available (1–3) for the chemical synthesis of the long-chain fatty acid esters of cholesterol: the reaction of cholesterol and fatty acid at high temperature (180 to 200°C) in the presence of a stream of CO_2, or the reaction (with heat) of the fatty acid chloride in the presence of pyridine. These procedures are suitable for the synthesis of the saturated fatty acid esters of cholesterol. However, the unsaturated fatty acids are sensitive to heating and the preparation of their acid chlorides offers some difficulty. Also, the chemical procedures are not readily adaptable for the preparation of very small amounts of cholesteryl esters in good yield. Previous studies (2, 4) have shown that the enzyme pancreatic cholesterol esterase is very active in catalyzing the esterification of cholesterol with the long-chain unsaturated fatty acids. With this as a basis, an enzymic procedure for the synthesis of those esters (in amounts of 1 to 75 mg) was developed which is simple and rapid, and gives esters of high purity and in good yield.

EXPERIMENTAL

Materials

1. Pancreas Powder and Enzyme Solution. Pancreas powder, steapsin (Fisher Scientific Co., Nutritional Biochemicals Corp., or any commercially available pancreas powder), is used as the source of the enzyme pancreatic cholesterol esterase. During the development of the

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procedure for the synthesis of small amounts of cholesteryl esters, utilizing 1 mg of cholesterol and 3 mg of a fatty acid, it was noted that small amounts of contaminating fatty acids were present in the synthesized cholesteryl ester. Analysis of the lipid extracted from the pancreas powder indicated that it was the major source of these extraneous fatty acids. Therefore, extractions with various solvents were carried out to determine which would most efficiently remove these fatty acids or fatty acid donors without impairing enzymic activity. The following procedure was found to give the best results. The pancreas powder (1 gm) is extracted with 10 ml of 2:1 chloroform-methanol at 0°C for 10 min and the solution centrifuged for 5 min at 1500 r.p.m. The supernatant solution is discarded. The residue is then extracted two additional times in the same manner. The washed pancreas powder is air-dried and stored at room temperature until use. The enzyme solution is prepared by adding 0.5 gm of the dried extracted pancreas powder to 5 ml of saline and shaking the mixture at room temperature for 1 hr. The mixture is then centrifuged at 1500 r.p.m. for 5 min and the supernatant solution used as the source of the enzyme. The enzyme solution is prepared fresh before each enzymic run.

2. Sodium Taurocholate. One gram of sodium taurocholate (Fisher Scientific Co.) is extracted three times with 10-ml portions of chloroform at room temperature in the same manner as described above for pancreas powder to remove small amounts of residual fatty acids, fatty acid donors, and sterols. The bile salt is then air-dried, pulverized, and stored in a vacuum desiccator.

3. Potassium Phosphate Buffer, pH 6.2 (0.154 *M*).

4. Fatty Acids. The fatty acids (98 to 99% pure), obtained from Hormel Foundation, were palmitoleic, oleic, linoleic, linolenic, and elaidic acids.

5. Cholesterol. Cholesterol-4-C^{14} (Nuclear-Chicago Corp.) was purified by silicic acid column chromatography (5) before use and the unlabeled cholesterol was purified by passage through the dibromide (6).

6. 2:1 Chloroform-Methanol. The chloroform and methanol were of reagent grade and were distilled before use.

Procedure

A. Cholesteryl Ester: 1 to 5 mg. The following solutions or multiples of these are required for the preparation of small amounts of labeled cholesteryl esters: (a) sodium taurocholate-potassium phosphate buffer solution (5.6 mg/ml), (b) cholesterol-4-C^{14}-fatty acid solution (1 mg cholesterol plus 3 mg fatty acid per 0.2 ml acetone), (c) a 10% pancreatic enzyme solution prepared as described above.

One milliliter of the sodium taurocholate-buffer solution is placed in a small incubation flask (10 ml). To this is added 0.2 ml of the cholesterol-fatty acid solution and 0.2 ml of the enzyme solution. The enzyme digest is incubated for 2 hr in an atmosphere of nitrogen with shaking. At the end of that time the digest is added to 25 ml of 2:1 chloroform-methanol; the mixture is filtered and the protein residue washed twice with 10-ml portions of 2:1 chloroform-methanol. The combined chloroform-methanol extracts are then washed with 25 ml of water. The methanol-water layer is discarded and the chloroform phase carefully taken to near dryness under nitrogen. The lipids are taken up in 50 ml of petroleum ether (b.p. 35 to 60°); the petroleum ether extract is dried for several hours over sodium sulfate. The extract is then transferred to a prepared silicic acid column (5). The synthesized cholesteryl esters are eluted with 350 ml of 1% ethyl ether in petroleum ether. If it is desired to recover the unreacted cholesterol and fatty acid these can be eluted with 350 ml of 25% ethyl ether in petroleum ether. Quantitative recovery and separation of cholesteryl esters from free cholesterol and fatty acid were checked with known C^{14}-lipids (cholesterol-4-C^{14} oleate, cholesterol-4-C^{14}, and oleic acid-1-C^{14}).

B. Cholesteryl Ester: 50 to 100 mg. Larger amounts of cholesteryl esters are prepared as follows: 75 mg of cholesterol and 225 mg of the appropriate fatty acid are added to a glass homogenizing tube, 130 × 150 mm. One milliliter of diethyl ether is added followed by 10 ml of phosphate buffer (pH 6.2) and 140 mg of sodium taurocholate, and the mixture is homogenized for 1 min with a ground-glass pestle (diameter 8 mm). The digest is preincubated at 37° for 1 hr to remove the ether; then 1 ml of the enzyme solution is added. The incubation is carried out as described above. At the end of 2 hr the digest is added to 200 ml of 2:1 chloroform-methanol; the mixture is filtered and the protein residue washed twice with 50 ml of 2:1 chloroform-methanol. The combined chloroform-methanol extracts are then washed with 150 ml of water and the cholesteryl ester isolated as described above.

Determination of Yield and Purity

The yield of cholesteryl esters, based on the original cholesterol added, was determined by both chemical (7) and C^{14} activity measurements. The C^{14} activity was determined on aliquots plated directly and also on the sterol digitonides (8) after hydrolysis of the esters. All of the C^{14} activity present in the ester fraction could be recovered as C^{14}-sterol digitonide.

The purity of the individual enzymically prepared C^{14}-cholesteryl esters was evaluated by (*a*) the observation mentioned above that

neither free cholesterol-4-C^{14} nor oleic-1-C^{14} acid was eluted in the 1% ethyl ether-petroleum ether fraction, (*b*) analyses by gas-liquid chromatography of the fatty acids esterified with cholesterol (9), (*c*) comparison of the chemically determined cholesterol content of the isolated ester with the cholesterol content calculated from C^{14} activity of the ester and the specific activity of the original cholesterol. The close agreement found indicated that the synthesized ester was entirely derived from the added cholesterol.

RESULTS AND DISCUSSION

The results are shown in Table 1. Starting with 1 mg of cholesterol-4-

TABLE 1

YIELD AND FATTY ACID COMPOSITION OF SYNTHESIZED CHOLESTERYL ESTERS

Fatty acid component[a]	Yield[b] (%) based on		Purity (%) of fatty acid	
	C^{14} activity	Chem. anal.	Orig.	Ester
	1 mg cholesterol-4-C^{14} + 3 mg fatty acid[c]			
Palmitoleic	69.2 ± 3.5	73.2 ± 3.0	98.8	94.5 ± 1.3
Oleic	74.7 ± 2.0	76.1 ± 2.5	99.1	94.8 ± 1.5
Linoleic	72.4 ± 3.6	72.5 ± 3.1	97.7	95.5 ± 1.8
Linolenic	73.7 ± 4.1	74.0 ± 3.7	98.3	93.2 ± 0.8
Elaidic	73.8 ± 4.0	75.3 ± 2.9	99.3	95.0 ± 1.0
None	3.3 ± 0.3	—	—	—
	75 mg cholesterol-4-C^{14} + 225 mg fatty acid			
Palmitoleic	57.4 ± 2.8	62.3 ± 3.5	98.8	98.8 ± 0.5
Oleic	62.3 ± 3.7	67.5 ± 2.9	99.1	98.7 ± 0.4
Linoleic	57.4 ± 4.0	58.2 ± 4.0	97.7	97.3 ± 0.5
Linolenic	52.7 ± 3.1	52.2 ± 3.7	98.3	98.0 ± 0.6
Elaidic	51.2 ± 4.0	49.2 ± 3.5	99.3	98.6 ± 0.5

[a] Represents the average of 4 to 5 digests ± standard deviation; see text for preparation of digests.

[b] The yield was calculated based on the C^{14} activity of the esterified cholesterol fraction, precipitated as the digitonide (cholesterol-4-C^{14} added per digest was 2×10^5 cpm), and also by chemical determination of the ester fraction.

[c] Very small amounts of myristic, palmitic, palmitoleic, and oleic acids were detected in the ester fraction.

C^{14} and 3 mg of fatty acid, the yield of a number of different unsaturated cholesteryl esters varied from 69 to 75% as determined by measurements of C^{14} activity of the sterol digitonides, and from 73 to 76% as evaluated by chemical determination of the ester. All of the fatty acids tested (palmitoleic, oleic, linoleic, linolenic, and elaidic) were esterified to approximately the same extent. The trans isomer of oleic acid (elaidic acid) was esterified to the same extent as were the other fatty acids. In other experiments the enzymic esterification of cholesterol with saturated fatty acids (palmitic and stearic was also checked and found to be on the order of 10 to 20%. This is in agreement with the earlier findings (2) that the saturated fatty acids are esterified by pancreatic cholesterol esterase at a much slower rate than are the unsaturated fatty acids.

A small amount of esterification (3.3%) was noted in the absence of added fatty acid; this may be attributed to the presence of a small amount of free fatty acids or fatty acid donor still present in the pancreas powder, bile salt, or both, which was not removed by the solvent extractions. The purity of the enzymically synthesized cholesteryl esters, based on the previously mentioned criteria, ranged from 93 to 95%. Determination of the fatty acid composition of the synthesized cholesteryl esters indicated (*a*) the presence of very small amounts of contaminating fatty acids (see Table 1), and (*b*) that the major peak was identical with that of the starting acid.

The synthesis of larger amounts of cholesteryl esters (starting with 75 mg of cholesterol) was also achieved in good yield (51 to 60%). The fatty acid composition of the different cholesteryl esters showed that there were virtually no contaminating fatty acids. It should be pointed out that a large amount of fatty acid (225 mg) was added to the original digest, which would greatly dilute the effect of any residual fatty acids or fatty acid donors still remaining in the pancreas powder and bile salt. Therefore, as shown in Table 1, the purity of the esters in this respect is limited only by the purity of the original starting fatty acids.

The procedure as described for the synthesis of cholesteryl esters can also be used to prepare the esters of other sterols known to be esterified by pancreatic cholesterol esterase, such as sitosterol, stigmasterol, ergosterol, and dihydrocholesterol. However, the yields, particularly of the plant sterol esters, will be less since they are esterified at a slower rate by the enzyme (10). The present procedure has been utilized to prepare cholesteryl esters with the fatty acid labeled and also with both the cholesterol and the fatty acid moieties labeled. However, the yield, based on the added C^{14}-fatty acid, will be lower because an excess of fatty acid is used in the procedure.

SUMMARY

An enzymic procedure is described which utilizes pancreatic cholesterol esterase for the synthesis of unsaturated fatty acid esters of cholesterol in amounts from 1 to 75 mg. The procedure is particularly suitable for the synthesis of very small amounts of labeled, high specific activity cholesteryl esters. A number of cholesteryl-4-C^{14} esters were obtained in good yield (from 70 to 75% starting with 1 mg of cholesterol and 50 to 60% starting with 75 mg of cholesterol) and with a degree of purity ranging from 93 to 99%.

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ANALYTICAL BIOCHEMISTRY 4, 341–345 (1962)

A Spectrophotometric Assay for Iodide Oxidation by Thyroid Peroxidase[1]

NICHOLAS M. ALEXANDER

From the Radioisotope Service, Veterans Administration Hospital, West Haven, Connecticut, and the Department of Biochemistry, Yale University, New Haven, Connecticut

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Manometry is useful, but inexpedient, for measuring thyroid peroxidase activity (1) and recently the enzyme was assayed by a colorimetric guaiacol method (2). This report describes a rapid, sensitive spectrophotometric assay with the natural substrate, iodide ion, rather than an artificial hydrogen donor.

Peroxidase catalyzes the formation of I_2 as depicted in Eq. (1) and periodide formation is then instantaneous (Eq. 2), when there is an

$$2\ KI + H_2O_2 \xrightarrow[\text{peroxidase}]{\text{iodide}} I_2 + 2\ KOH \qquad (1)$$

$$I_2 + KI \quad \leftrightarrows \quad KI_3 \qquad (2)$$

excess of iodide substrate. Nearly all of the enzymically generated I_2 is bound as I_3^- because the equilibrium constant for $(I_3^-)/(I^-)(I_2) = 714$ at 25° and the hydrolysis of I_2 or polyiodide formation is negligible (3, 4). Periodide can be spectrophotometrically determined at either of its absorption peaks 353 (5–7) or 287.5 mμ and the yield of I_3^- is directly proportional to peroxidase concentration in reaction mixtures containing approximate amounts of H_2O_2 and enzyme.

METHODS

Absorbancy of I_3^- in Buffered Solutions

Solutions containing varying amounts of periodide ion were prepared by adding aliquots (10 to 400 μl) of 0.001 *M* I_2 in ethanol to 1 ml of 0.133 *M* KI, 1.66 ml of 0.3 *M*, pH 7.4 sodium phosphate when indicated, and made up to 10 ml with water. The final concentrations of I_3^- were 90.4% of the added I_2 as calculated from the equilibrium $(I^-)(I_2)/(I_3^-)$

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$= 0.0014\,M$ (3). The optical density of each solution was determined in Beckman cuvettes with a 1-cm light path at room temperature (24–26°) using blank solutions that contained everything but iodine.

Preparation of Digitonin Solubilized Thyroid Mitochondria

Pig thyroid homogenate was prepared by homogenizing 10 gm of tissue with 40 ml of 1.1% KCl in a Waring Blendor for 1 min. The homogenate was centrifuged for 15 min at $1000 \times g$ and particles in the supernatant fluid that sedimented at $15{,}000 \times g$ in 30 min were collected and are referred to as mitochondria. One ml of 1% digitonin in $0.02\,M$, pH 7.4 phosphate was added for every 7.5 mg of mitochondrial protein and the suspension was mixed with the aid of a Potter-Elvehjem homogenizer. After 20 min at 3° with occasional stirring, the suspension was centrifuged at $40{,}000 \times g$ for 20 min and the brown supernatant solution was collected. It contained 4 mg protein/ml and could be stored several weeks in the freezer without loss of peroxidase activity.

One per cent digitonin in buffer was brought into solution with the aid of heat and cooled before use. Protein determinations were carried out by the method of Lowry *et al.* (8).

Procedure for the Peroxidase Assay

The assay for iodide oxidation by peroxidase is performed in the following manner (cf. 9): The reaction mixture contains 40 μmoles KI, 150 μmoles pH 7.4, $0.3\,M$ sodium phosphate, and an appropriate amount of enzyme in a total volume of 3 ml in a 1-cm Beckman cuvette. The optical density at 287.5 or 353 mμ is set at zero and 10 μl of $0.08\,M$ H_2O_2 added to the end of a stirring rod is dipped into the solution with a swirling motion. Optical density readings are recorded every 10 or 15 sec. Correction for the spontaneous oxidation of iodide by H_2O_2, which amounts to about 2 mμmoles I_3^-/ml in 60 sec, is obtained by performing the assay in the absence of enzyme. Periodide production in mμmoles/ml is calculated by multiplying the optical density by 10^6 and dividing by the molar extinction coefficient.

RESULTS AND DISCUSSION

Molar Extinction Coefficients for I_3^-

It is clear from Fig. 1 that I_3^- absorption obeys Beer's law at either 287.5 or 353 mμ and that pH 7.4, $0.05\,M$ phosphate buffer, unlike strong acid (6), has no effect on the absorbancy of I^-_3. Free I_2 ($<10^{-4}\,M$) does not absorb at these wavelengths and 4% ethanol has no effect on I_3^- absorbancy. The molar extinction coefficients for I_3^- calculated from the

thirty-two measurements in Fig. 1 are 34,690 ($\sigma = \pm 1461$) at 287.5 mμ and 22,900 ($\sigma = \pm 855$) at 353 mμ. These values agree quite well (within 2 to 4%) with the data of others (5–7) although one paper (10) reports

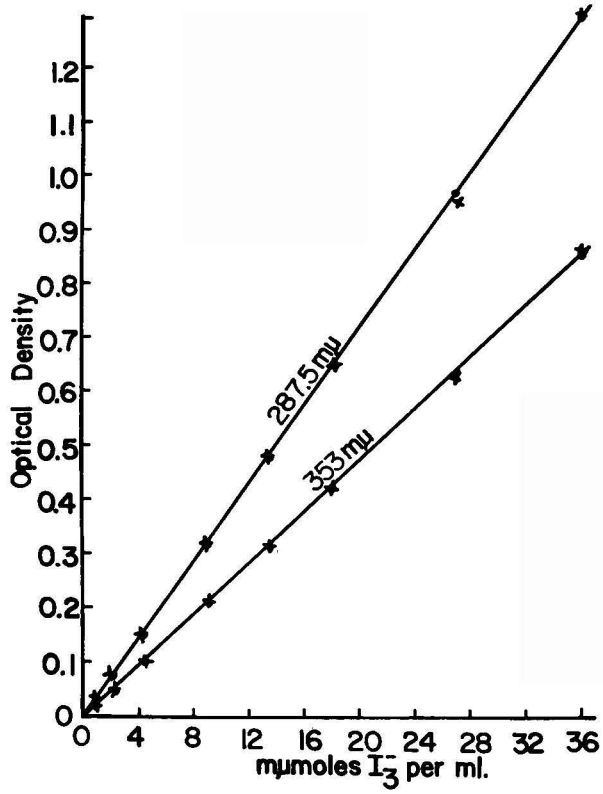

FIG. 1. Absorption of varying concentrations of periodide ion at 353 and 287.5 mμ obtained in 0.05 *M*, pH 7.4 sodium phosphate (solid circles) and in the absence of buffer (×).

extinctions for I_3^- that are 15% higher. In any event, I_3^- can be determined with about a 4% coefficient of variation.

Kinetics of Pig Thyroid Peroxidase Activity

In reaction mixtures containing appropriate concentrations of iodide, H_2O_2, and enzyme, I_3^- formation is zero order for a convenient interval of time and the formation of I_3^- is proportional to the concentration of iodide peroxidase as shown in Fig. 2 [consult Chance (11) for a general discussion of the desired conditions for peroxidase assays]. For the first 40 sec the rate of I_3^- formation is linear and dependent on enzyme con-

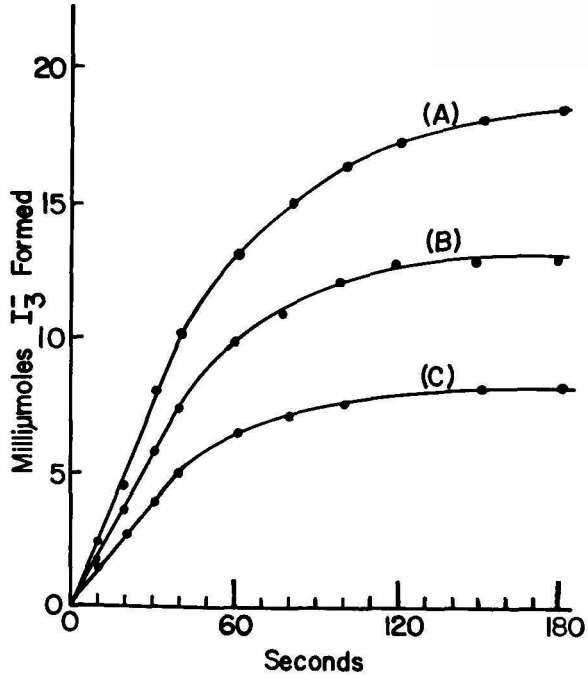

FIG. 2. Rate of periodide formation with varying amounts of iodide peroxidase. Curves (A), (B), and (C) were obtained with digitonin solubilized pig thyroid mitochondria containing 200, 150, and 100 μg of protein, respectively. The points were from duplicate determinations at 287.5 mμ and at room temperature (25°).

centration as seen with three different amounts of a digitonin extract from pig thyroid mitochondria. The rates decrease after 40 sec and curve (C), which contains lesser amounts of enzyme, reaches a plateau sooner than (A) or (B), probably because iodide peroxidase is inactivated by H_2O_2, a phenomenon typical of other peroxidases (11). Identical results are obtained at 353 mμ. The amount of I_2 actually formed is 1.1 times greater because 9.5% of the generated I_2 is not bound as I_3. Obvious alternatives are to measure the time required to obtain an arbitrarily chosen optical density or to use a recorder attached to the spectrophotometer. The assay is performed at 353 mμ if materials that strongly absorb in the ultraviolet are present in the reaction mixture and, although the enzyme preparation absorbs at 287.5 mμ, it does not interfere with the assay at this wavelength because the optical density can be set at zero prior to adding the H_2O_2. All enzymic activity is destroyed by immersing the enzyme in a boiling water bath for 5 min. When supplied with H_2O_2 (12, 13) digitonin solubilized mitochondria incorporate I^{131}-

iodide into tyrosine in amounts that correlate with the peroxidase activity assessed by this spectrophotometric method. Whole thyroid extracts cannot be assayed by this method because they contain considerable quantities of catalase and materials that react with I_2. Catalase does not oxidize iodide and inhibits thyroid peroxidase by decomposing H_2O_2. However, crude lacrimal and submaxillary gland extracts that readily peroxidize iodide can be assayed by this method.

Consistent with an earlier investigation (12), hematin increases the peroxidase activity of dialyzed (against 0.02 *M*, pH 7.4 phosphate) digitonin extracts by 40%. Other fractions that are obtained by treating the dialyzed extract with butanol show an absolute requirement for hematin. Detailed studies on hematin activation of various thyroid preparations are being carried out and will be reported later.

This method offers the obvious advantages of speed and sensitivity. It is at least 1000 times more sensitive than the standard manometric assay and can be performed in 2 min. For these reasons, it should be adequately suited for studies directed toward the purification and isolation of thyroid peroxidase.

SUMMARY

A rapid, sensitive spectrophotometric assay for iodide oxidation by thyroid peroxidase is described. The method depends upon the measurement of periodide at either of its absorption peaks, 353 mμ or 287.5 mμ, and is adequately suited for enzyme purification studies.

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ANALYTICAL BIOCHEMISTRY 4, 346–347 (1962)

SHORT COMMUNICATION

Reducing Power by the Dinitrosalicylic Acid Method

The dinitrosalicylic acid method for measuring reducing power as described by Bernfeld (1) overestimates the molar concentration of disaccharides when glucose is used as the standard reducing substance. An attempt was made to find conditions for the dinitrosalicylic acid method under which glucose and various disaccharides would exhibit equimolar absorbancies; this was achieved by modifying reagent concentration and temperature of reaction.

The dinitrosalicylic acid reagent was prepared as described by Bernfeld (1) except that 0.5 gm dinitrosalicylic acid/100 ml was used (0.5 gm 3,5-dinitrosalicylic acid, 20 ml 2 *N* NaOH, and 30 gm Rochelle salts in a total volume of 100 ml). One milliliter of solution to be assayed was added directly to 2 ml of color reagent. The sample was heated for 5 min at 75°C and cooled to room temperature in a cold water bath. Nine milliliters of distilled water was added and the absorbance measured at 500 mμ in a B & L Spectronic 20.

The effect of heating temperature on the molar absorbancies of glucose and cellobiose at 500 mμ with the samples contained in 19-mm diameter test tubes is as follows:

	Molar Absorbancy at 500 Mμ $\times 10^{-5}$					
Sugar	67°C	70°C	75°C	80°C	85°C	90°C
Glucose	1.57	2.23	2.62	2.92	3.36	3.56
Cellobiose	1.04	1.92	2.63	3.62	4.43	4.98

The absorbancies represent the slopes of curves prepared using five levels of the respective sugars. The values at 75°C are the average of the slopes of four curves.

Lactose, maltose, and gentiobiose also showed molar absorbancies equal to glucose when assayed by the procedure at 75°C. In all cases approximately 3.3×10^{-7} mole of sugar was consumed in side reactions before absorbance above the reagents alone was observed.

The principal disadvantage which may be encountered with this modification is the high initial absorbancy of the color reagents at 500 mμ. The absorbances vs. water were 0.5 in a B & L Spectronic 20 and 1.1 in a Coleman Jr. Model 6D. If the Coleman is to be used the

initial absorbancy must be lowered. This can be achieved by decreasing the amount of dinitrosalicylic acid to 0.25 gm/100 ml. However, the heating temperature must be increased to 76°C to maintain equimolar absorbancies for glucose and the disaccharides (2.4×10^{-5} under these conditions). It was observed that the molar absorbancies decreased with dinitrosalicylic acid concentration even though the response to sugar concentration was linear. The absorbance of the reagents may also be lowered by reading at higher wavelengths. However there is a concurrent loss of response to sugar. The molar absorbancies of the sugars at 535 mμ are approximately half their absorbancies at 500 mμ.

Miller and co-workers (2) reported that the molar concentrations of cellodextrins could be measured if they were heated for 55 min at 50°C in a "Modified Meyers Dinitrosalicylic Acid Reagent." Attempts to duplicate the results of Miller and co-workers with respect to glucose and cellobiose, using their conditions, were unsuccessful. A relative molar absorbancy of 0.83 for cellobiose (glucose = 1.00) was obtained with a 55-min heating at 50°C rather than the 1.11 for cellobiose reported in their Table 2 (2). With a 70-min heating at 50°C a relative molar absorbancy of 0.91 was obtained for cellobiose. A value of greater than one would be expected from the results in their Fig. 3.

In a private communication, Dr. Miller reported to us that repetition of their original experiments with cellobiose and glucose yielded in his hands a relative molar absorbancy for cellobiose of 0.88 after a 55-min heating and 1.00 after 70 min. The discrepancy between these values and the values reported previously by Miller and co-workers is unexplained.

ACKNOWLEDGMENTS

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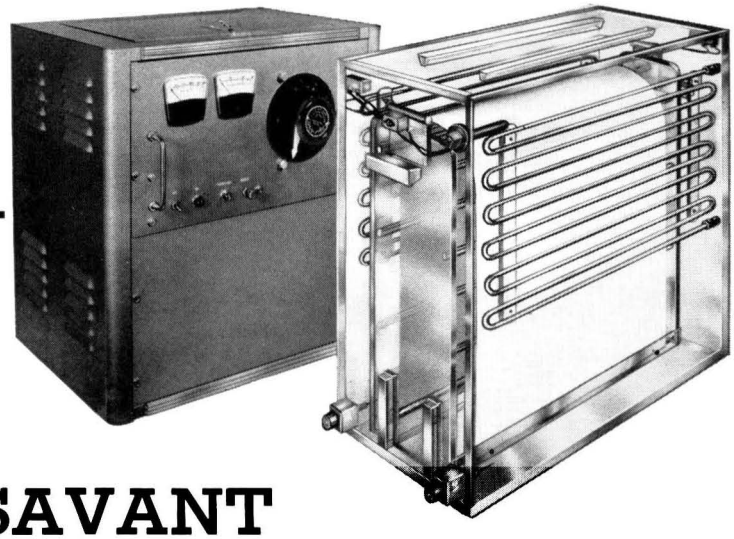
Department of Agricultural Biochemistry and Nutrition
West Virginia University
Morgantown, West Virginia
Received April 12, 1962

WAYNE W. LUCHSINGER
ROBERT A. CORNESKY



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ANALYTICAL BIOCHEMISTRY 4, 349–357 (1962)

Fluorescence Spectra of Some Simple Coumarins

DONALD G. CROSBY[1] AND ROBERT V. BERTHOLD

From the Research Department, Union Carbide Chemicals Company, South Charleston, West Virginia

Received November 9, 1961

INTRODUCTION

Perhaps the most obvious physical property of most natural coumarins is the brilliant fluorescence they display under ultraviolet illumination or even in daylight. This feature frequently has been employed for detection of coumarins on paper chromatograms (1) and for the quantitative estimation of compounds such as the pharmaceutical agent esculin (2), and, recently, scopoletin and its glycoside scopolin (3).

Although Goodwin and Kavanagh (4) described the use of pH-fluorescence curves as an aid in identification of a variety of coumarin derivatives, employment of fluorescence spectra for this purpose has received little attention. Wheelock (5) has published limited data for several coumarins, and Mattoo (6) has reported spectral properties of others.

The fact that a number of the most common coumarins to occur in nature exhibit remarkably similar ultraviolet absorption spectra (7), color of fluorescence, and chromatographic properties makes desirable still another type of data characteristic of their individual identities. The need for a simple method by which specific members of this series could be distinguished from their close homologs resulted in the investigation described here.

EXPERIMENTAL

Umbelliferone was prepared by reaction of resorcinol and malic acid in concentrated sulfuric acid (8). Scopoletin was prepared from scopoletin-4-carboxylic acid obtained through reaction of 2,4-dihydroxyanisole and sodium diethyl oxalacetate (9). Esculetin was obtained by reaction of phenenyl triacetate with malic acid in concentrated sulfuric acid (10) or by acid hydrolysis of commercial esculin; methylation gave scoparone (11), while reaction with methylene iodide gave ayapin (12). Iso-

[1] Present address: Pesticide Residue Research Laboratory, University of California, Davis, California.

scopoletin was derived from vanillin by a series of reactions (13), and the product was compared with that obtained by methylation and hydrolysis of the glycoside esculin. All melting points were measured on a Vanderkamp block and are corrected.

Chromatography was carried out in the ascending direction on Whatman No. 3 paper with freshly prepared solvents in commercial Chromatocabs. Fluorescent bands were detected by brief exposure to weak ultraviolet light, but all other manipulations were conducted in subdued light or darkness due to the frequently observed degeneration of chromatogram fluorescence in light.

Measurements of fluorescence and excitation maxima were made first with a Farrand Series 123 spectrofluorophotometer equipped with a high intensity 150-watt Hanovia xenon arc as source and a 1P28 photomultiplier tube as detector. The solutions under examination were held in 10×20 mm silica cells. Fluorescence intensities were recorded in terms of μamp with an RCA WV-84B microammeter and converted later to "quinine units."

Data obtained from the Farrand instrument at an excitation wavelength of 365 mμ were checked with both a Beckman fluorescence attachment (No. 22850) to a Model DK-2 ultraviolet spectrophotometer and the fluorescence attachment to the Cary Model 14 spectrophotometer. Ultraviolet spectra were measured in methanol with the Cary instrument.

Fluorescence measurements were made at a number of concentrations in each experimental solvent in the range of 10^{-4} to $10^{-7}\,M$ or below for preparation of concentration-intensity curves. Solvent methanol was an "AAA" grade distilled shortly before use. Other media were $0.1\,N$ sulfuric acid and $0.1\,M$ carbonate-bicarbonate buffer at pH 10.0. In no case did blanks show any fluorescence at the highest sensitivity level of the instruments.

C.P. quinine sulfate dihydrate in $0.1\,N$ aqueous sulfuric acid was employed as a standard. For purposes of comparison, a "quinine unit" (Q.U.) was defined as the fluorescence of the solution at a concentration of 0.1 μg/ml when irradiated at 365 mμ and detected at 445 mμ.

RESULTS AND DISCUSSION

Coumarins are becoming recognized as common and widespread minor constituents of plants. In particular, the simple hydroxylated coumarins and their alkyl ethers have received increasing attention; scopoletin, for example, has been implicated in a wide variety of plant functions.

The brilliant fluorescence of most of these compounds has been used frequently for their detection on paper chromatograms. Although there

are many references in the literature to the separation and identification of coumarins by virtue of their chromatographic characteristics, it was the confusion caused by subtle structural differences not easily detectable through paper chromatography which led to the present investigation.

Fluorescence spectra present yet another means by which many compounds may be distinguished from closely related substances without the necessity of isolation. Because the method is not directly useful on mixtures of unknown fluorescing materials, chromatographic techniques are important for separation of fluorescent components of extracts of plant and animal tissue. Table 1 lists R_f values for the coumarins used in this study; the solvents shown have been found to be the most useful through long experience.

The fluorescent chromatogram areas generally are best extracted with methanol. Fluorescence spectra may be measured directly in this solution or in other solvents after evaporation of the methanol. Only limited success was achieved through attempts to measure spectra of the compounds still adsorbed on the paper. The spectral curves of simple coumarins uniformly exhibited a single, almost symmetrical peak at 420–450 mμ corresponding to the blue-green color noted upon ultraviolet irradiation of each of the compounds.

The data presented in the tables represent corrected fluorescence maxima. Factors which might be expected to influence the location and degree of maxima (14), such as absorption overlap, recorder-pen response, variation of detector sensitivity, scattered light, monochromator calibration, solvent fluorescence, and sample purity have been taken into consideration. However, difficulty in assigning "maximum" values on the broad, almost flat fluorescence peaks and in interpolation of recorder readings and monochromator settings is responsible for further error not so readily corrected.

Fluorescence maxima measured with the Cary Model 14 spectrophotometer were within ± 3 mμ of those obtained with the Farrand instrument; this is about the limit of accuracy to be expected (15). The data obtained with the Beckman DK-2 were about 7 mμ above the Farrand values, due principally to recognized but generally unreproducible errors in the instrument's recording system. For determination of fluorescence spectra, the Cary appears to be superior to the others, while the Farrand is more versatile for quantitative fluorescence measurements and permits the recording of excitation spectra.

Quinine sulfate in 0.1 N sulfuric acid was used for a fluorescence standard as frequently reported in the past. The results of our measurements on the three instruments agreed with those of Sprince and Rowley

TABLE 1
PHYSICAL PROPERTIES OF COUMARINS

Coumarin	Common name	M.p. (°C)	R_f[a]			UV max., mμ (log ε)[b]
			A	B	C	
7-Hydroxy-	Umbelliferone	226	0.62	0.66	0.54	216(4.06), 324(4.09)
6,7-Dihydroxy-	Esculetin	270 (dec.)	—	0.53	0.38	229(4.03), 256(3.56), 262(3.53), 299(3.62), 348(3.96)
6-Methoxy-7-hydroxy-	Scopoletin	204–205	0.52	0.55	0.42	228(4.18), 252(3.71), 259(3.66), 297(3.69), 344(4.07)
6-Hydroxy-7-methoxy	Isoscopoletin	184	0.38	0.62	0.47	229(4.20), 253(3.74), 259(3.66), 296(3.77), 346(4.00)
6,7-Dimethoxy-	Scoparone	138	0.40	0.67	0.53	230(4.23), 251(3.72), 258(3.65), 294(3.71), 342(4.057)
6,7-Methylenedioxy-	Ayapin	223–224	0.52	0.72	0.50	233(4.26), 252(3.82), 258(3.77), 294(3.64), 344(4.11)

[a] Solvent A, water. Solvent B, 10% aqueous acetic acid. Solvent C, 10% aqueous *tert*-butyl alcohol. Ascending chromatograms.
[b] In methanol.

(16); the excitation maximum of quinine under these conditions lies at 350 mμ and the fluorescence maximum at 450 mμ. Although the recorded intensity of fluorescence for a fixed concentration of experimental compound varied considerably from day to day, the ratio of this value to the fluorescence of a standard quinine concentration measured concurrently generally remained almost constant. Consequently, our data are presented in terms of a "quinine unit," the fluorescent intensity of a solution of 0.1 μg/ml quinine sulfate in 0.1 *N* sulfuric acid when excited at 365 mμ (the "mercury green line" generally used in fixed wavelength excitation sources) and detected at 450 mμ, the quinine fluorescence maximum. In preparing the standard quinine solution, it must be recognized that severe concentration quenching occurs above about 10 μg/ml; this factor, of course, does not affect the use of "quinine units" as a measure of any degree of fluorescence.

Fluorescence spectral data are presented in Tables 2, 3, and 4 for

TABLE 2
FLUORESCENCE SPECTRA OF COUMARINS IN METHANOL

Coumarin	λ_{max} (mμ)		Fluor. intensity (Q.U.)[a]	
	λ_{excit}	λ_{fluor}	2.0-mm slit	0.5-mm slit
Umbelliferone	330	440[b]	4.0	14
	365	440	0.7	6.0
Esculetin	350	440	1.5	37
	365	440	1.4	29
Scopoletin	350	420	10	42
	365	420	8.0	40
Isoscopoletin	350	440	1.0	11
	365	440	0.8	10
Scoparone	350	420	4.7	18
	365	420	3.3	16
Ayapin	350	415	5.0	20
	365	415	3.7	17

[a] Coumarin concentration, 8×10^{-7} *M*.
[b] Shoulder.

coumarin solutions in methanol, dilute sulfuric acid, and dilute alkali, respectively. Comparison of the excitation wavelengths in methanol (Table 2) with the wavelength of maximum ultraviolet absorption in the same solvent (Table 1) indicates the close relationship of these values and the accuracy of Farrand spectrofluorometer readings. No fluorescence excitation was observed at wavelengths corresponding to other ultraviolet absorption maxima.

In addition to irradiation of the fluorescent solutions at the wave-

TABLE 3
FLUORESCENCE SPECTRA OF COUMARINS IN 0.1 *N* SULFURIC ACID

Coumarin	λ_{max} (mμ)		Fluor. intensity (Q.U.)[a]	
	λ_{exeit}	λ_{fluor}	2.0-mm slit	0.5-mm slit
Umbelliferone	325	470	6.4	10
	365	470	0.75	1.4
Esculetin	350	455	0.05	0.02
	365	455	0.05	0.02
Scopoletin	350	430	9.3	32
	365	430	6.7	23
Isoscopoletin	350	430	0.10	<0.1
	365	430	0.10	<0.1
Scoparone	350	425	9.3	29
	365	425	6.7	22
Ayapin	350	430	12	36
	365	430	9.4	32

[a] Coumarin concentration, 8×10^{-7} *M*.

TABLE 4
FLUORESCENCE SPECTRA OF COUMARINS IN BUFFER AT pH 10.0

Coumarin	λ_{max} (mμ)		Fluor. intensity (Q.U.)[a]	
	λ_{exeit}	λ_{fluor}	2.0-mm slit	0.5-mm slit
Umbelliferone	370	450	35	62
	365	450	35	62
Esculetin	390	465	6.9	18
	365	465	5.2	13
Scopoletin	390	460	39	160
	365	460	26	100
Isoscopoletin	370	460	0.13	<0.1
	365	460	0.13	<0.1
Scoparone	350	430	10	28
	365	430	7.1	20
Ayapin	350	430	11	29
	365	430	8.8	23

[a] Coumarin concentration, 8×10^{-7} *M*.

length of maximum excitation, corresponding measurements were made with the 365 mμ light most common to fixed wavelength instruments. Although, of course, the wavelength of maximum fluorescence is unaltered, the consequent loss in intensity is apparent from the tables.

The tabulated data represent a coumarin concentration of 8×10^{-7} *M*, a convenient point on the linear portion of the concentration-fluorescence curves. Figure 1 shows that meaningful measurements in the coumarin

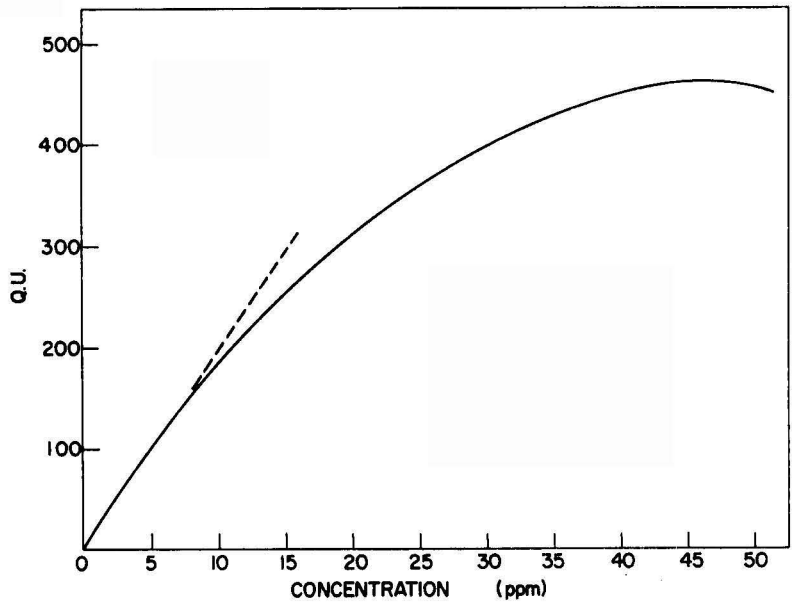

FIG. 1. Relationship of scopoletin concentration and fluorescence.

series may be made down to very low concentrations, while measurements at concentrations above about $10^{-5}M$ are distorted because of quenching.

The fluorescence intensities recorded in the tables generally are accurate to $\pm 5\%$. Values were obtained with both a 2.0-mm slit width (30-mμ band pass) and a 0.5-mm slit width (5-mμ band pass). The wider slit, of course, permits greater sensitivity; the fluorescence of a 0.1-ppm solution of quinine sulfate gave a galvanometer reading of 0.075 μamp, while the reading with a 0.5-mm slit was only 0.010 μamp. The relatively greater quinine unit values shown for the smaller slit therefore are a reflection of the slopes of the concentration-fluorescence curves for quinine and the coumarins at identical slit widths rather than the actual intensities. However, most of the compounds investigated were at least as fluorescent as quinine sulfate on a molar basis under identical conditions, and the particularly high values for scopoletin undoubtedly reveal it to be one of the most intensely fluorescent substances known.

The changes of fluorescence intensity when the test solutions were allowed to stand for 2 hr at room temperature are summarized in Table 5. The data represent averages of four to six independent determinations.

TABLE 5
CHANGE OF COUMARIN FLUORESCENCE WITH TIME (2 HR)

Coumarin	Intensity (% of initial value)[a]		
	Methanol	0.1 *N* H_2SO_4	Buffer pH 10.0
Umbelliferone	94 ± 6	98 ± 4	100 ± 3
Esculetin	90 ± 3	85 ± 2	83 ± 6
Scopoletin	90 ± 4	104 ± 4	98 ± 2
Isocopoletin	94 ± 6	94 ± 8	61 ± 4
Scoparone	61 ± 8	72 ± 2	66 ± 2
Ayapin	82 ± 7	96 ± 4	116 ± 3

[a] Change in Q.U. intensity at λ_{max} for both excitation and fluorescent energy.

The solutions were held in ordinary laboratory light in stoppered volumetric flasks during this period, and no special precautions were taken with regard to exposure to light and air. Although the methanol solutions gave rise to the most erratic data observed, it appears that most of the coumarins were fairly stable under these conditions. Likewise, the fluorescence of sulfuric acid solutions generally suffered little variation, but an alkaline medium led to several distinct changes. The general instability of the nonhydroxylic coumarins is surprising, and the significant increase in the fluorescence of ayapin in buffer, repeated on several occasions, remains unexplained.

Several brief discussions of the effect of coumarin structure on fluorescence have appeared in print (4, 6, 7). It is to be concluded from the present investigation that most of the coumarins studied, and the broad classes which each represents, may be characterized by their fluorescence spectra in one or more solvents. The closely related scoparone and ayapin may be differentiated by the change of fluorescent intensity with time, although this aspect requires further study. Undoubtedly, these methods will be extended and found useful for characterization of many types of natural coumarins.

SUMMARY

The fluorescence spectra of six representative coumarins containing hydroxyl and/or alkoxyl substituents have been determined and compared on several commercial instruments. Measurements in methanol, dilute sulfuric acid, and dilute alkaline buffer permitted characterization of coumarin type, and observation of the change of fluorescent intensity with time allowed further refinement. Application of the method to paper chromatogram eluates, combined with an inherently high degree of sensitivity, should make it especially useful in the investigation of natural products.

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Determination of Reducing Sugars by Oxidation in Alkaline Ferricyanide Solution[1]

THEODORE E. FRIEDEMANN, CHARLES W. WEBER, AND NORMAN F. WITT

From the Department of Chemistry, University of Colorado, Boulder, Colorado

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Of the many methods for the determination of reducing sugars, those depending upon oxidation by cupric-tartrate complex in alkaline solution are the best known and most widely used at present. Methods which are based upon oxidation by ferricyanide in alkaline solution are not among the officially accepted methods, and they receive little attention in the majority of standard handbooks of sugar and food analysis. This is somewhat surprising, since both categories of methods have been known for more than a century (1, 2) and, even more so, since the ferricyanide oxidation has several advantages over the cupric-tartrate oxidations. First, the oxidizing reagent contains only inorganic salts, the single reagent is simply and quickly prepared, it has a uniformly standard concentration, and it is stable when protected from light. Second, the reduction of ferricyanide is approximately proportional to the sugar content over a wide range (3–7), thus eliminating elaborate tables of conversion factors. Third, the resulting ferrocyanide is not as subject to air oxidation as is Cu_2O and, therefore, precautions against reoxidation during heating and after cooling need not be so rigorously observed (3). Finally, the residual ferricyanide, or the ferrocyanide, may be determined by a variety of oxidimetric, iodometric, photometric, gasometric, or potentiometric procedures (8). The extent of oxidation of sugars, and therefore the sensitivity, is about the same as that which results from the cupric-tartrate complex under similar conditions of alkalinity and heating. Both reagents oxidize amino acids, aldehydes, and other fairly readily oxidizable substances. However, ferricyanide does so to a greater extent than the copper reagents. This interfering effect

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in both reagents, which may be considerable with hydrolyzed or autolyzed plant and animal tissues, may be minimized or eliminated entirely by proper choice of precipitating reagents.

The preference for cupric over ferricyanide oxidation methods is due, in a large measure, to the detailed studies by many authors during the past century of the optimal conditions for the oxidation of sugars by alkaline cupric-tartrate solutions. This has resulted in a general acceptance of such reagents as the century-old Fehling solution (1, 9) and the more recent Shaffer-Somogyi reagent (10). Similar detailed studies have not been made of the ferricyanide method.

There is considerable difference of opinion concerning the optimal concentrations of carbonates for oxidation in the semimicro determination of sugars. Thus, Hagedorn and Jensen (11), Folin (12), Whitmoyer (13), Miller and Van Slyke (14), Hanes (3), Widdowson (4), Schales and Schales (6), Snell and Snell (15), and many others recommend widely differing concentrations of Na_2CO_3. A few authors recommend the use of both carbonate and bicarbonate. Thus, Van Slyke and Hawkins (16) use 7.5% each of K_2CO_3 and $KHCO_3$, and Shaffer and Williams (17) use 5% of Na_2CO_3 and 1% of $NaHCO_3$. In the majority of these methods the oxidation is carried out in a boiling water bath. Since the boiling point varies with altitude, different degrees of oxidation may be obtained when procedures that have been satisfactory at sea level are used in regions of higher altitude. In Boulder only about one-half as great reduction is obtained as that which occurs at sea level during the prescribed 15 min of heating with the Shaffer-Somogyi (10) reagent. Similar results are obtained with a ferricyanide solution of the same concentration of carbonates.

The procedures of the published ferricyanide methods vary widely. As a result, the experimental data upon which they are based, although applicable to a particular method, are not suitable for prediction of the degree of ferricyanide reduction under a wider range of conditions. Standardization of technique, and uniformity and predictability of results, have not been achieved as in the case of copper-tartrate methods.

The principal purpose of this paper is to present data on the effect of the concentration of ferricyanide and alkaline carbonates, either of Na_2CO_3 alone or in combination with $NaHCO_3$, on the reduction of ferricyanide by glucose at the boiling point of water in Boulder (about 95°C, at 5300 ft altitude), and at a standard temperature of 80°C. At the latter temperature, reproducible results should be obtained within ±2% of those of the authors', regardless of the altitude up to 19,000 ft. A suggested standard procedure, applicable from 0.3 to 13 mg of reducing sugar, is described in detail. The sensitivity may be increased

or decreased by altering the size of sample and the concentrations of ferricyanide, and from the data given in this paper the approximate degree of reduction may be calculated.

METHOD

Reagents

1. *Ferricyanide reagent,* 0.04 *M*. Dissolve 50 gm anhydrous Na_2CO_3 and 13.20 gm C.P. potassium ferricyanide in about 500 ml water and dilute to 1000 ml. Protect the reagent from light by storing it in a bottle which is completely covered with black paint or with heavy aluminum foil. Although the reagent thus stored may be used indefinitely, fresh preparation after six months is recommended. As a check on the purity of reagents 5 ml unheated reagent, when treated with KI and the zinc reagent listed below, should yield iodine equivalent to 20 ml 0.01 *N* thiosulfate.

2. *Potassium iodide solution,* 15 gm in 100 ml of water. Prepare frequently, protect from strong light, and keep cold when not in use.

3. *Zinc sulfate-acetic acid reagent.* Dissolve 60 ml glacial acetic acid and 60 gm $ZnSO_4 \cdot 7H_2O$ in water and dilute to approximately 1000 ml.

4. *Starch indicator.* Suspend 10 gm soluble starch in cold water; add the suspension to 500–700 ml hot water. Boil for 1–2 min, dilute to about 1000 ml with hot water, and cool. Prepare frequently; keep cold when not in use.

5. *Standard thiosulfate solutions,* 0.1 and 0.01, 0.02, or 0.03 *N*. Dissolve 50 gm C.P. $Na_2S_2O_3 \cdot 5H_2O$ in about 1000 ml water; add 4 ml approximately 1 *N* NaOH and dilute to 2000 ml. At intervals, standardize the approximately 0.1 *N* solution against 0.1 *N* KIO_3 solution. Larger volumes, 12–18 liters, may be prepared. After the first week, the normality remains virtually constant, decreasing less than 0.0001 *N* per month. Prepare 0.01–0.03 *N* thiosulfate, preferably on each day of use.

6. *Glucose standard solution.* Standard Sample, National Bureau of Standards, carefully dried *in vacuo* at 25–40°C, is recommended.

Procedure

Transfer 5 ml neutral, carbonate-free sample solution into thin-walled test tubes of 29 × 200 mm dimensions. Add 5, 10, or 15 ml ferricyanide reagent according to the reducing sugar as follows in the measured sample: 1–4.5, 2–7, or 3–13 mg, respectively. Mix immediately by gently rotating the contents; cover the tubes with small beakers or, preferably, with large glass bulbs. The blank tubes contain 5 ml

water. Place the rack, containing not more than 12 tubes, in an electrically heated, mechanically stirred water bath maintained at 80 ± 0.5°C. The bath should be large enough to hold several racks at one time if necessary. The water should cover the tubes to a depth of 4 to 5 in.

After exactly 30 min of heating at 80°C, place the rack in a cooling bath of running water, preferably at 20–25°C. Determine the residual ferricyanide either photometrically or titrimetrically. The photometric method is preferred.

Photometric method. Add about 50 ml water to each tube and quantitatively transfer the contents to volumetric flasks as follows: for 5 ml ferricyanide reagent used, dilute to 500 ml (about $0.0004\,M$); for 10 and 15 ml, dilute to 1000 ml (about 0.0004 and $0.0006\,M$). The Beckman DU spectrophotometer, using 1-cm square Pyrex cuvettes, is preferred. Determine the absorbance at 418 mμ wavelength.

Iodometric method. To each tube add in turn 1 ml KI solution and 5 ml zinc sulfate reagent for each 5 ml of ferricyanide reagent used. Mix by gently rotating the contents after each addition. In order to prevent loss of iodine, the tubes should be covered immediately after adding the zinc reagent, and they should remain covered during mixing and until ready for the thiosulfate titration. Let stand 5 min or more, during which time the contents are mixed twice again. Add dilute standard thiosulfate (0.01, 0.02, or $0.03\,N$ thiosulfate, respectively, for 5, 10, or 15 ml ferricyanide reagent used) until the yellow color of iodine is almost gone. Add the first few milliliters of thiosulfate around the sides of the tube in order to absorb any iodine vapors. Mix vigorously by rotation throughout the titration. Now add about 0.5 ml starch indicator and wash down the walls with a fine stream of water. Continue the titration drop-by-drop, mixing after each addition, until the color is pure white.

Comments on Procedure

Size of tube and volume of sample and reaction mixture. Within quite wide limits, these do not significantly affect the degree of reduction, provided that the heating is rapid and uniform and, further, that the relation of ferricyanide and Na_2CO_3 concentrations per unit volume is maintained under the various conditions. Thus, within the limits of experimental error, identical degrees of reduction are obtained per mole of glucose (in accordance with Eqs. 1 to 4) whether 2 and 2 ml ferricyanide reagent and glucose solution are heated in 18×180 mm tubes, or 5 and 5 ml in 29×200 mm tubes, or 10 and 10 ml in 31×200 mm tubes. The results may deviate at greater total volumes than 20

ml. Tubes of 29 × 200 mm dimensions are recommended, since mixing of sample and reagent, and the thiosulfate titration, can be done so conveniently; larger tubes are not readily handled.

The procedure may be varied greatly from that given above, depending upon the amount of sugar to be determined. The effect of ferricyanide concentration should be carefully considered (see Eq. 10). The sample solution should be neutral or slightly alkaline; it should not contain appreciable quantities of bicarbonate, since this affects the rate of reaction and the reduction per mole of sugar.

Standing at room temperature before incubation. The reduction appears to be slow at room temperature. In two series of experiments, no significant differences in reduction were noted between tubes which had been placed immediately in the water bath after mixing and those which had stood as long as 30 min before heating. Nevertheless, incubation as soon as possible after mixing is recommended.

Covering of tubes and timing. The tubes should be covered to prevent loss of water vapor during the incubation. Changes in volume affect the ferricyanide concentration which, in turn, affects the degree of reduction. The incubation should be accurately timed.

Photometric determination. The reduction yields ferrocyanide ion, exactly equivalent to the ferricyanide lost. Although either the ferro- or the ferricyanide may thus be determined, the latter is most conveniently determined photometrically. The maximal absorption is at approximately 418 mμ wavelength, at which point ferrocyanide shows no measurable interference. At this wavelength some colored samples may have considerable light-absorbing materials, in which case a sample blank may be prepared consisting of 5 ml sample solution plus 5 ml 5% Na_2CO_3 in 500 ml. This blank probably overcorrects for the colored substances, since most of the latter are undoubtedly oxidized by the ferricyanide. We find that the colored substances, along with aldehydes, ketones, and amino acids, are most effectively removed by precipitation with mercury salts.

The calculated molar absorbance of ferricyanide is about 1020 at 418 mμ wavelength, when the potassium salt is recrystallized from a saturated solution at 55°C, dried *in vacuo* at 40°, and determined in dilute Na_2CO_3 as in the analytical procedure.

Reoxidation of ferrocyanide does not appear to occur within several hours even when diluted with oxygen-saturated distilled water and allowed to stand 24 hr at room temperature. The reoxidation has been less than 2%, as measured by increased absorbance. However, the possibility of catalysis, due to light, metallic ions, sugar oxidation products, or

other substances from the sample, cannot be ruled out. No study has been made of this possibility. Immediate determination after short cooling is recommended.

Titrimetric determination. The method for iodometric determination of ferricyanide (3, 11, 18) depends upon the oxidation of iodide ion to elementary iodine in neutral or slightly acid solution in the presence of a zinc salt. Both ferri- and ferrocyanide form precipitates of zinc salts. As the granular, relatively more soluble, zinc ferricyanide is reduced by the iodide, the less soluble, gelatinous zinc ferrocyanide is precipitated. In the absence of carbonate, the reaction proceeds rapidly and quantitatively, even though nonhomogeneous, in spite of the possibility that gelatinous zinc ferrocyanide may be precipitated around the zinc ferricyanide precipitate. However, *in the presence of carbonate under the conditions of the procedure,* we have noted a tendency toward variable and progressively decreasing yields of iodine as the quantity of sugar is increased—and hence as the quantity of ferrocyanide is increased. We shall state briefly the conditions for quantitative yield of iodine under these conditions.

First, sufficient acetic acid must be added to neutralize the carbonate; pH 6.5–4.0 of the acidified reaction mixture is satisfactory. Second, the quantity of KI should be great enough to reduce all of the ferricyanide and to give maximum sensitivity to the starch-iodine color. Third, at least 2 moles of $ZnSO_4$ should be added for each mole of ferrocyanide to be precipitated. With smaller quantities, the thiosulfate titration to a permanently colorless end point is greatly prolonged. A very large excess of zinc salt, although giving a sharp and apparently permanent end point, tends to yield low results. This is due, perhaps, to decreased solubility of the zinc ferricyanide and decreased permeability of iodide ion through the zinc ferrocyanide, which is precipitated around the insoluble ferricyanide as the reduction of the latter proceeds. The recommended 5 ml 0.04 *M* ferricyanide reagent, plus 1 ml KI solution and 5 ml zinc reagent, contain the following approximate quantities: 5 mmoles acetic acid to neutralize 2.4 mmoles Na_2CO_3; 0.9 mmole KI, 10.4 mmoles $ZnSO_4$, and 0.20 mmole $K_3Fe(CN)_6$.

Finally, the temperature of the cooled tubes and the time of reaction with KI and zinc reagent are important. At low temperatures the starch-iodine end point may not be permanent, indicating incomplete reaction of iodide with ferricyanide. The reaction proceeds rapidly above 20°C and appears to be complete within 10 or more minutes. At elevated temperatures, considerable loss of iodine from the effervescing reaction mixture may occur. A temperature of 20–25°C is recommended.

Calculations

The quantity of reducing sugar, expressed as glucose, may be calculated from the following equations, which are derived from data obtained under the usual conditions of routine analysis in our laboratory. Let g = mg glucose in the 5-ml volume of sample solution taken for analysis;

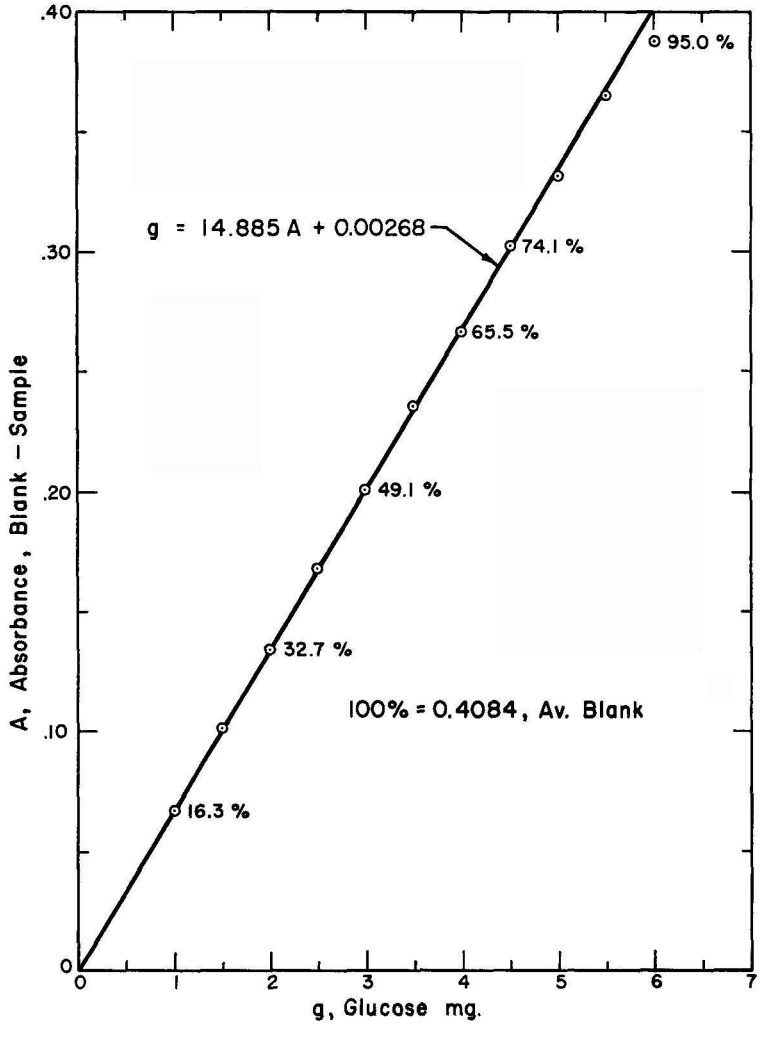

FIG. 1. Relation between glucose oxidized (mg) and absorbance at 418 mμ in 1-cm square cuvettes. Reaction mixture consisted of 5 ml glucose standard solution and 5 ml 0.04 M ferricyanide in 5.0% Na_2CO_3; it was incubated 30 min at 80°C. Final dilution was 500 ml.

A = difference of absorbance between the blank and sample determination at 100-fold dilution, measured in 1-cm square cuvettes at 418 mμ wavelength, using the Beckman DU spectrophotometer; and T = ml N thiosulfate titration difference between blank and sample determination.

When 5 ml 0.04 M ferricyanide and 5 ml sample solution are used, then, within the range of 15–75% reduction of ferricyanide (Figs. 1 and 2):

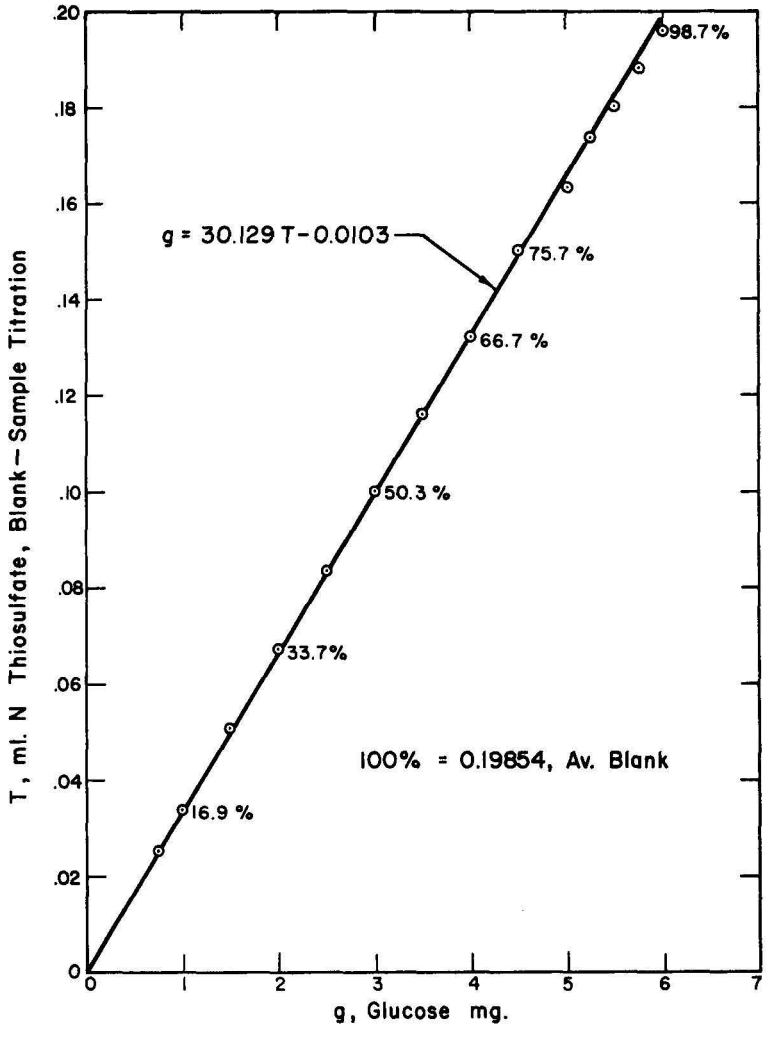

FIG. 2. Relation between glucose oxidized (mg) and ml N thiosulfate equivalent to mmoles ferricyanide reduced. Conditions of oxidation as in Fig. 1.

$$g = 14.88A + 0.0027, \quad \text{or} \tag{1}$$

$$g = 30.13T - 0.0103, \quad \text{or} \tag{2}$$

$$g = 30.00T \tag{3}$$

Equation (3) yields a fair approximation.

Within the range of 1–6 mg glucose in 5 ml, resulting in 15–98% reduction of ferricyanide:

$$g = 28.43T + 0.0991T^2 + 0.0464 \tag{4}$$

When 10 ml 0.04 M ferricyanide and 5 ml of sample solution are used, then, within the range of 15–75% reduction of ferricyanide:

$$g = 28.89T - 0.0488 \tag{5}$$

When 15 ml 0.04 M ferricyanide and 5 ml of sample solution are used, then, again within the range of 15–75% reduction:

$$g = 28.71T - 0.0531 \tag{6}$$

The ratio of absorbances of ferricyanide in Beckman 1-cm square cuvettes and Beckman 15 and 25 mm round cuvettes is approximately 1.000:1.305:2.242, which values may be applied to Eq. (1) when the round cuvettes are used.

EXPERIMENTAL

Comparison of Ferricyanide with Cupric-Tartrate Reduction by Sugars

The reduction of ferricyanide is quite similar to that of the cupric complex in the Shaffer-Somogyi reagent when the reaction is carried out in the boiling water bath and both reagents contain the same concentrations of alkaline salts and oxidizing agent. The Shaffer-Somogyi reagent contains 2.5% Na_2CO_3 and 2.0% $NaHCO_3$ (about 1:1 molar ratio), 0.03 mole $CuSO_4$, and 0.09 mole sodium potassium tartrate per liter. This reagent and a second one, containing the same salts except $NaHCO_3$, were prepared. Comparable 0.03 M ferricyanide reagents, with 2.5% Na_2CO_3 and 2% $NaHCO_3$, and 2.5% Na_2CO_3 only, were also prepared.

Reaction at 95°C. At the temperature of the boiling water bath at Boulder, the reduction of ferricyanide in Na_2CO_3 and $NaHCO_3$ by glucose was almost as rapid as that of the copper reagent (Fig. 3). The reaction was almost complete in 30 min, and approximately equal quantities of ferricyanide or cupric complex, 8.1 moles, were reduced per mole of glucose (Fig. 3). In previous experiments in Chicago (altitude about 600 ft) only 15 min was required to attain the same degree of

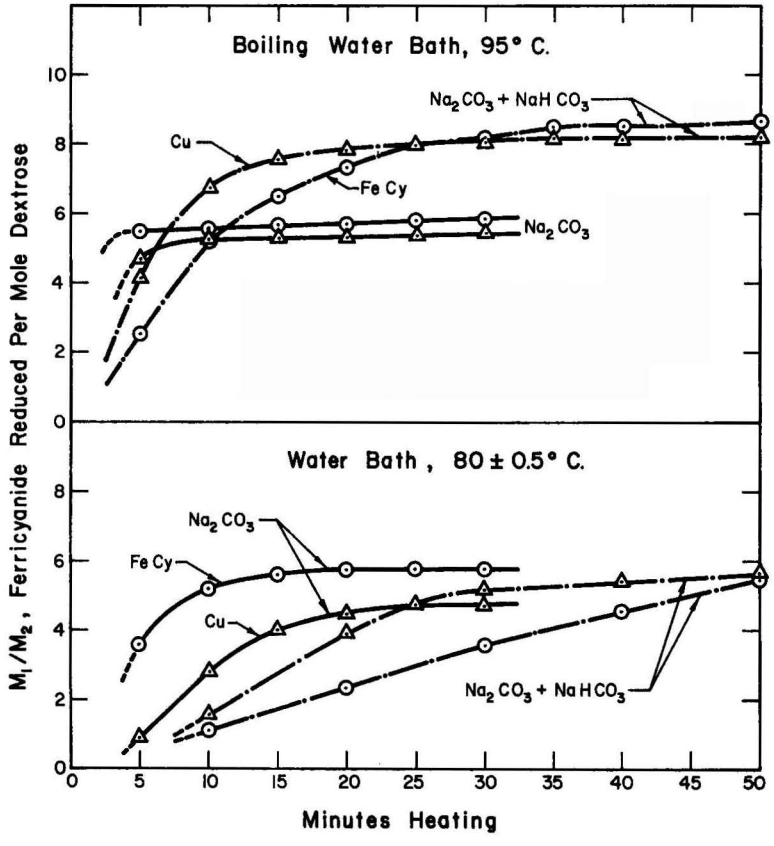

FIG. 3. Rate of reduction of Shaffer-Somogyi and ferricyanide reagents by 1.5 mg glucose in the boiling water bath at 95° and at 80°C under comparable conditions of alkalinity. Reagents contained either 2.5% Na_2CO_3 plus 2.0% $NaHCO_3$ or 2.5% Na_2CO_3 alone. Concentration of oxidizing agent was 0.03 M in both reagents.

reduction of both reagents. Within 30 min, the ratio of reductions of ferricyanide per mole of glucose, fructose, and galactose was 100:99:75; and that of the Shaffer-Somogyi reagent, 100:97:80.

In Na_2CO_3, both reagents exhibited similar parallel rates of reduction; both reactions were more than 95% complete within 5–10 min, and, at 30 min, the moles of oxidizing agent (M_1) reduced per mole of glucose (M_2) were 5.85 of ferricyanide and 5.4 of cupric ion (Fig. 3). The slightly lower rate and total reduction of cupric complex were most likely due to the separation of some of the cupric complex as a flocculent, semicolloidal, greenish precipitate. The ratio of reductions of ferricyanide by glucose, fructose, and galactose within 30 min was

about the same as in Na_2CO_3–$NaHCO_3$ solution, namely, 100:100:81. Because of the precipitation of cupric ion, the reduction of Shaffer-Somogyi reagent was not carried out.

In addition to showing the similarities of ferricyanide and copper-tartrate reductions by sugars under the same conditions of alkalinity, these experiments demonstrate that the reduction of Shaffer-Somogyi reagent, and the comparable ferricyanide reagent, is so greatly influenced by the temperature of the boiling water bath that the limit of usefulness of these carbonate-bicarbonate reagents is approached at about 5000 ft altitude, the altitude of Boulder. The necessity is indicated for a "standard" temperature and a reagent whose reduction becomes constant and reproducible within less than 30 min, regardless of altitude. The temperature of 80°C is suggested; it is the boiling point of water at about 19,000 ft altitude.

Reaction at 80°C. The same general trends were noted as at 95° in the boiling water bath, but at greatly decreased rates of reaction (Fig. 3). As before, the Shaffer-Somogyi reagent was reduced somewhat more rapidly than the corresponding ferricyanide reagent; the reduction of both reagents was still incomplete after 90 min (see Table 2, reagent 3A). On the other hand, the ferricyanide reagent in Na_2CO_3 was again reduced more rapidly than the cupric complex in Na_2CO_3; the reduction of ferricyanide was complete in 20 min, that of the cupric reagent in about 25 min; M_1/M_2 at 30 min was 5.8 of ferricyanide, and 4.7 of cupric complex. The considerably lower reduction of copper at 80° than at 95° may have been due to the greater volume of greenish precipitate. The degree of reduction of 0.03 M ferricyanide in Na_2CO_3 thus was about the same at 80° as at 95°. Ferricyanide in Na_2CO_3 solution appears to be the reagent of choice.

Relative Reducing Capacities of Sugars

The following sugars gave approximately the same *relative* reduction per milligram (glucose = 100%) by the authors' and the Shaffer-Somogyi method: glucose, fructose, galactose, sorbose, xylose, lyxose, and arabinose (Table 1). The following gave greater relative reduction per milligram by the authors' than by the Shaffer-Somogyi method: mannose, rhamnose, fucose, glucosamine, glucuronic acid, ribose, and the disaccharoses. Ferricyanide appears to oxidize both of the sugar moieties of the disaccharoses, whereas the copper reagent oxidizes only the reducing moiety. This behavior of ferricyanide has been mentioned by Sobotka and Reiner (5) in the case of maltose and lactose, and by Widdowson (4) in the case of maltose. This property confers several advantages to the ferricyanide methods. First, the polysaccharides and

TABLE 1

RELATIVE REDUCING CAPACITIES PER UNIT WEIGHT OF VARIOUS CARBOHYDRATES AS DETERMINED BY AUTHORS' FERRICYANIDE METHOD AT 80°C AND BY SHAFFER-SOMOGYI METHOD WITH 30 MIN HEATING IN BOILING WATER BATH AT 95°C

Hexoses and derivatives			Pentoses			Disaccharoses		
Compound	Ferricyanide	Cupric-tartrate	Compound	Ferricyanide	Cupric-tartrate	Compound	Ferricyanide	Cupric-tartrate
D-Glucose	100	100	D-Xylose	97	96	Cellobiose	97	54
D-Fructose	99	95	D-Lyxose	96	96	Lactose	82	48
D-Mannose	97	85	L-Arabinose	88	87	Maltose	78	42
D-Sorbose	86	86	D-Arabinose	88	—	Melibiose	61	52
D-Galactose	78	80	D-Ribose	86	77			
L-Rhamnose	94	66						
D-Fucose	77	52						
D-Glucosamine	105	97						
D-Glucuronic acid	95	76						

disaccharoses of plants and animal tissues need not be as completely hydrolyzed as is considered necessary when cupric-tartrate methods are used. Complete hydrolysis is time consuming and, when carried out with acid, involves considerable loss of reducing power. Second, the total carbohydrate expressed as glucose can be calculated more accurately, since the reducing power of the various sugars in a hydrolyzate is greater with ferricyanide than that obtained with cupric-tartrate reagents.

Effect of Ratio of Na_2CO_3 to $NaHCO_3$

Five reagents (Fig. 4 and Table 2) were prepared, all containing 13.20

TABLE 2

REDUCTION OF 0.02 *M* FERRICYANIDE IN Na_2CO_3 SOLUTION WITH VARYING QUANTITIES OF ADDED $NaHCO_3$ (Water bath temperature 80°C)

Reagent No.	Na_2CO_3 / $NaHCO_3$ Molar ratio	Time in water bath[a] (min) 30	60	90	120	Remarks
		M_1/M_2	M_1/M_2	M_1/M_2	M_1/M_2	
1A	1:0	6.00	5.95	6.07	6.14	Complete in 30 min
2A	1:0.5	6.01	7.49	8.05	8.17	Complete in 90 min
3A	1:1	4.63	7.00	8.14	9.98	Incomplete in 120 min
4A	1:1.5	3.85	6.12	7.55	8.96	Same
5A	1:2	2.88	5.59	6.42	8.06	Same

[a] M_1/M_2 = moles ferricyanide reduced per mole glucose.

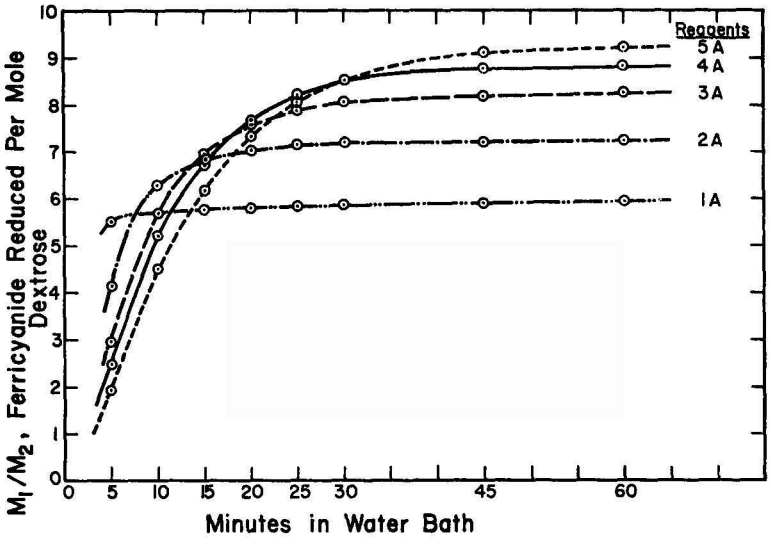

FIG. 4. Effect of alkalinity on rate of reduction of ferricyanide by 3 mg glucose in the boiling water bath, at approximately 95°C, at Boulder, Colorado. Reaction mixture consisted of 5 ml glucose standard solution and 5 ml 0.04 *M* ferricyanide in 2.5% Na_2CO_3 plus 0, 1.0, 2.0, 3.0, or 4.0% $NaHCO_3$ in the respective reagents 1A to 5A.

gm ferricyanide (0.04 *M*) and 25 gm Na_2CO_3 (0.24 *M*) per liter. Reagents 2A to 5A also contained $NaHCO_3$, 10, 20, 30, 40 gm, respectively, per liter. The molar ratios of Na_2CO_3 to $NaHCO_3$ thus varied from 1:0 to 1:2.

Reaction at 95°C. The presence of bicarbonate greatly reduced the reaction rate, but increased the reduction per mole of sugar (Fig. 4). Only reagents 1A to 3A had fairly complete reactions within 30 min, and M_1/M_2 were 5.9, 7.2, and 8.0, respectively, at this time (Fig. 4). With reagents 4A and 5A, at the Na_2CO_3–$NaHCO_3$ ratios of 1:1.5 and 1:2, the reactions were complete at about 60 min, and M_1/M_2 were about 8.8 and 9.2. Complete oxidation requires 12 equivalents of oxidizing agent. The results indicate again that the limit of usefulness of reagents containing 1:1 molar ratio of carbonates is at about 5000 feet altitude. On the basis of completeness of reaction and maximal reduction within a reasonable period of time, 30 min, reagent 3A is the reagent of choice at 95°C.

Reaction at 80°C. The reaction with reagent 1A (Table 2) was fairly complete within 30 min. About 90 min was required with reagent 2A, and more than 120 min with reagents 3A to 5A, which contained greater

than 1:0.5 molar ratio of carbonates. Thus, at 80°, the reaction was complete within a reasonable period of time only with ferricyanide in Na_2CO_3 solution.

Effect of Na_2CO_3 Concentration

Seven reagents were prepared, each containing 13.20 gm (0.04 *M*) ferricyanide and increasing quantities of Na_2CO_3, from 12.5, 25, 37.5, etc., to 87.5 gm, per liter. Thus the molar ratio of Na_2CO_3 to ferricyanide varied from 2.9, 5.9, 8.8 etc., :1 to 20.6:1. The 10-ml reaction mixture contained 3 mg glucose; ferricyanide was 0.02 *M*; and the Na_2CO_3 concentrations varied from 0.625 to 4.37%. The reaction mixture was heated 30 min at 80°C. Ferricyanide was determined iodometrically.

The 3 mg of glucose reduced approximately 50% of the ferricyanide with all of the seven reagents. The reduction was increased slightly with increasing carbonate concentrations, according to the equation:

$$\frac{M_1}{M_2} = 5.93 + 0.0284C \tag{7}$$

in which M_1/M_2 = moles ferricyanide reduced per mole glucose, and C = % Na_2CO_3 in the reaction mixture. Since $M_1/M_2 = 180T/g$:

$$T = g(0.03294 + 0.000158C) \tag{8}$$

It is evident that the absolute concentration of carbonate is not important. For example, with the second and last reagents, 3 mg glucose reduced 0.0994 and 0.1009 mmole ferricyanide, a difference of only 0.0015 mmole, or 0.15 ml 0.01 *N* thiosulfate, in two series of titrations.

However, the molar ratio of Na_2CO_3 to ferricyanide is important for a consistent linear relation between the reduction and glucose concentration over a wide range of the latter. With the first reagent, the reduction departed markedly from approximate linearity at greater levels than 3 mg glucose; with the second reagent, the departure was less marked. With the other five reagents, a linear relation was obtained up to 75% reduction of the ferricyanide; occasionally, the regression curve of a series of determinations intersected the origin, and often the reduction was linear up to almost 90% of the ferricyanide. Thus, the minimal molar ratio of Na_2CO_3 to ferricyanide for attainment of linearity over a wide range was 8.8:1.

Effect of Ferricyanide Concentration

Increasing ferricyanide concentration; Na_2CO_3 constant. Seven reagents were prepared, containing 0.01, 0.02, 0.03 etc., to 0.07 *M* ferricyanide.

All contained 5% Na_2CO_3. The molar ratios of Na_2CO_3 to ferricyanide in the first four reagents varied from 47.2:1 to 11.8:1, and in the last three, 9.4:1 to 6.7:1. Four to six levels of glucose in 5 ml of solution were analyzed, resulting in approximately 20–75% reduction of the ferricyanide in 5 ml of reagent. The quantities of glucose analyzed varied from 0.3–1.0 mg with the 0.01 *M* reagent to 2–7 mg with the 0.07 *M* reagent. The reaction mixture was heated 30 min at 80°C. Ferricyanide was determined iodometrically.

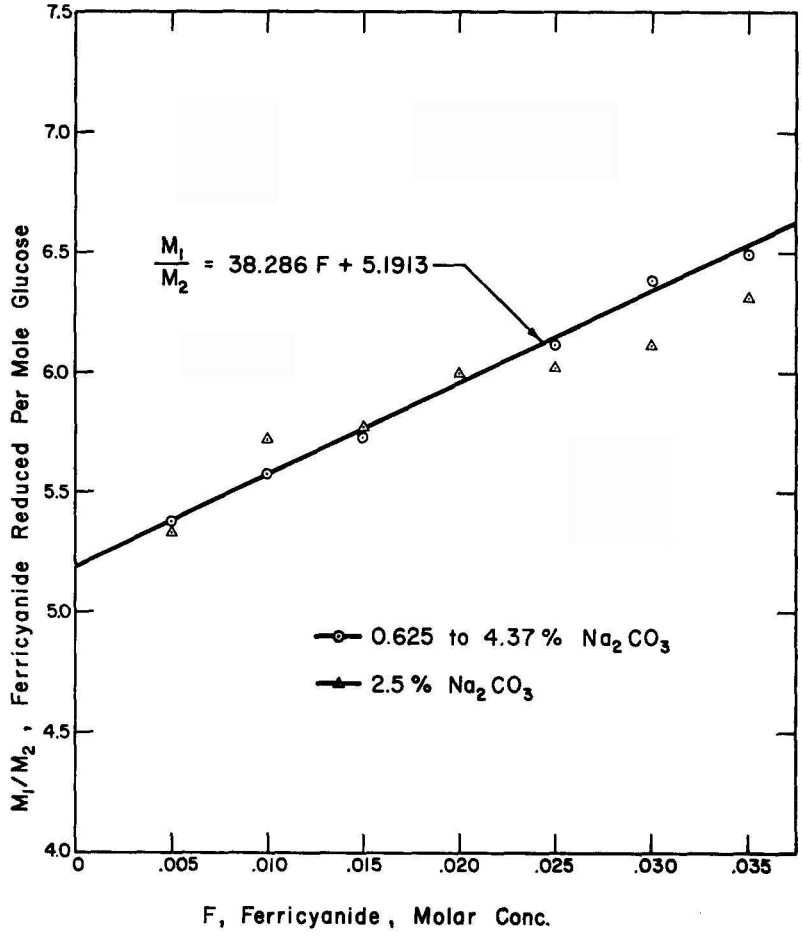

FIG. 5. Effect of ferricyanide concentration on average reduction of ferricyanide per mole glucose at 0.005–0.035 *M* ferricyanide concentration in reaction mixture at (a) proportionately increasing Na_2CO_3 concentrations, from 0.625–4.37%, and (b) at constant Na_2CO_3 concentration, 2.5%, in reaction mixture.

The ratio, M_1/M_2, was calculated for each analysis, and from this the mean was obtained. The ratio was not the same at all levels of glucose analyzed, since the curve for reduction, although linear with each reagent, did not pass through the origin. Thus, the relative reduction was generally greater at the lower levels of glucose analyzed, and smaller at the higher levels, than in the middle of the range. Occasionally, M_1/M_2 was constant over the entire range of sugar concentrations. The extreme differences were small, never greater than $\pm 1.5\%$ from the mean over the entire range. We believe, therefore, that the mean reduction per mole of glucose thus calculated provides a measure of the reducing capacity of a given reagent.

The concentration of ferricyanide markedly affected the oxidation of glucose (Fig. 5). The reducing capacity, as indicated by the mean M_1/M_2, increased approximately linearly at 0.005–0.020 M concentration of ferricyanide in the reaction mixtures. With the first four reagents, M_1/M_2 was 5.327, 5.725, 5.773, and 6.000. With the last three reagents, M_1/M_2 continued to increase linearly, but the slope of the curve was decreased. The departure from the initial slope was obtained with reagents in which the ratios of Na_2CO_3 to ferricyanide were less than 9.4:1.

Increasing ferricyanide concentration; proportionately increasing Na_2CO_3. Seven reagents were prepared, containing 0.01, 0.02, 0.03 etc., to 0.07 M ferricyanide and 12.5, 25, 37.5 etc., to 87.5 gm of Na_2CO_3 per liter. The molecular ratio of Na_2CO_3 to ferricyanide was 11.8:1 in all reagents. The methods of analysis and calculation of results were the same as before.

Throughout the entire range of concentrations (Fig. 5), from 0.005 to 0.035 M ferricyanide and from 0.625 to 4.375% Na_2CO_3 *in the reaction mixture*, the average reduction was proportional to the ferricyanide concentration.

$$\frac{M_1}{M_2} = 5.1913 + 38.286F \tag{9}$$

or, since $M_1/M_2 = 180T/g$:

$$g = \frac{T}{0.02884 + 0.2127F} \tag{10}$$

in which F = molar ferricyanide concentration *in the reaction mixture*. The results with the first four reagents were identical, within the limits of error, with those obtained from the first four reagents of the previous experiment.

Equation (10) is the general equation which applies to a wide range

of ferricyanide concentrations in the reaction mixture, regardless of Na_2CO_3 concentration or volume of reaction mixture. Results of analysis of 0.3–13.0 mg glucose, and application of general Eq. (10), are shown in Table 3.

SUMMARY

A suggested procedure is described which is applicable to 0.3–13 mg quantities of glucose. Five milliliters of sample solution and 5–15 ml 0.04 M potassium ferricyanide in 5.0% Na_2CO_3 are heated 30 min in a water bath at 80°C. The reasons for choice of 80° are discussed. Ferricyanide is determined spectrophotometrically at 418 mμ wavelength, or iodometrically by the Mohr method. Ferricyanide solutions in Na_2CO_3, when well protected from light, have been kept at least six months at room temperature without any evidence of decomposition whatsoever. The molecular absorbance of ferricyanide is 1020 under the conditions of alkalinity of the method. The factors which affect the accuracy of the Mohr method were studied; the conditions which must be met for accurate determination of ferricyanide are summarized. A linear relation between glucose and ferricyanide reduction is obtained up to about 75% reduction of ferricyanide, and often the relation is linear to almost 90% reduction. Equations are given for calculation of glucose under prescribed conditions of procedure.

In the boiling water bath, about 95°C at Boulder, Colorado, altitude 5300 ft, the reduction of ferricyanide is quite similar to that of the cupric-tartrate complex of the Shaffer-Somogyi reagent when both reagents contain the same concentration of Na_2CO_3, $NaHCO_3$, and oxidizing agent. The rates are parallel and the reaction is more than 95% complete at about 30 min; approximately 8.1 moles of both oxidizing agents is reduced per mole of glucose, M_1/M_2. At the altitude of Chicago (600 ft) the same degree of reduction is attained in 15 min. But when both reagents contain Na_2CO_3, and no $NaHCO_3$, the reactions of both approach completion within 5–10 min in the boiling water bath, 95°C; M_1/M_2, although of approximately the same magnitude with both reagents, is considerably reduced. At 80°C, only the ferricyanide reagent, with Na_2CO_3 alone, gives satisfactorily complete reduction within less than 30 min; the reaction is complete within about 20 min, and M_1/M_2 agrees closely with that at 95°C.

The following sugars give approximately the same relative reduction per milligram (glucose = 100%) respectively by the authors' and the Shaffer-Somogyi methods: glucose 100, 100; fructose 99, 95; galactose 78, 80; sorbose 86, 86; xylose 97, 96; lyxose 96, 96; and arabinose 88, 87. Greater relative reductions per milligram are given by the authors'

TABLE 3
APPLICATION OF GENERAL EQUATION (10)

Reagent		Reaction mixture		Glucose	
Vol. (ml)	Ferricyanide concn. (M)	Tot. vol. (ml)	Ferricyanide concn. (M)	Analyzed (mg)	Recovered (%)
			$g = 33.44T$		
5	0.01	10	0.005	0.30	100.3
				0.50	99.8
				0.75	100.0
				1.00	99.7
			$g = 30.22T$		
5	0.04	10	0.020	1	101.5
				2	101.1
				3	100.6
				4	100.1
				4.5	100.9
			$g = 28.97T$		
10	0.04	15	0.0267	2	102.5
				3	101.8
				4	101.4
				5	100.9
				6	101.5
				7	101.5
				8	100.6
			$g = 28.39T$		
15	0.04	20	0.030	3	98.6
				4	99.6
				8	100.5
				9	100.4
				12	99.1
				13	98.6
			$g = 27.56T$		
5	0.07	10	0.035	2	99.7
				4	100.0
				5	99.3
				6	99.3
				7	98.6

method than by the Shaffer-Somogyi by the following: mannose 97, 85; rhamnose 94, 66; fucose 77, 52; glucosamine 105, 97; glucuronic acid 95, 76; ribose 86, 77; cellobiose 97, 54; lactose 82, 48; maltose 78, 42; and melibiose 61, 52. Ferricyanide appears to oxidize both sugar moieties of

the disaccharoses whereas the copper reagent apparently oxidizes only the reducing moiety. The advantages of the greater relative reducing ability of ferricyanide are pointed out.

The following variables do not significantly affect the reduction of ferricyanide in Na_2CO_3 solution: (*1*) size of test tube, from 18 to 31 mm diameter, and volume of reaction mixture, from 4 to 20 ml, provided that the rate of heating is rapid and uniform; (*2*) time of standing of the reaction mixture up to as long as 30 min *before* heating; (*3*) concentration of Na_2CO_3 from 0.625 to 4.37% in the reaction mixture, provided the molar ratio of Na_2CO_3: ferricyanide is greater than about 10:1; (*4*) the time of standing of the reaction mixture *after* heating and cooling. Immediate determination of residual ferricyanide is recommended, although ferrocyanide reoxidation is extremely slow, if at all, less than 2% occurring within 24 hr.

Three factors greatly affect the oxidation: (*1*) presence of $NaHCO_3$, either in the reagent or produced by unneutralized acid in the sample; (*2*) ratio of Na_2CO_3: ferricyanide; (*3*) concentration of ferricyanide. The first two are ruled out when the authors' recommended reagent is used and the sample is properly neutralized. The third factor, therefore, is the most important variable. The following general equation may be used for approximate calculation of milligrams glucose, g, in the sample when the milliequivalents of ferricyanide reduced, T, and the molar concentration of ferricyanide, F, in the reaction mixture are known:

$$g = \frac{T}{0.02884 + 0.2127F}$$

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Colorimetric Determination of Hypotaurine

KOICHIRO SUMIZU

From the Department of Chemistry, Faculty of Science, Kyoto University, Kyoto, Japan

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INTRODUCTION

During studies on the enzymic oxidation of hypotaurine, an improved method for the determination of hypotaurine was developed. The method is based on the following principle: hypotaurine is oxidized by the addition of an excess of HIO, the HIO remaining is converted to iodine by acid, and the iodine liberated is assayed by a colorimetric method.

MATERIALS AND APPARATUS

Hypotaurine was prepared from taurine by the method of Bricas (1).[1] Iodine was purified by sublimation from commercial iodine. NaOH, KI, trichloroacetic acid, metaphosphate, uranium acetate, serum albumin, taurine, and soluble starch were all commercial products. Perchloric acid (30%) and H_3PO_4 (85%) were also commercial products. Optical density was measured with a Beckman Model DU spectrophotometer.

METHOD

Conditions for Colorimetric Determination of Iodine

The well-known purple-colored solution was obtained by addition of a starch solution to iodine (2). Although the color varied with pH and temperature, the purple-colored solution at room temperature in 1 *N* HCl exhibited maximum absorbance at 590 mμ. Iodine produced from KI in acidic solution by autoxidation (4) or by irradiation with daylight was also examined (3); the results are shown in Fig. 1. Variations in absorption were observed with the different acids used and at various acid concentrations. H_2SO_4 and HCl were found to increase the formation of iodine, while the same molar solution of organic acids such as acetic acid or citric acid had a tendency to suppress its liberation. Among the acids tested, only H_3PO_4 was found not to increase or decrease the color intensity.

[1] The melting point and R_f value (paper chromatography) were identical with those in the Bricas report (1).

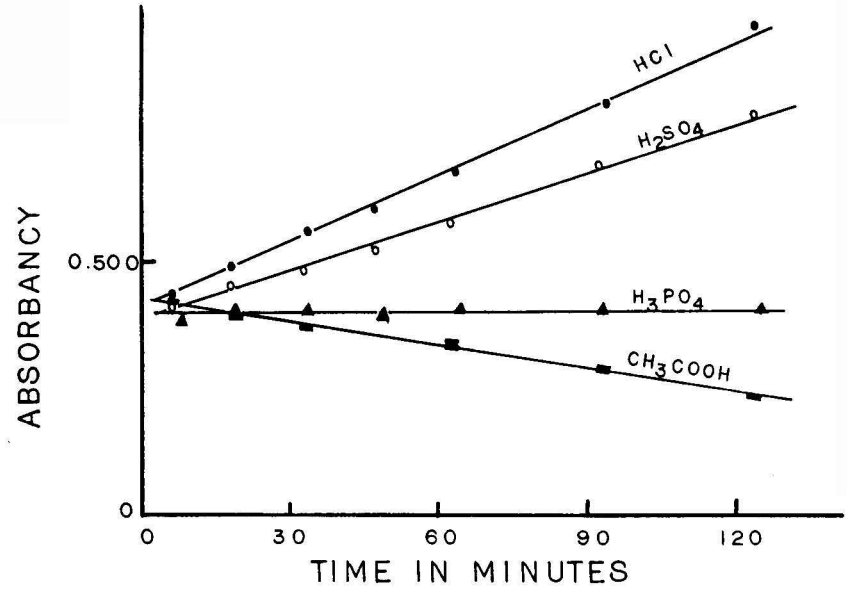

FIG. 1. Effect of acids on color development. Final concentration of acids was 1.0 *M*. Reaction mixture composition and assay procedure: 1.0 ml 1 *N* NaOH, 50 μg I_2, and 1.0 ml 0.1 *M* KI were mixed and allowed to stand for 5 min; 1.0 ml 0.2% starch and 1.0 ml 10 *N* acid were added to this mixture; after the total volume was made to 10 ml with distilled water, optical density was measured at various times.

Since the liberation of iodine from KI and the evolution of iodine were dependent on the concentration of KI (5), 0.01 *M* KI in 1 *N* H_3PO_4 was chosen as the optimum concentration to give almost the same amount of iodine as the standard.

Reaction

When the solutions of NaOH and I_2 were mixed together, NaIO was produced by reaction (I). Then hypotaurine was added to the mixture

$$I_2 + 2\,NaOH \underset{\text{acidic}}{\overset{\text{basic}}{\rightleftharpoons}} NaI + NaIO + H_2O \qquad (I)$$

and was oxidized by NaIO (II). In this assay, a large excess of mixture

$$NaIO + HO_2SCH_2CH_2NH_2 = NaI + HO_3SCH_2CH_2NH_2 \qquad (II)^2$$

[2] After the reaction was complete, the mixture was evaporated under 22 mm Hg pressure and the residue extracted four times with 10 ml absolute ethanol. The remaining white crystals were subjected to paper chromatography (1) in the solvent system phenol:H_2O (saturated) and butanol:acetic acid:H_2O (4:2:1). Only one spot corresponding to authentic taurine appeared on the chromatogram.

(I) was used. The HIO remaining was completely decomposed into iodine by addition of H_3PO_4 and the liberated iodine was determined by measuring the absorbance of the purple color (590 mμ) developed by addition of starch solution. The amount of hypotaurine was determined by subtraction of the amount of iodine liberated from the standard iodine originally added in the first step. The time needed for color development was not important in this procedure.

Procedure for Hypotaurine Assay

The standard procedure is described below: 1 ml 1 *N* NaOH, 0.1 ml 0.5 mg/ml I_2 solution, and 1.0 ml 0.1 *M* KI were first mixed and to each such solution was added a sample containing hypotaurine. The reaction mixture was maintained for 5 min at room temperature and then 1.0 ml 0.2% starch solution was added. The blue-colored solution thus formed was transferred to a 10-ml flask, diluted to the mark with distilled water, and the optical density measured at 590 mμ. This procedure was shown to be reliable for the determination of hypotaurine in the range from 5 to 30 mμmoles/ml.

RESULTS

In Table 1 the results are given of hypotaurine determinations and

TABLE 1

HYPOTAURINE DETERMINATIONS AND RECOVERIES BY ANALYTICAL PROCEDURE

Hypotaurine added (mμmoles)	Hypotaurine found (mμmoles)	Recovery (%)
350	348	99.5
300	300	100
250	253	102
200	198	99.0
150	152	101

Analytical procedure, as described in text. Total volume of reaction mixture, 10 ml.

recoveries made on samples that contained hypotaurine in rat liver homogenate deproteinized with trichloroacetic acid.

Effect of Metal Ions

The effect of metal ions on this color development was examined and the results are shown in Table 2. Mg^{++}, Cd^{++}, Al^{+++}, Zn^{++}, and molybdate ion (footnote *a*, Table 2) had no effect, while Mn^{++}, VO_3^-, Co^{++}, and Ni^{++} were observed to increase the color development. Addition of EDTA

TABLE 2
EFFECT OF METAL IONS FOR COLOR DEVELOPMENT

Metal ion added	Final concn. (M)	Absorbance at 590 mμ[a]
None	0	0.240
Mg^{++}	10^{-3}	0.242
Zn^{++}	10^{-3}	0.238
Cd^{++}	10^{-3}	0.235
Molybdate	10^{-3}	0.242
VO_3^{--}	10^{-3}	0.790
Mn^{++}	10^{-3}	2.400
Co^{++}	10^{-3}	1.385
Ni^{++}	10^{-3}	0.950
Co^{++} plus EDTA	$Co^{++} = 10^{-3}$, EDTA $= 10^{-2}$	1.380
Ni^{++} plus EDTA	$Ni^{++} = 10^{-3}$, EDTA $= 10^{-2}$	0.900
Mn^{++} plus EDTA	$Mn^{++} = 10^{-3}$, EDTA $= 10^{-2}$	2.400

[a] These values show the absorbancy of iodine-starch solution. Composition of reaction mixture and assay procedure, as described in text except for addition of metal ion and no addition of hypotaurine. Mg^{++}: $MgCl_2$. Zn^{++}: $ZnCl_2$. Cd^{++}: $CdCl_2$. Mn^{++}: $MnSO_4$. Ni^{++}: $NiCl_2$. Co^{++}: $CoCl_2$. VO_3^-: $NaVO_3$. Molybdate: $(NH_4)_{14}Mo_{24}O_{27}$.

did not reverse the enhancement of the color development by Mn^{++}, Vo_3^-, Co^{++}, and Ni^{++}.

Influence of Protein and Deproteinizing Reagent

The influence of the deproteinizing reagent on this procedure was also investigated. Perchloric acid, metaphosphate (both final concentrations were $1.0M$), uranium acetate (final concentration was $0.08M$), and Somogyi's deproteinizing reagent were shown to have almost no effect. The use of lead acetate for deproteinization should be avoided in this procedure because it resulted in precipitation of PbI_2. The effect of trichloroacetic acid on color development was also examined, with the results shown in Fig. 2. Interference was noted with a protein[3] concentration of 3 μg/ml.

Other Substances Which Interfere with Color Development

When sulfurous oxide, cysteine, cysteinesulfinic acid, cysteamine, formaldehyde, acetaldehyde, or acetone was mixed with hypotaurine, the observed value of the color intensity was found to exceed the amount of hypotaurine previously added.

DISCUSSION

Efficient exclusion of sulfurous oxide from the reaction mixture can

[3] The protein used was human serum albumin.

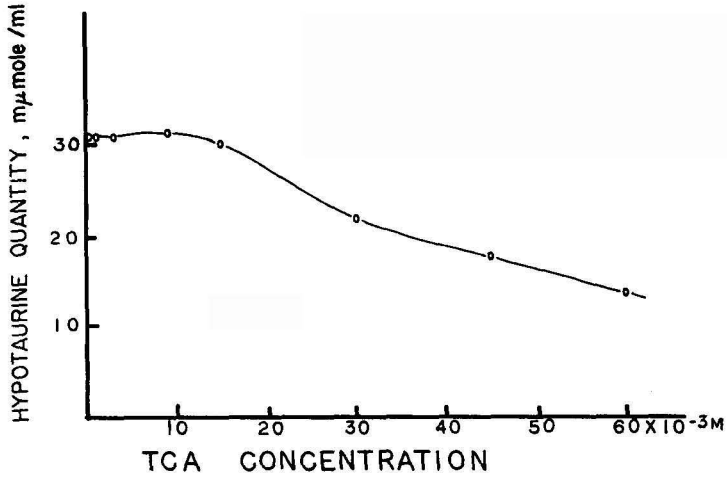

FIG. 2. Influence of trichloroacetic acid (TCA) on color development. Composition of the reaction mixture and assay procedure are as described in the text except for addition of TCA.

be achieved by use of the method of Fromageot (6). When the reaction mixture contains volatile compounds such as acetone, formaldehyde, and acetaldehyde, hypotaurine should be determined after excluding the volatile compounds by microdiffusion (7) or by evaporation under reduced pressure. Hypotaurine is determined by the author's procedure with a correction for cysteinesulfinic acid, cysteine, or cysteamine content. Cysteinesulfinic acid is assayed by a modification of Lavine's method (8), and cysteine and cysteamine are measured by the method of Boyer (9). Hypotaurine is found not to be determined by these methods.

SUMMARY

Hypotaurine was determined colorimetrically by oxidizing with HIO and converting the excess of HIO into I_2, followed by the iodine-starch reaction. Determination of hypotaurine in the range 5–40 mμmoles/ml was found to be possible by this method. Sulfurous oxide, cysteine, acetone, aldehyde, protein, and organic acids interfered with hypotaurine determination.

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Self-absorption Corrections for C^{14}-Labeled Protein[1]

MASAMI MURAMATSU[2] AND HARRIS BUSCH

From the Department of Pharmacology, Baylor University College of Medicine, Houston, Texas

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One of the critical problems in metabolic studies on proteins is the determination of their specific activities after introduction of tracers into biological systems. At the present time, it is not possible to provide true specific activities at infinite thinness for most protein preparations and it is difficult to find precise data on the limits of applicability of counting procedures employed. Despite the theoretical explorations of self-absorption characteristics of C^{14}-labeled compounds (1–6), most laboratories use empirically derived tables or graphs for correction of experimental results to "infinite-thickness" or "infinite-thinness." In part, this procedure stems from the frequency with which only small amounts of protein are available. One of the problems in determination of absolute specific activities stems from the fact that extrapolation of curves of "apparent specific activity" or "self-absorption" curves to the intercept representing activity is extremely imprecise because of the increased back-scattering with small amounts of sample on the planchet. This problem is increased when planchets made of metals with large atomic weights are used (4). The curves for "apparent specific activity" resemble hyperbolic rather than exponential curves and the approach to the ordinate representing activity may appear to be asymptotic. Another problem is evenness of plating protein samples; in this study, this problem was largely overcome by using planchets with small wells (7).

The present study was undertaken to define the limits of accuracy of the methods employed in this laboratory for plating and counting protein samples. In order to determine the absorption coefficient (μ) for proteins, 26 preparations were made with varying weights of a single protein preparation in wells of two sizes. With the aid of apparent specific activities determined from these experiments and the weights of

[1] Supported by grants from U. S. Public Health Service, American Cancer Society, and Jane Coffin Childs Fund.

[2] Post-doctoral Trainee in Pharmacology.

the proteins, μ was determined by the least square method indicated in Eq. (1). In this equation, h_i is each mg of weight of protein per cm^2,

$$-\mu = \frac{\sum_{i=1}^{n}\left[h_i \cdot \ln\left(1 - \frac{I}{I_\infty}\right)\right]}{\sum_{i=1}^{n} h_i^2} \tag{1}$$

I is the activity[3] found, and I_∞ is the activity at infinite thickness. The large number of calculations involved were simplified by the use of an IBM 1620 data-processing system from which μ was obtained. Using this value for μ with a completely new set of unknowns, it was possible to define the limits at which the specific activity could be determined with an accuracy of $\pm 5\%$.

MATERIALS AND METHODS

Preparation of Protein Samples. Proteins of rat liver were labeled by injection of 10 μc of L-lysine-U-C^{14} intravenously into normal rats (Holtzman Co., Houston, Texas) 1 hr prior to sacrifice of the animals. The livers were homogenized in 0.25 *M* sucrose (9 : 1, v/w). The proteins were precipitated and their lipids were extracted as described previously (7). Two protein preparations were made which were used for determination of μ for the small and large wells, respectively. The plating apparatus, which consists of a stainless-steel planchet with a well in the center and a snugly fitting stainless-steel pestle, differed from that described previously (7) in that the diameter of the well of the planchets was either 0.650 or 0.990 cm. The cross sectional areas of the wells were 0.332 and 0.770 cm^2, respectively. The depth of the well from the top of the planchet and the distance of the bottom of the well from the anode wire were kept constant in these experiments.

Counting of the samples. The radioactivity was determined in an automatic Nuclear-Chicago counting apparatus with a D-47 gas-flow counter equipped with a Micromil window. Each planchet was counted for 10–20 min to a total of 1280–5120 counts. The background counting rate was 14–18 counts/min.

Data processing. Twenty-six samples ranging in weight from 0.2 mg (0.6 mg/cm^2) to 8.2 mg (24.7 mg/cm^2) were prepared and counted in small wells. Twenty-two samples ranging in weight from 0.5 mg (0.6

[3] In this report, counting rate is the parameter used to determine activity and the terms are used interchangeably.

mg/cm^2) to 19.1 mg (24.6 mg/cm^2) were prepared and counted in the large well. From the basic equations derived earlier (1, 2), the standard exponential equation is:

$$I/I_\infty = 1 - e^{-\mu h} \tag{2}$$

The logarithmic form of this equation is:

$$\ln\left(1 - \frac{I}{I_\infty}\right) = -\mu h \tag{3}$$

Equation (1) is derived from Eq. (3) by the least square method and provides the basis for calculation in the IBM 1620 data-processing system. Using the FORTRAN System, the following instructions and data were inserted to obtain μ and the correction factors based upon this μ. Similar programs were developed for small and large wells; the program for the small well is as follows:

	FORTRAN statement[4]	Explanation of instruction
	DIMENSION A (26), Y(26), X(26)	A is mg/planchet; X is h_i, or A/0.332, and Y is $1 - I/I_\infty$
4.	SUMX2 = 0	
	SUMNU = 0	
	DO 2 I = 1, 26	
1.	READ, A(I), Y(I)	26 sets of A and Y are inserted
	X(I) = A(I)/0.332	
	SUMX2 = SUMX2 + X(I)**2	Σh_i^2
2.	SUMNU = SUMNU + X(I)*LOG(Y(I))	$\Sigma(h \times \ln [1 - (I/I_\infty)])$
	D = −SUMNU/SUMX2	D is μ
	TYPE, D	Type μ
	B = 0.1	B is mg/planchet
	DO 3 M = 1, 200	
	C = (0.332/D)* (1. − EXP (−D*B/0.332))	C is correction factor
	TYPE, B, C	
	B = 0.1 + B	
3.	J = M	In this step, the system types the correction factors as shown in Table 1.
	GO TO 4	
	END	

RESULTS

Relationships Defined by Basic Counting Data. Figure 1 is a graph of apparent specific activity plotted against the thickness of the sample in mg/cm^2. This type of graph is commonly the basis for preparation of self-absorption curves. It is apparent that precise determination of the intercept is not feasible. Figure 2 is a semi-log plot of the same data

[4] The letters indicated represent the tabs activated to insert instructions into the IBM 1620 data-processing system and are not to be confused with abbreviations in formulas in the text.

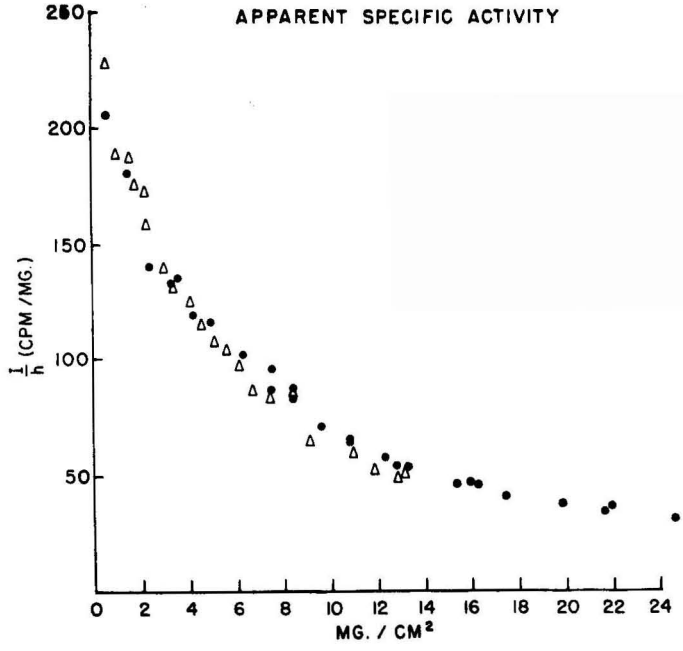

FIG. 1. Apparent specific activity plotted against thickness of sample. Various amounts of proteins labeled with lysine-U-C^{14} were plated on planchets with large wells (△) and small wells (●). Radioactivity was determined in a gas-flow counter with a Micromil window. I is observed counts/min and h is mg protein/cm². Specific activity of the protein of Figs. 1 to 3 was 182 at infinite thinness as determined by the value for μ derived by the least square method.

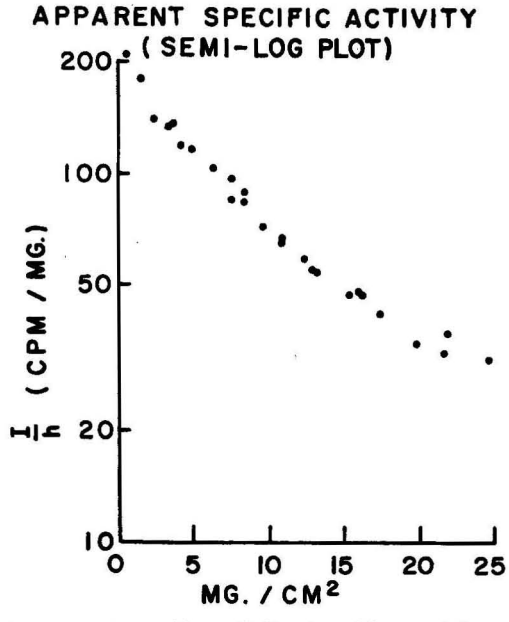

FIG. 2. Plot of apparent specific activity (semi-log scale) against thickness of sample. Data are the same as shown in Fig. 1 (●).

TABLE 1

SELF-ABSORPTION CORRECTION FACTORS FOR PROTEIN ON STAINLESS-STEEL PLANCHETS

mg	C_s	C_L	mg	C_s	C_L	mg	C_s	C_L
0.8	0.583		4.5	1.17	2.22	8.2		2.63
0.9	0.632		4.6	1.17	2.24	8.3		2.64
1.0	0.677		4.7	1.17	2.26	8.4		2.64
1.1	0.719		4.8	1.17	2.28	8.5		2.64
1.2	0.757		4.9	1.17	2.30	8.6		2.65
1.3	0.792		5.0	1.18	2.32	8.7		2.65
1.4	0.824		5.1	1.18	2.33	8.8		2.66
1.5	0.854		5.2	1.18	2.35	8.9		2.66
1.6	0.881		5.3	1.18	2.36	9.0		2.67
1.7	0.907		5.4	1.18	2.38	9.1		2.67
1.8	0.930		5.5	1.18	2.39	9.2		2.67
1.9	0.951		5.6	1.18	2.40	9.3		2.68
2.0	0.970	1.43	5.7	1.18	2.42	9.4		2.68
2.1	0.988	1.47	5.8	1.18	2.43	9.5		2.68
2.2	1.00	1.52	5.9	1.19	2.44	9.6		2.69
2.3	1.02	1.56	6.0		2.46	9.7		2.69
2.4	1.03	1.61	6.1		2.46	9.8		2.69
2.5	1.05	1.65	6.2		2.48	9.9		2.69
2.6	1.06	1.69	6.3		2.49	|		
2.7	1.07	1.73	6.4		2.50	10.0		
2.8	1.08	1.76	6.5		2.51	|		2.70
2.9	1.09	1.80	6.6		2.52	10.3		
3.0	1.10	1.83	6.7		2.53	10.4		
3.1	1.11	1.87	6.8		2.54	|		2.71
3.2	1.11	1.90	6.9		2.54	10.7		
3.3	1.12	1.93	7.0		2.55	10.8		
3.4	1.12	1.96	7.1		2.56	|		2.72
3.5	1.13	1.98	7.2		2.56	11.3		
3.6	1.14	2.02	7.3		2.57	11.4		
3.7	1.14	2.04	7.4		2.58	|		2.73
3.8	1.14	2.07	7.5		2.59	11.9		
3.9	1.15	2.10	7.6		2.60	12.0		
4.0	1.15	2.12	7.7		2.60	|		2.74
4.1	1.16	2.14	7.8		2.61	12.9		
4.2	1.16	2.16	7.9		2.61	13.0		
4.3	1.16	2.18	8.0		2.62	|		2.75
4.4	1.16	2.20	8.1		2.62	14.0		
						14.1		
						|		2.76
						19.0		
						19.1		2.77

The values in this table were obtained by direct readout from the IBM 1620 data-processing system, using the formula:

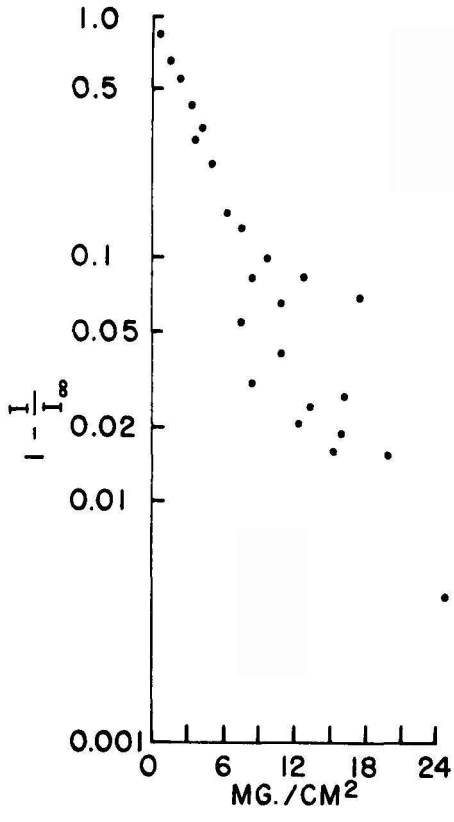

FIG. 3. Plot of $1 - (I/I_\infty)$ on a log scale against thickness of sample. According to Eq. (3) these data should theoretically form a straight line. Data are the same as in Fig. 1.

which has the same deficiencies as that of Fig. 1, somewhat diminished by the logarithmic Y axis. In an effort to obtain a straight line for the experimental points Eq. (3) was used as the basis for Fig. 3. The ordinate is $1 - (I/I_\infty)$ plotted on a log scale and the abscissa is mg/cm² of

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$$J' = \frac{A}{0.278}\left(1 - e^{-0.278m/A}\right)$$

in which J' is the quotient of measured activity divided by specific activity at infinite thinness, A is the area, and m is the weight of the protein in mg. The symbols C_s and C_L stand for the correction factors for the small and large wells, respectively. To determine the specific activity for a given weight of protein in one of these two wells, the observed count rate is divided by the appropriate correction factor (J'). The values of J' at infinite thickness for C_s and C_L were 1.19 and 2.77, respectively.

protein. The slope should be $-\mu$. However, as is shown by the graph, above 6 mg/cm^2 the differences between I and I_∞ become smaller and marked variability results. While the points obtained with less than 6 mg/cm^2 seem satisfactory for defining the line, the effects of back-scattering increase in this region.

Test of μ Determined by Least Square Method. As derived from the procedure indicated in the section on Materials and Methods, the values for μ for the large and small wells were 0.276 and 0.279 cm^2/mg, respectively. Three series of experiments were carried out with 20 or more different samples in each series. The deviation of the μ was 0.0017 from the average. From the average value of μ derived by the least square method, Table 1 was prepared by a direct readout from the IBM data-processing system based on the formula:

$$J' = A/0.278(1 - e^{-0.278m/A}) \tag{4}$$

In this equation, J' is the quotient of measured activity divided by specific activity at infinite thinness, A is the area, and m is the weight of the protein in mg.

Two tests of the self-absorption coefficient, μ, were made with 24 and 21 new samples, respectively, in the small and large wells (Figs. 4 and

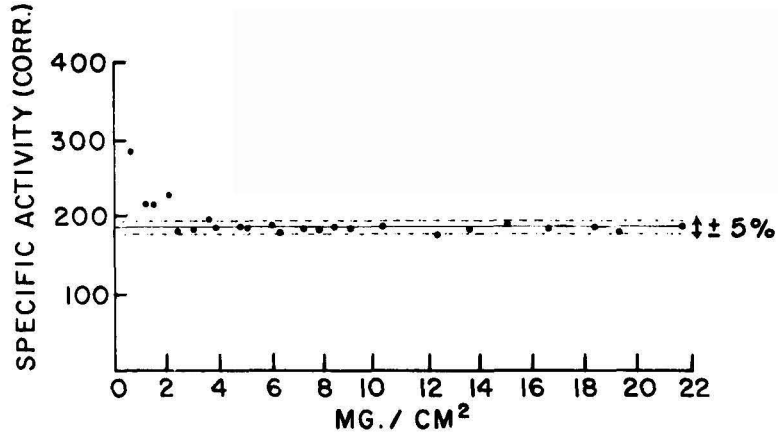

FIG. 4. Test of validity of μ determined by least square method using Eq. (1). Specific activities calculated by Table 1 derived from IBM 1620 data-processing system (see text) are plotted against thickness of sample, using different weights of the same protein preparation. Black dots show data for planchet with small well. Specific activity is counts/min/mg dry weight of protein. The solid line is the mean of specific activities of protein samples weighing from 2.2 to 22 mg/cm^2, using value for (μ) derived from initial studies.

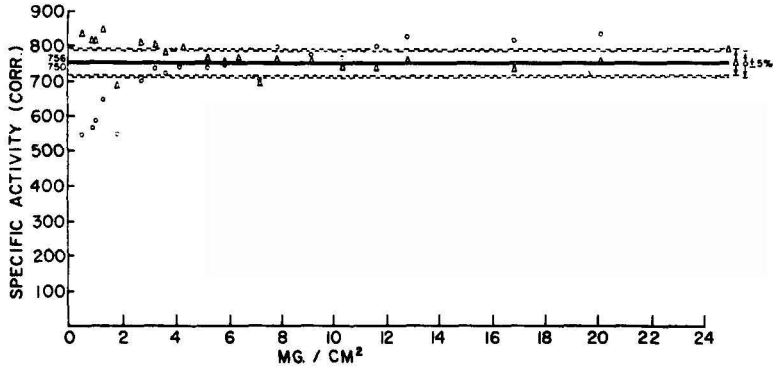

FIG. 5. Similar plot as Fig. 4 for planchet with large well (△).[5] The values calculated by "wax curve" are plotted in white circle (○). The white circles tend toward high specific activities as amount of sample increases. Specific activity is counts/min/mg dry weight of protein. The upper solid line is the mean of specific activities of protein samples weighing 3.6 to 24.5 mg/cm², using the value for (μ) derived from initial studies. The lower solid line is the mean of specific activities of protein samples weighing 3.2 to 11.6 mg/cm² obtained from the "wax curve."

5). From this data, it is apparent that specific activity calculated for most samples differ from the theoretical value by less than 5% when more than 2.4 mg/cm² is on the planchet.

DISCUSSION

The value of μ obtained in these studies is very similar to that (0.285 ± 0.008 cm²/mg) obtained by other investigators (3, 8) for barium carbonate. Figure 5 shows that the data are also in good agreement with the data calculated from the empirically determined absorption curve for waxy substances ("wax curve") (5), except for the results with thick samples. The results with thin samples are variable regardless of the method employed for determination of the specific activity.

The implications of the present findings with regard to the validity of the mathematical expression of such data as hyperbolic or exponential curves (6, 9, 10) are indicated in Fig. 6. The F value, proposed by Hendler (6), which is the fraction, reference specific activity divided by observed specific activity, is plotted in Fig. 6 against the thickness of the sample; the reference specific activity used here is the specific activity at infinite thinness derived from the exponential equation. The solid curve is derived from the exponential equation. The broken line

[5] These values were multiplied by 1.8 so as to compare the specific activities calculated by this procedure to those calculated from "wax curve."

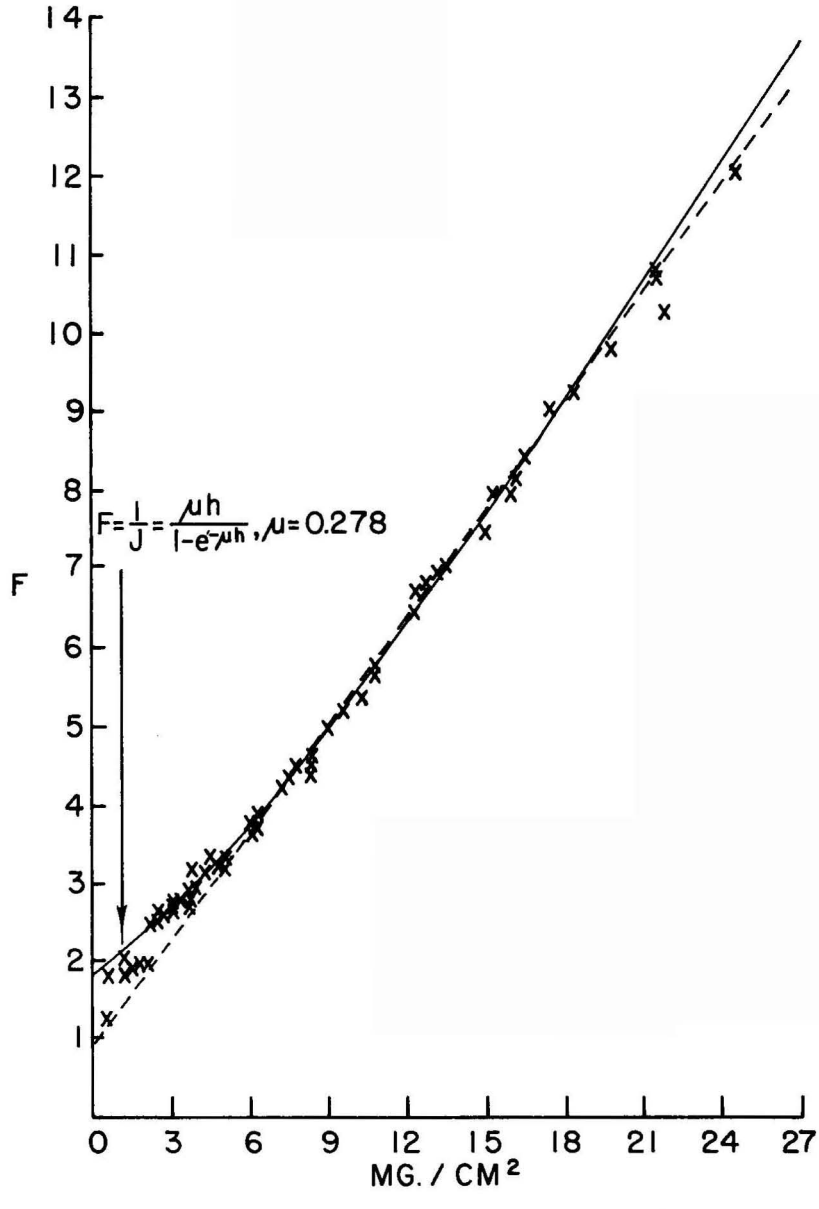

FIG. 6. Plot of F against thickness of sample. $F = \mu h/(1 - e^{-\mu h})$ is a representation of an exponential equation. The broken line represents the straight line which best fits the experimental points.

is the straight line which best fits the experimental points and can be represented by the equation:

$$F = \frac{R}{I_\infty} h + b$$

(R is reference specific activity, h is thickness of the sample, and b is the intercept on the ordinate), which is one representation of a hyperbolic curve. When more than 5 mg/cm^2 was on the planchet, either curve was a satisfactory representation of the data within the limits of experimental error. With thinner samples, ranging in weight from 2.4–5.0 mg/cm^2, the exponential curve seemed more satisfactory. The discrepancies between the theoretical curves and the data may result from back-scattering or unevenness of plating. However, in the range from 2.4–5.0 mg/cm^2, the plating was satisfactory because of the small size of the wells. With samples of smaller weight, the experimental values fell between two curves, and it was not possible from these data to select one curve which provided a more satisfactory representation of the data.

SUMMARY

The present study was undertaken to define the limits of accuracy of methods employed in this laboratory for plating and counting protein samples. The absorption coefficient (μ) for proteins was found to be 0.278 cm^2/mg with the aid of the least square method. Two tests made with this self-absorption coefficient indicated that a specific activity with an error of less than 5% could be obtained when the sample weight was 2.4 mg/cm^2, or greater. When lesser amounts were plated there was increasing error in the calculated value for the specific activity. Both hyperbolic and exponential treatment of the data were satisfactory for defining the relationship between the weight of the sample and the radioactivity when the weight was in excess of 2.4 mg/cm^2, but neither was completely satisfactory when smaller amounts were plated.

ACKNOWLEDGMENT

The authors wish to acknowledge the generous aid of Dr. Walter P. Abbott, Assistant Professor of the Department of Epidemiology, Baylor University College of Medicine, Houston, Texas, in instruction on procedures and evaluation of results obtained with the IBM 1620 data-processing system.

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ANALYTICAL BIOCHEMISTRY 4, 395–399 (1962)

Resolution of Mixtures of Sugars by Means of Partition Chromatography on Columns: Resolution of a Mixture of the Four Aldopentoses and 2-Deoxyribose and of Certain Hexoses[1]

ROSS H. HALL

From the Department of Experimental Therapeutics, Roswell Park Memorial Institute, Buffalo, New York

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Nucleic acids contain 2-deoxy-D-ribose and D-ribose as major constituents. Speculation has long existed, however, that other sugars might exist in nucleic acids (1). Recently, Smith and Dunn found 2(3)-*O*-methylribose in ribonucleic acid isolated from plant and animal sources (2) and Bergmann found D-arabinose containing nucleosides in sponges (3). These observations coupled with the fact that relatively few nucleic acid samples have been examined critically for the presence of other sugars reinforces the speculation that other sugars may exist in nucleic acids. Possibilities for making such observations would be increased if more adequate methods of analysis were available. One requirement of an improved analytical technique is facile resolution of sugars on a preparative scale, as previously described methods present certain problems. For example, separation of the aldopentoses by ion-exchange procedures suffers from the inherent disadvantages of low column capacity and a requirement for borate buffers (4). Partition chromatography on starch columns, while demonstrating an ability to resolve multicomponent mixtures of sugars, proved to be very slow in operation and also to have a low capacity for the sugars (5). The partition column technique, however, does appear to be basically superior for certain classes of compounds. An improved technique for resolving multicomponent mixtures of nucleosides on columns of diatomaceous earth by partition chromatography has been developed (6). This technique is also applicable to the separation of free sugars and this paper describes

[1] This work was partially supported by a grant (C-4640) from the National Cancer Institute of the U. S. Public Health Service.

resolution of a mixture of the pentoses and also resolution of a mixture of certain hexoses.

EXPERIMENTAL

Resolution of the Mixture of Pentoses. Celite-545[2] (50 gm) purified as previously described (6), was thoroughly mixed with 22 cc of the lower phase of a well-equilibrated mixture of ethyl acetate: *n*-propanol: water, 4:1:2 (solvent 1). This Celite-solvent mixture was tamped into a precision bore glass tube (¾″ diameter) with the aid of a close-fitting plunger machined from a Teflon rod. The height of the

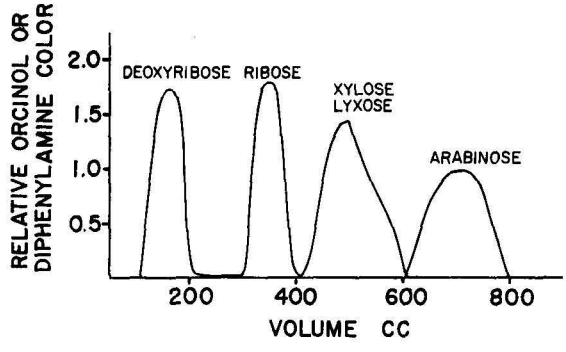

FIG. 1. Resolution of a 50-mg mixture of equal parts of 2-deoxy-D-ribose, D-ribose, D-lyxose, D-xylose, and L-arabinose separated on a 50-gm column of Celite-545. Solvent: ethyl acetate: *n*-propanol: H_2O, 4:1:2.

packed column was 45 cm and the volume of upper phase which was retained by this column was 70 cc. Full details for preparing and operating these columns are given in Reference 6.

A mixture consisting of 10 mg each of 2-deoxy-D-ribose, D-ribose, D-lyxose, D-xylose, and L-arabinose was dissolved in 2 cc of the lower phase of the above solvent system. The solution was mixed with 4 gm of Celite-545 and tamped on top of the column. Flow rate of the upper phase of solvent 1 through the columns was 40 cc per hour. The eluate was collected in 15-cc fractions.

Separation of Xylose and Lyxose (Fig. 2). A 50-gm column of Celite-545 was prepared as described above with the exception that the lower phase of a mixture of 2-butanone: water, 3:1 (solvent 2) was used. A mixture of D-xylose and D-lyxose (20 mg of each) was dissolved in 2 cc of the lower phase of this solvent mixture mixed with 4 gm of Celite-545 and tamped on the top of the column. The flow rate of the upper phase

[2] Trade name for a brand of diatomaceous earth, Johns-Manville Co.

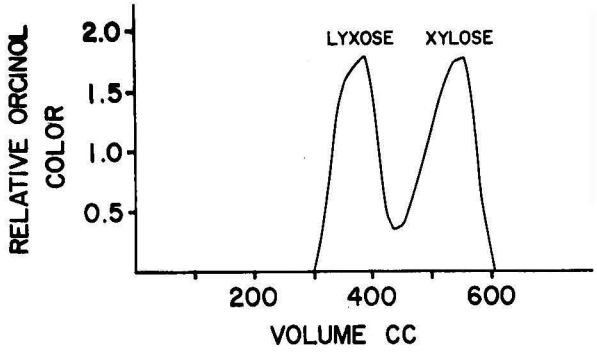

FIG. 2. Resolution of a 40-mg mixture of equal parts of D-lyxose and D-xylose separated on a 50-gm column of Celite-545. Solvent: 2-butanone: H_2O, 3:1.

of solvent 2 through the column was 60 cc/hr. The eluate was fractionated as above.

Separation of Hexoses (Fig. 3). The same procedure was used as with

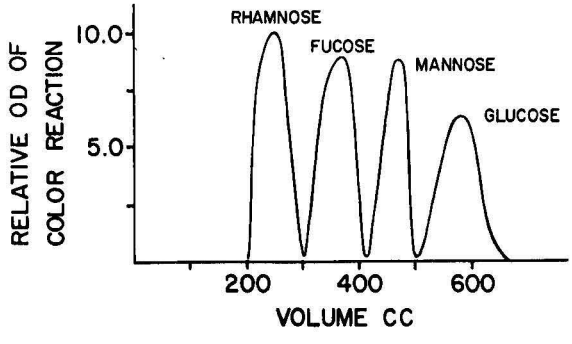

FIG. 3. Resolution of a 200-mg mixture of equal parts of L-rhamnose, L-fucose, D-mannose, and D-glucose separated on a 50-gm column of Celite-545. Solvent: ethyl acetate: *n*-propanol: H_2O, 4:1:2.

the five-carbon sugars. A starting mixture consisting of 50 mg each of L-rhamnose, L-fucose, D-mannose, and D-glucose was dissolved in 3 cc of the lower phase of the solvent. This solution was mixed with 6 gm of Celite-545, which was packed on top of a 50-gm column of Celite-545 prepared as above. The flow rate of the solvent was 60 cc/hr.

Paper Chromatography. Paper chromatography was conducted using Whatman No. 1 paper in a descending direction. The upper phase of solvent 1 (ethyl acetate: *n*-propanol: H_2O, 4:1:2) was placed in the trough and the lower phase was placed in the bottom of the tank.

Papers were developed for 18 hr; however, if an unequivocal differentiation of D-xylose and L-lyxose was necessary, the papers were left for 48 hr. The sugars were visualized by the aniline hydrogen phthalate spray (7).

Analysis. The fractions from the columns were analyzed for 2-deoxyribose by the diphenylamine method (8) and for the pentoses by the orcinol method (8). The hexoses in the fractions of column 3 were determined by the method of Gardell (9). Location and identification of the peaks was confirmed by paper chromatography.

RESULTS AND DISCUSSION

Complete resolution of all five sugars of the pentose series by a single system proved to be difficult. The high-capacity system of ethyl acetate: *n*-propanol: water which most nearly attained this goal was selected as the main system. Although xylose and lyxose were poorly separated by this system, resolution was achieved by passing these two sugars through a second column. Separation of the four hexose sugars (shown in Fig. 3) indicates the potentialities of this system for separating sugars of the hexose series. Prediction of possible separations can be made by an examination of the R_f values of the sugars on paper developed in solvent system 1. For example, Fig. 4 shows the R_f position

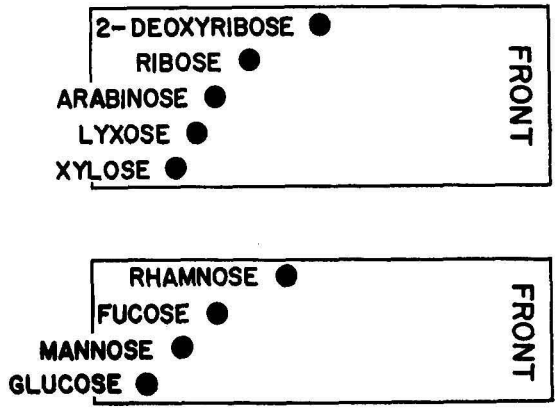

FIG. 4. Chromatography of sugars on Whatman No. 1 paper (descending 18 hr). Solvent: ethyl acetate: *n*-propanol: H_2O, 4:1:2.

of the sugars on a paper strip developed 18 hr in this solvent. The order of R_f values is identical with the order in which the same sugars were eluted from the columns. The maximum capacity of these columns was not ascertained accurately. However, experience with the resolution of

nucleoside mixtures (6) indicates that a starting mixture equivalent to approximately 1% of the weight of Celite-545 could be successfully resolved by this technique.

SUMMARY

A preparative partition column technique has been developed which will resolve mixtures of monosaccharides of the pentose and hexose series. The sugars are quantitatively eluted from the columns and the use of volatile salt-free solvents simplifies final recovery.

ACKNOWLEDGMENT

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ANALYTICAL BIOCHEMISTRY 4, 400–406 (1962)

A Method for the Simultaneous Measurement of the Radioactivity of Proline-C^{14} and Hydroxyproline-C^{14} in Biological Materials

BEVERLY PETERKOFSKY AND D. J. PROCKOP[1]

From the Laboratory of Clinical Biochemistry, National Heart Institute, Bethesda, Maryland

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Isotopic studies on the conversion of proline to hydroxyproline frequently require the measurement of the specific activities of both imino acids. In a previous report (1), a procedure was described for the determination of the specific activity of radioactive hydroxyproline. The method involved the oxidation of hydroxyproline to pyrrole with chloramine-T and extraction of the pyrrole into toluene for the determination of radioactivity and colorimetric assay. Since proline is also oxidized by chloramine-T (1), it was possible to modify the previous method so that both proline-C^{14} and hydroxyproline-C^{14} radioactivity can be estimated on a single aliquot.

In the modified procedure, relatively large amounts of carrier proline and hydroxyproline are added to ensure consistent recoveries of the radioactivity from both imino acids. The product derived from the oxidation of proline is extracted into toluene and then several more extractions are carried out to remove traces of this compound remaining in solution. Upon heating the extracted reaction mixture, the hydroxyproline oxidation product is converted to pyrrole, which is then extractable into toluene. The recovery of carrier proline is determined by comparison with a radioactive standard; the recovery of carrier hydroxyproline is determined colorimetrically. An outline of the entire procedure is presented in Fig. 1.

PROCEDURE

All operations are carried out at room temperature unless otherwise stated and the pH of the solution is maintained at about 8. Culture tubes, 20×2.5 cm, with Teflon-lined screw caps are used.

[1] Present address: Departments of Medicine and Biochemistry, University of Pennsylvania, Philadelphia, Pa.

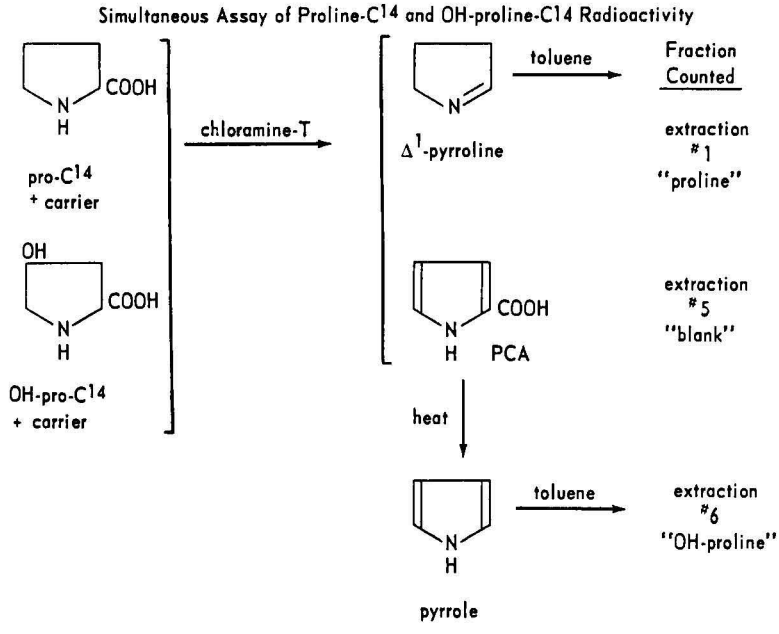

FIG. 1. Outline of the assay procedure. PCA represents pyrrolecarboxylic acid.

Transfer the sample to a tube and make up the volume to 9.0 ml with water. Add a drop of 0.2% phenolphthalein in ethanol and 1.0 ml of a solution containing L-proline, 10.0 mg/ml, and L-hydroxyproline, 1.00 mg/ml. Adjust the pH to about 8 (pink to phenolphthalein) with NaOH and add 4.0 ml of 0.25 *M* sodium pyrophosphate buffer, pH 8.0. A separate proline-C^{14} assay standard is prepared by adding an exact amount of uniformly labeled[2] L-proline-C^{14} to a tube containing everything but the sample for analysis.

Add 1.0 ml of freshly prepared 0.25 *M* chloramine-T in water to oxidize the sample, and after 20 min stop the reaction with 0.5 ml of 2 *M* sodium thiosulfate. Saturate the solution with sodium chloride by adding an excess of the solid salt and add 2 ml of the sodium pyrophosphate buffer. Extract the proline oxidation product by adding 11.0 ml of

[2] Uniformly labeled L-proline-C^{14}, 10 μc/μmole, was obtained from Nuclear-Chicago Corporation. It was found that the dilute solutions used as standards deteriorated upon constant freezing and thawing, leading to apparently low proline recoveries. It is advisable to freeze small aliquots of a standardized solution in tubes, so that a tube may be used once and discarded. Several of the experiments cited in this report were carried out before this was discovered, accounting for the apparent variability of the proline recoveries. With fresh solutions, recoveries range between 60–70%.

toluene, tightly capping the tube and shaking vigorously[3] for 5 min. Centrifuge briefly at a low speed to separate the two phases and then transfer 10.0 ml of the toluene phase to a glass counting vial (*proline assay*).

Remove the residual toluene from the reaction mixture by suction. Carry out three or four additional extractions with 30 ml of toluene to remove the remaining radioactivity of the proline oxidation product and discard the toluene from each of these. Add 11.0 ml of toluene, extract as before, and then transfer a 10.0-ml aliquot of the toluene to a counting vial (*hydroxyproline blank*).[4]

Remove residual toluene from the reaction mixture, tightly cap the tube, place it in a boiling water bath for 20 min to form pyrrole, and then cool it to room temperature. Add 11.0 ml of toluene and carry out an extraction as before. Remove a 0.10-ml aliquot of the toluene for the colorimetric determination of pyrrole and transfer a 10.0-ml aliquot to a counting vial (*hydroxyproline assay*).

Add 1.0 ml of fluorophor solution (1) to the counting vials from the various extractions and count in a Packard Tri-Carb Liquid Scintillation Counter.

The recovery of radioactivity derived from proline is calculated from the recovery observed in the proline-C^{14} assay standard. The specific activity may then be determined if a quantitative assay for proline (2) has been performed on the original sample solution.

The recovery of hydroxyproline is determined by colorimetric assay using the 0.10-ml aliquot of toluene removed. Add 4.4 ml of toluene to the aliquot and prepare a reagent blank with 4.5 ml toluene. Then add 2.0 ml of *p*-dimethylaminobenzaldehyde solution (3), stir, and allow the color to develop at room temperature for 15 to 30 min. Measure the optical density at 560 mμ and compare it to a standard prepared from redistilled pyrrole. The specific activity of the hydroxyproline may then be determined if a quantitative assay (3) has been performed on the original sample solution. In calculating the specific activity, it should be noted that one carbon from hydroxyproline is lost during the conversion to pyrrole (3).

[3] An Equipoise horizontal shaking machine (Precision Scientific Co.) was employed.

[4] This vial measures, and is used to correct for, traces of radioactivity due to the proline oxidation product (presumably Δ^1-pyrroline) which remain in the reaction mixture after extracting with large volumes of toluene. The radioactivity observed here should be subtracted from the radioactivity in the pyrrole extracts. It is only necessary to use as many toluene washes as will reduce the residual proline radioactivity to an amount which is insignificant compared to that of the radioactivity present in the hydroxyproline.

The amount of $C^{14}O_2$ produced during the oxidation of proline-C^{14} was determined by the Hyamine absorption method (4). After a typical oxidation was stopped with $Na_2S_2O_3$, aliquots were placed in Warburg flasks with Hyamine in the center well and HCl in the side arm. The flasks were stoppered, the acid tipped in, and the flasks shaken at 37° for 30 min. The Hyamine was then transferred to counting vials, toluene and fluorophor were added, and the vials were counted in the liquid scintillation counter.

RESULTS AND DISCUSSION

Some of the details of the general method have already been described with respect to hydroxyproline (1). Proportionality is obtained when various amounts of proline-C^{14} and hydroxyproline-C^{14} are added to the assay system. This is illustrated for proline-C^{14} in Fig. 2.

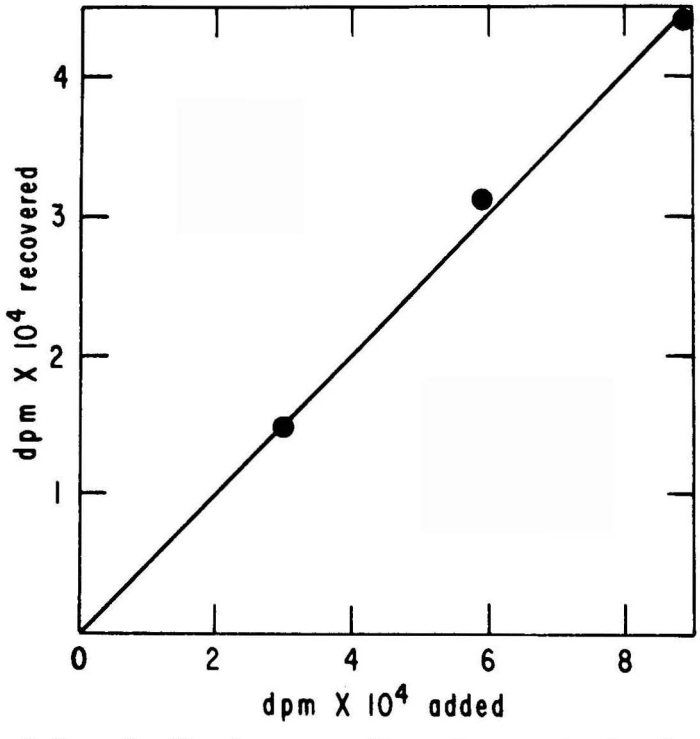

FIG. 2. Proportionality of recovery with varying amounts of proline-C^{14}.

It appears that one carbon of proline is lost during oxidation. It was found that 21.3% of the uniformly labeled proline-C^{14} radioactivity was released as $C^{14}O_2$ while 65.2% was recovered in the first toluene extrac-

tion of a duplicate sample. This result is in agreement with those of Skursky (5), who isolated Δ^1-pyrroline after oxidation of proline with periodate or chloramine-T. Bragg and Hough also isolated Δ^1-pyrroline after oxidation of proline with periodate (6). Although the proline oxidation product was not isolated by us, the loss of a carboxyl group and the extractability of the product from an alkaline medium suggest that the product is Δ^1-pyrroline.

Relatively large amounts of other nonradioactive amino acids may be added to the system with no significant effect on the recoveries of either proline or hydroxyproline. A series of determinations was performed in which various amounts of casein hydrolyzate were added. The results of this experiment (Table 1) indicate that as much as 15 mg of casein

TABLE 1
EFFECT OF ADDED AMINO ACIDS ON RECOVERIES OF PROLINE AND HYDROXYPROLINE

Casein hydrolyzate (mg)	Recovery (%)	
	Proline[a]	Hydroxyproline[b]
0	49	52
2.5	52	49
5	51	54
10	49	47
15	49	49

[a] Determined by radioactivity.

[b] Determined colorimetrically as pyrrole.

The average recovery of hydroxyproline here is 50% while in Table 2 it is 62%. In this experiment the $Na_2S_2O_4$ concentration was 20 mmoles/tube as used in the previous procedure (1). It was later found that, by lowering the concentration to 1.0 mmole/tube, hydroxyproline recoveries could be proportionately increased to values such as those observed in Table 2.

hydrolyzate has no effect.

In order to determine the specificity of the method, several C^{14}-labeled amino acids were tested by adding them to the assay system in place of proline-C^{14}. Of particular interest were glutamic acid, ornithine, and hydroxyproline, which might become labeled from proline-C^{14} in various biological systems (7). It was found that a portion of hydroxyproline radioactivity appeared in the first toluene extract (*proline assay*), in addition to the major portion, which was finally extracted as pyrrole (Table 2). The amount appearing in the *proline assay* was determined to be about 4–5% of that appearing in the final pyrrole extract. This figure of 4–5% was found to be constant with the reagents used. A cor-

TABLE 2
BEHAVIOR OF SEVERAL C^{14}-AMINO ACIDS IN THE ASSAY SYSTEM

C^{14} amino acid[a]	Radioactivity recovered (%)		Hydroxyproline recovery (colorimetric)
	Proline extract	Pyrrole extract	
Proline	54	0.05	58
Hydroxyproline	2.4	61[b]	64
Glutamic acid	0.13	0.03	64
Lysine	23	1.4	63
Ornithine	13	5.0	63

[a] 0.1 μc (1.0 mg) of L-glutamic acid-C^{14}, 0.0025 μc (1.0 mg) of L-ornithine-C^{14}, 0.026 μc (1.0 mg) of L-lysine-C^{14}, 0.012 μc DL-hydroxyproline-C^{14}, and 0.0044 μc of L-proline-C^{14} were used. All were uniformly labeled. Four toluene extractions were carried out between the first extraction and heating.

[b] This value has been corrected for the loss of 20% of the radioactivity as CO_2, which occurs during the conversion of PCA to pyrrole.

rection factor for hydroxyproline contamination in the proline radioassay should be redetermined with each set of reagents.

It may also be noted in Table 2 that only 0.05% of the proline radioactivity appeared in the pyrrole extract (extraction #6). Approximately the same amount appeared in the hydroxyproline blank (extraction #5). This differs from the results obtained in the previous procedure (1) where, after four toluene extractions, 1% of the proline radioactivity could still be extracted. It should also be pointed out that in the previous procedure only 15% of the proline radioactivity was present in the first toluene extract. The present procedure was modified with respect to reagent concentrations so as to give the maximum recovery of proline while retaining a good recovery of hydroxyproline. Since such a small amount of the proline radioactivity appears in the blank, it is possible to assay accurately samples which contain up to 100 times more proline activity than hydroxyproline activity.

In Table 2 it can be seen that glutamic acid-C^{14} did not give rise to a toluene-extractable product, but that ornithine-C^{14} did. Lysine-C^{14} behaved much like ornithine, with the exception that less of its radioactivity was present in the final toluene extract after heating. The hydroxyproline recovery was not affected by the presence of these amino acids.

It is interesting that the diamino acids were both oxidized to toluene-extractable compounds; most likely both were decarboxylated (8) to form cyclic compounds. This property may lend itself to devising procedures for the determination of the radioactivity of these amino acids. Interference from ornithine-C^{14} and lysine-C^{14} is not a problem

if the method is limited to studying proteins or biological systems where there is a minimum formation of ornithine from proline. It is not likely that lysine would become highly labeled from proline-C^{14} in most instances. By limiting the use of this method to the study of proline-C^{14} and hydroxyproline-C^{14} in proteins, we have found it to be a useful tool with many advantages over time-consuming paper or column chromatographic separations of these two imino acids.

SUMMARY

A method is described by which radioactivity in proline-C^{14} and hydroxyproline-C^{14} derived from biological materials may be determined simultaneously. The method involves oxidizing both imino acids with chloramine-T and then selectively extracting the oxidation products into toluene. Of the amino acids derived from proline in biological systems, glutamic acid does not interfere at all while ornithine gives rise to an oxidized product which is toluene extractable to some extent. The method is extremely useful in studying the metabolism of hydroxyproline-containing proteins, where interference from ornithine is of no major importance.

ACKNOWLEDGMENT

We gratefully acknowledge the helpful suggestions and guidance of Dr. Sidney Udenfriend.

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ANALYTICAL BIOCHEMISTRY 4, 407–412 (1962)

Modified Fibrinolytic Technique for Analysis of the Plasmin System

RICHARD E. MAXWELL AND VERA LEWANDOWSKI

From the Research Laboratories, Parke, Davis & Co., Ann Arbor, Michigan

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INTRODUCTION

Plasmin, plasminogen, and related activators and inhibitors are traditionally analyzed by determination of the time required for visually observed lysis of a fibrin clot. Among the notable disadvantages of this method are: (*1*) the all-or-none end point precludes conventional kinetic measurements of fibrinolysis; (*2*) the decision as to when a clot is actually lysed may be subjective and variable, particularly in many situations which prevent sharp and sudden lysis; (*3*) the limited activity range permissible may necessitate repeated concentration adjustments and reassays; and (*4*) the necessary addition of all materials to be investigated before fibrin formation obviates study of certain factors separately from thrombin action and fibrin monomer polymerization. The proposals of Alkjaersig *et al.* (1) for the use of I^{131}-labeled clots, and of Lüscher and Käser-Glanzmann (2) for the preparation of fibrinogen coupled with a fluorescent dye, offer approaches which avoid these problems. However, they present additional methodological or theoretical difficulties with respect to complexity and modification of the substrate. We have found that the inherent fluorescence of the soluble products of fibrin digestion by plasmin is sufficient for the development of a simple, convenient, and sensitive analytical method.

METHODS

Plasminogen-deficient bovine thrombin and fibrinogen were prepared as previously described (3). Bovine plasmin and antiplasmin were obtained essentially as described by Loomis *et al.* (4, 5); these preparations do not have high specific activity, but their purity is not relevant to this discussion. The buffer used for all solutions and dilutions was 0.22 *M* tris(hydroxymethyl)aminomethane·HCl containing 0.155 *M* NaCl, pH 7.4.

Thin and uniform films of fibrin were formed on the bottoms of

25-ml Erlenmeyer flasks by first adding 0.35 ml containing 6 units of thrombin, and then 0.15 ml 1% fibrinogen, and allowing 15 min for fibrin polymerization. After each addition, the fluid was thoroughly but gently maneuvered over the entire bottom surface to ensure uniform coverage and complete mixing. The flasks were then set on a level surface at 25°C for the 15-min period. Before use, each film was visually checked and any showing inhomogeneity or unevenness was discarded. The flasks were placed in a New Brunswick Gyrotory shaker bath at 37°, 1.5 ml supernatants containing desired materials were added to each flask, and incubation was carried out at 72 excursions per minute. Although there was only rarely any solid material in the supernatants, they were routinely poured through sintered-glass filters before analysis. The fluorescence values of appropriate dilutions were determined in an Aminco-Bowman spectrophotofluorometer at the activation wavelength of 280 mμ and fluorescence wavelength of 360 mμ, which had been shown to be the maxima. Blanks and controls were, of course, included to permit corrections for fluorescence not due to action of plasma on fibrin. All determinations were run in duplicate. The response of the instrument was also checked at each use with a standard tyrosine solution.

RESULTS AND DISCUSSION

An experiment indicating the characteristic time course of plasmin action is shown in Fig. 1. It has been established in different studies that the plasmin activity deteriorates, presumably by autodigestion, at an accelerating rate after about one hour; this may account in part for the

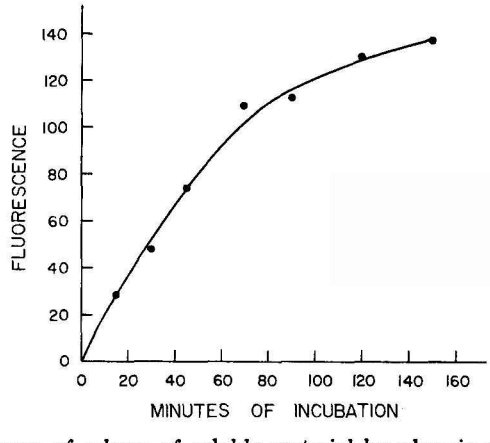

FIG. 1. Time course of release of soluble material by plasmin solution, 50 μg/ml. In this case, fluorescence values of 1:10 dilutions are expressed at a photomultiplier sensitivity of 0.3.

decreasing rate of reaction. Depletion of substrate was not a likely factor since at this plasmin level, for example, less than 20% of the available fibrin was dissolved after 2 hr. Similar curves with this type of plasmin preparation have been obtained over a range of 10 to 100 μg/ml, with the approximately linear duration of the reaction being from about 10 to 70 min. For this reason, we have selected 50 min as a standard period of lysis, and the results at this time are shown in Fig. 2.

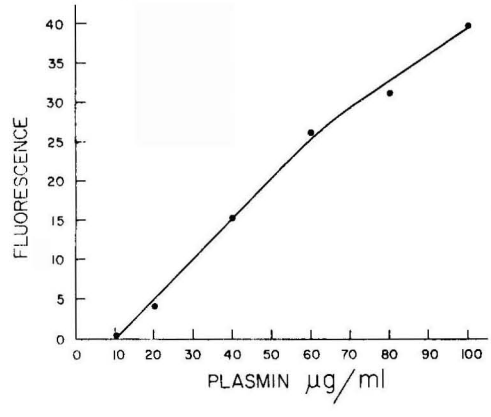

FIG. 2. Fluorescence values of 1:10 dilutions of supernatants at various plasmin levels, all incubated for 50 min, and expressed at a photomultiplier sensitivity of 1.0. The curve does not pass through the origin because values of lower enzyme levels, in this period of incubation, are not greater than blank corrections.

As a matter of convenience in comparing various preparations, we define one unit of activity as that amount which produces a fluorescence value of 25.0 at a photomultiplier sensitivity of 1.0, and at a 1:10 dilution of the supernatant fluid. In the case of this preparation the unit amount would correspond to approximately 89 μg.

Aside from the restrictions of this definition, it is apparent that the high sensitivity of the detection method and the wide range of photomultiplier settings make possible the analysis of a few μg of a crude plasmin preparation, or of activities up to those which bring about complete dissolution of the fibrin. This flexibility is of great convenience in evaluating numerous fractions of unknown potency in purification procedures. If digestion of the film is extensive, the resulting volume increase of the supernatant must be taken into account in making calculations.

The precision of duplicate determinations is not as high as one might desire; experience with several hundred duplicates indicates reliability

to about ±15%. This may be related to the impossibility of bringing about instantaneous mixing of the thrombin and fibrinogen, or perhaps the fibrin polymerization is sufficiently random that no two films can be actually identical, and this method may be sensitive enough to reflect such inhomogeneities. However, in our experience, this precision is at least as satisfactory as that of visually observed lysis methods. In both methods, quantitatively different results may be obtained with different lots or types (e.g., human vs. bovine) of thrombin and fibrinogen, so that it is advisable to establish various plasmin preparations as reference standards.

Additional variables that may be of interest are surface area and fibrin concentration of the film. Over the range of about 2 to 5.5 cm^2, the rate of reaction was almost directly proportional to area, and then showed a more gradual increase to about 11 cm^2, which is approximately the area of the bottoms of the 25-ml Erlenmeyer flasks. Between 0.2 and 0.5% fibrin, the rates were not significantly different; 0.1% was not enough to give a rigid gel. The amount of thrombin used with 0.3% fibrinogen was optimum with regard to time available for mixing and desired gel rigidity. Another observation of importance was that, after separation of the supernatants from the gels, no significant change in their fluorescence was found after standing 3 hr at 25°C.

Preliminary studies with antiplasmin indicated that its interaction with plasmin was a slow reaction which proceeded even more slowly in the presence of the fibrin film. Furthermore, the degree of inhibition was strongly influenced by the relative concentrations of the two proteins; since different levels of plasmin may spontaneously deteriorate at different rates, it was necessary to select arbitrarily a single concentration of a standard plasmin preparation against which to titrate different antiplasmin levels, and to limit the total period of incubation to a time in which no spontaneous decrease of the plasmin activity would be likely. In view of considerations stated previously, this time was set at 50 min.

A standard plasmin preparation at 50 μg/ml was incubated for 20 min at 37°C with different amounts of antiplasmin, and then 1.5-ml aliquots were added to previously prepared films for a 30-min period of incubation as described above for plasmin analysis, and measurement of fluorescent material was made in the same fashion. All necessary blanks and controls were added, of course, including plasmin incubated in the absence of antiplasmin to confirm its maintenance of activity.

For comparisons of different antiplasmin preparations, we define one unit of activity as that amount which produces 50% inhibition. A typical analysis is shown in Fig. 3, and the unit amount in this case would be about 25 μg.

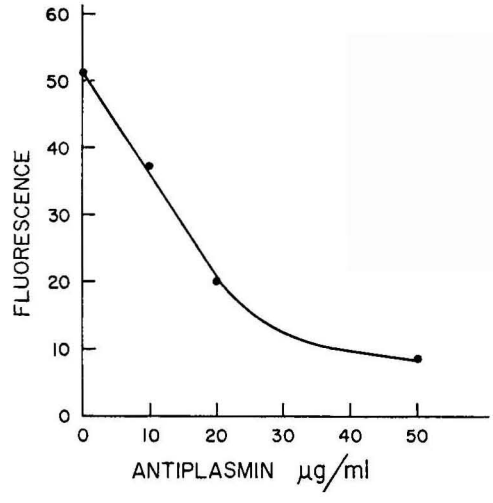

FIG. 3. Antiplasmin analysis. Fluorescence values of 1:10 dilutions of the supernatants were obtained at a photomultiplier sensitivity of 0.3.

It is obvious that this technique may be used also for the analysis of plasminogens and their activators; these analyses involve numerous complexities which are beyond the scope of this discussion. The two-phase arrangement of the system and the ability to follow the time course of the reactions facilitate mechanism studies with alternate combinations of various precursors, activators, and inhibitors distributed between the phases, as the case may be *in vivo*.

In many studies we have been concerned with activities which would not be reliably estimated by measurement of light absorption at 280 mμ in a spectrophotometer. However, it is possible that the latter in conjunction with a photomultiplier arrangement might have adequate sensitivity. It must be emphasized that the conventional technique of acid precipitation of a proteolytic digest and determination of the 280 mμ absorbance of the supernatant affords only a fractional measurement of the soluble products of fibrin digestion by plasmin, at least under the conditions we are using; 1 *M* perchloric acid will remove practically all of the fluorescent material from the supernatants described above.

Finally, it may be noted that this technique is equally useful for the analysis of other proteolytic systems for which fibrin can serve as a substrate; for example, we have found it very satisfactory for the study of chymotrypsin, trypsin, papain, bromelin, and ficin.

SUMMARY

A convenient and sensitive method for the analysis of plasmin and

antiplasmin is described, based on a two-phase system of fibrin film and supernatant, and measurement of the inherent fluorescence of soluble products released into the supernatant.

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ANALYTICAL BIOCHEMISTRY 4, 413–417 (1962)

A Simple Scintillation Counting Technique for Assaying $C^{14}O_2$ in a Warburg Flask

DONALD R. BUHLER

From the Biochemistry Department, The Upjohn Company, Kalamazoo, Michigan

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When large numbers of $C^{14}O_2$ samples must be counted, as the result of Warburg respirometry, a simple, rapid sample preparation technique is necessary. The ease of sample preparation, high counting efficiencies, and automatic features make liquid scintillation counting the method of choice.

In metabolism studies, it is often desirable to measure oxygen uptake and $C^{14}O_2$ production simultaneously. However, the methanolic Hyamine hydroxide commonly employed to trap the evolved $C^{14}O_2$ for liquid scintillation counting is toxic toward most biological systems. Consequently, it is necessary to add the organic base at the end of the incubation period, eliminating the possibility of simultaneous measurement of oxygen uptake. Thus, Snyder and Godfrey (1) employed a Warburg flask with a removable center well containing Hyamine injected through a rubber cap upon termination of the incubation. Wakil (2) moistened a paper strip suspended in the Warburg flask with methanolic Hyamine at the end of the incubation period. Passmann *et al.* (3) eliminated this difficulty by trapping the respiratory CO_2 in NaOH and then, after acidification, retrapping in Hyamine hydroxide. Although these procedures are fairly satisfactory, it is difficult to transfer quantitatively the viscous Hyamine hydroxide to the counting vials without specially constructed Warburg flasks (1, 4). Recently, mention was made of a technique by Mirsky (5) for trapping $C^{14}O_2$ on papers moistened with NaOH (injected through a vial seal into the conventional Warburg center well). The strips then were removed, dried, and counted in the liquid scintillation counter.

The present communication describes a simple procedure for trapping the respiratory $C^{14}O_2$ on KOH-moistened paper in the Warburg center well in a manner which permits simultaneous measurement of oxygen uptake and subsequent liquid scintillation counting of the resulting

moist paper strip. This procedure eliminates the cumbersome drying of papers and injection of alkali apparently employed by Mirsky (5).

METHODS

The main compartment of the conventional Warburg vessel contained the tissue sample or enzyme preparation in a suitable buffer along with the C^{14}-labeled substrate. The flasks were incubated for an appropriate period (0.5–4 hr) at 37°C using 120 oscillations per minute. Following incubation, the enzymic processes were terminated by addition of 0.2 ml 10 N H_2SO_4 from a side arm and the evolved CO_2 trapped in the center well which contained 0.1 ml of 10% CO_2-free KOH and a 2×2 cm piece of fluted Whatman No. 1 filter paper. With this volume of KOH, the base was almost entirely absorbed by the filter paper.

After shaking 1 hr to allow equilibration, moist papers were removed carefully from the center wells and dropped directly into counting vials containing 10 ml of scintillator solution made up of 2 parts toluene and 1 part absolute ethanol and containing 0.4% 2,5-diphenyloxazole (PPO) and 0.01% 1,4-bis-2-(5-phenyloxazolyl)benzene (POPOP). Subsequent investigation indicated that diotol [toluene-dioxane-methanol-naphthalene (350:350:210:73), 0.46% PPO, 0.008% POPOP] gave slightly greater counting efficiencies (Table 3).

Counting was performed in a Tri-Carb Model 314X liquid scintillation spectrometer (Packard Instrument Co.) at −8°C operating at 980 volts with channel settings of 6–70 and 70–100. No attempt was made to maintain reproducible geometry of the papers; however, the papers inevitably lay flat at the bottom of the counting vials.

RESULTS AND DISCUSSION

To test the reliability of the trapping and counting technique, aliquots of standard $Na_2C^{14}O_3$ [1] were pipetted into the main compartments of duplicate Warburg flasks and carrier Na_2CO_3 added to bring the total Na_2CO_3 content to 2.5 mg (equivalent to 528 μl of CO_2, the maximum CO_2 evolution to be expected during a typical Warburg run). Water then was added to bring the total volume to 3 ml. After incubation, acid was tipped into the flasks and the released $C^{14}O_2$ trapped on the KOH-paper in the center well. Table 1 demonstrates that the recovery of $C^{14}O_2$ was constant over a wide range of activities. Similar studies carried out at lower Na_2CO_3 concentrations gave comparable results.

When the toluene-ethanol counting solution was carefully decanted from the papers and recounted in fresh vials, it was found to be essentially nonradioactive. This indicated that the trapped $K_2C^{14}O_3$ was still

[1] Purchased from Nuclear-Chicago Co. and containing 2.30×10^5 dpm/mg $Na_2C^{14}O_3$.

TABLE 1
LIQUID SCINTILLATION COUNTING OF $C^{14}O_2$ RADIOACTIVITY FROM WARBURG FLASKS CONTAINING STANDARD $Na_2C^{14}O_3$[a]

Initial radioactivity/flask (dpm)	Net counting rate (cpm)[b]	Counting efficiency (%)
105	38.8	36.9
525	216	41.1
1,050	428	40.8
5,250	1,940	37.0
10,500	4,252	40.5
21,000	8,817	42.0
36,800	14,883	40.5
52,500	21,257	40.5
	Average	39.9

[a] Total Na_2CO_3 adjusted to average 2.5 mg/flask.
[b] Average of duplicate flasks.

largely absorbed by the paper and was not eluted appreciably by the counting solution. Addition of 0.1-ml aliquots of water to each vial did not affect materially the observed counting rate.

The absorption of $K_2C^{14}O_3$ on the KOH-papers accounted for the decreased counting efficiency (39.9%) observed in the present studies as compared to the normal 61% (determined by internal toluene standard) usually obtained for soluble C^{14}-labeled materials in this counting solution. In fact, the observed 33% reduction in counting efficiency is very similar to that observed by Wang and Jones (6) when counting C^{14}-labeled compounds uneluted from paper chromatograms by the scintillator solution.

Although no attempt was made to control the position of the papers in the present study, the normal horizontal orientation assumed by the papers at the bottom of the vials apparently gave a satisfactory geometry in view of the observed reproducibility of counting efficiencies and replicate counts. Davidson (7), measuring hypoxanthine-C^{14} on paper, has observed that a horizontal orientation of papers gave a 40% absolute counting efficiency with a significantly smaller average variation for repetitive counts than did an orientation perpendicular to the bottom of the vials.

The recovery of $C^{14}O_2$ radioactivity was independent of the amount of carbon dioxide in the Warburg flask, as may be seen in Table 2. The straight-line relationship between the quantity of CO_2 liberated and the observed counting rate provides an indication that the $C^{14}O_2$ is absorbed quantitatively by the moist KOH-paper.

TABLE 2
EFFECT OF TOTAL CO_2 CONCENTRATION ON RECOVERY OF RADIOACTIVITY FROM WARBURG FLASKS CONTAINING STANDARD $Na_2C^{14}O_3$
(Initial radioactivity = 10,500 dpm)

Total Na_2CO_3/flask (mg)	Net counting rate[a] (cpm)	Counting efficiency (%)
0.050	3712	36.2
0.300	4072	38.8
0.500	3912	37.2
1.000	4154	39.6
2.000	3972	37.8
3.000	4154	39.6
5.000	3982	37.9

[a] Average of duplicate flasks.

Originally, the center wells of the Warburg flasks were rinsed with two 0.5-ml portions of toluene-ethanol counting solution and the washings transferred to the appropriate vials containing the KOH-papers. Subsequent investigation demonstrated that the washing step could be eliminated with no appreciable loss in recovery (Table 3). The re-

TABLE 3
LIQUID SCINTILLATION COUNTING OF $C^{14}O_2$ RADIOACTIVITY FROM WARBURG FLASKS UNDER VARIOUS COUNTING CONDITIONS

Scintillator solution	Comments	Net counting rate[a,b] (cpm)	Counting efficiency
Toluene-ethanol[c]	Center well washed twice with fresh toluene-ethanol	4258	40.6
Toluene-ethanol[c]	Paper alone (no center well washing)	4150	39.5
Diotol[d]	Center well washed twice with fresh diotol	4922	46.9
Toluene[e]	Center well washed twice with fresh toluene	586	5.6
Toluene[e]	Center well washed twice with fresh toluene; paper dried before adding scintillator solution	4744	45.1

[a] Average of triplicate flasks.

[b] Each flask contained 1.04×10^4 dpm $Na_2C^{14}O_3$ and a total Na_2CO_3 content of 2.5 mg.

[c] Toluene-ethanol (2:1), 0.4% PPO, 0.01% POPOP.

[d] Toluene-dioxane-methanol-naphthalene (350:350:210:73), 0.46% PPO, 0.008% POPOP.

[e] Toluene, 0.4% PPO, 0.01% POPOP.

producible recoveries observed under a variety of CO_2 concentrations and radioactivities (Tables 1 and 2) suggest that most of the $C^{14}O_2$ was indeed absorbed directly by the KOH-paper and that negligible $C^{14}O_2$ losses occurred in the well itself.

When several common scintillator solutions were compared, diotol was found to give the highest counting efficiency (Table 3).

When the moist KOH-papers were counted in a scintillator solution containing toluene, 0.4% PPO, and 0.01% POPOP, only 5.6% of the radioactivity initially present in the Warburg flask was detected (Table 3). However, if the strips were first dried *in vacuo* and then counted in the same counting solution, the counting efficiency increased to 45.1%. This suggests that the water on the paper strip either prevented complete penetration of the scintillator solution or else produced a very high localized quenching effect when the papers were counted in scintillator solutions that did not remove water from the paper. If counting solutions such as toluene-ethanol or diotol were employed, the water was dissolved in the counting solution and much higher counting efficiencies were obtained.

The present studies demonstrate that $C^{14}O_2$ can be trapped effectively on paper moistened with KOH and the wet papers then counted directly in the liquid scintillation counter without further processing. The procedure is simple, rapid, and quite reproducible, and results in high counting efficiencies.

SUMMARY

Radioactive carbon dioxide can be trapped readily in Warburg vessels on paper moistened with KOH and the damp papers counted directly without further manipulation in the liquid scintillation counter in a reproducible manner with high counting efficiencies.

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ANALYTICAL BIOCHEMISTRY 4, 418–421 (1962)

SHORT COMMUNICATIONS

A Simple Technique for Scanning Thin Layer Chromatograms

Although thin-layer chromatography offers several advantages as a means of separating mixtures of biological compounds, its application to tracer studies is hampered by the lack of a convenient method for detecting closely adjacent labeled substances on the developed chromatograms. The following technique enables such chromatograms to be scanned with an efficiency and resolution comparable to that obtained using paper chromatograms.

The method involves an adaptation of a procedure described for removal and preservation of thin-layer chromatograms (1). The developed chromatogram is sprayed thoroughly with Neatan, an aqueous dispersion of polyvinyl propionate developed by E. Merck (Germany) and distributed in the United States by Brinkmann Instruments, Inc. The saturated layer is dried for a few minutes with the aid of a hair dryer and then a piece of colorless transparent tape (1) is applied firmly to the surface. If the chromatogram is subjected to prolonged drying (for example, by heating at 105°C in an oven) it must be immersed in water before the chromatographic material can be removed cleanly from the glass plate. This procedure is satisfactory only for water-insoluble compounds. If not overdried the adsorbent can be readily peeled off along with the tape.

At this stage the chromatogram is unsuitable for scanning owing to the loss during handling of small particles of adsorbent which contaminate the scanner. This problem can be obviated by a light application of Neatan to the underside of the chromatogram, with subsequent drying. The resulting chromatogram is completely dust-free, the emulsion surface having the appearance and texture of filter paper. Strips may be prepared for scanning by use of a sharp knife, cutter, or scissors. The scanning technique is identical to that used for paper chromatograms. For weak emitters like carbon-14, however, only 2π geometry is obtain-

able, the emissions on one side being absorbed by the adhesive tape. It is therefore necessary to place the strip in the scanner with the emulsion side adjacent to the detector. The scanning sensitivity with respect to carbon-14 has been found to be at least 80% of that obtained under comparable conditions using untreated No. 1 Whatman paper. A sample scanogram is shown in Fig. 1.

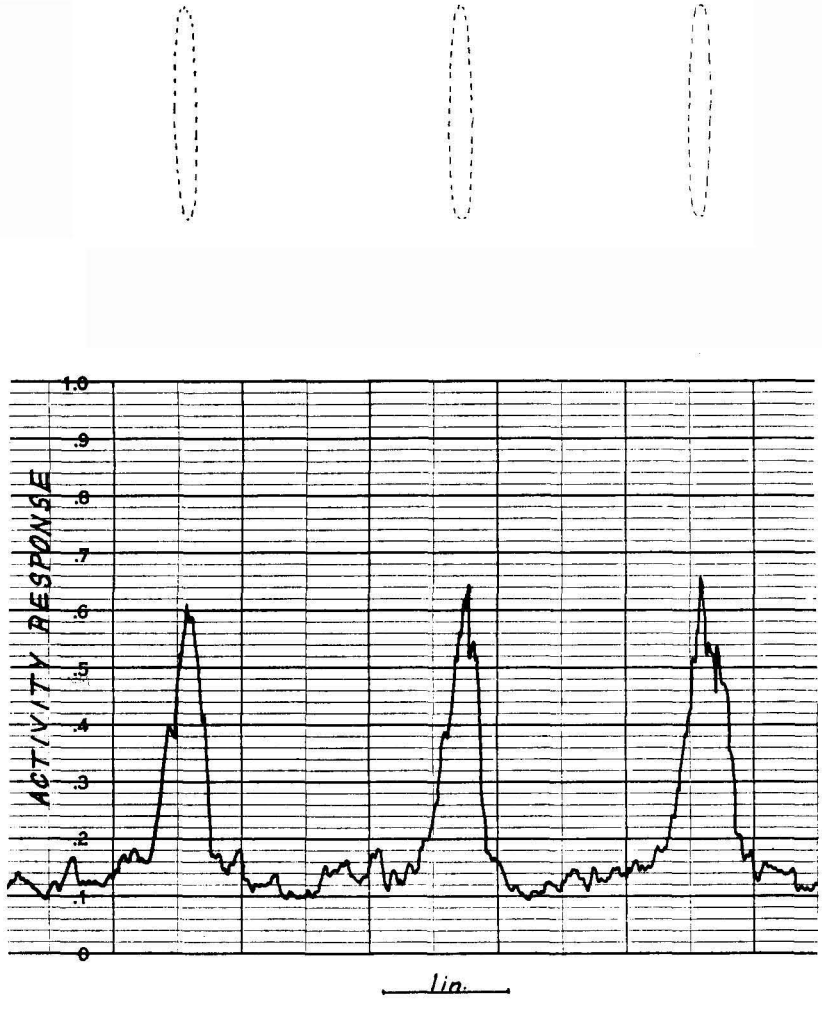

FIG. 1. Photograph of scanogram obtained from a thin-layer strip prepared by the technique described. Total activity 5500 disintegrations per minute. Scanned in Vanguard Model 800 Autoscanner, time constant *D*, range 100, medium slit width, chart speed 6 in./hr, 2π geometry. Chromatogram attached to top of chart paper.

ACKNOWLEDGMENT

This investigation was carried out with the aid of a grant from the National Science Foundation (G-21374).

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A. Saari Csallany
H. H. Draper

Division of Animal Nutrition
University of Illinois
Urbana, Illinois
Received July 19, 1962

Citrate Interference in Inorganic Phosphate Analysis[1]

The determination of inorganic phosphate is a routine analysis in biological investigations. A standard optical procedure is the Fiske-SubbaRow method (1), which is based on the formation first of phosphomolybdate followed by reduction to a blue color. We have recently found that the presence of citric acid interferes with the formation of phosphomolybdic acid and consequently the observed values for phosphate are lower than the actual values.

The Fiske-SubbaRow method for determining inorganic phosphate was carried out on known quantities of phosphate with varying amounts of citrate. Reduction was generally accomplished with 1,2,4-aminonaphthol-sulfuric acid in a sulfite-bisulfite solution; however, other reducing agents gave the same results. The results obtained are given in Table 1.

The effect of the order of addition of citrate was also investigated; these results are given in Table 2. The additions were 100 μmoles of citrate. All optical density measurements were made at 6660 A in a Beckman DU spectrophotometer.

The results in Table 1 show that citrate interferes with the determination of phosphate and, at relatively high concentrations, citrate

[1] This investigation was aided by grants from the National Multiple Sclerosis Society and The U. S. Public Health Service.

TABLE 1
INFLUENCE OF CITRATE ON OPTICAL DENSITY

Pi, μmole	Citrate, μmole	OD
0.50	0.0	0.560
0.50	12.5	0.492
0.50	25.0	0.482
0.50	50.0	0.470
0.50	75.0	0.085
0.50	100.0	0.000

TABLE 2
INFLUENCE OF ORDER OF CITRATE ADDITION

Pi μmole	Citrate addition	OD
0.50	No citrate	0.560
0.50	After color development	0.560
0.50	After NH_4 molybdate	0.560
0.50	Before NH_4 molybdate	0.000

completely obscures the presence of phosphate. Order of addition studies indicate that citrate interferes with the formation of phosphomolybdic acid. This was confirmed by the absence of the characteristic yellow hue of phosphomolybdic acid and the absence of absorption in the 3600–4600 A region which phosphomolybdic acid solutions exhibit.

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AMEDEO F. D'ADAMO, JR.
JOHN R. BROICH

Department of Biochemistry
Seton Hall College of Medicine
Jersey City, New Jersey
Received July 29, 1962

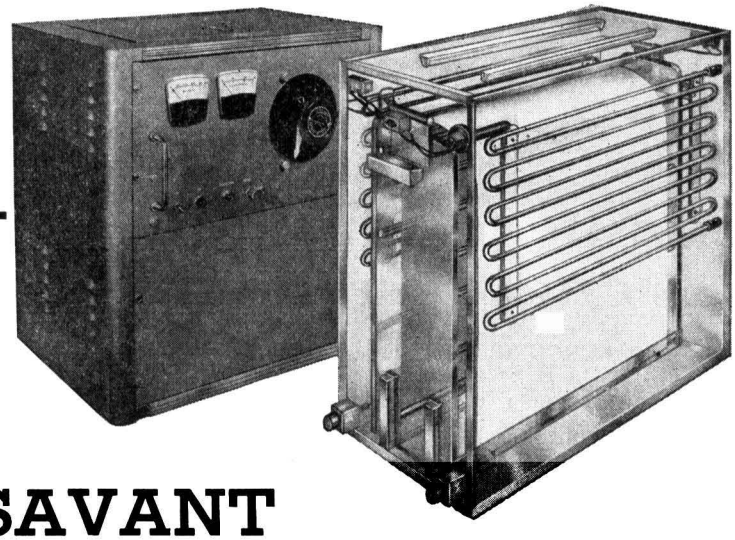
Errata

D. Watson, "Enzymic Determination of Glucose and Easily Hydrolyzable Glucose Esters in Blood." *Anal. Biochem.* **3**, 131–144 (1962).

PAGE *140:* line 2 from the top, delete "1 vol."
line 5 from the top, "215 ml Fermcozyme" should read "2.5 ml Fermcozyme."

Shlomo Burstein and Howard L. Kimball, "Quantitative Paper Chromatography of $C_{21}O_5$ and $C_{21}O_6$ Corticosteroids from Guinea Pig Urine Extracts." *Anal. Biochem.* **4**, 132–142 (1962).

PAGE *133:* line 15 from the top, "1% solution of BT" should read "0.1% solution of BT."



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ANALYTICAL BIOCHEMISTRY 4, 423–432 (1962)

Gas Chromatography of Urinary Aromatic Acids

CLYDE M. WILLIAMS

From the Department of Radiology, University of Florida College of Medicine, Gainesville, Florida[1]

Received February 13, 1962

INTRODUCTION

The physiologically important aromatic acids found in urine, after concentration by ethyl acetate extraction from acidic solution, may be separated and identified adequately by well-established techniques of two-dimensional paper chromatography [(1) phenolic acids, (2) indolic acids], but their quantitative determination by this method is laborious and time consuming. It is also difficult to determine more than one acid at a time by this method. Other techniques for the determination of such compounds as 5-hydroxyindoleacetic acid and 3-methoxy-4-hydroxymandelic acid, involving solvent extraction and colorimetric or fluorometric estimation, are subject to errors arising from the interference of closely related urinary compounds which may not be adequately separated by the solvent extraction. In the case of other acidic metabolites such as phenylacetic acid, no satisfactory method of measurement has yet been devised. The feasibility of separating and determining quantitatively many of the closely related aromatic acids in a single sample by gas-liquid partition chromatography using polar and nonpolar liquid stationary phases was demonstrated recently (3, 4). The following investigation was undertaken to determine optimal conditions for the separation and qualitative identification of some of the physiologically important indolic, phenolic, and other aromatic acids found in urine.

METHOD

A Barber-Colman Model 10 gas chromatographic unit containing an argon ionization detector with a high-temperature foil of 50 μc of Ra^{226} as the radioactive source was used. The temperature of the cell was maintained about 25°C above the column operating temperature to prevent condensation of the sample vapor and "bleeding" from the

[1] Supported by grants from the American Heart Association (61 G 57) and the National Institutes of Health (B-3221) and an Institutional Grant from the American Cancer Society (1N-62).

column. The inlet pressure of the column was maintained at pressures varying from 18 to 45 psi of argon. Outflow rates were measured with a soap bubble flowmeter. The flash heater was maintained at about 300°C to ensure complete and immediate volatilization of the injected sample. Column operating temperatures varied from 170° to 225°C. The columns were U-shaped borosilicate glass tubing with an internal diameter of 6 mm and an over-all length of 6 ft. They were packed with stationary liquid phases coated on acid-washed Chromosorb W in mesh size 80–100 obtained from Applied Science Laboratories, Inc., State College, Pa., or Analabs, Hamden, Connecticut. The columns were packed by gentle vibration and conditioned at optimal operating temperatures and pressure for at least 12 hr prior to use. The aromatic acid standards were obtained from commercial and private sources and were not purified further. The source of each acid is indicated in Table 1.

Because of the low vapor pressure of the free acids, it was necessary to prepare suitably volatile derivatives. Aromatic carboxylic acids react with an ethereal solution of diazomethane to form the methyl ester preferentially. The diazomethane reagent (5) is prepared by slowly adding 20 gm of *N*-methyl-N^1-nitro-N^1-nitrosoguanidine (Aldrich Chemical Company, Milwaukee) to a 1-liter Erlenmeyer flask containing 400 ml of 20% KOH and 400 ml of ether, the whole mixture being chilled in an ice bath. After complete evolution of the gas into the ether phase, the two layers are separated by a separatory funnel and the ethereal solution of diazomethane is distilled, caution being taken not to let the ether in the distillation flask go dry. The diazomethane ether solution is collected in an Erlenmeyer flask in an ice bath and is stored over anhydrous sodium sulfate or potassium hydroxide. *Both of these operations must be carried out in a hood because of the toxicity of inhaled diazomethane.* The diazomethane solution may be stored at —4°C for several weeks satisfactorily. The reaction of the diazomethane solution with aromatic acid standards is complete when the evolution of nitrogen bubbles ceases (ordinarily a few minutes); if the solution becomes colorless before this time, another 1 ml of the diazomethane solution is added so that, when the reaction is complete, a faint yellow color persists. Ethyl acetate extracts of urine containing aromatic acids (1) are best methylated before evaporating to dryness and in this case the methylation is carried out for 15 min at room temperature. The mixture is then evaporated to dryness with a stream of nitrogen in a hood. In the case of phenolic acids, the *O*-methyl derivatives are formed in small yield (1–5%) when the above procedure is carried out. Formation of the (methyl) esterified *O*-methyl phenolic ether is carried out by treatment

of the phenolic acid with the diazomethane reagent and methanol (1:1, v/v) overnight at room temperature in a hood (6).

The most suitable solvent for injection of the standards was found to be methanol or ethyl ether. In the case of ethyl acetate extracts of urine, the oily residue following methylation did not always dissolve in methanol or ethyl ether alone but did dissolve in a mixture of the two (1:1, v/v). The injections were carried out with a 10-μl syringe equipped with a Chaney adapter (Hamilton Company). The needle was always kept within the septum for at least 5 sec. Quantitative conversion to the derivatives was carried out by treating 100–200 mg samples with diazomethane or diazomethane-methanol overnight as described. The samples were then dried to constant weight by evaporation under a stream of nitrogen at room temperature. The weights of the derivatives ranged from 96–102% of the theoretical yield, with the exception of protocatechuic, homoprotocatechuic, and 3,4-hydroxymandelic acid (see Table 1). Reproducibility of the mass response for the other derivatives by these techniques was $\pm 2\%$ for samples containing 1–20 μg.

RESULTS AND DISCUSSION

The results are summarized in Table 1. Retention times of the derivatives are referred to the methyl ester of hippuric acid which was chosen because it is found in relatively large quantities in most specimens of normal urine and is therefore easily identified. Three polar columns were examined in detail: 3% neopentyl glycol adipate, 3% neopentyl glycol succinate, and 3% neopentyl glycol sebacate. The relative retention times of all the aromatic acids examined were essentially the same on these three polar columns at comparable operating conditions and for this reason relative retention times are given for only one polar column (adipate).

Veratric acid, after reaction with ethereal diazomethane, or protocatechuic acid, vanillic acid, and veratric acid after reaction with diazomethane-methanol, all yielded a common product with a peak having a relative retention time of 0.22 on the polar adipate column. The identity of the common derivative was established by infrared analysis as methyl 3,4-dimethoxybenzoate. After reaction with ethereal diazomethane, vanillic acid yielded a product with a single peak having a relative retention time of 0.29. A sample of authentic methyl 3-methoxy-4-hydroxybenzoate (Bios Laboratories Inc.) had a relative retention time of 0.29. On this column methyl 3-methoxy-4-hydroxybenzoate would be expected to have a longer retention time than methyl 3,4-dimethoxybenzoate because of its greater polarity due to the phenolic

TABLE 1

RELATIVE RETENTION TIMES OF METHYLATED DERIVATIVES OF AROMATIC ACIDS ON POLAR AND NONPOLAR COLUMNS

Aromatic acid	3% adipate[a]		2% silicone[b]	
	Diazomethane	Diazomethane-methanol	Diazomethane	Diazomethane-methanol
Hippuric(a)	1.00	1.00	1.00	1.00
Phenylacetic(b)	0.02	0.02	0.14	0.14
o-Hydroxyphenylacetic(c)	0.07	0.07	0.18	0.29
m-Hydroxyphenylacetic(c)	0.60	0.09	0.43	0.32
p-Hydroxyphenylacetic(c)	0.60	0.10	0.45	0.35
3,4-Dihydroxyphenylacetic (homoprotocatechuic)(a)	0.54(1)	0.28	0.61(4)	0.69
3-Methoxy-4-hydroxyphenylacetic (homovanillic)(a)	0.37	0.28	0.61	0.69
3,4-Dimethoxyphenylacetic (homoveratric)(b)	0.28	0.28	0.69	0.69
Benzoic(d)	0.02	0.02	0.10	0.10
o-Hydroxybenzoic (salicylic)(d)	0.03	0.06	0.15	0.25
m-Hydroxybenzoic(b)	0.49	0.06	0.34	0.24
p-Hydroxybenzoic(b)	0.64	0.08	0.38	0.28
3,4-Dihydroxybenzoic (protocatechuic)(a)	0.47(2)	0.22	0.61(5)	0.63
3-Methoxy-4-hydroxybenzoic (vanillic)(a)	0.29	0.22	0.49	0.63
3,4-Dimethoxybenzoic (veratric)(a)	0.22	0.22	0.63	0.63
p-Hydroxyphenylpyruvic(h)	0.62	0.53	1.15	1.48
Phenylpropionic (hydrocinnamic)(b)	0.04	0.04	0.19	0.19
p-Hydroxyphenylpropionic(c)	0.77	0.13	0.63	0.52
β-Phenyllactic(e)	0.11	0.11	0.30	0.30
p-Hydroxyphenyllactic(e)	2.37	0.41	1.09	0.78
Mandelic(d)	0.09	0.09	0.20	0.20
p-Hydroxymandelic(f)	0.42	0.35	0.82	0.56
3,4-Dihydroxymandelic(a)	1.85(3)	0.94	1.15(6)	1.15
3-Methoxy-4-hydroxymandelic (vanilmandelic)(a)(g)	1.31	0.94	0.98	1.15
Indoleacetic(h)	2.16	2.16	1.52	1.52
Indolepropionic(b)	2.83	2.83	2.23	2.23
Indolelactic(h)	>3.00	>3.00	3.56	3.56
5-Hydroxyindoleacetic(i)	>3.00	>3.00	5.06	3.81

(a) California Foundation for Biochemical Research. (b) Distillation Products, Inc. (c) Aldrich Chemical Co., Milwaukee. (d) Fisher Scientific Co. (e) Mann Research Laboratories. (f) Gift from Dr. K. N. F. Shaw. (g) Nutritional Biochemicals Corp., Cleveland. (h) Sigma Chemical Corp. (i) Regis Chemical Co., Chicago.

[a] Al neopentyl glycol adipate 3% on acid-washed Chromosorb W 80-100 mesh (Analabs). Argon inlet pressure 30 Outlet psi. flow 80 ml/min. Column temperature 187°C. Approximate retention time of methyl ester of hippuric acid 60 min.

(1) Major peak 44%, minor peaks at 0.37 (40%) and 0.28 (15%). (2) Major peak 75% minor peaks at 0.29 (15%) and 0.22 (10%). (3) Major peak 55%, minor peaks at 0.94 (37%) and 1.31 (8%). (4) Major peak 50%, several minor peaks (decomposition products). (5) Major peak 80%, minor peak at 0.49 (20%). (6) Major peak 70%, minor peak 0.98 (30%).

[b] Silicone (SE-30) 2% on 80-100 mesh Chromosorb P (Applied Science Laboratories). Argon inlet pressure 20 psi. Outlet flow 75 ml/min. Column temperature 180°C. Approximate retention time of methyl ester of hippuric acid 6.5 min.

group. This difference is undoubtedly analogous to the significant decrease in retention time of hydroxy steroids caused by *O*-methyl ether formation and is attributed partly to decrease in polarity and partly to an increase in volatility of the compound (7). The reaction of protocatechuic acid with diazomethane gave a mixture of three products, the relative retention times of which were 0.47, 0.29, and 0.22. The 0.47 peak is the relatively more polar methyl 3,4-dihydroxybenzoate. Apparently the presence of 2 adjacent hydroxyl groups on the benzene ring favors *O*-methyl formation and repeated attempts to obtain a single product, the methyl 3,4-dihydroxybenzoate derivative, were unsuccessful. A mixture of three products by diazomethane methylation was also observed for homoprotocatechuic acid and 3,4-dihydroxymandelic acid on the polar column. The identity of the common product obtained by the reaction with diazomethane-methanol of homoprotocatechuic, homovanillic, and homoveratric acids was established by comparison of their infrared spectra. A similar confirmation was made for the results of diazomethane-methanol methylation of 3,4-dihydroxymandelic acid and vanilmandelic acid. Following reaction of mandelic acid, vanilmandelic acid and 3,4-dihydroxymandelic acid with diazomethane-methanol, a free hydroxyl absorption band at 3600 cm^{-1} in the infrared remains, indicating that the hydroxyl group on the side chain remains unmethylated by this procedure.

Methylation of the carboxylic acid group in aromatic acids without *O*-methyl ether formation from phenolic acid hydroxyls was also carried out by reaction with 1 ml of BF_3-methanol reagent (125 gm/liter, Applied Science Laboratories, Inc.) (8) for 1 hr at room temperature, at which time the reaction was complete. No evidence was obtained for methylation of phenolic hydroxyl groups with this reagent, but, in the presence of dihydroxyphenolic aromatic acids, dark brown pigments (presumably polymerization products) are found which do not yield identifiable peaks on the columns used. Attempts to methylate ethyl acetate extracts of urine with this reagent have been generally unsuccessful.

For aromatic acids with a retention time between veratric and hippuric, the 3% adipate column is quite satisfactory. Figure 1 shows two chromatographs of a standard mixture of aromatic acids. The upper graph shows a mixture treated with ethereal diazomethane for 15 min at room temperature. Of this group, only *p*-hydroxyphenylacetic and *p*-hydroxyphenylpyruvic cannot be distinguished from one another. Another aliquot of the same mixture of aromatic acids treated with diazomethane and methanol gave the results shown on the lower part of Fig. 1. Hippuric, veratric, and homoveratric are unchanged in reten-

tion times by this treatment. Vanilmandelic is converted from a derivative with a relative retention time of 1.31 to one with a relative retention time of 0.94. Homovanillic acid is now found in the homoveratric peak

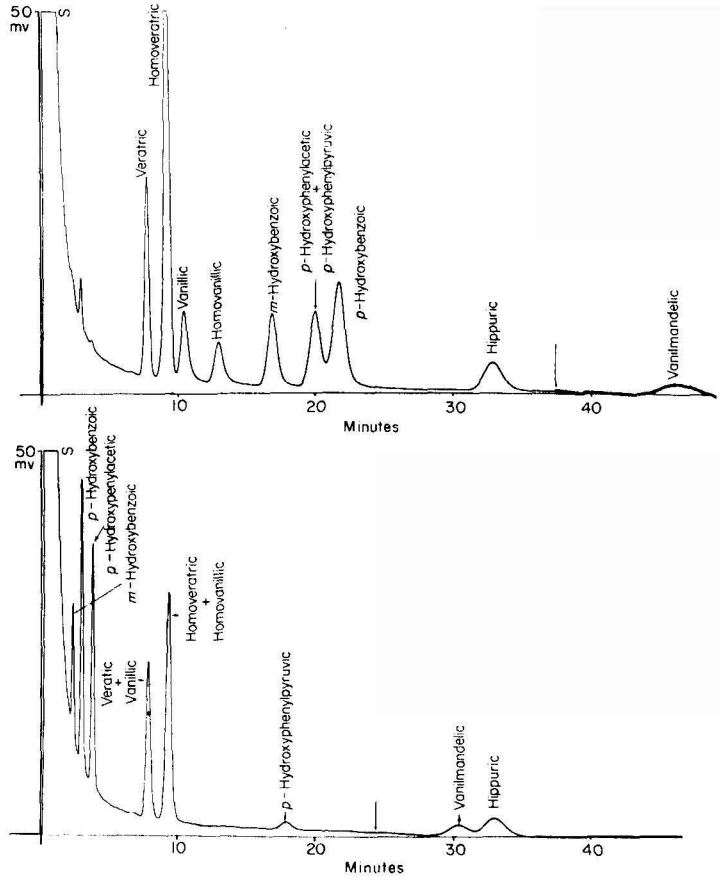

FIG. 1. Analysis of standard mixture of aromatic acids. 3% neopentyl glycol adipate. Argon inlet pressure 30 psi. Outlet flow 80 ml/min. Column temperature 200°C. Upper record shows the results of diazomethane methylation. Arrow indicates change of relative gain from 3×10^{-6} to 3×10^{-7} amp. Lower record shows the same mixture after reaction with diazomethane-methanol. Arrow indicates change of relative gain from 1×10^{-6} to 3×10^{-6} amp. S = solvent front.

and vanillic acid is now found in the veratric acid peak. *m*-Hydroxybenzoic acid, *p*-hydroxybenzoic acid, and *p*-hydroxyphenylacetic acid are converted to derivatives with very short retention times. Provided that no evidence of homoprotocatechuic acid (relative retention time of

methyl 3,4-dihydroxyphenylacetate derivative 0.54) or protocatechuic acid (relative retention time of methyl 3,4-dihydroxybenzoate derivative 0.47) is observed, homovanillic and vanillic acid may be measured in a sample methylated with diazomethane. *p*-Hydroxyphenylacetic and *p*-hydroxyphenylpyruvic are estimated by diazomethane-methanol methylation. If no *p*-hydroxyphenylpyruvic is observed at a relative retention time of 0.53 the *p*-hydroxyphenylacetic acid may be calculated from the diazomethane graph. Confirmation of the identity of the methyl ester derivative of homovanillic, *m*-hydroxybenzoic, vanillic, *p*-hydroxyben-

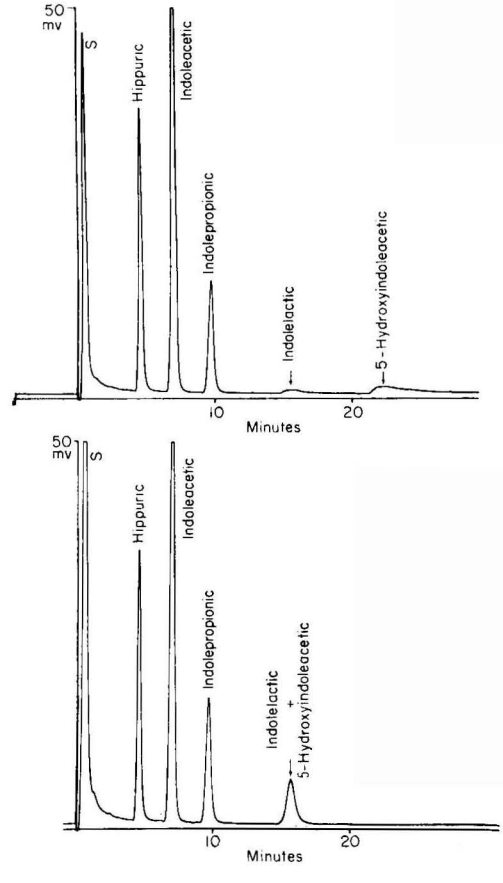

FIG. 2. Analysis of standard mixture of aromatic acids. 2% silicone (SE-30). Argon inlet pressure 20 psi. Outlet flow 70 ml/min. Column temperature 192°C. S = solvent front. Upper record shows results of diazomethane methylation. Lower record shows same mixture after reaction with diazomethane-methanol. Relative gain on both records was 1×10^{-7} amp.

zoic, *p*-hydroxyphenylacetic, and vanilmandelic acids is provided by the conversion to another derivative. In the determination of vanilmandelic acid by this method, the methyl 3,4-dimethoxymandelate derivative with a relative retention time of 0.94 on the polar columns is usually obscured by the very large methyl ester of hippuric acid [approximately 500 μg/mg creatinine (4)]. For this reason, it is necessary to measure it as the methyl 3-methoxy-4-hydroxymandelate with a retention time of 1.31. However, at 180–190° this derivative has a relatively long retention time (90 min) with an inconveniently broad base, so that it is difficult to measure when present in normal amounts (1–2 μg/mg creatinine). For this reason it is convenient to determine this compound at a column temperature of 210–225°C. Other acids such as *p*-hydroxyphenyllactic acid, indoleacetic acid, and *p*-coumaric acid are also conveniently determined at this temperature.

The indolic acids have long retention times on polar columns and it is usually preferable to chromatograph these on nonpolar columns. Figure 2 shows two chromatographs of a standard mixture of five aromatic acids, four of which contain an indole nucleus. The upper graph shows a mixture treated with diazomethane for 15 min at room temperature. All the acids are clearly distinguished from one another on this graph. Another aliquot was treated with diazomethane and methanol overnight and the result is seen on the lower graph. The peaks are the same with the exception of methyl 5-hydroxyindoleacetate, which has been converted to the methyl 5-methoxyindoleacetate derivative having a retention time overlapping that of methyl indolelactate. There is considerable trailing of methyl 5-hydroxyindoleacetate on the nonpolar column and it is preferable to measure this as the esterified *O*-methyl ether if no indolelactic acid is present in the sample. For those acids with short retention times such as phenylacetic, *O*-hydroxyphenylacetic, and phenyllactic, separation is best carried out at a lower temperature with a heavier coating of the stationary liquid phase. Table 2 gives retention times relative to homoveratric acid for a number of aromatic acids on such a column. Such acids as phenylpyruvic and phenyllactic which are so close together as to be indistinguishable on a 3% adipate column at 187°C may be adequately separated at 170°C on an 8% adipate column. Figure 3 illustrates this with a mixture of standards. Of the many aromatic acids in urine in variable amounts some might well have relative retention times similar to the compounds described. For this reason it is important to identify any aromatic acid found in the urine with as many derivatives as possible on at least two columns.

Two technical improvements would simplify the analysis. It would be preferable as well as more economical to determine most of the acids

TABLE 2
RELATIVE RETENTION TIMES OF METHYLATED DERIVATIVES OF AROMATIC ACIDS ON LOW TEMPERATURE POLAR COLUMN[a]

Aromatic Acid	Diazomethane	Diazomethane-methanol
Phenylacetic	0.06	0.06
Phenylpropionic	0.09	0.09
m-Hydroxybenzoic	>1.00	0.16
p-Hydroxybenzoic	>1.00	0.22
o-Hydroxyphenylacetic	0.24	0.20
m-Hydroxyphenylacetic	>1.00	0.27
Mandelic	0.28	0.28
p-Hydroxyphenylacetic	>1.00	0.31
Phenylpyruvic[b]	0.33	0.33
Phenyllactic	0.36	0.36
Veratric	0.80	0.80
Vanillic	>1.00	0.80
p-Hydroxyphenylpropionic	>1.00	0.41
Homoveratric	1.00	1.00
Homovanillic	>1.00	1.00

[a] 8% ethylene glycol adipate on acid washed Chromosorb W 80-100 mesh (Applied Science Laboratories). Argon inlet pressure 30 psi. Outlet flow 75 ml/min. Column temperature 170°. Approximate retention time of methyl homoveratrate 45 min.

[b] Mann Research Laboratories.

described in a temperature-programed run instead of the isothermal runs, thus clearing the column of long retention time compounds which might interfere with subsequent runs. The determination of acids such as protocatechuic, homoprotocatechuic, and 3,4-dihydroxymandelic by this method is unsatisfactory from an analytical standpoint because of the

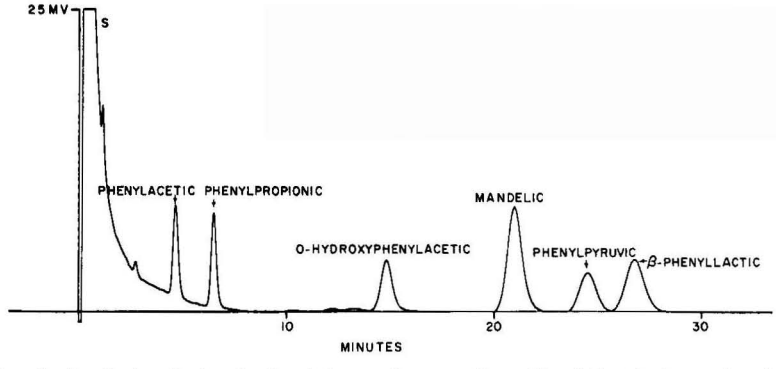

FIG. 3. Analysis of standard mixture of aromatic acids. 8% ethylene glycol adipate. Argon inlet pressure 12 psi. Outlet flow 100 ml/min. Column temperature 170°C. S = solvent front. Relative gain was 1×10^{-7} amp.

conversion by diazomethane to a mixture of three derivatives. Investigation of the above problem as well as attempts to produce a single methyl ester derivative of these acids is in progress.

SUMMARY

A method is described for the separation and identification of 29 aromatic acids by gas chromatography. Aromatic acids without phenolic groups are converted to their methyl esters by reaction with ethereal diazomethane and chromatographed on an 8% ethylene glycol adipate column at 170°C. Phenolic acids are converted to their methyl ester derivatives by ethereal diazomethane and chromatographed on a 3% neopentyl glycol adipate column at 187°C. When other compounds in the urinary extracts overlap with these peaks, a second derivative, the methyl esterified *O*-methyl ether is prepared by reaction with diazomethane and methanol overnight, and chromatographed on the same column. Indolic acids are converted to their methyl esters with diazomethane and chromatographed on a 2% silicone (SE-30) column at 180°C. When the indolic hydroxyl groups are present, as in 5-hydroxyindoleacetic acid, the methyl esterified *O*-methyl ether derivative may be prepared if necessary and chromatographed at the same temperature. In certain cases such as vanilmandelic acid, the preferred derivative has an inconveniently long retention time at 187°C on the polar column and an inconveniently short retention time at 180°C on the nonpolar column. This derivative may best be determined at a higher temperature (210–225°) on the polar column.

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Simultaneous Determination of Glycerol, Inositol, Serine and Ethanolamine in a Phospholipid Hydrolyzate[1]

L. W. WHEELDON, M. BRINLEY, AND D. A. TURNER

From the Biochemistry Research Division, Department of Medicine, Sinai Hospital of Baltimore, Inc., Baltimore, Maryland

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INTRODUCTION

Standard procedures for the analysis of phospholipids require a separate sample to be taken for each of the constituents analyzed, not the least because conditions of hydrolysis differ for the quantitative recovery of, for example, inositol as compared with ethanolamine or serine (1–3). By the use of silicic acid chromatography it is generally a simple matter to separate without loss (4) the choline-containing and non-choline-containing classes of phospholipid. The non-choline phospholipids include the polyglycerol phospholipids, aminophospholipids, and phosphatidyl inositols and these can be completely resolved only with difficulty, if at all (4–6). Consequently there is a need for a sensitive analytical procedure for checking the composition of "cephalin" fractions obtained by silicic acid chromatography of mixed phospholipids. While the present procedure was designed specifically for use in conjunction with silicic acid column chromatography, it is sufficiently sensitive to be applied to the identification of cephalin fractions obtained by thin-layer chromatography.

The main steps in the method are as follows: *1.* Removal of fatty acids by brief acid hydrolysis, followed by hydrolysis of resulting phosphoric esters with alkaline phosphatase. *2.* Paper chromatography of the hydrolyzate. *3.* Elution of the hydrolyzate constituents and determination by reaction with periodate. In the case of serine, ethanolamine, and glycerol, formaldehyde formed by periodate oxidation was estimated with the chromotropic reagent; inositol was estimated from periodate consumption, measured spectrophotometrically.

REAGENTS AND MATERIALS

Reference Materials

Monomethylol dimethylhydantoin (Glyco Chemicals, Williamsport,

[1] This work was supported by PHS Grant #H-5283.

Pennsylvania) was recrystallized from ethanol until it gave a constant color yield with the chromotropic reagent.

Ethanolamine (Eastman) was distilled to remove water and impurities which caused it to streak on chromatograms.

DL-α-*Dipalmitoyl phosphatidyl ethanolamine* (Mann Research Laboratories) contained 4.58% phosphorus.

Phosphatidyl serine and *phosphoinositide* were prepared from ox brain by the method of Folch (7). The former was further purified on the ammonium silicate column of Rouser *et al.* (8) and the latter by conventional silicic acid chromatography.

Cardiolipin was prepared from ox heart lipids by silicic acid chromatography (5). It was free of amino nitrogen.

Other reference standards were the purest commercially available materials.

Reagents for Hydrolysis

Hydrochloric acid, 0.50 *N*.

Sodium carbonate buffer: 0.1 *M* sodium bicarbonate solution was brought to pH 9.3–9.5 by addition of 0.1 *N* sodium hydroxide.

Alkaline phosphatase: A relatively simple method was used for the preparation of soluble bone phosphatase, incorporating the extraction procedure of Martland and Robison (9) and the butanol treatment of Morton (10). Lamb fore-leg bones were freed of adhering tissue and marrow and broken into small pieces. These were leached in the proportion of 1 kg to 5 liters of water for a period of 7 days at room temperature, with daily addition of a few drops of chloroform and shaking. The extract was poured through a Büchner funnel to remove bone fragments, then filtered through celite. To a 2.5-liter filtrate, 1 liter of *n*-butanol was added with stirring; the temperature was raised to 40°C with constant shaking for 5 min, after which the flask was left to cool at 0°. When the phases separated (2–3 hr), the upper (butanol) layer was siphoned off as completely as possible and the remaining butanol as well as the protein at the interphase removed by centrifugation. A clear yellow aqueous phase was first obtained by filtration through celite and acetone (60%, v/v) and diethyl ether (10%, v/v) were then added. The resulting precipitate was allowed to settle out overnight at 0°, collected by centrifugation, washed with cold acetone (−10°C) and ether, and dried over H_2SO_4 *in vacuo*. The dried protein was finally dispersed in 0.02 *M* $NaHCO_3$ and dialyzed overnight against 5 liters of water containing 0.1 mole $NaHCO_3$ and 0.05 mole $MgCl_2$. The soluble portion of the dialyzed protein was recovered by centrifugation and lyophilized, yielding 1.2 gm soluble phosphatase per kilogram starting material.

Phosphatase solution: A 10% solution of bone phosphatase in sodium carbonate buffer was prepared as needed.

Trichloroacetic acid, 10%.

Phosphorus reagents: The procedure of Bartlett (11) was used for the determination of phosphorus.

Solvents for Chromatography

Isopropyl alcohol, reagent grade, was redistilled from sodium hydroxide.

Acetic acid, certified reagent, was redistilled from potassium dichromate at weekly intervals. Caution must be exercised here since a mild explosion may occur if the distilling flask is allowed to go dry.

Spray Reagents

Reagents (*a*) and (*b*) were prepared essentially according to Cifonelli and Smith (12). (*a*) *Sodium periodate:* Sodium metaperiodate, 0.30 gm, was dissolved in 50 ml of water and added to 50 ml of 0.20 *M* phosphate buffer, pH 7.0. (*b*) *Benzidine:* 10 vol of 0.1 *M* benzidine dihydrochloride in 50% ethyl alcohol was added to 2 vol of acetone.

Ninhydrin: Triketohydrindene hydrate, 0.1 gm, was dissolved in 100 ml of ethyl alcohol.

Eluting Solutions

Sodium bicarbonate, 0.50 *M*.

Sodium phosphate, 0.10 *M*, pH 6.0.

Reagents for Glycerol, Serine, and Ethanolamine

Formaldehyde standard: Monomethylol dimethylhydantoin, 0.158 gm, was dissolved in 500 ml of water and 10 ml of this solution diluted to 100 ml; 1 ml of dilute solution contained 6.00 μg (0.200 μmole) available formaldehyde.

Glycerol standard: The purity of glycerol should be checked by titrimetric assay (13). This standard was diluted in water to contain approximately 0.1 μmole in 0.50 ml.

Sodium periodate: Sodium metaperiodate, 0.21 gm, was dissolved in 10 ml of water immediately before use.

Sodium sulfite: A 10% solution was prepared immediately before use.

Sulfuric acid, 66%.

Chromotropic reagent: 4,5-Dihydroxy-2,7-naphthalenedisulfonic acid, disodium salt, 0.25 gm, was dissolved in 2 ml of water and made to 250 ml with 66% sulfuric acid. This reagent was kept in a dark bottle and stored in a refrigerator.

Thiourea, 4% in water.

Reagents for Inositol

Inositol standard: Mesoinositol dihydrate, 0.108 gm (0.50 mmole) was dissolved in 500 ml of water; 10.0 ml of this solution was diluted to 100 ml with 0.10 *M* sodium phosphate, pH 6.0, to give 0.020 μmole inositol/0.20 ml.

Sodium phosphate, 0.10 *M,* pH 6.0.

Sodium periodate: 0.0030 *M* sodium metaperiodate was freshly prepared by dissolving 0.160 gm of $NaIO_4$ in 250 ml of water; 0.1 ml of this solution, when diluted to 3.0 ml in 0.10 *M* sodium phosphate, pH 6.0, should have an absorbance at 223 mμ in the range 0.95–1.00.

METHODS

Hydrolysis

A sample of the phospholipid solution (in chloroform, for example) containing 2–5 μmoles phosphorus (2–8 mg phospholipid) was taken to dryness in a narrow tube of about 5-ml capacity and tapered to the bottom, using a gentle stream of air or nitrogen so as to concentrate the lipid residue at the bottom of the tube. Two-tenths of 1 ml of 0.50 *N* HCl was pipetted on to the residue and the tube sealed with a glass stopper moistened with octyl alcohol and placed in an oven at 100°C for 3 hr. (Alternatively, 0.50 ml of 0.20 *N* HCl may be added without sealing the tube, provided that care is taken to remove the tubes before they become completely dry.) The hydrolyzates were then gently extracted twice with diethyl ether (2–3 ml) employing a Pasteur pipet to remove the ether phase. The last traces of ether were removed by warming. The tubes were then transferred to a small desiccator containing calcium chloride and placed in an oven at 70°. The hydrolyzates were usually dried overnight. The dry residues were next taken up in 0.050 ml of 10% alkaline phosphatase in sodium carbonate buffer. The pH was checked by placing 2–3 μl on indicator paper and adjusted if necessary with a few microliters of 1 *N* NaOH so as to fall in the range 8.5–9.5. The hydrolyzates were then allowed to stand at room temperature for at least 24 hr. In some cases (phospholipids containing calcium and magnesium), a fine precipitate was deposited which contained phosphorus. Agitation with an eccentrically rotating cork was sufficient to produce a uniform dispersion, from which a 10-μl sample was withdrawn and transferred to a small piece of ashless filter paper, which was then dropped into 2.0 ml of 10% trichloroaceitc acid. After standing 5–10 min, the trichloroacetic acid extract was filtered and 0.50-ml samples of filtrate analyzed separately for inorganic phosphorus and total phosphorus ac-

cording to Bartlett (11). Agreement should be found between the two values, which form a reference basis for the subsequent determination of glycerol, etc.

Paper Chromatography

Three 10-μl samples of the final hydrolyzate were each spotted on a strip (1.5 × 16 in.) of Whatman No. 1 chromatography paper. Chromatograms were run by the descending procedure in a solvent mixture consisting of isopropyl alcohol, acetic acid, and water in the proportions 40:10:5 (v/v) for 16–18 hr under isothermal conditions. Two strips served as marker chromatograms: after drying in air for 30 min one of these was first sprayed very lightly with a fine spray of reagent (*a*), allowed to stand for 7 min, then sprayed with reagent (*b*). Periodate-consuming substances appeared as white spots on a blue background. More precise definition of the serine and ethanolamine areas was obtained by application of the ninhydrin reagent to the second chromatogram, followed by heating at 80–100°C for 3–4 min. By comparison with the marker strips, inositol, glycerol, serine, and ethanolamine were located on the third strip. Blank strips were run with each series of analyses.

TABLE 1
ELUTION OF HYDROLYZATE CONSTITUENTS

Component	Paper area (in.)	Eluent (ml)
Glycerol	2 × 1.5	Water (2.5)
Inositol	1.5 × 1.5	0.1 *M* sodium phosphate, pH 6.0 (1.0)
Serine	1.5 × 1.5	0.5 *M* $NaHCO_3$ (2.0)
Ethanolamine	2 × 1.5	0.5 *M* $NaHCO_3$ (2.5)

Table 1 shows the elution procedures found to be adequate for the quantitative recovery of the chromatogram spots. Each section of the chromatogram was cut into the minimum number of pieces necessary to accommodate it in a 20-mm internal diameter test tube and allowed to stand 30 min before analysis. It was neither necessary nor desirable to agitate or disrupt the paper pieces in order to effect elution.

Analysis of Eluates

Glycerol, serine, and ethanolamine: Duplicate 0.50-ml samples of the eluates, including eluates from the blank strips, were pipetted into glass-stoppered, 10-ml test tubes. To each tube 0.10 ml of 0.10 *M* sodium periodate was added and, after 5 min at room temperature, 0.10 ml of 10 *N* sulfuric acid and 0.30 ml of 10% sodium sulfite, followed by 4.0

ml chromotropic reagent. The same quantity of chromotropic reagent was added to 1.0 ml of standard formaldehyde solution and to 1.0 ml of water (the formaldehyde standard was read against the water blank.) The contents of the tubes were mixed by inversion and the tubes placed in a boiling water bath for 30 min. After cooling, 1.0 ml of thiourea solution was added to each tube, the contents mixed, and the extinction recorded at 570 mμ.

Inositol: Periodate solutions should be protected from bright light during this analysis. All extinctions were read against water in matched 1-cm silica cuvettes using the Beckman Model B spectrophotometer. A 0.20-ml sample of eluate, as well as 0.20 ml eluate from the blank strip, were pipetted into separate cuvettes. Then 2.70 ml of 0.10 *M* sodium phosphate (pH 6.0) and 0.10 ml of 0.0030 *M* sodium metaperiodate were added in turn. The contents of the cuvettes were stirred with a polyethylene rod and the extinction at 223 mμ recorded as the initial reading (*I*). Another 0.20-ml sample of each eluate was pipetted into a stoppered tube and mixed with 0.10 ml of 0.0030 *M* sodium metaperiodate. These tubes were incubated at 50°C in the dark for 1.5 hr, then diluted with 2.70 ml of 0.1 *M* sodium phosphate (pH 6.0) and transferred to the cuvettes. The extinction was recorded as the final reading (*F*). Similar readings were obtained for 0.20 ml of reference inositol solution and 0.20 ml of phosphate buffer. When the value *F* for the unknown was lower than 0.25, the procedure was repeated with a smaller sample of the eluate, e.g., 0.050 ml, diluted to 2.90 ml with phosphate buffer before the addition of periodate. The difference, $I - F$, corrected for the appropriate blank, was proportional to the inositol concentration when $I - F$ was within the range 0.00–0.75.

RESULTS

Treatment of the following compounds with 0.50 *N* HCl at 100°C for 3 hr released all constituent phosphorus in water-soluble form: dipalmitoyl phosphatidyl ethanolamine, cardiolipin, cephalin fractions from ox heart, and phosphoinositide from ox brain. Serine and ethanolamine were completely stable to the acid treatment. In the case of the phosphoinositide, free glycerol and inorganic phosphorus were formed. The formation of free glycerol necessitated caution in taking the acid hydrolyzate to dryness, as glycerol was readily lost if an oil pump vacuum was applied at room temperature, or in the presence of phosphorus pentoxide as drying agent. A water pump vacuum did not cause loss of glycerol under the conditions specified, but was dispensed with as a matter of caution. Significant loss of ethanolamine was also noted if an

HCl-trapping agent (NaOH) was present in the desiccator, presumably due to hydrolysis of the hydrochloride. Splitting of the phosphoric esters in the presence of alkaline phosphatase at pH 8.5 was complete in less than 5 hr in the case of phosphatidyl ethanolamine and phosphatidyl serine, while the hydrolysis of cardiolipin and phosphoinositide required 24 hr for completion. A commercial intestinal alkaline phosphatase preparation with approximately ten times the activity of the bone phosphatase greatly reduced the reaction times but the hydrolyzates were found to be contaminated with peptides due, presumably, to incomplete removal of trypsin. Both *O*-phosphoethanolamine and *O*-phosphoserine were almost as susceptible to phosphatase as glycerophosphate, thus eliminating the difficulties arising from the occurrence of small amounts of these esters in acid hydrolyzates (3). After complete hydrolysis of dipalmitoyl phosphatidyl ethanolamine, ethanolamine was the only ninhydrin-reacting constituent on the chromatogram.

The R_f values of inositol, serine, ethanolamine, and glycerol were, respectively, 0.07, 0.20, 0.55, and 0.73. Choline did not separate from glycerol but did not, if present, interfere with the estimation of glycerol.

The reactions of periodate with glycerol, inositol, serine and ethanolamine proceed according to the following equations (13):

$$CH_2OHCHOHCH_2OH + 2\ HIO_4 = 2\ HCHO + HCOOH + 2\ HIO_3 + H_2O \quad (1)$$

$$(CHOH)_6 + n\ HIO_4 = n\ HCOOH + n\ HIO_3\ (n = 4\text{–}6) \quad (2)$$

$$CH_2OHCHNH_2COOH + HIO_4 = HCHO + OHCCOOH + NH_3 + HIO_3 \quad (3)$$

$$CH_2OHCH_2NH_2 + HIO_4 = 2\ HCHO + NH_3 + HIO_3 \quad (4)$$

The oxidation of glycerol and inositol occurs only in neutral or acid solution but serine and ethanolamine require alkaline conditions (14). For reproducible results, the reaction with inositol must be carried out in buffer (15). Equations (1), (3), and (4) show that the chromotropic procedure, which measures formaldehyde, is only half as sensitive for serine as it is for ethanolamine and glycerol. Under the present conditions of assay, 0.10 μmole formaldehyde gave an optical density of approximately 0.30, using a Coleman Junior Spectrophotometer. The yield of formaldehyde from serine and ethanolamine was quantitative with reference to glycerol as the standard. Recoveries on paper chromatography were quantitative for serine and inositol, but minor losses were observed with ethanolamine (90–95% recovery) and glycerol (95% recovery). A recovery correction was obtained by running a series of standards on each substance spotted on the paper in the range 0.10–0.50 μmole (Fig. 1). The only important source of blank values in the chro-

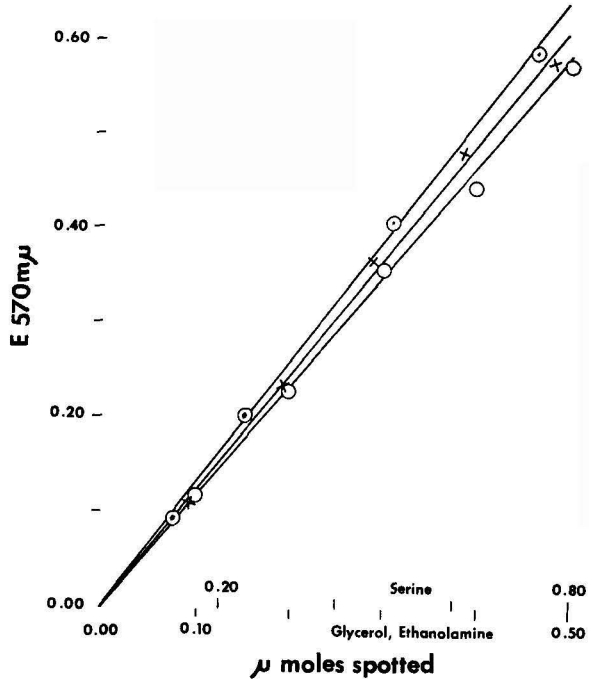

FIG. 1. Standard curves for chromatographed serine (⊙), glycerol (×), and ethanolamine (○).

motropic procedures was formaldehyde derived from acetic acid employed in the solvent for chromatography. With newly purified acetic acid, the blank was of the order of 0.030 OD unit and uniform over the paper strip. The blank did not exceed 0.060 OD unit after one week.

Inositol may consume either more or less than the theoretical six equivalents of peridate, according to the pH and duration of the reaction (15). Under the present conditions, periodate consumption ranged from 4.7–5.1 equivalents/mole inositol in ten controls. As the reliable range of optical density change is limited, due to interference from residual absorption of iodate below the value $F = 0.20$ (16), the sample of eluate analyzed should contain no more than 0.050 μmole inositol, preferably 0.02–0.04 μmole for maximum accuracy. A standard series for chromatographed inositol is shown in Fig. 2. The blanks varied between 0.01–0.05 OD unit, and were partly due to variable stability of periodate solutions on heating.

The results of analyses of representative phospholipids are shown in Table 2. Cardiolipin, or diphosphatidyl glycerol (17), contains theoretically 1.50 moles glycerol/mole phosphorus. Replicate analyses of syn-

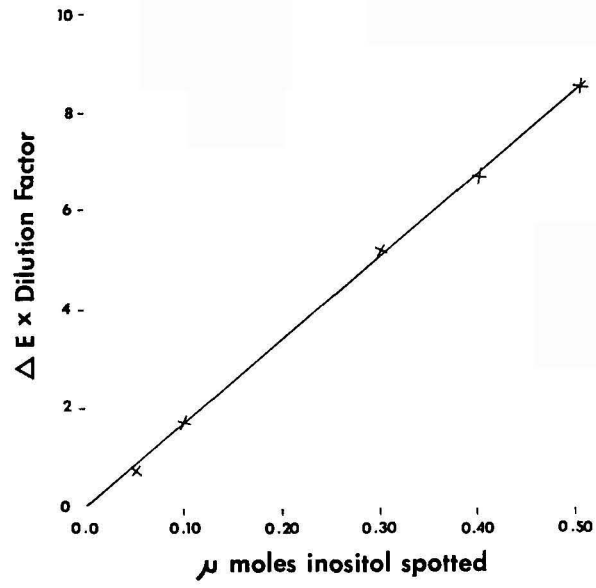

FIG. 2. Standard curve for chromatographed inositol.

TABLE 2

PHOSPHOLIPID ANALYSIS

Phospholipid	Sample for anal.[a] (μmoles)	Molar ratios				Recovery (%)
		Glycerol/P	Ethanolamine/P	Serine/P	Inositol/P	
Cardiolipin	1.32	1.55	—	—	—	—
Dipalmitoylphosphatidyl-ethanolamine	2.86	1.07	1.06	—	—	—
	3.04	1.03	1.04			
	2.92	1.00	0.98	—	—	—
Dipalmitoylphosphatidyl-ethanolamine + serine	1.92 / 0.50	1.00	1.05	0.25	—	97
	1.92 / 1.0	—	—	0.54	—	104
"Phosphatidyl-serine"	2.82	0.90	0.00	0.76	0.090	—
"Phosphatidyl-serine" + inositol	2.82 / 0.067	—	—	0.73	0.115	102
	2.82 / 0.335	—	—	—	0.220	109
	2.82 / 0.67	—	—	—	0.342	106
"Phosphoinositide" (brain)[b]	3.00	0.46	—	—	0.39	—
[c]	3.00	0.71	0.26	0.08	0.39	—

[a] Samples were sufficient for a single analysis of each hydrolyzate.

[b] Results of total acid hydrolysis (48 hr at 100° in 2 *N* HCl).

[c] Results of hydrolysis according to text.

thetic phosphatidyl ethanolamine were performed on three samples of this phospholipid and showed good agreement with the expected ratios. In the recovery tests, serine and inositol were added to the lipid samples before hydrolysis. Verification of the complete liberation of inositol was obtained by a separate analysis of brain phosphoinositide, hydrolyzed by prolonged heating in 2 *N* HCl. The prolonged acid and enzymic hydrolyzates each gave the same inositol value; however, there was partial loss of glycerol on prolonged acid hydrolysis. Destruction of serine and ethanolamine would also be expected under these conditions (3).

DISCUSSION

Paper chromatography has been applied in a variety of ways to the determination of phospholipid constituents. Susler (18) recommended quantitative planimetry, a procedure which was examined critically by Olley (19). The ninhydrin reagent, which has been applied to the paper chromatographic determination of serine and ethanolamine (20, 21), suffers from lack of quantitative yield of colored product, influenced in part by metal impurities in the paper. Procedures using fluorodinitrobenzene (21, 23) do not permit the simultaneous determination of glycerol and inositol. Burmaster (24) introduced periodate for the estimation of lipid serine and ethanolamine, using the microdiffusion technique for measurement of ammonia released. Periodate has also been applied to the determination of inositol in phospholipids using paper chromatography and titrimetry (25) and spectrophotometrically following the removal of interfering bases and glycerol (26, 27). No method has previously been described for the simultaneous determination of all four constituents. The particular value of paper chromatographic analysis, as pointed out by Olley (19), is that it offers a qualitative analysis of a lipid hydrolyzate and at the same time may reveal unexpected constituents which react with periodate or ninhydrin.

SUMMARY

A method has been described for the simultaneous analysis of glycerol, inositol, serine, and ethanolamine in a 2–8 mg sample of phospholipid. Acid hydrolysis is employed for removal of fatty acids, enzymic hydrolysis for degradation of the phosphoric esters, and paper chromatography for the separation of the hydrolyzate constituents. Periodate oxidation of each constituent eluted from the chromatogram is performed. The formaldehyde formed from glycerol, serine, and ethanolamine is estimated with the chromotropic reagent: inositol is estimated from periodate consumption, measured spectrophotometrically.

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Continuous Scintillation Counting of Carbon-14 and Tritium in Effluent of the Automatic Amino Acid Analyzer

KARL A. PIEZ

From the Section on Protein Chemistry, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland

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Ion-exchange chromatography of amino acids has become a widely accepted tool, particularly since the introduction of automatic instrumentation which can accomplish the complete resolution and analysis of a complex amino acid mixture in a single day with high precision (1, 2). An obvious adjunct to these methods would be a practicable procedure to measure radioactivity continuously in the effluent. Since the effluent from the ion-exchange column is aqueous and because of the low energy of the disintegrations of carbon-14 and tritium, the isotopes of greatest interest in amino acid studies, continuous scintillation counting in a two-phase system (solid flour in contact with aqueous solution of the isotope) appears to have the greatest potential (3–6). Since amino acid concentration is determined in the effluent by the color produced with a ninhydrin reagent, the counting must be done prior to the addition of the reagent to the flow stream; at a later stage the scintillations would be largely absorbed by the chromophors. The counting system must not affect the later quantitation of color by changing the composition of the effluent or by the introduction of mixing with a loss of resolution. An additional requirement is introduced by consideration of the distance between amino acid peaks and the rate of change of concentration. Loss of resolution between adjacent radioactive peaks could become significant if radioactivity were measured in increments much larger than about 1 ml. This can also be considered a minimum volume, since the highest possible sensitivity is desirable. The cell must be leak-free at the pressure of the system, which may reach 20 lb/sq in., and it should also not introduce a significant back pressure.

There appear to be only two solid fluors which have been sufficiently well investigated to be considered for use in the present application. These are plastic impregnated with a scintillator and crystalline anthracene. The plastic scintillator has been used in sheet form by Schram and

Lombaert (4) to construct a spiral flow cell. The efficiency for carbon-14 was 5–6% with a background greater than 100 counts per minute (cpm). The same plastic scintillator in tubing form, 0.6 mm i.d., gave about the same efficiency, also with a high background (5). In a static system Steinberg (3) found that beads of a similar plastic permitted 12.5% efficiency. A considerably higher efficiency, 35%, was obtained with close-packed filaments of the plastic. The short mean free path between solution and fluor was undoubtedly responsible for the improved efficiency; it also severely restricted the volume of solution which could be counted. In the same system tritiated water was counted with 0.7% efficiency. Steinberg (3) also investigated several crystalline organic compounds for counting weak beta emitters in aqueous solutions. He concluded that anthracene was the best and superior to the plastic scintillator. Efficiencies of 54% for carbon-14 and 2.3% for tritium were observed when an excess of anthracene was used to absorb a small volume of radioactive solution. The background was about 100 cpm.

On the basis of these results anthracene was chosen as the fluor in a flow cell to be inserted in the flow stream of the automatic amino acid analyzer. This report is concerned with the design of the cell, the method of presentation of the data, and the sensitivity and precision obtainable. Schram and Lombaert (6) have also reported the use of powdered anthracene for the same application.

EXPERIMENTAL

Flow Cell

The flow cell and cell holder that were selected are shown in detail in Fig. 1.[1] The design of the cell was based in part on a plastic cell employed in the flow counter manufactured by Packard Instrument Co. (La Grange, Ill.). It was constructed of quartz for minimal background although Pyrex was nearly as good. The lower surface of the cell holder and the steel supporting rod were painted white with a titanium dioxide pigment in a lacquer base (Nuclear-Chicago Corp., Chicago, Ill.) for maximum reflectivity.

Connections were made to the cell as follows: The end of a suitable length of black Teflon tubing, $\frac{1}{32}$ in. i.d., $\frac{1}{16}$ in. o.d. (Pennsylvania Fluorocarbon Co., Philadelphia, Pa.), was slipped into a 2-in. piece of

[1] A quartz cell was recently constructed for us by Optical Cell Company (Kensington, Md.) similar to the one in Fig. 1, but designed to take Swagelok fittings, P-200-6-1 (Crawford Fitting Co., Cleveland, Ohio), for the connection of the Teflon tubing. It is necessary to reduce the diameters of the body hex and the larger hex nut on the fittings to $\frac{3}{8}$ in.

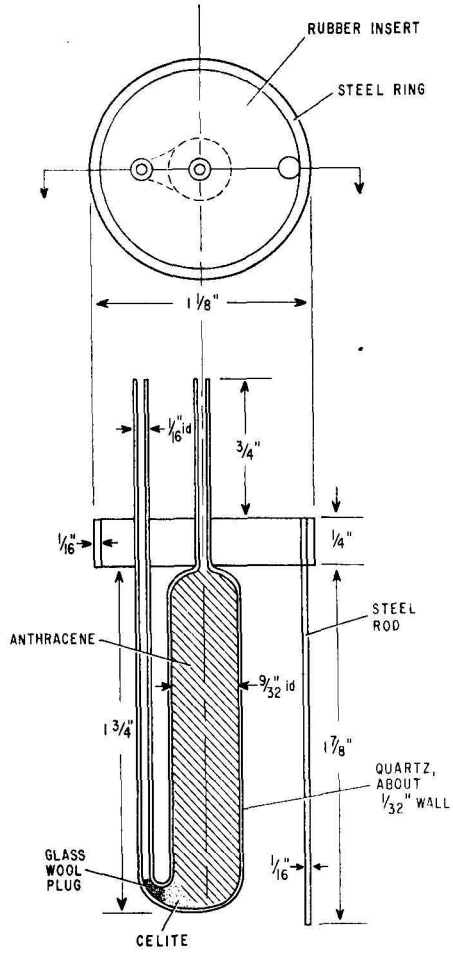

FIG. 1. Quartz flow cell and cell holder for continuous determination of carbon-14 and tritium.

Tygon tubing, R-3603, 1/32 in. i.d., 3/16 in. o.d. (United States Stoneware Co., Akron, Ohio), for a distance of at least 1 in. (This can be done readily if the Teflon tubing is grasped with a small piece of sandpaper or steel wool. It may be necessary to stiffen the Teflon tubing by inserting a fine piece of steel wire through the Tygon tubing and into the lumen of the Teflon tubing. The wire is withdrawn after the Teflon tubing is in place.) The free end of the Tygon tubing was softened in boiling water and then placed over the outlet of the cell, while the cell was in

place in the holder. After the cell had been filled with anthracene a similar connection was made to the inlet side.

Before filling the cell, a small piece of glass wool was pushed through the cell and into the outlet arm (Fig. 1). The free end of the Teflon tubing on the outlet side was then connected temporarily in the manner described above to a piece of Tygon tubing in a finger pump. Diatomaceous silica (Celite Filter-Aid, Johns Manville Co.) was suspended in water containing an efficient detergent such as Triton G-5 (Rohm and Haas, Inc., Philadelphia, Pa.) and allowed to settle for a few minutes. The supernatant was discarded and the coarse residue was resuspended and introduced into the cell with a pipet with a capillary tip. The pump was then started and allowed to pull water through the cell for sufficient time to deposit a layer of Celite on the glass wool plug (Fig. 1). It was then stopped, excess Celite was withdrawn with the pipet, and the cell was completely filled with water containing detergent. A small funnel with a short stem about $\frac{1}{16}$ in. i.d. was connected to the inlet side of the cell with a short piece of tubing. Anthracene (blue-violet fluorescence grade, Eastman Kodak Company, Rochester, N. Y.) was suspended in water containing detergent, stirred gently, and allowed to settle, and the supernatant was discarded. This was repeated several times to remove all fine particles of anthracene. The suspension was then poured into the funnel over the cell. The pump was restarted to pull water through the cell at about 60 ml/hr. A piece of wire was moved up and down in the neck of the cell to prevent clogging. The cell was held upright and tapped gently on the bench top as it filled to help pack the anthracene crystals. Anthracene and water were added as needed into the funnel until the cell was full and did not pack further. The pump was stopped, the funnel was removed, and black Teflon tubing was connected to the inlet side of the cell as described above. The pump was then reversed and water was pumped backward through the cell at a very slow flow rate so as not to disturb the packing until the inlet tubing was filled with water. The cell was then connected to the amino acid analyzer through connectors similar to those provided on most commercial instruments making certain that flow would be downward through the cell and taking care not to allow air to enter the cell. (A small bubble will do no harm and will eventually dissolve.) The cell was then lowered into place in the counter through the elevator shaft. The cell was oriented with the outlet tube to one side of a line between the photomultiplier tubes.

Instrumentation

A manually operated, room-temperature scintillation counter with

anticoincidence circuitry and a two-channel output (Model 701, Nuclear-Chicago Corp., Chicago, Ill.) was used in all experiments. The only modifications necessary were to remove the shutter assembly to provide access to the counting chamber and to cover or disconnect the switch which operates the elevator to prevent accidental raising of the elevator. After the cell was in place a black rubber stopper with two grooves to allow the black Teflon tubing to pass was inserted in the top of the elevator shaft. The assembly was then made light-tight by covering with a black photographers cloth. (The photomultiplier voltages must be off and the room partially darkened whenever the counting chamber is open to precent damage to the tubes by light.)

In the present studies a logic scaler (Model 8250), a logic timer (Model 8600), a ratemeter (Model 1620A), and a logic printer with analog output (Model 8400) from Nuclear-Chicago Corp. were employed. The chart recorder used with the ratemeter was a Sargent Model SRL (E. H. Sargent and Co., Chicago, Ill.) with linear gears and a chart spread of 3 in./hr. When the recorder on the amino acid analyzer [Speedomax Type G, triple-point, Leeds and Northrup Co., Philadelphia, Pa.; see (1)] was used to record the analog output of the printer it was necessary to increase the range of the analog output from 100 to 200 mv to obtain full scale deflection because of the low impedance of the recorder. This was done by changing resistor R58 from 37 to 18 megohm. A 20-megohm resistor placed across the output terminals restored the normal output for other applications.

The amino acid analyzer was constructed in the laboratory as previously described (2). It is a modification of the design of Spackman, Stein, and Moore (1), differing significantly only in the use of a single column and a continuous gradient for the resolution of all the common amino acids. It is equipped with the valve manifold manufactured by Phoenix Precision Instrument Co. (Philadelphia, Pa.) that has provision for insertion of a counting cell in the flow stream at a point prior to the addition of ninhydrin reagent.

Labeled Amino Acids

The cell was tested with appropriately diluted samples of glycine-1-C^{14} and DL-proline-3,4-T dissolved in water, pH 2.9 citrate buffer (2), or 0.8 *M* sodium citrate (2). It was calibrated with two different standard solutions of sodium carbonate-C^{14} ($\pm 3\%$) and tritiated water ($\pm 10\%$) and averages were taken. The standard mixture used on the amino acid analyzer contained DL-proline-3,4-T and a number of carbon-14 labeled amino acids with estimated activities ranging from 1100–44000 disintegrations per minute (dpm) per milliliter (see Table 1). A few experi-

TABLE 1

	Standard solution					1:2 dilution		1:4 dilution		1:10 dilution	
	Activity found[a] (dpm)	Exptl. error[b] (%)	Counting error[c] (%)	Activity added[d] (dpm)	"Recovery"[d] (%)	Activity found (dpm)	Counting error[c] (%)	Activity found (dpm)	Counting error[c] (%)	Activity found (dpm)	Counting error[c] (%)
DL-Serine-1-C^{14}	2320	2.2	2.6	2200	105	1040	4.4	624	5.6	190	15
DL-Glutamic acid-1-C^{14}	13770	1.4	1.0	17600	78	6760	1.5	3530	2.1	1390	3.9
DL-Proline-3,4-T	247600	2.4	1.5	440000	56	119900	2.4	67200	3.3	22210	7.1
Glycine-1-C^{14}	12710	2.0	1.0	11000	115	6190	1.5	3260	2.1	1240	4.0
L-Alanine-1-C^{14}	1440	4.9	3.5	1100	131	690	6.1	430	8.2	130	20
DL-Valine-1-C^{14}	6120	1.6	1.5	8800	70	3120	2.3	1520	3.5	590	6.7
L-Isoleucine-1-C^{14}											
allo	3450	0.9	2.2	11000	115	1680	3.4	860	5.5	330	11
normal	9150	2.1	1.2			4400	1.8	2240	2.8	980	4.9
L-Leucine-1-C^{14}	47670	1.3	0.5	44000	108	23190	0.7	12180	1.0	4550	1.8
DL-Phenylalanine-1-C^{14}	21090	1.2	0.8	17600	120	8870	1.2	5300	1.7	2210	3.1
DL-Lysine-1-C^{14}	9400	1.9	1.2	11000	85	4400	1.8	2380	2.7	920	5.1

[a] dpm $= K(N_T - N_B)$, where N_T is total counts, N_B is background counts, and K is 1.212 for C^{14} and 51.7 for T; see text. The averages of six replicate analyses are presented.

[b] The experimental error expresses the over-all error as measured by the standard deviation of six analyses.

[c] Standard counting error (%) $= 100(N_T + N_B)^{1/2}/(N_T - N_B)$. The errors for single analyses are presented.

[d] Calculated on the assumption that the commercial samples were pure and correctly labeled as to activity.

ments were performed with DL-valine-4-C^{14} and DL-phenylalanine-2-C^{14}. The amino acids were obtained from a variety of commercial sources and had specific activities between 1 and 10 mc/mmole. Unlabeled amino acids were added to give a concentration of each 0.5 μmole/ml. One-milliliter portions, or dilutions, of the standard solution were chromatographed.

RESULTS

Cell Design

The first type of cell tried consisted of coiled plastic tubing packed with anthracene. It was found that these cells required too high a pressure, about 20 lb/sq in., to maintain a flow of 30 ml/hr even when carefully packed. Another type was machined from a cylinder of Lucite to contain a chamber of approximately the same dimensions as the cell in Fig. 1. Though its flow characteristics were good, the background was about twice that obtained with the quartz cell. A cell constructed of Pyrex glass also performed well and had a background only about 30% higher than the quartz cell. The fluid volume in contact with packed anthracene was estimated by suspending dry anthracene in a measured volume of water containing detergent, centrifuging at low speed, and measuring the increase in volume and the packed volume of the anthracene. It was found that about 60% of the packed volume was fluid, giving an approximate fluid volume of the quartz cell used in the present studies of 1.1 ml. It is not necessary to know the volume precisely, since a correction factor can be determined which includes both the fluid volume in effective contact with the fluor and the counting efficiency (see below).

Flow Characteristics

With the cell in position in the counter and the inlet connected to a finger pump operating at 9.5 ml/hr the cell was first equilibrated with pH 2.9 citrate buffer. A solution of glycine-1-C^{14} in the same buffer was pumped through the cell for about 15 min followed by buffer alone. The count rate was followed; typical results appear in Fig. 2. It is apparent that the cell filled and flushed with very little mixing. About 80% of the total radioactivity was removed in one cell volume and essentially all the radioactivity in less than two cell volumes. In addition, no significant differences could be seen in the resolution on chromatograms obtained with and without the cell in the flow stream.

The pressure required to maintain 30 ml/hr was 1–3 lb/sq in., varying with the particular packing and the solvent. Highest pressures were obtained with 0.8 *M* sodium citrate, which is used as the limiting solution

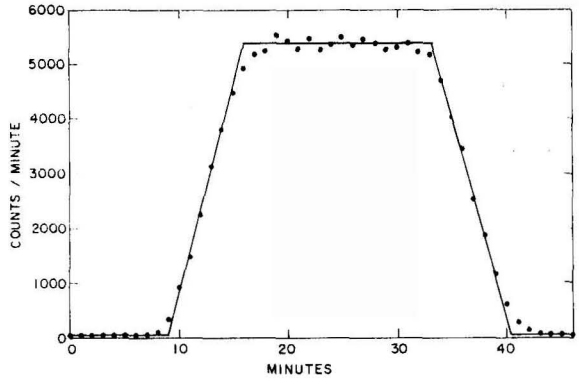

FIG. 2. Flow characteristics of anthracene-packed quartz cell (Fig. 1). The count was measured in 1-min increments as solvent, solvent containing glycine-1-C^{14}, and solvent again was pumped through the cell at 9.5 ml/hr. The solid line shows ideal flow characteristics of a 1.1-ml cell.

in the elution gradient of the ion-exchange column (2). The back-pressure did not change even after repeated use with the analyzer.

Operating Conditions

Photomultiplier tube voltages and discriminator settings were selected in the usual manner to give the lowest statistical counting error [maximal ratio of $(cpm)^2$/background]. Typical results appear in Fig. 3 for glycine-1-C^{14} and DL-proline-3,4-T dissolved in the pH 2.9 citrate buffer. It was found that the ratio $(cpm)^2$/background, though somewhat higher with the upper discriminator set in the 7–10 volt range, was

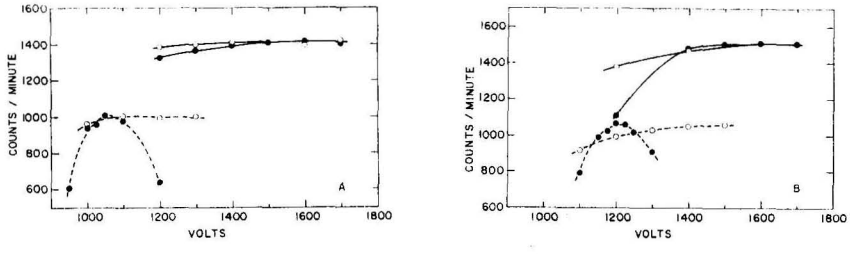

FIG. 3. Operating characteristics of quartz cell (Fig. 1) when counting glycine-1-C^{14} (A) and DL-proline-3,4-T (B). Data were obtained as follows: with a base-7 volt window (dash line) and voltage on the gate photomultiplier at 1500 volts, the count rate was determined for various voltages on the data photomultiplier (●). The voltage giving maximal count rate was selected and the count rate was then determined at different voltages on the gate photomultiplier (○). Similar data were obtained for a base-∞ window (solid line).

nearly maximal with no upper discriminator. It was therefore decided to use integral counting (base-∞ window) with voltages on both tubes of 1600 volts for all the experiments described here. This has an important advantage. The voltages on the photomultiplier tubes are not critical, since the count rate is independent of small voltage changes under these conditions (see Fig. 3). Since this is true for both carbon-14 and tritium, the two isotopes can be counted under balance point conditions in the same experiment using a single channel. For experiments with a single isotope, slightly better precision would be obtained with the upper discriminator set at about 7 volts.

Possible quenching effects in the present application were looked for by measuring the count rate of glycine-1-C^{14} and proline-3,4-T dissolved in water and in the two eluting solutions used in the amino acid analyzer (pH 2.9 citrate buffer and 0.8 *M* sodium citrate). Identical rates were obtained in all three solvents, indicating the absence of variable quenching. The count rate was independent of amino acid concentration and directly proportional to absolute activity.

It was found that some amino acid solutions left activity in the cell that could not be removed, requiring cleaning and repacking of the cell. This residual activity was apparently related to the presence of impurities that were adsorbed on the anthracene, for other labeled amino acid preparations left no activity in the cell even after many hours of contact. Amino acid mixtures or protein hydrolyzates which had first passed through the ion-exchange column in no case caused contamination. The background did not increase even after more than twenty runs through the same cell.

The cell was calibrated with sodium carbonate-C^{14} dissolved in water and with tritiated water. Assuming an effective fluid volume of 1.1 ml, the efficiencies were 38% for carbon-14 and 0.9% for tritium in a base-∞ window and 27% and 0.7% in a base-7 volt window. The corresponding backgrounds were 18 and 7 cpm for both isotopes.

Data Presentation

The simplest way to record the radioactivity data is with the use of a ratemeter and recorder. The type of record that is obtained is shown in Fig. 4. Activities can be calculated from the areas under the peaks.

A procedure which has been found to be more useful is to use a timer, scaler, and printer. With the instruments employed, any time interval can be selected (2 min in the present experiments) and at the time set the printer prints the counts collected by the scaler in the previous interval and resets the scaler to collect the counts in the next interval. This continues for as long as desired. The time lost in printing and re-

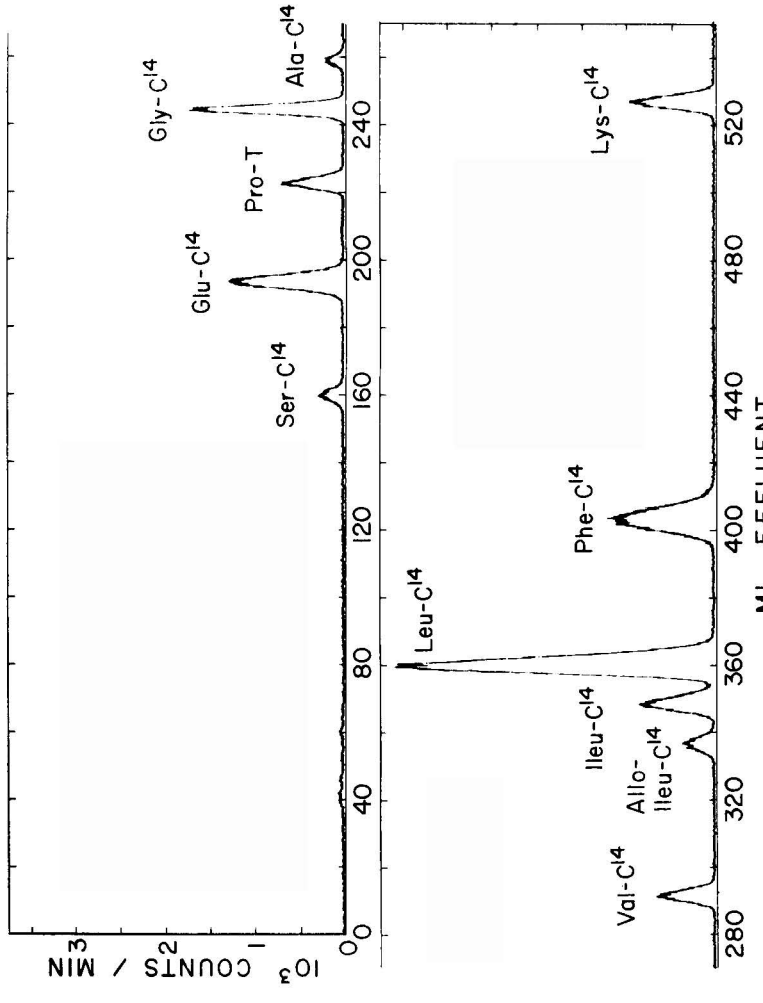

FIG. 4. Record obtained by use of a ratemeter and recorder with a scintillation counter containing a flow cell (Fig. 1) inserted in effluent stream of an amino acid analyzer. The standard mixture described in Table 1 was analyzed. Ratemeter was set for a range of 0–3000 cpm (80% of full scale on the recorder) and a time constant of 10 sec. Integral counting was employed.

setting is a fraction of a second and can usually be ignored. The data appears on a tape as a series of counts which are simply added to obtain total counts in the amino acid peak. The absolute activity can be calculated from the following formula:

$$\text{dpm} = K\ (\text{total counts} - \text{background counts})$$

where

$$K = \frac{100 \times \text{flow rate}}{\text{cell volume} \times \text{efficiency}}$$

K can be determined experimentally by adding an amino acid of known activity to the sample, selecting one which would not otherwise be radioactive. If the flow rate is known precisely, which is the case with the amino acid analyzer, K can also be calculated from a value for cell volume $\times$ efficiency determined in a static experiment. The latter procedure was used in the present experiments so that reproducibility could be estimated. However, in actual use the former procedure will be preferable because it constitutes an internal control.

In addition to providing the data in the form of numbers on a tape, the printer has an analog output. The voltage at this output is proportional to the total counts in the preceding time interval and changes at each printout. When recorded on a chart, the data appears as short dashes the length of the time interval, at a height proportional to the total counts in the interval. Different ranges in multiples of 10 can be selected. It is convenient and saves extra instrumentation to use the third point on the recorder of the amino acid analyzer to record the analog output.[2] A typical record of this type appears in Fig. 5. The counts are recorded from the top to the bottom of the chart on a linear scale while the amino acid concentration is recorded in the conventional manner on a logarithmic scale. The radioactive peaks precede the corresponding concentration peaks by about 18 min. This is the time required for the effluent to move from the counter through the reaction coil where color development occurs to the photometer where the color in measured. This type of record permits rapid and nearly unequivocable identification of each radioactive peak. Although quantitative data can also be obtained from the chart, it is simpler and more precise to use the printed counts obtained simultaneously.

[2] This point normally indicates absorbancy at 570 mμ in a cell with a short light path for reduced sensitivity. The data is useful only in those instances in which high amino acid concentrations are encountered and point 1 is off the portion of the chart that is easily read. However, even in these cases the absorbancy at 440 mμ (point 2) can be used in the same manner since it generally has a lower sensitivity and bears a readily determinable relationship to the absorbancy at 570 mμ (point 1).

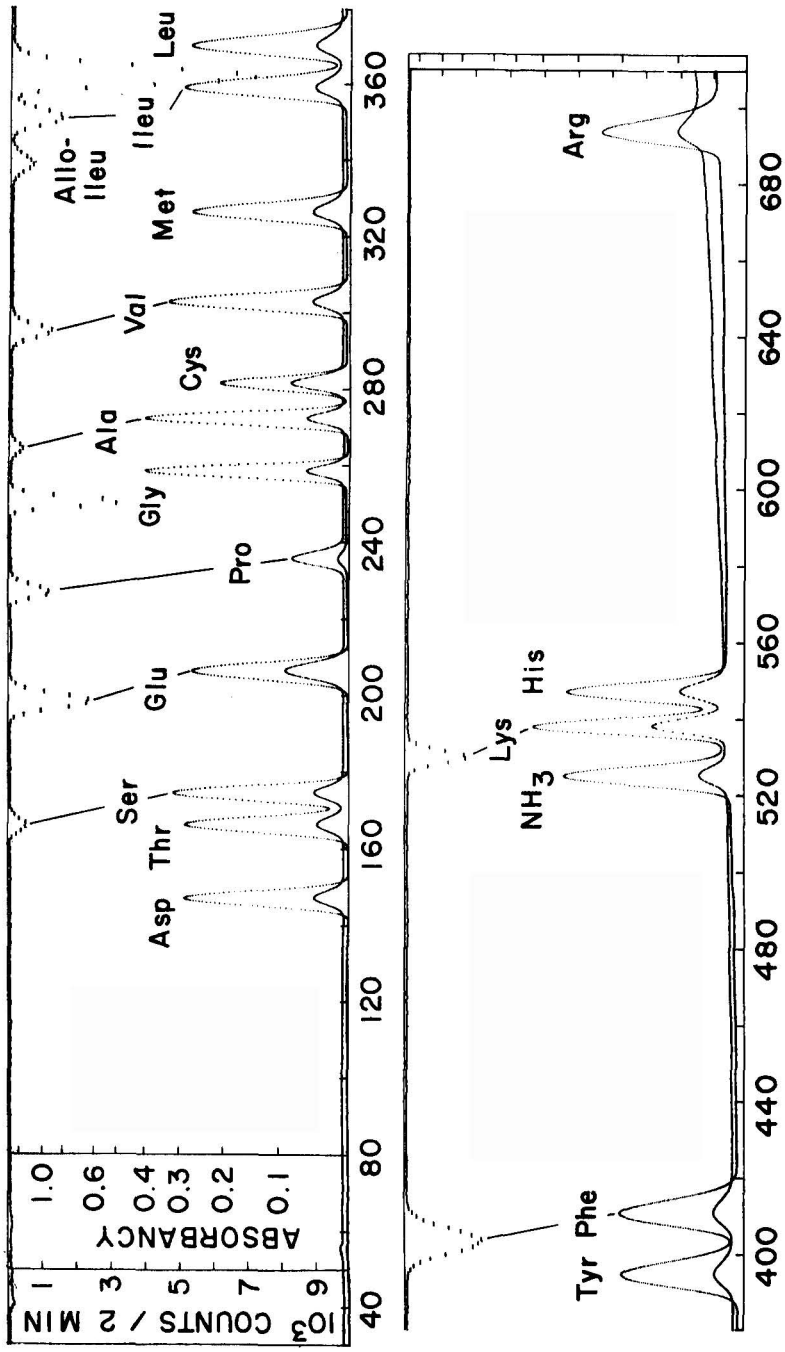

FIG. 5. Combined chromatogram of radioactivity and concentration obtained with automatic amino acid analyzer. A standard mixture of amino acids (0.5 μmole of each), some of which were labeled (see Table I), was analyzed. One point of the recorder, at top of chart, was used to indicate counts collected in 2-min intervals. Integral counting was employed. The other points indicate concentration measured at 570 and 440 mμ after reaction with ninhydrin.

Precision and Sensitivity

To determine the over-all precision of the method a standard sample containing nine carbon-14 labeled amino acids with activities ranging from approximately 1500 to 50000 dpm and proline-3,4-T at 250000 dpm was analyzed six times. In addition, single analyses were performed on several dilutions. The results appear in Table 1. It was found that, for carbon-14 activities greater than about 3000 dpm, the counting error, based only the number of counts collected and the background, was less than the over-all error of 1–3% expected of the amino acid analyzer (1, 2). Below about 2000 dpm the counting error was predominant, becoming about 5% at 1000 dpm, 10% at 400 dpm, and 20% at 130 dpm. The minimum activity which could be detected in a single peak was less than 100 dpm. For tritium about forty times as much activity was required for the same errors owing to the lower counting efficiency.

It was possible in these same experiments to compare the absolute activities with the values estimated by the supplier. The "recoveries" are listed in Table 1. It is apparent that most of the samples were as good or better than claimed though a few (glutamic acid, proline, and valine) probably contained impurities. This was also indicated by the presence of activity in the effluent at positions not corresponding to amino acids. Much of this was unretarded by the column appearing at about 40 ml of effluent with tailing (Figs. 4 and 5). The isoleucine sample, though stated to be L-isoleucine-1-C^{14}, contained about 25% of a substance that behaved like the diastereoisomer alloisoleucine-C^{14}. Since no unlabeled isomer was added to the sample there was no corresponding peak on the concentration curve (Fig. 5).

Isotope Effect

It was previously reported that carbon-14 labeled amino acids show an isotope effect when chromatographed on Dowex 50 using manual methods (7). The labeled species follow the unlabeled species by a small interval, resulting in an increase in specific activity from the leading to trailing sides of the concentration peak. This effect was also observed in the present studies. Although the maxima of the radioactivity peaks (Fig. 5) could be located only within a few tenths of a milliliter, since the counts were recorded in 2-min increments, the following approximations of volume displacement were made assuming valine-4-C^{14} to have no isotope effect as previously found (7): proline-3,4-T, 0 ml; serine-3-C^{14}, 0.5 ml; glutamic acid-1-C^{14}, glycine-1-C^{14}, valine-1-C^{14}, isoleucine-1-C^{14}, leucine-1-C^{14}, phenylalanine-2-C^{14}, and lysine-1-C^{14}, 1.0–1.5 ml;

phenylalanine-1-C^{14}, 2.5 ml. These results agree qualitatively with the previous findings (7). The differences in magnitude are probably related to the different chromatographic conditions. Of particular interest is the relatively large isotope effect observed with phenylalanine-1-C^{14}, an amino acid not included in the earlier study (7). If, as previously indicated (7), the isotope effect is an expression of a small change in ionization constant of the group adjacent to the heavier isotope, the large effect may be explained by the fact that phenylalanine shows adsorption independent of ion exchange and is therefore eluted at a higher pH than other amino acids with a similar isoelectric point. At the higher pH phenylalanine would carry a larger net negative charge and be eluted in part by exclusion of negative ions. The isotope adjacent to the negative charge might then be expected to have a larger effect than usual.

The only tritium-labeled amino acid examined here, proline-3,4-T, showed no isotope effect. This cannot be considered to be a general conclusion, for Pisano[3] has shown that tritiated histidine has an isotope effect in the same direction as carbon-14 labeled amino acids.

DISCUSSION

It has been demonstrated that the system described here is practicable for determining activities of carbon-14 exceeding about 1000 dpm. This level of activity is attained in most experiments. When it is not and sufficient sample is available, it is possible to analyze one portion for concentration and a larger portion for radioactivity alone. In the latter case the amino acid analyzer should be operated for counting alone without the addition of ninhydrin reagent, to avoid the possibility of precipitation of chromophor and clogging of the reaction coil.

Although tritium can be counted only at a much lower sensitivity than carbon-14, the forty-fold difference in efficiency is the same order of magnitude as the differences in cost and available specific activity. Therefore, if the use of tritium-labeled amino acids is indicated, the same counting system can be employed. Perhaps more important is the fact that double-labeling experiments can be counted using conventional procedures which employ two channels, one being set to discriminate against one isotope and the other against the other isotope.

It should be remembered that the ability of the amino acid analyzer and associated counting equipment to determine the specific activity of any of the common amino acids in the presence of others means that certain types of multiple-labeling experiments can be performed by the

[3] Pisano, J. J., personal communication.

use of two or more carbon-14 labeled amino acids. It is only necessary to know that the amino acids selected are equivalent measures of the variable of interest.

In an independent study of an anthracene-packed cell used with an amino acid analyzer, Schram and Lombaert (6) recently obtained similar results. They found efficiencies of 44 and 2% for carbon-14 and tritium with a background of 60 cpm in a cell constructed of 2.2 mm i.d. polyethylene tubing 60 cm long.

SUMMARY

The construction of a quartz cell containing crystalline anthracene for continuous determination of carbon-14 and tritium in the effluent of the amino acid analyzer is described. The cell can be used with standard scintillation counters permitting efficiencies of 38% at 18 cpm background or 27% at 7 cpm background for carbon-14 and 0.9 and 0.7% with the same backgrounds for tritium. Depending on the choice of instrumentation the results can be indicated by a ratemeter and recorder, or they can be simultaneously printed on tape and indicated on the recorder of the amino acid analyzer. Carbon-14 and tritium can be determined in the same experiment in different amino acid peaks or, with the use of two channels in the conventional manner, in the same peak. A carbon-14 activity of about 1000 dpm can be determined with a 5% standard error. Above 3000 dpm the error is 1–3%. About forty-fold higher activities are required for tritium.

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Determination of 9-*cis* Isomers of Vitamin A by Reaction with Opsin[1, 2]

DAVID C. HERTING, EMMA-JANE E. DRURY, AND PHILIP L. HARRIS

From the Distillation Products Industries Division of Eastman Kodak Company, Rochester, New York

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The natural occurrence of the 9,13-di-*cis* isomer[3] of vitamin A in marine crustacea was postulated several years ago (e.g., 1–3), but recent reports of both the 9,13-di-*cis* and 9-*cis* isomers in fish liver oils and rat liver oil (4) and in stored aqueous multivitamin preparations (5, 6) suggest a more generalized distribution in nature and in supplements of importance in human nutrition. The relatively low biopotency of these isomers (9-mono and 9,13-di-*cis*) compared with the all-*trans* and 13-*cis* isomers (7) increases the need for an analytical method specific for the 9-*cis* isomers, since neither the antimony trichloride blue color procedure (8) nor the USP XVI spectrophotometric procedure (9) makes full allowance for their lower biopotency.

Two methods have been proposed for measuring the content of 9-*cis* and 9,13-di-*cis* isomers. Both require that the isomers be in the aldehyde form and therefore require preliminary saponification and oxidation. Infrared spectrophotometry has been used for samples of sufficient quantity (5 mg or more) and with relatively high purity (4). Reaction of the 9-*cis* and 9,13-di-*cis*-retinals (vitamin A aldehydes) with opsin, a protein of the retina, to give isorhodopsin ($\lambda_{max} = 487$ mμ) was proposed as an analytical method by Hubbard *et al.* in 1953 (10) and was described briefly by Brown *et al.* in 1959 (4). The only other isomer which reacts to any extent with opsin, the 11-*cis* isomer, has been found only in the eye (4).

The opsin procedure promised advantages of both greater sensitivity and less concern about purity, thus providing a useful technique for

[1] Communication No. 291 from the laboratories of Distillation Products Industries.

[2] Presented in part at the meetings of the Federation of American Societies for Experimental Biology, April 14–18, 1962, Atlantic City, New Jersey.

[3] Nomenclature is that officially adopted by I.U.P.A.C., *J. Am. Chem. Soc.* 82, 5575 (1960).

assessing the natural occurrence of the 9-*cis* and 9,13-di-*cis* isomers. Unfortunately the various steps in the preparation and analysis of vitamin A isomers have not been correlated and described in detail with supporting evidence. The objective of this work was to evaluate the various steps and to present a unified analytical procedure. We were assisted in certain phases of the work by Brown, Brown, and Wald.[4]

METHODS

Procedures described hereinafter are those adopted for use. Wherever possible, all work was carried out in red glassware, under red light, and/or in the dark.

In brief, the method involves saponification of the lipid concentrate, purification of the retinols (vitamin A alcohols) by desterolization and by chromatography on deactivated alumina, oxidation of the retinols to retinals with MnO_2, and solution of the retinals in digitonin. The retinals are incubated with opsin at 20–25°C for 2 and 6 hr, at which times the reaction is stopped and the amount of isorhodopsin is measured spectrophotometrically. From these measurements, the amounts of 9-*cis* isomers are calculated. Opsin is prepared from cattle retinas and is pretested with 11-*cis* retinal.

Materials and Reagents

1. *Vitamin A isomers.* Eastman Organic Chemicals (except for 9,13-di-*cis*-retinal from Chemistry Research Dept., DPI).
2. *Ethanol,* U.S.I.
3. *Diethyl ether,* redistilled from KOH pellets and aluminum.
4. *Methanol,* Eastman Organic Chemicals, 467.
5. *n-Hexane,* Buffalo Solvents and Chemical Corp., b.p. 66–69°C, shaken with concentrated H_2SO_4, washed with water, dried over $CaCl_2$, and redistilled from KOH pellets and Zn flakes.
6. *d-γ-Tocopherol,* Eastman Organic Chemicals, 6685.
7. *Aluminum oxide,* Merck reagent 71707, suitable for chromatographic adsorption.
8. *Acetone,* Eastman Organic Chemicals, 297.
9. *Skellysolve F,* Skelly Oil Co., b.p. 35–60°C, redistilled from KOH pellets and Zn flakes.
10. *Celite 545,* Johns Manville Corp.
11. *Digitonin solution,* 2% digitonin (w/v) (S.A.F. Hoffman-LaRoche

[4] We are deeply indebted to Professor George Wald and his co-workers at Harvard University for their initial guidance and advice on our work with opsin (personal communication, 1959).

& Co. Ltd.) dissolved in phosphate buffer and centrifuged to remove undissolved material.

12. *Phosphate buffer,* pH 6.8 (1/15 *M*), mixture of equal parts of $Na_2HPO_4 \cdot 2H_2O$ solution (11.876 gm/liter) and KH_2PO_4 solution (9.078 gm/liter).

13. *Sucrose solution,* 40% sucrose (w/v) dissolved in phosphate buffer.

14. *Hydroxylamine,* 6.95 gm of $NH_2OH \cdot HCl$ (Eastman Organic Chemicals, 340) dissolved in distilled H_2O, diluted to 100 ml, and titrated to neutrality just before use.

Preparation of Sample

Extraction. Fresh tissues are frozen, dried by grinding with anhydrous Na_2SO_4, and extracted with diethyl ether as described by Ames *et al.* (11).

Saponification. Saponification of the lipid concentrate containing vitamin A isomers and extraction of the nonsaponifiable materials are carried out according to the USP XVI method (9). An aliquot is analyzed for total vitamin A with antimony trichloride ($\lambda_{max} = 620$ mμ) (8).

Desterolization. The ether solution of nonsaponifiable material is carefully evaporated to dryness with nitrogen in a 15-ml centrifuge tube. A 5- to 10-ml portion of methanol is added and the residue dissolves during heating under nitrogen in a hot water bath. The methanol is then evaporated with nitrogen to a final volume of 1 or 2 ml/gm of original oil. The tube is chilled in a dry ice-acetone bath for 15 min and is then centrifuged at $3075 \times g$ for 15 min at $-10°C$. If the contents of the tube solidify, the solution is warmed, more methanol is added, and the precipitation and centrifuging are repeated. The supernatant is decanted and evaporated carefully with nitrogen, and the residue is immediately dissolved in 1 ml or less of hexane containing sufficient γ-tocopherol to provide about 200 μg tocopherol/1000 units (300 μg) of retinol (12).

Chromatography on alumina. Aluminum oxide is deactivated as described by Olson (13). A 0.5×20 cm column (with glass wool plug) is about half-filled with the alumina, with gentle tapping and application of weak suction.

The hexane solution of retinols from the desterolization is transferred quantitatively to the dry column, and the column is developed essentially as described by Olson (13). Weak suction is used during passage through the column of a 10-ml portion of hexane, followed by a 10-ml portion of 2% acetone in hexane. The retinols are then eluted by passing 5% acetone in hexane through the column without suction. Movement of this fraction can be followed by observing a cream yellow area with an

ultraviolet light. From 10 to 20 ml of solvent is usually required for complete elution.

The eluate is carefully evaporated with nitrogen, and the residue is dissolved in about 0.5 ml of Skellysolve F in readiness for oxidation by MnO_2.

Oxidation. Retinols are oxidized to retinals as follows: A column (0.6 × 20 cm with stopcock) is prepared by inserting a glass-wool plug and adding successively, with slight tapping, a thin layer of Celite 545, a mixture of activated MnO_2 (14) (20 times the weight of retinol) made up to 0.4 gm with Celite 545, and another thin layer of Celite 545. The adsorbent is then wetted with Skellysolve F.

The retinols in Skellysolve F are immediately adsorbed on the column in the dark (red light) and the flask and sides of the column are rinsed with about 0.5 ml of Skellysolve F. After 30 min, the retinals are eluted from the column with a total of about 10 ml of Skellysolve F, using slight nitrogen pressure.

Solution of retinals in digitonin. The solution of retinals in Skellysolve F is evaporated carefully with nitrogen, and the residue is dissolved in the minimum amount of ethanol (use no more than necessary). Sufficient digitonin solution is added to give the concentration desired. The resultant solution is usually murky and is clarified by centrifuging.

As much as 50% of the retinal is occasionally lost in the precipitate in this step. The final concentration of the solution is determined by taking 1 part of the digitonin solution, adding 3 parts of ethanol, and extracting 3 times with 15 parts of hexane each time. The combined hexane extracts are dried with anhydrous Na_2SO_4, and an aliquot is analyzed by the antimony trichloride method ($\lambda_{max} = 664$ mμ).

Preparation of Opsin

The following procedure, based on reports from Wald's laboratory[4] (15) and by Plack (16), has been the most satisfactory of those we have employed.

1. Thaw 15 cattle eyeballs and cut around the equators. (It is important that the eyeballs be frozen as soon as possible after removal from the cattle and that they be kept frozen until used.)
2. Strip out retinas with fine forceps and place in Petri dish with small amount of phosphate buffer, pH 6.8.
3. Bleach rhodopsin to opsin by exposure of retinas to light (60-watt bulb) for 30 to 45 min.
4. Grind bleached retinas thoroughly in a mortar with 5 ml sucrose-phosphate buffer and centrifuge for 5 min at 390 × g (room temperature) to precipitate larger pieces of tissue.
5. Decant suspension (bright scarlet) into another centrifuge tube.

6. Centrifuge for 1-hr at 20,000 × *g* (5°C) to sediment rods of the retina.

7. Discard supernatant and grind rod residue with 5 ml sucrose-phosphate buffer. Centrifuge 5 min at 560 × *g* (room temperature) to precipitate epithelium and heavier tissue.

8. Decant suspension, treat residue as above, and combine suspensions.

9. Dilute suspension with 5 vol of phosphate buffer. Centrifuge for 30 min at 20,000 × *g* (5°C).

10. Discard supernatant and suspend rod residue in 4 ml of phosphate buffer. Centrifuge for 30 min at 20,000 × *g* (5°C). [A tanning step can be included at this point (15), but we have not found this step to be of value.]

11. Discard supernatant. Extract opsin from residue by suspending in 4 ml of digitonin solution for 1 hr (room temperature).

12. Centrifuge for 30 min at 20,000 × *g* (5°C). Dispense supernatant into small vials and store at —20°C.

13. Repeat extraction twice. (The third extract usually has low potency.)

Testing of Opsin

The procedure is carried out, in general, as described by Brown and Wald (17).

1. Work in red light. Measure rhodopsin with a Beckman Model DU spectrophotometer using microcells (3 mm wide, 10-mm light path).

2. Put 1.0 ml of digitonin solution in the control cell. Put in the experimental cell 0.5 ml of opsin solution plus excess 11-*cis*-retinal (in digitonin solution), plus sufficient digitonin solution to give a total volume of 1.0 ml. In our experience, 2.0 μg of 11-*cis*-retinal is usually adequate. In order to conserve opsin, we do not use it in the control cell.

3. Stir contents of cell with a fine glass rod.

4. Incubate solutions in the dark for 2 hr at 20–25°C.

5. Add 0.1 ml of freshly neutralized hydroxylamine to each cell and let stand in the dark for 15 min.

6. Measure absorbance (A_{ru}) of the experimental cell at 500 mμ (rhodopsin). Remove cells from spectrophotometer and bleach for 15 min in the light of a 160-watt tungsten lamp passed through Jena GG3 and Corning 3962 filter glasses to decrease ultraviolet and infrared radiation, respectively.

7. Replace cells in spectrophotometer and measure absorbance (A_{rb}) of the experimental cell at 500 mμ.

8. Calculate the amount of 11-*cis*-retinal converted to rhodopsin:

$$\mu g\ 11\text{-}cis\text{-retinal} = \frac{1.1^a \times 10^4 (A_{ru} - A_{rb})}{1.87^b \times 764^c}$$

a. Volume (ml) in cell.

b. Correction for the 87% increase in molar extinction of rhodopsin relative to that of 11-*cis*-retinal (17).

c. $E_{1cm}^{1\%}$ (380 mμ) of 11-*cis*-retinal = 764 (in 2% digitonin solution) (17).

Alternatively, the opsin concentration can be determined by substituting 9-*cis*-retinal for 11-*cis*-retinal, by measuring absorbance (isorhodopsin) at 487 mμ, and by using for calculation the appropriate factors listed in the following section.

Assay of 9-cis-Retinals

The method for analysis of 9-*cis*-retinals is similar to that for testing opsin but is somewhat more extensive:

1. Work in red light. Measure isorhodopsin with a Beckman Model DU spectrophotometer using microcells (3 mm wide, 10-mm light path).

2. Put 1.0 ml of digitonin solution in the control cell. Put in each of two experimental cells excess opsin solution (usually 0.5 ml), an aliquot of the mixed isomers in digitonin solution (supplying 0.25 to 0.90 μg of 9-*cis* isomers/ml cell solution) and sufficient digitonin solution to give a total volume of 1.0 ml.

3. Stir contents of cells with a fine glass rod, and incubate in the dark for 2 hr at 20–25°C.

4. Stop the reaction in cell I by adding 0.1 ml of freshly neutralized hydroxylamine. Stir and let stand in the dark. Add hydroxylamine also to the control cell.

5. After 15 min, measure absorbance (A) of cell I at 487 mμ (isorhodopsin). Remove control cell and cell I from spectrophotometer and bleach as described above.

6. Replace cells in spectrophotometer and measure A of cell I at 487 mμ. The difference in A before and after bleaching cell I is called A_{I}.

7. Follow same procedure with cell II after incubating for 6 hr. The difference in A before and after bleaching cell II is called A_{II}.

8. Calculate the content of 9-*cis* isomers by assuming 100% reaction of 9-*cis*-retinal and 21% reaction of 9,13-di-*cis*-retinal (by isomerization to 9-*cis*-retinal) (4) in 2 hr and 100% reaction of both isomers in 6 hr:

$$\mu\text{g 9,13-di-}cis\text{-retinal} = \frac{1.1 \times 10^4(A_{\mathrm{II}} - A_{\mathrm{I}})}{0.79^a \times 1.25^b \times 1017^c}$$

$$\mu\text{g 9-}cis\text{-retinal} = \frac{1.1 \times 10^4\{(A_{\mathrm{I}}) - [1.27^a(A_{\mathrm{II}} - A_{\mathrm{I}}) - (A_{\mathrm{II}} - A_{\mathrm{I}})]\}}{1.25^b \times 1150^c}$$

a. Correction for the fact that the equivalent of 21% of the 9,13-di-*cis*-retinal had resulted in isorhodopsin after 2 hr.

b. Correction for the 25% increase in molar extinction of isorhodopsin relative to that of the 9-*cis* isomers.

c. $E_{1cm}^{1\%}$ (377 mμ) of 9,13-di-*cis*-retinal = 1017 (in 2% digitonin solution). $E_{1cm}^{1\%}$ (379 mμ) of 9-*cis*-retinal = 1150 (in 2% digitonin solution).

The latter equation can be simplified to the following:

$$\mu g\ 9\text{-}cis\text{-retinal} = \frac{1.1 \times 10^4(1.27A_I - 0.27A_{II})}{1.25 \times 1150}$$

No correction is made [as was done by Brown *et al.* (4)] for the small apparent reaction of opsin with the all-*trans*- and 13-*cis*-retinals (about 0.5 to 1.0% with up to 80 μg in the cell). The "standard" compounds may contain other isomers in trace amounts which are not detectable by any known chemical or physicochemical technique; and, in any case, such a correction would have a negligible effect within the limits of the assay.

RESULTS AND DISCUSSION

The relative ease of isomerization of vitamin A requires that care be taken during the preparation of samples. Procedures should be carried out as rapidly as possible and in red glassware or with a red safelight. Solvents should be evaporated carefully, and the residues should be taken up immediately in solvent. Standard compounds may isomerize slowly with time and it is desirable to make up fresh solutions frequently.

The *storage* of solutions of retinols in Skellysolve F should be avoided because this solvent seems to accelerate formation of anhydrovitamin A (λ_{max} = 365 mμ in Skellysolve F).

With proper precautions, the procedures described in this report give satisfactory results (see Table 1 for typical results). The saponification step routinely gives recoveries in the range of those illustrated (average value for over 50 saponifications was 96%), even with quantities as small as 14 μg.

The over-all average recovery of retinols from the desterolization is 85% (26 samples), slightly lower than suggested by Table 1. The recovery can be boosted either by increasing the amount of methanol used for this step or by precipitating a second time from methanol and combining the supernatants. However, it is usually preferable to sacrifice the small amount of retinol which could be recovered in order to avoid the proportionately greater amount of other nonsaponifiable material which also remains in the final solution.

We have tested alumina from several sources and deactivated by several procedures. That described by Olson (13) was the best. Average recovery from the column before the use of tocopherol (a recent modi-

TABLE 1

EFFICIENCY OF TECHNIQUES FOR PURIFYING VITAMIN A ISOMERS

	Vitamin A added			Vitamin A recovered		
Step	Compound[a]	Amt. (units)[b]	Maleic value[c]	Amt. (units)[b]	%	Maleic value[c]
Saponification	All-*trans*-retinyl palmitate	1,648	1.2	1,650	100.1	2.4
		2,269	2.7	2,160	95.2	2.6
		5,450	3.7	5,540	101.7	3.0
		19,689	0.1	20,000	101.6	1.1
		28,779	0.1	—	—	0.7
	All-*trans*-retinyl palmitate plus 9-*cis*-retinyl acetate	2,776 3,164	3.1	6,000	101.0	4.1
	9-*cis*-Retinyl acetate	3,234	9.2	3,220	99.6	12.1
		6,174	8.9	5,800	93.9	7.9
		30,307	7.7	31,000	102.3	6.0
	13-*cis*-Retinyl acetate	951	104.9	—	—	57.8
		1,902	104.9	—	—	74.4
		4,756	104.9	—	—	70.9
	13-*cis*-Retinyl acetate	12,591	96.4	12,600	100.1	76.2
Desterolization	All-*trans*-retinol	4,986	3.0	4,240	85.0	3.9
		19,333	1.1	19,333	100.0	3.3
		29,333	0.7	29,333	100.0	1.0
	All-*trans*-retinol plus 9-*cis*-retinol	2,524 2,876	4.1	5,000	92.6	3.5
Alumina column	All-*trans*-retinol	3,816	3.9	3,120	81.8	3.3
		28,666	1.0	20,500	71.5	1.6
	All-*trans*-retinol plus 9-*cis*-retinol	2,210 2,290	3.5	3,320	77.2	5.8
	9-*cis*-Retinol	428	2.6	400	93.5	2.4
		856	2.6	810	94.6	2.6
		1,284	2.6	1,267	98.7	2.9
		29,008	11.2	29,600	102.0	11.3
Desterolization plus alumina column	Mixed retinol isomers	6,320	28.4	5,375	85.0	28.0
		6,900	21.6	3,125	45.3	20.7
		7,200	23.9	6,300	87.5	23.1
		7,560	28.4	6,645	87.9	27.6
		124,000	30.2	77,004	62.1	26.9
		(μg)		(μg)		Opsin assay[d] (% 9-*cis* isomers)
Oxidation	All-*trans*-retinol	749	3.3	490	65.4	2
		2,772	1.6	1,200	43.3	4
		11,400	1.5	8,250	72.4	2
	All-*trans*-retinol plus 9-*cis*-retinol	372 425	5.8	680	85.3	52

Table continued

TABLE 1 (*Continued*)

Step	Vitamin A added			Vitamin A recovered		
	Compound[a]	Amt. (μg)	Maleic value[c]	Amt. (μg)	%	Opsin assay (% 9-*cis* isomers)
Oxidation (contd.)	9-*cis*-Retinol	1,494	0	1,100	73.6	102
		4,200	11.3	1,798	42.8	95
		12,000	0	10,400	86.7	100
	13-*cis*-Retinol	389	92.7	230	59.2	1
		1,944	92.7	1,350	69.4	0
		3,399	76.2	2,073	61.0	1

[a] Experiments were carried out either with standard compounds or with fractions derived originally from the appropriate ester.

[b] Measured by $SbCl_3$ test.

[c] Maleic value expresses percentage of isomers which contains a *cis* bond in the 13 and/or 11 position.

[d] Six-hour assay.

fication) was 78% (26 samples). Most of the loss was probably due to the formation of anhydrovitamin A, which appeared as an orchid-purple area under the ultraviolet light and which was eluted by the 2% acetone in hexane. Losses during this step are virtually nil (about 5%, 13 samples), when γ-tocopherol is added prior to chromatography (12).

A 1:1 (w/w) mixture of $ZnCO_3$ (Merck Reagent 75192, precipitated): Celite 545 also gives recoveries of 70 to 80% of the retinols,[5] but the alumina is preferred because the speed of elution is greater, the bands on the column are more discrete, and the progress of the fractions is easier to follow. We have not determined whether the addition of γ-tocopherol would also enhance the recovery of retinols from the $ZnCO_3$-Celite adsorbent.

Recovery of retinals from the oxidation step is not quite so complete; but the usual range is 60 to 85%, and quantities as small as 100 μg can be oxidized satisfactorily.

The over-all average recovery of retinals from the preparative steps (exclusive of solution in digitonin) was 45 to 50% with standard compounds. The spectra of the final products showed that the vitamin A was present as the retinals with an extinction at least 4 times that of any other material absorbing between 250 and 400 mμ.

Use of the maleic anhydride procedure (18) (to distinguish 11,13-*trans* isomers from others) indicated that neither isomerization nor selective loss of isomers is a problem during these procedures. If the all-*trans*

[5] G. Fletcher, Distillation Products Industries, personal communication, 1961.

compound isomerizes at any stage, only the 13-*cis* compound appears. If the 9-*cis* compound (the acetate was impure originally) isomerizes, only the 9,13-di-*cis* isomer is produced. The 13-*cis*-retinyl acetate isomerizes during saponification of small amounts,[6] but the absence of 9-*cis* isomers from the ultimate aldehyde indicates that only all-*trans*-retinol is produced. Neither this change nor any of the others which conceivably could have occurred have any effect on the per cent of total 9-*cis* isomers and therefore on practical application of the procedure.

Studies with 9-*cis*-retinal (Table 2) showed that the recovery averaged

TABLE 2

OPSIN ANALYSIS FOR 9-*cis*-RETINAL[a]

Retinal isomer added			Retinal isomer recovered	
9-*cis* (μg/ml)	All-*trans* (μg/ml)	13-*cis* (μg/ml)	9-cis (μg/ml)	(%)
0.222	—	—	0.229	103
0.222	0.889	—	0.222	100
0.222	—	—	0.236	106
0.222	—	1.12	0.236	106
0.238	—	—	0.247	104
0.242	—	—	0.236	98
0.242	2.72	—	0.236	98
0.242	—	—	0.236	98
0.242	—	3.27	0.236	98
0.303	—	—	0.306	101
0.303	—	—	0.306	101
0.303	0.544	—	0.306	101
0.351	—	—	0.371	106
0.369	—	—	0.356	96
0.454	—	—	0.452	100
0.591	—	—	0.605	102
0.605	—	—	0.611	101
0.605	—	—	0.590	98
0.605	0.135	—	0.570	94
0.700	—	—	0.671	96
0.740	—	—	0.716	97
0.751	—	—	0.696	93
0.820	—	—	0.764	93
0.871	—	—	0.871	100
0.900	—	—	0.870	96
0.909	—	—	0.905	100
				99 ± 4% S.D.

[a] Run for 90–120 min. Extending the time of the assay did not significantly increase recovery at any level.

[6] Larger amounts, i.e., 8 to 10 mg, show no isomerization during saponification.

99 ± 4% S.D. within 2 hr and with concentrations ranging from 0.222 μg to 0.909 μg/ml of solution in the cell. Recoveries of amounts less than 0.222 μg/ml were high. The upper limit was established by the volume and concentration of opsin solution that we can add to the cell under practical conditions. Addition to the cell of all-*trans*-retinal or 13-*cis*-retinal had no effect on the analysis.

TABLE 3
OPSIN ANALYSIS FOR 9,13-DI-*cis*-RETINAL

Retinal isomer added			9,13-Di-*cis*-retinal estimated			
			2 hr		Final[a]	
9,13-Di-*cis* (μg/ml)	All-*trans* (μg/ml)	13-*cis* (μg/ml)	(μg/ml)	(%)	(μg/ml)	(%)
0.265	—	—	0.079	30	0.259	98
0.332	—	—	0.102	31	0.322	97
0.493	—	—	0.157	32	0.471	96
0.493	—	—	0.157	32	—	—
0.493	4.00	—	0.149	30	—	—
0.591	—	—	0.157	27	—	—
0.591	—	7.78	0.173	29	—	—
0.625	—	—	0.164	26	—	—
0.738	—	—	0.189	26	—	—
0.738	—	7.78	0.189	26	—	—
0.935	—	—	—	—	0.951	102
0.935	5.45	—	0.314	34	0.904	97
0.960	—	—	0.267	28	—	—
0.960	6.67	—	0.267	28	—	—
				29 ± 3% S.D.		98 ± 2% S.D.

[a] Five to seven hours.

Table 3 indicates that 29 ± 3% S.D. of 9,13-di-*cis*-retinal isomerized to 9-*cis*-retinal and formed isorhodopsin within 2 hr and that formation of isorhodopsin was complete in 5 to 7 hr (98 ± 2% S.D.). Neither all-*trans*-retinal nor 13-*cis*-retinal affected the reaction. We have no explanation for the apparently "normal" recovery after 2 hr of 9,13-di-*cis*-retinal (as 9-*cis*-retinal)in amounts significantly less (e.g., 0.079 μg/ml) than could be recovered satisfactorily when 9-*cis*-retinal alone was added to the cell (see Table 2).

The results in Table 3 differ in two respects from those of previous workers. First, our observation of 29% reaction of 9,13-di-*cis*-retinal in 2 hr, at least in the absence of added 9-*cis*-retinal, is slightly higher than the value of 21% reported to us by Brown, Brown, and Wald.[4] Second, we found that the reaction of 9,13-di-*cis*-retinal is complete in 6 hr,

TABLE 4

OPSIN ANALYSIS FOR 9-*cis*- AND 9,13-DI-*cis*-RETINALS[a]

Retinal isomer added				Recovery of isomers					
				29% calculation			21% calculation		
9-*cis* (μg/ml)	9,13-Di-*cis* (μg/ml)	All-*trans* (μg/ml)	13-*cis* (μg/ml)	9-*cis* (%)	9,13-Di-*cis* (%)	Total (%)	9-*cis* (%)	9,13-Di-*cis* (%)	Total (%)
0.215	0.201	2.73	1.91	113	110	112	126	99	113
0.215	0.201	3.64	1.27	96	110	103	105	99	102
0.215	0.201	1.09	0.636	92	115	104	103	104	104
0.236	0.244	—	—	95	120	108	108	108	108
0.236	0.244	—	—	84	100	92	94	90	92
0.333	0.343	3.82	1.91	94	107	100	105	96	100
0.333	0.343	1.64	1.27	91	110	100	102	99	100
0.342	0.344	—	—	92	112	102	101	97	99
0.342	0.344	—	—	88	112	100	99	101	100
0.289	0.382	—	—	87	113	99	99	100	99
0.389	0.383	1.64	1.27	85	113	99	96	101	99
0.454	0.403	—	—	74	129	100	81	121	100
0.473	0.442	—	—	74	102	88	84	92	88
0.473	0.443	5.45	3.18	79	107	93	85	97	91
0.473	0.442	—	—	87	109	97	95	99	97
0.473	0.443	5.45	3.18	87	110	98	97	99	98
0.473	0.442	—	—	87	107	97	97	97	97
0.473	0.443	2.73	1.27	87	110	98	97	99	98
0.524	0.493	—	—	83	92	88	92	83	88
				—	—	—	—	—	—
				88	110	99	98 ± 10	99 ± 7	99 ± 6% S.D.

[a] All mixtures about 1:1.

whereas Brown *et al.* (4) found that the reaction was complete only within about 24 hr. In fact, we are unable to incubate solutions for 24 hr without cloudiness developing in the experimental cells.

One of these differences was apparently resolved when known mixtures of approximately equal amounts of 9-*cis*- and 9,13-di-*cis*-retinals were analyzed (Table 4). Recovery of the 9-*cis*-retinal was consistently low and that of 9,13-di-*cis*-retinal consistently high when 29% reaction of the latter was assumed to take place in 2 hr. Assumption of 21% reaction gave values much closer to theoretical, and we have adopted this figure for use. This finding is not really surprising, because one might anticipate that the presence of 9-*cis*-retinal would inhibit the rate of isomerization of the 9,13-di-*cis*-retinal. Over-all recovery of 99 ± 6% S.D. after 6 hr was the same by both methods of calculation and was not influenced by the presence of the all-*trans* and 13-*cis* isomers in quantities as high as 10 times the amount of the combined 9-*cis* isomers.

TABLE 5

OPSIN ANALYSIS FOR 9-*cis*- AND 9,13-DI-*cis*-RETINALS IN VARYING PROPORTIONS

Retinal isomer added			Recovery of isomers					
			29% calculation			21% calculation		
9-*cis* (μg/ml)	9,13-Di-*cis* (μg/ml)	Proportion 9-*cis*:9,13-Di-*cis*	9-*cis* (%)	9,13-Di-*cis* (%)	Total (%)	9-*cis* (%)	9,13-Di-*cis* (%)	Total (%)
0.226	0.676	1.0:3.0	31	120	98	70	108	98
0.231	0.736	1.0:3.2	136	75	90	160	68	90
0.231	0.706	1.0:3.1	91	75	79	111	68	78
0.231	0.706	1.0:3.1	118	77	87	142	69	87
0.231	0.455	1.0:2.0	54	107	89	75	96	89
0.303	0.201	1.0:0.66	92	81	87	96	74	87
0.462	0.232	1.0:0.50	44	196	95	54	176	95
0.471	0.247	1.0:0.52	42	188	93	52	169	92
0.605	0.403	1.0:0.67	77	137	101	85	123	100
0.676	0.247	1.0:0.37	79	144	98	85	130	96
0.678	0.248	1.0:0.36	85	135	98	90	121	98
0.678	0.248	1.0:0.36	61	175	91	67	158	91
0.779	0.234	1.0:0.30	30	359	106	36	294	105
								93 ± 7% S.D.

Analysis of known mixtures in proportions other than 1:1 (Table 5) gave acceptable over-all recovery of 93 ± 7% S.D. after 6 hr but showed that this procedure cannot be applied to such mixtures for measuring the amounts of the individual 9-*cis* isomers. The isomer present in lower concentration usually showed high recovery, probably reflecting an effect of relative concentrations on the rate of isomerization.

TABLE 6

ANALYSIS OF RETINAL CONCENTRATES FOR 9-*cis* ISOMERS

Sample	Total 9-*cis* isomers			9-*cis* Isomer composition			
				DPI		Harvard	
	Infra-red[a] (%)	DPI opsin[b] (%)	Harvard opsin[c] (%)	9-*cis* (%)	9,13-Di-*cis* (%)	9-*cis* (%)	9,13-Di-*cis* (%)
1. E_{379} = 1215	3–5	4	—	2	2	—	—
2. E_{377} = 1260	22	21	23	9	12	19	4
3. E_{373} = 972	8	7	7	3	4	6	1
4. E_{374} = 1080							
Original[d]	48	—	45	—	—	34	10–11
Opened[e]	40	33	—	17	16	—	—
Unopened[f]	45	42	—	21	21	—	—
5. E_{376} = 1040	23	26	—	15	11	—	—
6. E_{375} = 1010	22	20	—	7	13	—	—
7. E_{377} = 1270	25	25	—	15	10	—	—
8. E_{379} = 1480	—	<1	—	—	—	—	—
9. E_{375} = 965	9	13	15	7	6	—[g]	—[g]
10. E_{373} = 1022	19	15	—	8	7	—	—

[a] Courtesy of W. Blum, Physical and Analytical Chemistry Department.

[b] Analyzed at DPI after November, 1960.

[c] Analyzed by Mrs. P. Brown, Harvard University, prior to January, 1959.

[d] Analyzed in 1958. Contained 9% all-*trans*-retinal and 43% 13-*cis*-retinal.

[e] Analyzed in December, 1960. Vial had been opened and resealed during interim. Contained 30–35% all-*trans*-retinal and 25–30% 13-*cis*-retinal.

[f] Analyzed in December, 1960. Vial had been intact since original sealing. Contained 20% all-*trans*-retinal and 35% 13-*cis*-retinal.

[g] Data not furnished by Harvard.

Table 6 compares data obtained by infrared analysis and by opsin analysis of relatively pure samples of vitamin A obtained in appreciable quantity from aqueous multivitamin dispersions and commercial concentrates. Several of the samples were analyzed previously by Brown *et al.* (4). The total 9-*cis* isomer contents determined in two laboratories and by two methods compared favorably, but values for the individual 9-*cis* isomers differed greatly for the few samples for which data were available. Sample 4 was also of interest because it demonstrated that such concentrates of aldehydes may slowly isomerize during storage for several years *in vacuo* at −20°C.

Although we do not know the detailed history of each fish oil that we tested, application of the opsin procedure to various samples of liver lipids strongly indicated the natural occurrence of 9-*cis* isomers. Table 7 shows that the content of total 9-*cis* isomers in 6 samples of fish liver oil ranged from 6 to 12%. Total 9-*cis* isomers increased as the maleic

TABLE 7
VITAMIN A RECOVERY FROM AND 9-*cis* ISOMER CONTENT OF LIVER LIPIDS

Sample and potency	Amt. of vitamin A[a]			Maleic value[d]	Total 9-*cis* isomers (%)
	Initial[b] (μg)	Final[c] (μg)	(%)		
Pharmaceutical-grade fish oil					
Cod (2100 units/gm oil)	1,896	1,359	71.7	28.4	11
Cod (1150 units/gm oil)	2,070	750	36.2	21.6	6
Cod (4700 units/gm oil)	2,268	1,458	64.3	28.4	9
Halibut (82,500 units/gm oil)	2,160	1,235	57.2	23.9	7
Halibut (55,000 units/gm oil)	37,200	11,234	30.2	30.2	9
Feed-grade fish oil (4375 units/gm oil)	2,625	1,079	41.1	39.2	12
Mammalian liver lipids					
Steer (12 units/gm liver)	924	265	28.7	37.2	30
Steer (56 units/gm liver)	2,700	745	27.6	12.2	3
Sheep (290 units/gm liver)	3,276	1,038	31.7	3.2	5
Sheep (127 units/gm liver)	2,580	846	32.8	9.3	1
Pig (61 units/gm liver)	2,880	1,181	41.0	15.3	9
Pig (35 units/gm liver)	2,023	1,226	60.6	21.4	15
Rat (704 units/liver)	422	239	56.6	—	11
Rat (650 units/liver)	420	196	46.7	—	4
Rat (423 units/liver)	274	121	44.2	12.8	<1
Rat (10,275 units/liver)	3,014	612	20.3	6.6	3

[a] Measured by $SbCl_3$ test.
[b] Calculated as retinols.
[c] Calculated as retinals.
[d] Determined after saponification for all samples except after saponification and desterolization for the last two samples.

value increased but never attained the levels of 19 to 26% reported for fish liver oil concentrates by Brown *et al.* (4).

Livers from several mammalian species were obtained at the time of slaughter and were frozen until analysis. Maleic values and 9-*cis* isomer contents of the vitamin A in these livers indicated wide variation in isomer composition both within and among species (Table 7). Our value of 11% total 9-*cis* isomers in one sample of rat liver lipids is similar to that of 14% reported for a rat liver oil concentrate by Brown *et al.* (4).

The variation within a species undoubtedly reflects to some degree the dietary history of the individual animals. The rats, for example, were of different ages and had consumed our stock diet for varying periods of time subsequent to being used in nutrition experiments. The relatively high value of 30% of 9-*cis* isomers in the vitamin A from one of the steer livers is of particular interest because it was associated with

a very low level of only 12 units of vitamin A/gm liver. We speculate that the deficient animal preferentially utilizes the most active isomers from its liver reserves, leading to an increase in the relative proportion of 9-*cis* isomers. We are currently investigating this hypothesis.

To illustrate the application of these methods to samples containing higher proportions of 9-*cis* isomers, lipids were recovered from the gastrointestinal tracts of rats about 5 hr after oral administration of 9-*cis*-retinyl acetate. The vitamin A remaining in the stomach and the small intestine showed 70 and 56%, respectively, of total 9-*cis* isomers. Details of these studies will be reported separately by Ames *et al.* (report being prepared).

The inability to analyze mixtures (other than 1:1) for the individual 9-*cis* isomers rules out use of a combination of this procedure with the maleic anhydride procedure (18) and the antimony trichloride method (8) for determining the individual components in a mixture of the all-*trans*, 13-*cis*, 9-*cis*, and 9,13-di-*cis* isomers.

However, the average total recovery figure of 96 ± 7% S.D. for all known mixtures of the 9-*cis*- and 9,13-di-*cis*-retinals (Tables 4 and 5) indicates a general usefulness of this technique for measuring the total amount of 9-*cis* isomers, and, in conjunction with a determination of total retinal by reaction with antimony trichloride, the per cent of 9-*cis* isomers in a mixture.

SUMMARY

A procedure is described for the determination of the 9-*cis* components in pure or concentrated mixtures of vitamin A isomers. The method includes: saponification of esters; low temperature desterolization; purification of retinols on alumina; oxidation of retinols with MnO_2; and specific reaction of 9-*cis*-retinals with opsin, a protein from the retina, to form isorhodopsin ($\lambda_{max} = 487$ mμ). As little as 0.25 μg of 9-*cis* isomers can be determined, with an average recovery of 96 ± 7% S.D. Application of the procedure to liver oils from fish, cattle, sheep, pigs, and rats showed 9-*cis* isomer contents ranging from 1 to 30% of total vitamin A.

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ANALYTICAL BIOCHEMISTRY 4, 476–488 (1962)

Analytical Applications of the Continuous Measurement of Reaction Rate:

Lactic Dehydrogenase in Blood Serum

W. J. BLAEDEL AND G. P. HICKS

From the Department of Chemistry, University of Wisconsin, Madison, Wisconsin

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Recognition of the diagnostic value of enzyme determinations in blood serum has resulted in much interest in the development of better enzyme assay procedures and related instrumentation. One serum enzyme frequently determined is lactic acid dehydrogenase (LDH). Ultraviolet spectrophotometric procedures for LDH measure the increase or decrease of reduced diphosphopyridine nucleotide (DNPH) (1, 2). A variety of colorimetric methods exists, some measuring the disappearance of pyruvate, the substrate, by a colorimetric reaction (3, 4), while others measure DPN reduction with a coupled dye reaction giving an absorbance change in the visible region (5, 6).

In general, assay procedures for serum enzymes are based on the measurement of the rate of the enzyme-catalyzed reaction, and the success of a method ultimately depends on the precise and accurate measurement of the rate. The major variables affecting the measurement of the rate of an enzyme reaction are temperature, incubation time, substrate concentration, pH, and enzyme stability. Errors in enzyme assays may be high unless these variables are carefully controlled, which is sometimes difficult in routine clinical procedures.

Complete or partial automation of some enzyme procedures involving DPN have been described (7, 8). One instrument permits continuous measurement of the rate of enzyme-catalyzed reactions (9), and its application to LDH in blood serum is the subject of this paper.

Figure 1 outlines the continuous method. The LDH reagent, which contains all of the components necessary for the reaction except the enzyme, flows at a constant rate to meet and mix with a sample stream containing the enzyme, also flowing at a constant rate. The reaction, which causes an absorbance change, is initiated at the mixing point. The resulting reaction stream flows through two photometer cells separated by a delay line. Since the flow rates are constant, the time interval

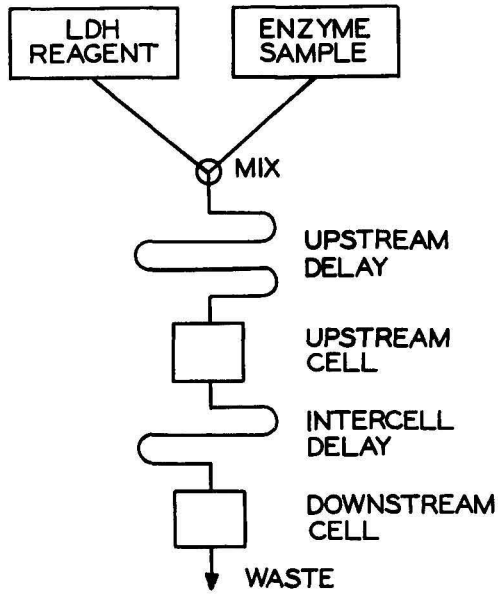

FIG. 1. Outline of continuous method for LDH.

between the cells is fixed, and the steady-state absorbance difference between the cells is proportional to the rate of reaction. The time intervals are small (about 30 sec), and good linearity between the enzyme concentration and the output from a recording differential photometer is obtained. After a simple calibration with reconstituted serum of known value, unknown sample values are read directly from the recorder chart with no calculation.

APPARATUS

Photometer and Electronics. The recording differential photometer, equipped with a 600 mμ interference filter, and associated electronics are essentially the same as previously described (9). Some minor changes in the equipment have been made for the sake of convenience and compactness. These changes, while desirable, are not essential and will not be discussed here. Details can be supplied upon request.

Flowing System. A diagram of the flowing system is shown in Fig. 2. The reagent and sample streams are metered with single-channel peristaltic pumps (Models PA-56, PA-6, New Brunswick Scientific Company, New Brunswick, N. J.). The PA-56 pump with 0.045-in. i.d. Tygon tubing gives a reagent flow rate of 4.6 ml/min. The PA-6 with 0.056-in. i.d. Tygon tubing gives a sample flow rate of 0.9 ml/min. Samples are

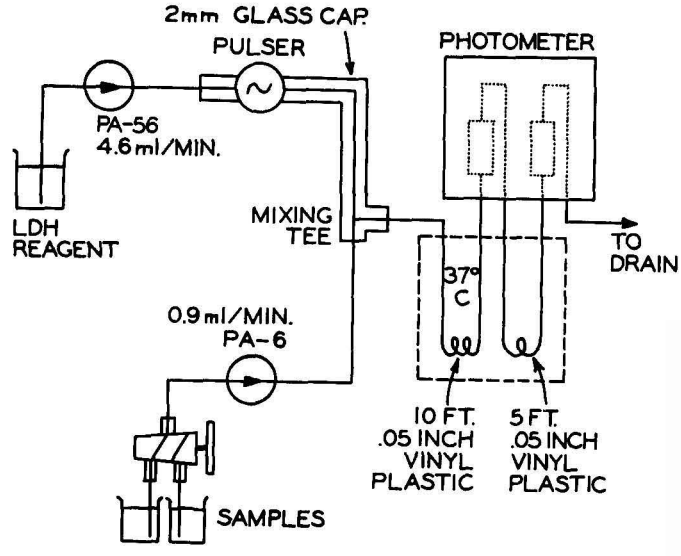

FIG. 2. Flowing system for LDH determination.

introduced through a 1-mm three-way stopcock threaded with 0.045-in. i.d. polyethylene tubing. No emphasis has yet been placed on automatic introduction of samples. The polyethylene tubing from the sample stopcock is inserted into the Tygon tubing of the PA-6 pump. All tubing before the sample pump is kept short in order to minimize sample holdup.

The pulser is located in the reagent stream, far enough from the mixing point so that the reaction stream is not pulled into the pulser.

The reagent and sample streams meet and mix in a 2-mm glass capillary T joint. Tygon to glass connections are made by dipping the Tygon into ethyl acetate and inserting it into successively larger pieces of Tygon until the outer piece of Tygon fits tightly over the glass tubing. The Tygon connection is slipped over the glass so that the small Tygon tubing butts against the glass capillary opening with little holdup.

The delay lines are 0.05-in. i.d. vinyl plastic tubing submerged in a 37 ± 0.05°C constant-temperature bath. The lengths of delay lines are selected to give an upstream delay of about 50 sec and an intercell delay of about 25 sec. Thus, the absorbance change is measured over a 25-sec interval within about 75 sec after the reaction is initiated. The upstream delay is more than enough to permit temperature equilibrium of the reaction stream. Some of the tubing and the cells which are not at 37° undoubtedly have an effect on the rate, but this effect is constant and

causes no error because of the method of standardization that has been developed.

The cells are made of 3-mm square-bore borosilicate glass tubing similar to those previously described (9). Tygon connections from the delay lines are made to 1-mm glass capillary tubing sealed to each end of the square tubing. The cells are installed in the photometer, and the delay lines are connected so that solution always flows upward through the cells. This prevents occasional air bubbles from being trapped in the optical path.

EXPERIMENTAL

Chemistry

Coupled dye reactions employing phenazine methosulfate (5, 6) or the enzyme diaphorase (10) as an electron transport system have been used for the assay of LDH in serum. Diaphorase is used in this work, the reactions being:

$$\text{L-Lactic acid} + \text{DPN}^+ \xrightarrow{\text{LDH}} \text{pyruvic acid} + \text{DPNH} + \text{H}^+$$

$$\text{DPNH} + \text{H}^+ + \text{dye}_{ox}\ (\text{blue}) \xrightarrow{\text{Diaphorase}} \text{DPN}^+ + \text{dye}_{red}\ (\text{colorless})$$

By making the concentrations of lactic acid, DPN, oxidized dye, and diaphorase sufficiently high, the rate of dye reduction and absorbance change depends only on the LDH concentration. Further, by measuring essentially an initial rate over a small time interval, the relationship between the measured absorbance change and the concentration of LDH is virtually linear, and the procedure is simple.

Reagents

Buffer: 0.05 *M* KH_2PO_4 is adjusted to pH 7.5 with 4 *N* NaOH.

Dye solution: 34 mg 2,6-dichlorophenolindophenol (Mann Research Laboratories, New York 6, N. Y.) is dissolved in 100 ml distilled water and stored at 2–5°C. This solution is stable for at least a month.

LDH reagent: To about 380 ml of buffer are added 150 mg DPN (Sigma Chemical Company, St. Louis 18, Mo.), 7 to 8 ml sodium lactate, 60% syrup (Sigma), 2 ml diaphorase (Worthington Biochemical Corporation, Freehold, N. J.), and 10 ml dye solution. To reduce the incidence of air bubbles in the flowing system, the buffer is aspirated several minutes to remove excess air before making up reagents. The addition of unbuffered sodium lactate causes the pH to drop to about 7.4, but pH is not critical in this procedure. LDH reagent has been used successfully after standing for a day at room temperature and overnight at 2–5°C.

Routinely, the reagent is made fresh daily and is quite stable for several hours at room temperature.

Reagent A: 4 ml dye solution is added to 160 ml buffer. This reagent is used to dilute routine serum samples. By including dye at about the same concentration in the sample as in the reagent, noise due to pump fluctuation is minimized. (Since the noise level corresponds to about 0.0001 absorbance unit, pump pulses are noticeable and contribute to the noise level when the reagent and sample streams differ greatly in absorbance.)

Reagent B: 4 ml dye solution is added to 120 ml buffer. This reagent was used to dilute enzyme samples in special studies.

Reconstituted serum: Enza-trol (Scientific Products, Evanston, Ill.) is reconstituted simply by adding distilled water, divided into aliquots, and frozen. Aliquots may be kept frozen for at least 4 days.

Analysis of Serum Samples

Serum samples were prepared by diluting 0.2 ml of blood serum with 4.0 ml of reagent *A*. When smaller amounts of serum were used, the total volume was adjusted to 4.2 ml with buffer. Blanks were prepared by substituting 0.2 ml of buffer for the serum. A typical chart recording which illustrates the procedure is shown in Fig. 3. The chart reads from right to left.

LDH reagent and blank solution were metered into the reagent and sample streams, respectively. After background was established, it was set with a bucking current control [R_9 in Ref. (9)] to the recorder zero, which had previously been set to 10 divisions with the recorder zero adjustment. A value of 10 divisions was chosen for illustrative purposes, to make any noise or drift fully perceptible in the record. A reconstituted serum sample of known value (Enza-trol) was introduced. At steady state the calibration control was adjusted so that each chart division represented 20 LDH units [conventional LDH units, defined as a decrease in OD of 0.001/min at 340 mμ (3)].

After calibration, a sample containing only 0.1 ml of reconstituted serum was introduced to check the linearity of the instrument. Then five fresh serum samples were run, followed by a blank and a second reconstituted serum sample. Finally, a high reconstituted serum sample and a normal fresh serum sample were run to show the response of a high value followed by a normal value.

After a day's work is completed, water is introduced into the sample and reagent streams to flush the instrument. Next, 1 *N* HCl and 1 *N* NaOH are each pumped in place of the sample and reagent streams for about 5 min, after which the instrument is again flushed with water.

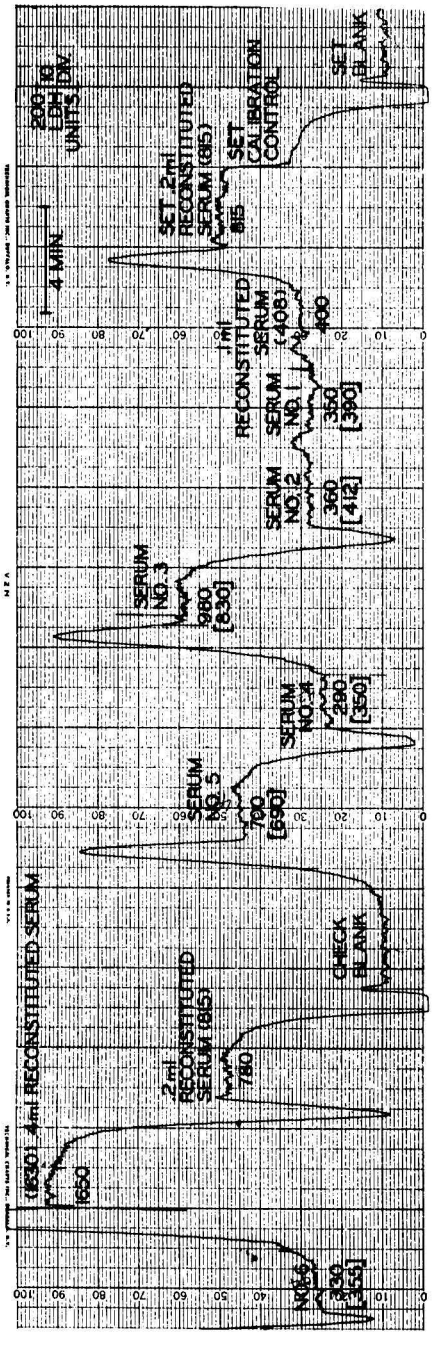

FIG. 3. Chart record for LDH in serum samples.

In shutdown, water is left in the flowing system. This simple washing procedure prevents gradual contamination of the flowing system.

Several characteristics in Fig. 3 are worth noting. First, the response time of the instrument from one steady state to another is about 2.5 min. This time may be slightly larger or smaller depending on the difference between two samples. With this type of response, samples may be run at the rate of 20 per hour with a readout time of about 4 min for any one sample.

The spikes on samples Nos. 3 and 4 are due to air bubbles which gradually build up and pass through the flowing system. These bubbles are infrequent and cause no error.

Transition peaks serve to distinguish consecutive samples. Such peaks exist even between two very similar samples (e.g., Nos. 1 and 2).

The overshoot on the steady state of sample No. 5 may be attributed to the alternating sample input system. Some of the high sample No. 3 remained trapped in the stopcock and was flushed in with the initial portion of sample No. 5. Because each sample is run to steady state, such overshoot causes no error. The overshoot could be avoided simply by altering the sample input system.

The second 815 unit control sample falls about 35 LDH units below the original calibration. In general, better checks than this are obtainable.

The noise level on recordings is routinely about 1% of full scale, but it may be as high as 2%, depending on the nature of the sample and reagents. The chart may be easily read to 1 scale division, or 20 LDH units. The reproducibility of replicate samples is 2% relative or 20 LDH units, whichever is larger. An experimental working curve prepared from different dilutions of reconstituted serum is shown in Fig. 4. Data for Fig. 4 are taken from Fig. 3. Known values for each reconstituted serum sample are indicated in parentheses in Fig. 3, while values obtained by direct readout (see below) are indicated directly below the chart trace. A linear working curve is obtained which misses the origin by about 30 LDH units when extrapolated. The broken line is the working curve which is assumed to be valid for direct readout, that is, when unknown sample values are read directly from the chart recording with no calculation after calibration with the 815 unit reconstituted serum. Around the calibration value, the direct readout and experimental curves coincide and no error is introduced by direct readout procedures. The error introduced by direct readout increases as the distance from the calibration point increases. Between about 300 and 1500 units, the error of direct readout does not exceed about 20 LDH units. For most samples encountered, the principal error is the chart reading error of about 20 LDH units, which is generally acceptable for routine clinical samples.

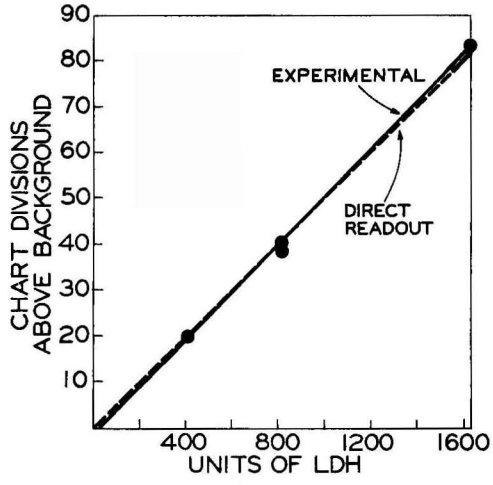

FIG. 4. LDH working curve prepared by quantitative dilution of reconstituted serum (data of Fig. 3).

For samples below 300 units, direct readout errors may reach 30 LDH units. If better accuracy is required for samples in this region, either a larger serum sample may be taken, or a more dilute control sample should be used to calibrate at a higher sensitivity.

The values obtained by direct readout are indicated under each sample in Fig. 3. The values obtained by an independent colorimetric procedure (3, 4) are indicated in brackets under the chart trace for each sample. The agreement between the two methods is as good as can be expected and is in accord with other studies that compare LDH methods (4). The data on the six samples in Fig. 3 are typical of about three dozen serum samples that have been compared by the two methods. The samples ranged from 290 to 1400 units, the root mean square difference between the two methods being 70 units.

The accuracy of the continuous method depends on how accurately the value of the reconstituted serum is known, and on the stability of the dried serum before reconstitution. Since the reconstituted serum is originally assayed by methods with errors (10–20%) comparable to the colorimetric method, no greater accuracy can be proved for the continuous method. It is felt, however, that the relatively high precision of the continuous method (about 20 LDH units) gives more reliable results than the colorimetric procedure. No trouble has been encountered with reconstituted serum stability, but cross checks between fresh and old reconstituted serum should be made occasionally to ensure quality control.

Discussion of Results

The great advantages of linear response and direct readout of the continuous method cannot be fully realized unless calibration can be performed with a standard enzyme solution. For LDH, which is a relatively stable enzyme, the use of a standard enzyme solution for calibration seems to involve less risk than other routine clinical methods that are based on a standard substrate concentration, and that require rigorous and absolute control of all conditions that affect the rate of reaction. The applicability of the continuous method will probably increase as more stable enzyme standards become available in the future.

REAGENT STUDY

A reagent study was made to establish nonlimiting concentrations of DPN, diaphorase enzyme, dye, and lactate. For each study, LDH reagent was prepared as described under *Reagents,* but the component under study was omitted. For example, in DPN studies, the LDH

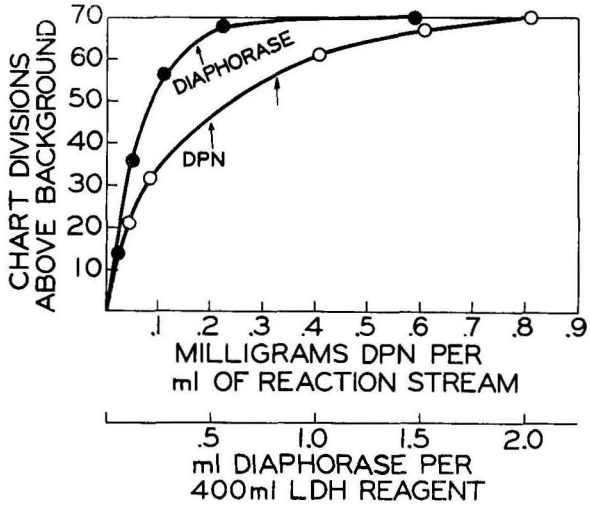

FIG. 5. Effect of diaphorase and DPN concentrations on reaction rate.[1]

reagent contained no DPN. Samples were prepared by adding the component under study and the desired amount of serum enzyme to 3.0 ml of reagent *B* and adjusting the total volume to 4.2 ml with buffer.

[1] The instrument was not calibrated with reconstituted serum for this experiment. Sensitivities may be different for different sets of data.

DPN. The DPN concentration in the reaction stream was varied from 0 to 0.8 mg/ml in the presence of about 1900 units of control serum. The results are shown in Fig. 5. A maximum rate was found around 1.0 mg/ml, which is not shown. While the rate increases rapidly up to about 0.4 mg/ml, it only increases slightly above this value.

Because of the rapid conversion of DPNH to DPN by the diaphorase enzyme, the concentration of DPN does not change appreciably during the reaction. Since DPN maintains a steady-state value during the reaction, a linear relationship between LDH enzyme concentration and the rate of absorbance change may be realized even with a limiting concentration of DPN. Further, because of the method of standardization used, the concentration of DPN from day to day is not critical. For these reasons, and for economy, the DPN concentration chosen for routine use was about 0.3 mg/ml, which is shown by the arrow in Fig. 5, and which gives about 80% of the maximum velocity. The chosen DPN concentration does in fact give linear working curves.

Diaphorase. The concentration of diaphorase, expressed as milliliters of commercial preparation added to 400 ml of LDH reagent, was varied from 0 to 1.5 ml in the presence of 1900 units of control serum enzyme. A maximum velocity is reached at about 0.5 ml. However, 2.0 ml of diaphorase is used routinely to assure a nonlimiting concentration of enzyme.

Dye and Lactic Acid. The dye and lactic acid concentrations used in the LDH reagent are known to be nonlimiting because linear working curves are obtained, and because the addition of more dye or lactic acid causes no increase in rate.

ATTEMPT TO USE PURIFIED LDH AS A STANDARD

Studies were made to determine whether or not a purified LDH preparation (Sigma, Stock No. 340–10) might be used as a secondary calibration solution after standardization against reconstituted serum.

The stock LDH solution was diluted about 40-fold with phosphate buffer to give a diluted purified LDH solution with an activity satisfactory for calibration (600 to 1000 units). The activity increases slightly after 6 to 8 hr at room temperature. Stored at 2–5°C, the diluted purified LDH solution loses 8% of its activity after 4 days, 17% after 8 days, and 50% after a month. Deterioration is somewhat faster when the solution is stored frozen. As far as stability is concerned, the diluted purified LDH would be satisfactory secondary calibration solution.

However, an activation of the diluted purified LDH by serum was noted. To study the activation, 0.1–0.4 ml portions of diluted purified enzyme were measured as previously described before and after the

addition of 0.3 ml of serum. In Fig. 6, activities are plotted as a function of sample size. The solid curve in the bottom half of Fig. 6 represents the activities of diluted purified LDH samples alone, while the broken

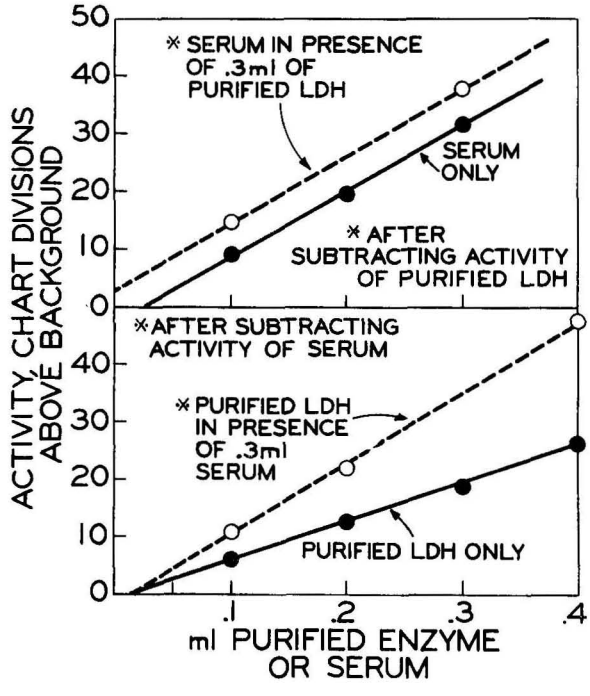

FIG. 6. Activation of diluted purified LDH by serum.[1]

curve represents the activities of diluted purified LDH in the presence of 0.3 ml of serum, after subtracting the activity of the serum (separately measured). The activating effect of serum on diluted purified LDH is clear. Both reconstituted and fresh serum give the activation effect.

In the top half of Fig. 6, the activities of varying amounts of serum were measured in the absence and presence of 0.03 ml of diluted purified LDH. All of the data together in Fig. 6 show that the activation is proportional to the amount of diluted purified LDH, and not to the amount of serum. It seems, therefore, that the diluted purified LDH is activated by serum, and not vice versa. This activation effect renders the diluted purified LDH unsuitable as a secondary calibration solution.

It was further found that the activating effect of serum extends to low serum concentrations. Thus, 0.2 ml of serum increased the activity

of 0.2 ml of diluted purified enzyme by 50%, but only 0.01 ml of serum increased the activity by 17%.

To establish that there was no interaction between reconstituted and fresh serum, varying amounts of reconstituted serum were measured in the presence and absence of fresh serum. Some typical results are shown in Fig. 7. No significant activation effect was observed between reconsti-

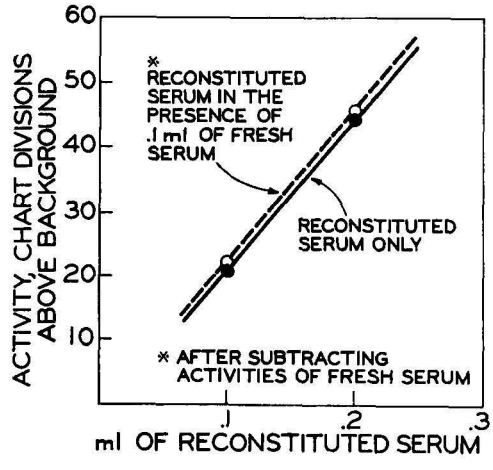

FIG. 7. Study of reconstituted serum in presence of fresh serum.[1]

tuted serum and various kinds of fresh sera (normal, abnormal, and pooled). No further study of the activation effect was made.

The activating effect of protein on LDH activity when measured by a dye reaction coupled through either phenazine methosulfate or diaphorase enzyme has been noted (5). The activation was observed when protein was added to a purified rabbit muscle enzyme or a rat liver homogenate, but not when added to serum. The effect was attributed to the increased catalytic efficiency of the electron transport system in the presence of protein.

Because diaphorase is nonlimiting in the continuous method, effects on the electron transport system alone cannot adequately explain the activation. From the data obtained with the continuous method, it seems probable that LDH enzyme itself is involved in the activation.

SUMMARY

The application of an instrument that continuously and automatically measures the rates of many enzyme-catalyzed reactions has been de-

scribed for the determination of lactic acid dehydrogenase in blood serum. Serum samples (0.2 ml) are analyzed at a rate of 20 per hour. Conservative amounts of reagents and enzyme are required, reagents are stable, and the method is feasible for routine application.

Because of the linear response and simple calibration procedure, routine serum samples may be read out directly, with an error of about 20 LDH units. No calculation is necessary.

At the present time, application of the technique is limited to procedures for which a suitable standard enzyme solution can be prepared. Application should increase in the future with increased availability of standards and the development of new standardization procedures.

ACKNOWLEDGMENTS

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ANALYTICAL BIOCHEMISTRY 4, 489–504 (1962)

Separation of Labeled from Unlabeled Proteins by Equilibrium Density Gradient Sedimentation

A. S. L. HU, R. M. BOCK, AND H. O. HALVORSON

From the Departments of Biochemistry and Bacteriology, University of Wisconsin, Madison, Wisconsin

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INTRODUCTION

Equilibrium sedimentation in a density gradient (1) has furnished biochemists with a sensitive and powerful tool for the study of nucleic acid structure and function. This method, if extended and applied to proteins, would provide a sensitive method for detection of natural differences in density among proteins and, when used in conjunction with stable isotopes, would permit the differentiation between newly synthesized and pre-existing proteins. We have examined the theory and performed experiments to determine which experimental conditions would give high resolution between two proteins differing 1 to 4% in density. The following parameters have been examined for their effect on resolution and on the time needed for effective resolution: density difference between labeled and unlabeled proteins, molecular weight of protein, sedimentation path length, centrifugal field, and the equilibrium density distribution given by various solvents.

The enzyme β-galactosidase was labeled with deuterium, N^{15}, and C^{13} and its separation from unlabeled enzyme is described here.

MATERIALS AND METHODS

RbCl was obtained from American Potash and Chemical Corporation in Los Angeles and was used without further purification. $N^{15}H_4Cl$ (99%) was obtained from Bio-Rad Laboratories, Richmond, California; $BaC^{13}O_3$ (54%) was obtained from Merck and Co., Ltd., Montreal, Canada; and D_2O was obtained from Stuart Oxygen Company, San Francisco. The chromogenic substrate *O*-nitrophenyl β,D-galactoside (ONPG) was purchased from Sigma Chemical Co., St. Louis, Missouri.

A Spinco model L preparative ultracentrifuge equipped with a SW 39 rotor was used in all the experiments.

Escherichia coli ML 308, which produces β-galactosidase constitu-

tively, was grown in synthetic medium C with Na_2-succinate as energy source (2) except when the cells were labeled with carbon-13. The carbon-13 label was introduced by first growing *Hydrogenomonas facilis* autotrophically (3), introducing the label as $C^{13}O_2$, then using an acid hydrolyzate of the harvested *Hydrogenomonas* as the sole carbon source in growing *E. coli*.

The *E. coli* cells were washed once by suspension and resedimentation in 0.02 *M* Tris-thioglycolate (TTG), pH 7.6, which was made 0.001 *M* in $MgSO_4$. Rupturing of the cells was accomplished by means of a Hughes press, and the supernatant, after centrifugation at 10,000 $\times$ *g* for 30 min, was used as the β-galactosidase preparation in our studies.

An exponential gradient [Fig. 1, Ref. (4)] of RbCl in TTG was created by using a mixing chamber containing 2 ml of 32% RbCl and forcing the solution out into a centrifuge tube by the addition of 4 ml of 37% RbCl through a syringe. Stirring was maintained by employing a small bar magnet and a magnetic stirrer. A 0.1-ml pipet was used to layer 0.1 ml of the material to be investigated over the preformed gradient. The tubes were immediately placed into a precooled SW 39 rotor and centrifuged for 75 to 80 hr at 30,000 rpm. The temperature during centrifugation was maintained at about 5°C. Tubes containing isotope-labeled enzyme, unlabeled enzyme, and a mixture of both species were examined in each experiment.

After centrifugation the tubes were carefully removed and clamped to a ring stand. A 25 gage hypodermic needle with its Luer adapter removed was forced through the bottom of the tube and fractions of about 0.2 ml were collected manually by the counting of drops.

The fractions were assayed for β-galactosidase activity, using the chromogenic substrate *o*-nitrophenyl β,D-galactopyranoside (5). The assays were conducted at 30°C and readings were made at 100-sec intervals in a Bausch and Lomb Spectronic 20 colorimeter.

EXPERIMENTAL DESIGN

(*a*) *Parameters Influencing Theoretical Resolution*

The condition where ρ_1 and ρ_0 exist within the equilibrium gradient will be considered first. The isopycnic densities of labeled and unlabeled macromolecules are designated ρ_1 and ρ_0.

The concentration of a macromolecular species which has sedimented to equilibrium in a density gradient may be described to a close approximation by a Gaussian distribution. The standard deviation of the concentration distribution about the level where the supporting electrolyte

is isopycnic with the macromolecule is a function of the properties of the macromolecular and the conditions of ultracentrifugation (1).

$$\sigma^2 = \frac{RT}{M\bar{v}(d\rho/dr)_r\omega^2 r} \tag{1}$$

where σ is the standard deviation of the concentration distribution, R is the gas constant, M is the molecular weight of the macromolecular species, $\bar{v}$ is the apparent specific volume of the macromolecular species; ρ is the density of the salt solution, r is the radius in the centrifuge cell at which the macromolecule is isopycnic, and ω is the angular velocity.

The resolution between two molecular species, 1 and 0, may conveniently be defined as $(r_1 - r_0)/(\sigma_1 + \sigma_0)$. At a resolution of 1.2 a minimum will exist in the center of the distribution if two macromolecular species are present in equal amounts. If the amounts of the two macromolecules differ by a factor of 5, then a resolution of 2 will permit recognition of two maxima and direct estimation of the amounts of each.

We have calculated the resolution attainable for protein of various molecular weights as a function of the density difference between the two macromolecules present. Figure 1 shows a plot of these data computed for conditions of $d\rho/dr$, ω, and r which correspond to the experi-

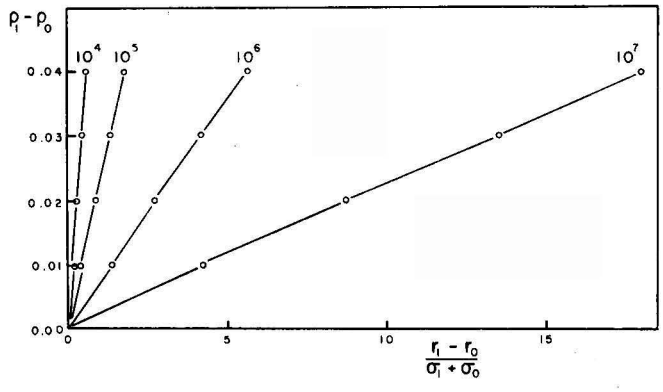

FIG. 1. Resolution of macromolecules as a function of density difference between labeled and unlabeled species. Values pertain to RbCl gradients at $\rho = 1.33$ gm cm^{-3} and are given for molecular weights 10^4, 10^5, 10^6, and 10^7.

mental conditions used of Figs. 4, 5, 6, and 7. The solvent systems listed in Table 1 permit a choice of resolution as high as double or as low as one-half that shown in Fig. 1. In Table 2 are listed the density differences which can be introduced into macromolecules by various procedures.

TABLE 1

PROPERTIES OF SOLUTES USED FOR EQUILIBRIUM DENSITY GRADIENT CENTRIFUGATION OF PROTEINS AND NUCLEIC ACIDS

Solute	Solvent (%)	(gm solute/ 100 gm soln.)	Density (20°C)	$10^{10}\, d\rho/\omega^2 r dr$	Remarks
CsCl	H_2O	30	1.288	6.25	$\eta_r = 1$[a]
CsCl	H_2O	35	1.352	6.91	
CsCl	H_2O	60	1.789	7.46	
CsCl	H_2O	65.1	1.91	8.0	Satd.
CsCl	D_2O	63	1.98	8.0	Satd.
Cs_2SO_4	H_2O	64.1	2.01	(30)	$\eta_r = 1.5$
Cs formate	H_2O	70	2.1	7	$\eta_r = 2$
Cs acetate	H_2O	70	2.0	(3)	$\eta_r = 3$
RbCl	H_2O	35	1.324	3.63	[b]
RbCl	H_2O	48	1.49	(5)	Satd.
Rb formate	H_2O		1.85	(3)	
RbBr	H_2O		1.63	8	Satd.,[b] UV absorber
KAc	H_2O	72	1.41	0.71	$\eta_r = 3$, satd.
KAc	D_2O	50	1.35	0.5	$\eta_r = 2$
K formate	H_2O	77	1.57	(1.5)	Satd.
K formate	D_2O	74	1.63	(1.5)	Satd.
K_2 tartrate	H_2O	60	1.49	?	$\eta_r = 11$
Na formate	H_2O	49	1.32	(1)	Satd.
Na formate	D_2O	45	1.40	(1)	Satd.
LiBr	H_2O	63	1.83	0.8	Satd.,[b] UV absorber
LiCl	D_2O	30	1.33	0.4	Satd., $\eta_r \sim 3$
Sucrose	H_2O	63	1.30	(4)	$\eta_r = 60$[b]

[a] Detailed data on this salt are given by Trautman (6).

[b] Detailed data for these solutes are given by Ifft, Voet, and Vinograd (7). The values in parentheses are estimates which may be in error by as much as 50%. Relative viscosities, η_r, are believed to be less than 2 except where noted. Data compiled from Robinson and Stokes (8), "International Critical Tables," and "Lange's Handbook of Chemistry," or measured directly in our laboratory.

An equation relating the density gradient obtained to the salt used has been published by Ifft, Voet, and Vinograd (7). Trautman (6) has also derived a similar equation. The equation of Vinograd *et al.* is:

$$\left(\frac{1}{\omega^2 r}\right)\frac{d\rho}{dr} = \frac{M(1 - \bar{v}\rho)}{\nu RT \dfrac{\delta \ln \gamma m}{\delta \rho}} \qquad (2)$$

where the terms are as defined for Eq. (1) except that they refer to the supporting electrolyte rather than the banded macromolecule. The symbols ν, γ, and m are the number of ions per molecule, the activity co-

TABLE 2

DENSITY DIFFERENCES EXISTING IN OR INTRODUCIBLE[a] INTO MACROMOLECULES

Macromolecule	Source of density shift	Max. density diff.
Protein,[b] av. dens. 1.33	Natural variations in amino acid compn. and salt binding	0.10
	N^{15} replacing N^{14}	0.013
	C^{13} replacing C^{12}	0.05
	D replacing all C-bound H	0.06
	O^{18} replacing O^{16}	0.03
	Fluorine replacing H in CH_2 and CH_3 groups of:	
	leucine	0.07
	valine	0.08
	alanine	0.04
	lysine	0.12
	glutamate	0.06
Nucleic acid, av. dens. 1.71 (DNA) and 2.0 (RNA)	Natural variations in AT/GC ratio in DNA	0.04 (obs.) (0.1 theor. max.)
	Bromouracil in DNA	0.08
	N^{15} replacing N^{14}	0.016
	C^{13} replacing C^{12}	0.036
	D replacing all C-bound H	0.035
	Replacement of bound Cs by Mg or spermine	0.3
	5-Fluorouridine in RNA	0.02
	Solvent interaction[c]	0.6

[a] The procedures for introduction of deuterium and N^{15} into DNA have been reported by Marmur and Schildkraut (9). Nucleoproteins are usually slightly less dense than predicted from their protein/nucleic acid composition. This probably reflects displacement of bound cations by the protein nucleic acid interaction. The introduction of fluorine analogs of amino acids has not yet been reported, but appears to be a promising route to high resolution. Sueoka (10) has listed many of the natural density variations in DNA.

[b] The protein composition used for these calculations was an average representing twenty pure proteins of known composition.

[c] Preferential interaction with components of the solvent are large when the salt includes a polyion of same sign of charge as the nucleic acid (11). This interaction causes small density shifts in proteins (12) but does not markedly alter the resolution calculated ignoring the interaction.

efficient and the molality, respectively. We have used this equation to calculate $(1/\omega^2 r)(d\rho/dr)$ for several salts. While tabulating the gradients produced by various salts, it became apparent that the term $(\delta \ln \gamma m)/\delta\rho$ was of major importance in producing extremely shallow or steep gradients in the salts available. The large value of this term for some

acetates encouraged us to determine the gradients on several salts for which no published activities or densities could be found.

Figure 2 illustrates the predicted equilibrium separation of the center of the bands for two proteins having a density difference of 0.01 gm/ml.

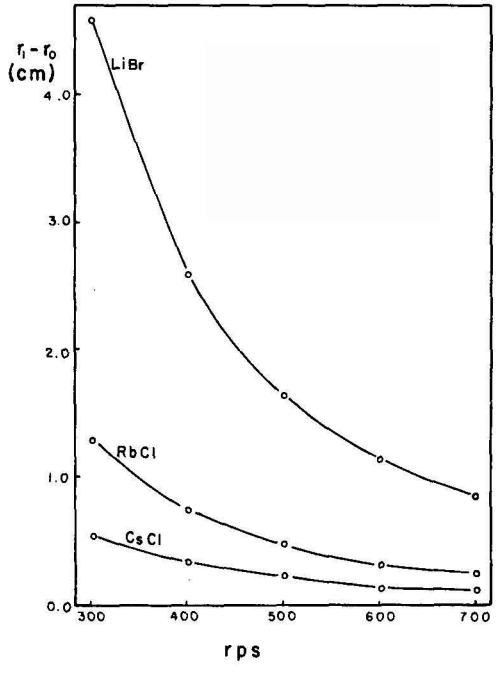

FIG. 2. Equilibrium band separations in various salts. Data pertain to a Spinco SW39 rotor where rps is revolutions per second and where the two macromolecules have densities 1.33 and 1.34.

These data are useful as a practical guide to decide on the size of samples to be removed from the gradient.

A combination of Eqs. (1) and (2) reveals that, for the condition that both macromolecules are isopycnic within the equilibrium gradient, resolution is independent of centrifuge speed for any single supporting solvent. However, variation of the solvent is useful because resolution is proportional to $[d\rho/dr]^{-1/2}$. Thus, one seeks a salt giving as small a density gradient as will permit banding of both labeled macromolecules within the solution column at the maximum available centrifuge speed. The compressibility of the solvent limits the minimum attainable gradient (13).

The interaction of salts with proteins causes density changes which

are small enough not appreciably to alter the theoretical resolution. Nucleic acids, however, bind sufficient cations that their molecular weight and densities are markedly altered. The binding of a heavy cation decreases the equilibrium band width approximately one-half as much as it decreases the band separation and thus can cause appreciable loss of resolution.

(*b*) *Time of Centrifugation*

Of practical importance in equilibrium sedimentation studies is the centrifugation time necessary to attain equilibrium. If the material to be banded is homogeneously dispersed throughout the solution, the time required to approach within 1% of equilibrium may be expressed by the equation of Meselson *et al.* (1):

$$t = \frac{\sigma^2}{D}\left(\ln\frac{L}{\sigma} + 1.26\right), \qquad L \gg \sigma \tag{3}$$

where t, D, and L are time, diffusion constant, and length of the column of solution, respectively.

However, if the macromolecules to be banded are initially layered in a narrow band over a gradient preformed to approximate the equilibrium gradient, the equation relating the time required to the other parameters may be derived from the Svedberg equation (14):

$$\frac{dr}{dt} = \frac{D(1 - \bar{v}\rho)M\omega^2 r}{RT} \tag{4}$$

if one assumes that migration of the narrow band is the time-dependent phenomenon which limits resolution.

From Eq. (2) we know that:

$$d\rho/dr = kr \tag{5}$$

Integrating Eq. (5), we have:

$$\rho = \frac{kr^2}{2} + c \tag{6}$$

Substituting Eq. (6) into (4), and integrating between limits:

$$\int_0^t dt = \int_{r_0}^{r_t} \frac{RT\,dr}{DM\omega^2\left[r - \dfrac{\bar{v}kr^3}{2} - \bar{v}cr\right]}$$

$$t = \frac{RT}{DM\omega^2(1 - \bar{v}c)} \ln\left[\left(\frac{r_t}{r_0}\right)^2 \left(\frac{1 - \dfrac{\bar{v}k\,r_0^2}{2(1 - \bar{v}c)}}{1 - \dfrac{\bar{v}k\,r_t^2}{2(1 - \bar{v}c)}}\right)\right] \tag{7}$$

We have made calculations using Eqs. (3) and (7), using values of ω, r, $d\rho/dr$, and L that were used in our experiments. The calculations using Eq. (7) involved the time required for a band to reach within 0.25 mm of its equilibrium position ($r_1 = 6.5$ cm) 1 cm away from the starting point ($r_0 = 5.5$ cm). We have assumed that $\bar{v}$ is independent of ρ. The numerical values of D were taken to be 5×10^{-7}, 4×10^{-7}, 3×10^{-7}, 2.4×10^{-7}, 1.8×10^{-7}, 1.4×10^{-7}, and 10^{-7} for molecular weights 10^5, 2×10^5, 5×10^5, 10^6, 2×10^6, 5×10^6, and 10^7, respectively.

Figure 3 presents some of the calculations in graphic form. The time required to approach equilibrium is markedly less when the material to be banded is layered over a preformed gradient than when the material has been dispersed throughout the solution. The calculated time required

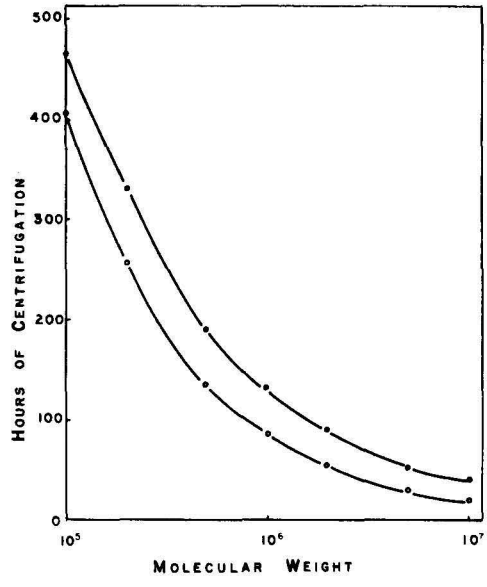

FIG. 3. Time required to attain sensible equilibrium in RbCl gradients at 500 rps in Spinco rotor SW39: (—●—) protein homogeneously dispersed in tube at start of centrifugation; (—○—) protein layered over a preformed gradient. The criteria for "sensible equilibrium" are appropriate for the methodology of this study but do not represent a general comparison of the two procedures.

to sediment β-galactosidase within 0.25 mm of its equilibrium position was 129 hr. That useful resolution can be often obtained in much shorter periods is demonstrated by Figs. 4, 5, 6 and 7, in which the sedimentation

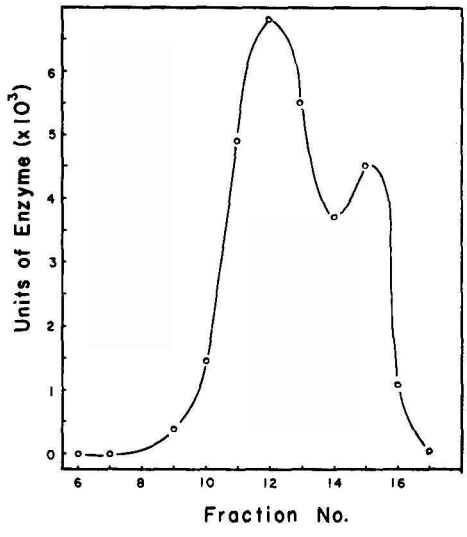

FIG. 4. Separation of N^{15}-labeled β-galactosidase from the unlabeled enzyme in a RbCl gradient. Duration of centrifugation was 75 hr at 500 rps. Density increases with increasing fraction number.

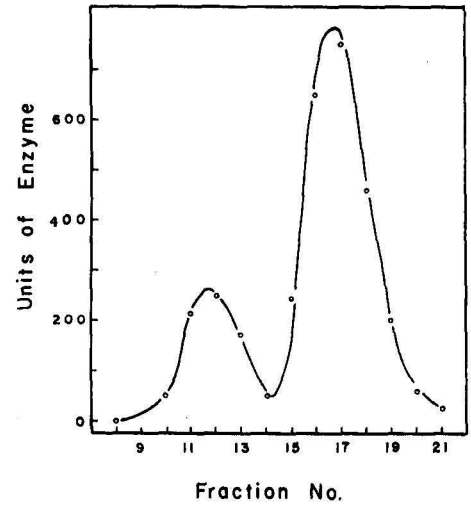

FIG. 5. Separation of C^{13}-labeled β-galactosidase from the unlabeled enzyme in a RbCl gradient.

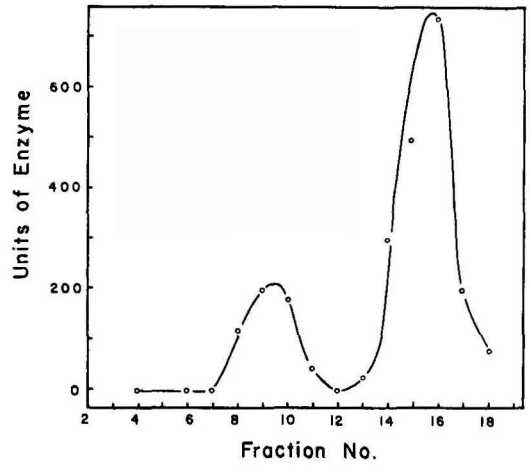

FIG. 6. Separation of C^{13}, N^{15} doubly labeled β-galactosidase from the unlabeled enzyme in a RbCl gradient after centrifugation for 76 hr at 500 rps.

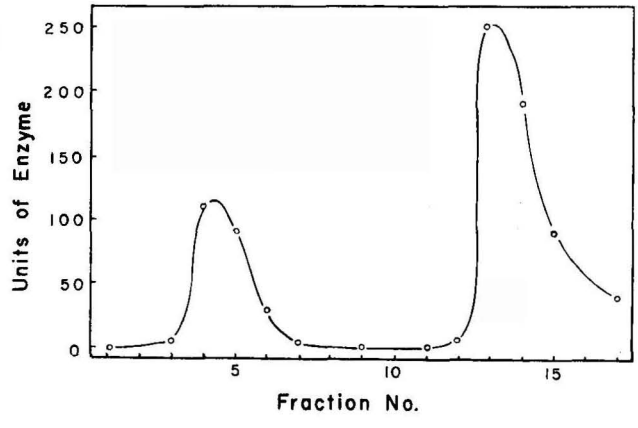

FIG. 7. Separation of D, N^{15} doubly labeled β-galactosidase from the unlabeled enzyme in a RbCl gradient.

times were 75 to 80 hr. The influence of solvent viscosity on D is sufficient to limit the value of solvents such as sucrose and potassium tartrate because of the increased time needed for equilibration.

RESULTS

Separation of Labeled from Unlabeled β-Galactosidase

Nitrogen-15 labeled β-galactosidase could be resolved from the normal, unlabeled enzyme, although the respective peaks overlapped con-

siderably (Fig. 4). The introduction of 54% carbon-13 into the enzyme markedly increased the resolution of labeled from unlabeled enzyme, although some overlapping was still present (Fig. 5). Complete separation of the doubly labeled nitrogen-15, carbon-13 enzyme was obtained, as shown in Fig. 6. The greatest separation was obtained between β-galactosidase from cells grown in nitrogen-15 plus 99.5% D_2O (with unlabeled succinate as carbon source) and the enzyme from cells grown in nonisotopic medium (Fig. 7).

The calculated value of σ for β-galactosidase, using a molecular weight of 750,000 (11), was 0.15 cm. The estimate of σ from the experimental data was 0.17 to 0.19 cm. The agreement is remarkably good considering the manipulations involved in the collection of fractions after centrifugation, and the fact that the systems had not been centrifuged sufficiently long to approach true equilibrium.

Calculations were made of the resolution and separation expected and compared with the experimental values obtained (Table 3). Here

TABLE 3

EQUILIBRIUM BAND SEPARATION AND RESOLUTION OF β-GALACTOSIDASE

Label	$r_1 - r_0$		$(r_1 - r_0)/(\sigma_0 + \sigma_1)$	
	Calc.	Obs.	Calc.	Obs.
N^{15}	0.69 ± 0.13	0.6 ± 0.1 cm	2.3 ± 0.5	1.55 ± 0.15
C^{13}	1.15 ± 0.15	1.1 ± 0.1	3.8 ± 0.5	2.75 ± 0.25
$C^{13} + N^{15}$	1.70 ± 0.30	1.4 ± 0.1	5.7 ± 1.0	3.5 ± 0.25

also, experimental values agree well with the calculated values.

Because deuterium incorporation depends on metabolic exchange of deuterium with hydrogen at stable positions on the protein, no calculations could be made on the expected separation and resolution between the labeled and unlabeled enzymes. However, the reverse calculation revealed that 35 to 50% of the hydrogen atoms have been metabolically replaced by deuterium atoms that were not easily exchangeable with the medium.

With the availability of the solutes listed in Table 1, it became apparent that there exists a second interesting condition for separation of macromolecules. In this condition the density range within the centrifuge cell is small compared to the density difference between the macromolecules and isopycnic conditions do not exist within the cell. The above equations no longer apply but the classical equation for equilibrium sedimentation or flotation:

$$d \ln C = \frac{M(1 - \bar{v}\rho)\omega^2 r dr}{RT} \tag{8}$$

or modifications of this equation which allow for variation of $1 - \bar{v}\rho$ with r, predict that separation increases with centrifuge speed. The equations predict that a density difference of 0.04 would permit isolation of 90% pure light component in the top fifth of the cell and 90% pure heavy component in the bottom fifth of the cell for a protein of molecular weight 50,000 centrifuged to equilibrium at full speed in either a Spinco SW39 rotor or an analytical Spinco AnD rotor. Rotor speeds of 10^5 rpm or density differences of 0.1 would be needed for similar separation of very small proteins such as pancreatic ribonuclease or egg white lysozyme.

TEST FOR *DE NOVO* ENZYME SYNTHESIS IN A CELL-FREE SYSTEM

In disrupted bacterial systems, studies involving amino acid incorporation, appearance of enzyme activity, and appearance of antigenically identifiable proteins have led to the conclusion that these disrupted systems were capable of carrying out *de novo* protein synthesis. The low degree of amino acid incorporation as well as the existence of latent forms of enzyme in bacterial particles have cautioned against the view that such observations are necessarily due to *de novo* protein synthesis. The equilibrium sedimentation procedure with heavy isotopes was applied to one such system to examine its usefulness in studying cell-free protein synthesis.

Preliminary experiments indicated that in disrupted cells of *Escherichia coli* active amino acid incorporation could be demonstrated both into the ribosomal and into the membrane fraction. The higher activity in the latter led to its use in the following experiment designed to test *de novo* β-galactosidase synthesis. A supernatant fraction containing activating enzymes was prepared as follows. *E. coli* ML 30 (i^+z^+) was grown in a peptone, tryptone, yeast extract medium, preinduced for 10 min with lactose (0.5%), washed with TTG buffer, and the cells (1 gm wet weight) broken by passing the suspension through a French pressure cell. The suspension was centrifuged in a Servall for 25 min at $15{,}200 \times g$, and the supernatant retained. This procedure yields a supernatant with reasonably low levels of β-galactosidase. *E. coli* ML308 was used as a source for membrane preparations. One liter of cells was grown, washed, and disrupted as above except that preinduction with lactose was omitted. The suspension was centrifuged in a Servall for 25 min at $15{,}200 \times g$, the supernatant was discarded, and the membrane fraction was resuspended in 10 ml of buffer without disturbing the whole cell pellet. The larger particles were removed by centrifugation for 5 min at $3{,}000 \times g$ and the membrane fraction collected by centrifugation and resuspended in 1 ml of buffer. Microscopic examination of this fraction indicated

it was free of intact cells. DNA was prepared from *E. coli* ML308 by the method of Marmur and Grossman (15).

The incubation for protein synthesis are indicated in Table 4. Tube 1

TABLE 4

AMINO ACID STIMULATION OF β-GALACTOSIDASE FORMATION BY MEMBRANE FRACTIONS OF *Escherichia coli*

Tube	System	Amino acid added	β-Galactosidase (units/ml)
1	—Cr P, —nucleotides	None	46
2	Complete	*H*-amino acids	870
3	Complete	*D*-amino acids	870

The reaction mixture (1.2 ml) contained 50 μmoles of Tris-HCl buffer (pH 7.2), 2.5 μmoles $MgSO_4$, 1 μmole each of GTP, ATP, CTP, UTP, 10 μmoles isopropyl thiogalactoside, 5 μmoles creatine phosphate, 100 μg creatine phosphokinase, 0.3 mg DNA, 2 ml crude supernatant from *E. coli* ML 30, and 0.3 ml membrane preparation (see *Note* below) from *E. coli* ML 308. Amino acids were added as indicated either as a mixture of 20 unlabeled L-amino acids (0.2 μmole each) or 8 mg of deuterated amino acids (protein hydrolyzate of algae) supplemented with 0.4 mg proline, 0.4 mg L-tryptophan, and 0.06 mg L-cysteine. The amino acid content of the supernatant fraction was less than 5% of the added deuterated amino acid mixture. The mixture was incubated for 1 hr at 30°C and centrifugated at 16,000 × *g* for 20 min, and supernatant was assayed for β-galactosidase activity.

Note: Incubation of washed ribosomes in a similar system showed no heavy labeled enzyme in experiments where 50 and 200% increases in β-galactosidase occurred during incubation.

served as a control, tube 2 was supplemented with unlabeled amino acids; and tube 3 received deuterated amino acids. The tubes were incubated for 60 min at 30°C and centrifuged and the supernatant was retained for analysis. As can be seen, the enzyme activity rose from 46 units in the control to about 870 units in each of the tubes incubated with amino acids. The supernatant fractions were then layered on a RbCl density gradient as shown in Fig. 8. The expected position of β-galactosidase synthesized *de novo* from *D*-amino acids (Fig. 7) is shown by the arrow. It is clear from inspection of Fig. 8 that the equilibrium position of the increased β-galactosidase is that of the unlabeled enzyme. The recovery of total enzymes from the gradient was 109% in the case of the unlabeled amino acid and 114% for the deuterated amino acid; thus, the newly synthesized enzyme was not lost during centrifugation. Since the shape of each peak is reasonably symmetrical, the incorporation of heavy amino acids was undoubtedly extremely small.

These data underscore the difficulties inherent in studies on protein

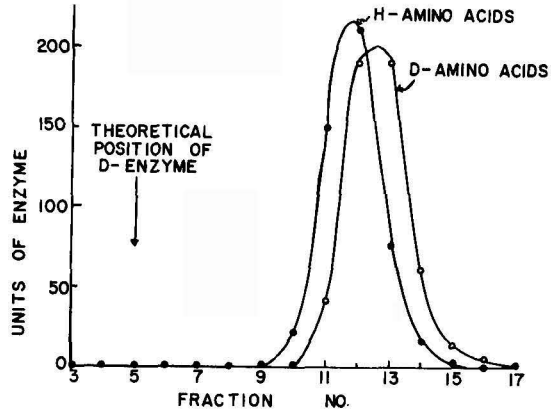

FIG. 8. Centrifugation of β-galactosidase synthesized in cell-free extract. Supernatant fractions (0.01 ml) from tubes 2 and 3 in Table 4 were centrifuged in a RbCl gradient. Duration of centrifugation was 45 hr at 620 rps.

synthesis and point out the necessity for critical evaluation of data purporting to demonstrate *de novo* protein synthesis *in vitro*. This same system actively incorporates amino acids, suggesting in the present experiment that the observed amino acid requirement may be for the completion of a polypeptide chain or the release of previously formed complex precursors of β-galactosidase.

DISCUSSION

At present the probable lower limit of molecular weight which would permit useful separation and resolution of two molecular species is slightly less than 100,000. However, it is likely that centrifuges with higher speeds than the Model L will make it practicable to perform equilibrium density gradient sedimentation of even smaller molecules.

The choice of a suitable salt is governed by a number of factors: (*1*) the solubility of the salt chosen must be sufficiently great to give a solution of sufficient density to permit equilibrium sedimentation; (*2*) the gradient of density must be suitable for good separation and resolution; (*3*) the proteins to be studied must be fairly stable in high concentrations of the salt; (*4*) the proteins should not be salted out of solution at the desired concentration of salt. We have found that LiBr and KBr would provide sufficiently shallow gradients to permit good separation and resolution of nitrogen-15 labeled β-galactosidase from unlabeled enzyme, but the enzyme was quite labile in both salts. Cox and Schumaker (12) found multiple bands in a mixed precipitate sedimented to

equilibrium, but coprecipitation of labeled with unlabeled protein occurs more readily than coprecipitaton of unlike proteins. At the time that the enzyme separations were done, we had not yet discovered the high resolution system K acetate in D_2O.

A source of ambiguity in experiments such as described here is the possibility that some proteins may polymerize during the course of the experiment, giving rise to highly skewed or possibly even multiple bands. The enzyme β-galactosidase is just such a protein, and will form polymers linked by disulfide bridges in the presence of oxygen. This difficulty was circumvented by the use of thioglycolate buffer. Experiments performed in the absence of thioglycolate have resulted in highly skewed bands. It is also possible that the high ionic strengths involved may degrade a protein into subunits with subsequent recombination, but the resolution attained here showed that such rearrangements were not significant for this enzyme.

Studies of protein synthesis, particularly those involving cell-free systems, would be made easier and more interpretable if newly synthesized protein could be separated from pre-existing protein. The employment of amino acids labeled with stable isotopes and density gradient centrifugation offers such a possibility. Such a method would, moreover, be an extremely sensitive one for the detection of a newly synthesized enzyme since most of the "background noise" would be eliminated. It is likely that, under the proper conditions, a net increase of a few per cent of an enzyme could unequivocally be proved to be due to the synthesis of new enzyme. In the cell-free system studied here, a several-fold increase in enzyme activity was shown to consist of enzyme which had existed in polypeptide form before incubation was carried out.

Since proteins as a class vary in density from about 1.27 to 1.43 gm/ml, it is also possible that equilibrium sedimentation in a density gradient could be applied to the fractionation of natural mixtures of proteins according to their densities. For example, our data predict that bovine serum albumin ($\rho = 1.33$) would be resolved from bovine fibrinogen ($\rho = 1.42$) by the procedures described in this paper.

SUMMARY

If equilibrium density gradient ultracentrifuge experiments are conducted under carefully designed conditions, the resolution of the method can be markedly improved or, alternatively, the experiment can be designed to describe the nature and amount of many widely varying macromolecules in a single experiment. The theoretical limits on resolution are examined for two types of experiments. Solutes have been found

which give high resolution and appropriate densities for protein, RNA, and DNA equilibrium centrifugation. The properties of these solutes are tabulated.

The theory has been tested by separation of normal β-galactosidase from samples labeled with N^{15}, C^{13}, and deuterium. It is shown that the method will resolve labeled from unlabeled proteins if an adequate, but attainable, density difference has been introduced.

ACKNOWLEDGMENTS

We are pleased to acknowledge the help of Mr. Richard Burnes in growing *Hydrogenomonas facilis,* and the measurement and calculation of several density gradients by Mrs. A. Benoit Kovacs. The fully deuterated algae from which we prepared deuterated amino acids were kindly provided by Dr. J. J. Katz of Argonne National Laboratory. This research received financial support from National Science Foundation Grant G9869 and U. S. Air Force Office of Scientific Research, Air Research and Development Command Contract AF 49(638)314.

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SHORT COMMUNICATIONS

Problems Involved in Dialyzing Low Concentrations of Inorganic Pyrophosphate

In seeking to establish the form in which phosphate occurs in the phosphomannan from *Hansenula holstii* NRRL Y-2448 (1), we found that periodate oxidation of the phosphomannan, followed by treatment with alkali, eliminated the phosphate in inorganic form (2). Although colorimetric analysis (2, 3) indicated this inorganic phosphate was *ortho*, confirmation by isolation was necessary. Dialysis was the logical choice for separating the small proportion of inorganic phosphate from the periodate-oxidized phosphomannan, but difficulties were encountered in dialyzing controls containing pyrophosphate. The results observed, which are significant in biochemical research, are reported here.

Visking[1] regenerated cellulose tubing was used for dialysis. The sizes were 1-7/8 S.S. and Nojax with wall thickness of 0.0016 in. and 0.0010 in. respectively. These membranes are known to contain glycerol plasticizer and sulfur (4); the presence of heavy metals is implied by some methods of pretreatment (5). Before use the tubing was soaked at least four successive times in distilled water; after the second time, conductivity measurements indicated that essentially all ionic matter had been removed. In addition, some tubing was boiled in distilled water as recommended by others (6, 7) for two 2-hr periods. Only a very slight amount of conducting material was released after the first boiling. The pH of the water was increased from 6.00 to 6.70 by the first soaking and to 7.1 by the first boiling. Subsequent treatments caused progressively smaller increases in pH. The ends of tubes were tied with string which had been boiled and autoclaved repeatedly until free of ionic matter.

One hundred milliliters of solution was dialyzed against 1 liter of distilled water at 4°C, with occasional manual agitation. Before analysis, dialyzates were concentrated *in vacuo* at temperatures not exceeding 30°C and with pH maintained in the range 6.5–8.5.

[1] Mention of a specific commercial product does not constitute endorsement by the U. S. Department of Agriculture over similar products.

Results of dialysis of solutions having initial concentrations comparable to that expected from alkali-treated periodate-oxidized phosphomannan (2) are shown in Table 1. At this low concentration, pyro-

TABLE 1

USE OF WASHED TUBING FOR DIALYSIS OF DILUTE PHOSPHATE SOLUTIONS

Initial solution composition[a,b]		Dialysis tubing wall thickness (in.)	Solution analyzed[c]	Orthophosphate found (% of total P)	Pyrophosphate by paper chromatography[d]
Phosphate	Phosphate concn. (%)				
$Na_4P_2O_7 \cdot 10H_2O$	0.013	0.0016	Dialyzate 1	0	Absent
			Dialyzate 2	0	Absent
			Residue	12.5	Present
$Na_4P_2O_7 \cdot 10H_2O$	0.013	0.0010	Dialyzate 1	0	Absent
			Dialyzate 2	2	Absent
			Residue	11	Present
Na_2HPO_4	0.0083	0.0016	Dialyzate 1	11	—
			Dialyzate 2	11	—
$NaH_2PO_4 \cdot H_2O$	0.0080	0.0016	Dialyzate 1	11	—
			Dialyzate 2	9	—

[a] The phosphorus concentration in each solution was 0.0018%. Also present was 0.0014% KCl.

[b] At initiation of dialysis the pH of each solution was in the range 9.3–9.5. After dialysis and concentration to 50 ml, dialyzates from pyrophosphate solutions showed pH 7.5–7.8, and residues showed pH 6.8–6.9.

[c] Dialyzate 1 was removed after 20 hr at 4°C. Membrane and contents were transferred to fresh distilled water (4°C), and after 20 hr dialyzate 2 was removed.

[d] Experimental conditions are reported in reference (2).

phosphate did not permeate either thickness of tubing. In both sizes of tubing, some pyro- was converted to orthophosphate, and a small proportion (2%) of it permeated the thinner walled tube. When orthophosphate was present in the initial solution, it permeated the thicker walled tubing slowly in marked contrast to the complete lack of movement of the pyrophosphate. Under these conditions, orthophosphate in the initial solution would be distinguished from pyrophosphate only by its initial rate of appearance in the dialyzate.

When alkali-treated, periodate-oxidized phosphomannan Y-2448 was handled under these same conditions, inorganic orthophosphate was found in both the first and the second dialyzates; pyrophosphate was not present in either.

Failure of pyrophosphate from controls to appear in dialyzates is not due to inherent impermeability of the membrane to this salt. By increasing the concentration of pyrophosphate 100-fold, rapid dialysis of pyrophosphate was observed (Table 2, first initial solution). This dialysis

TABLE 2

EFFECTS OF PHOSPHATE CONCENTRATION AND OF USING BOILED TUBING ON RATE AND PRODUCT OF DIALYSIS

Initial solution composition[a,b]			Dialysis tubing			P found (% of total)	
Phosphate	Phosphate concn. (%)	P concn. (%)	Wall thickness (in.)	Pretreatment	Solution analyzed[c]	Orthophosphate	Pyrophosphate
$Na_4P_2O_7 \cdot 10H_2O$	1.30	0.18	0.0016	Washed	Dialyzate 1	2	69
					Dialyzate 2	<1	15
					Residue	<1	13
Na_2HPO_4	0.825	0.18	0.0016	Washed	Dialyzate 1	85	—
					Dialyzate 2	10	—
					Residue	5	—
$Na_4P_2O_7 \cdot 10H_2O$	1.30	0.18	0.0016	Washed	Dialyzate 1	3	72
				and	Dialyzate 2	<1	11
				boiled	Residue	<1	13
$Na_4P_2O_7 \cdot 10H_2O$	0.013	0.0018	0.0016	Washed	Dialyzate 1	0	6
				and	Dialyzate 2	6	8
				boiled	Residue	50	30

[a] These solutions contained no KCl.

[b] At initiation of dialysis the pH of each solution was in the range 9.2–10.2.

[c] See Table 1, note *c*.

was accompanied by relatively small conversion to orthophosphate. A 100-fold increase in concentration of orthophosphate in the initial solution (Table 2, second initial solution) also resulted in a great increase in rate of dialysis through washed membrane.

The more rapid rate of dialysis of ortho- as compared with pyrophosphate might be expected since the rate of dialysis through Visking membranes has been shown to be related directly to molecular size (8). But the retardation or inhibition of dialysis of low concentrations of pyrophosphate appears to result from negative charges which the membrane acquires in water (4, 9). As already shown by others, these charges favor diffusion of cations (9), retard diffusion of anions (10, 11), and cause a separation of ions which leads to accumulation of H^+ within the dialysis membrane (9).

The higher charged pyrophosphate ions would be repelled more effectively than the orthophosphate ions. In keeping with the general principle already stated (9), the local concentration of H^+ apparently becomes sufficient to hydrolyze some pyro- to orthophosphate. When the pyrophosphate concentration is sufficiently high (Table 2, first initial solution), this hydrolysis seems to be accompanied by neutralization of the charge on the membrane so that the remaining pyrophosphate can dialyze normally.

When pyrophosphate solutions of the same composition as those dialyzed (Tables 1 and 2) were allowed merely to stand at 4°C and then concentrated and chromatographed, traces of orthophosphate were observed, but in much lesser amounts than those found in corresponding dialyzates.

When membrane that has been washed and also boiled as recommended (6, 7) is used with pyrophosphate solution of the higher concentration, both the initial rates of conversion to ortho- and of diffusion of pyrophosphate seem to increase somewhat (Table 2, third initial solution). These effects were much more pronounced when the lower concentration of pyrophosphate solution was employed (Table 2, fourth initial solution). These data indicate that boiling regenerated cellulose membranes produces changes not heretofore suspected (6, 7). These changes might be due to oxidation and consequent intensification of surface charge, and to modification of pore size.

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PAUL R. WATSON
JAY E. PITTSLEY
ALLENE JEANES

Northern Utilization Research and Development Division
Agricultural Research Service
United States Department of Agriculture
Peoria, Illinois
Received May 11, 1962

Periodation of the Glucan of *Agrobacterium tumefaciens* with Recovery of Cleavage Products[1]

McIntire, Peterson, and Riker (1) showed that *Agrobacterium tumefaciens* produces a low molecular weight extracellular polysaccharide consisting entirely of glucose and having a specific rotation in water, $[\alpha]_D$, of $-9°$ to $-10°$C. An upward shift in rotation during hydrolysis suggested a predominance of β linkages. The molecular weight estimated from sedimentation and diffusion constants was 3600, indicating that there were 22 glucose units per molecule. Reeves (2) suggested that the glucose units are linked chiefly through the 2-position on the basis of optical rotation studies in water and cuprammonium solution. The polysaccharide has been partially methylated and only 3,4,6-trimethylglucose was isolated after hydrolysis (3), indicating that a substantial portion of the glucopyranose residues are united through $1 \rightarrow 2$-glucosidic linkages.

Periodate oxidation of polysaccharides followed by reduction of the polyaldehyde to the polyalcohol, hydrolysis, and identification of the products has been used extensively in the determination of structure (4). The cleavage product containing the original sugar aldehyde group has not usually been quantitatively determined, or else the yields have been low, due to the lability of these compounds under the conditions of hydrolysis used. It may be noted that periodate oxidation of a glucan with $1 \rightarrow 2$-linkages would result in cleavage of the glucose molecule between carbons 3 and 4. Reduction of the polyaldehyde followed by hydrolysis would yield, per mole of polysaccharide of molecular weight 3600, 22 moles of glycerol and 20 moles of glyceraldehyde. In the work reported here the hydrolysis of the polyaldehyde was combined with distillation in 10% H_2SO_4, so that the glyceraldehyde was transformed into methylglyoxal and recovered as such in the distillate (5). Approximately 90% yields of both glycerol and methylglyoxal were recovered and no unchanged glucose could be found.

These results confirm previous suggestions that the low molecular weight glucan produced by *A. tumefaciens* is an unbranched polymer with the glucose units joined entirely by $1 \rightarrow 2$-linkages. In addition to its application to structural analysis, this method should be useful in

[1] Contribution No. 544, Microbiology Research Institute.

determining the distribution of radioactive carbon from variously labeled sugar precursors, since both halves of the periodate-cleaved monosaccharide units can be recovered, and both the glycerol and the methylglyoxal moieties are amenable to further degradation. While this polysaccharide is so far unique in having $1 \rightarrow 2$-linkages only, this linkage has been reported in other polysaccharides such as yeast mannan (6) and fucoidin (7) where the application of this method may prove useful.

The polysaccharide was isolated from cultures of *A. tumefaciens* (Strain A-6) essentially by the method of McIntire *et al.* (1). The dry white powder was assayed at 94% glucose and no other sugar was found in acid hydrolyzed samples. Two hundred milligrams of the powder in 5 ml of water was added to 400 mg of sodium metaperiodate in 5 ml of water and kept at 2°C for 11 days in the dark. At this time no glucose could be found in an acid hydrolyzate of a small sample.

Four hundred milligrams of sodium borohydrate was added to the periodation mixture and the solution left overnight at room temperature. Six milliliters of glacial acetic acid was added and the solution evaporated to a syrup *in vacuo*. Several volumes of methanol were added and the solution again evaporated to a syrup. This procedure was repeated twice more. The residue was neutralized and passed through a column of Dowex-45 in the carbonate form and then through a column of Amberlite IR-120 in the hydrogen form. The effluent was evaporated to a slightly yellow gum *in vacuo*, dissolved in water, and made up to a volume of 25 ml. Five milliliters of this polyalcohol solution was placed in a small still-pot with 0.5 ml concentrated H_2SO_4. Five milliliters was distilled over, with 3 ml water being added to the still pot during the distillation.

Part of the residue in the still pot was neutralized with $Ba(OH)_2$ and chromatographed on Whatman No. 1 paper in *n*-propanol–ethyl acetate–water (70:10:20) with glycerol and erythritol as standards. Glycerol was the only compound detectable with the periodate-starch spray (8), although a slight trace of an unknown substance was seen near the origin. Neither erythritol nor glucose was detectable. In this solvent system glycerol, erythritol, and glucose have R_f values of 0.57, 0.47, and 0.30, respectively.

The amount of glycerol in the distillation residue was measured directly by the colorimetric method described by Burton (9). Methylglyoxal in the distillate was determined by forming the di-2,4-dinitrophenylhydrazone, developing the color in 5% alcoholic sodium hydroxide as described by Entner and Doudoroff (5) and reading the color at 560 mμ. The di-2,4-dinitrophenylhydrazones of the unknown and au-

thentic methylglyoxal had identical spectra in 5% alcoholic sodium hydroxide, with maxima at 560 mμ. The melting point of the di-2,4-dinitrophenylhydrazone of the unknown after recrystallization from nitrobenzene was 303–306°C. The authentic compound melted at 304–306°C and the mixed melting point was 303–306°C, while Entner and Doudoroff (5) reported 305–307°C (all uncorrected). The data for the analyses for glycerol and methylglyoxal are shown in Table 1.

TABLE 1

RECOVERY OF CLEAVAGE PRODUCTS FROM PERIODATE OXIDIZED AND BOROHYDRIDE REDUCED POLYSACCHARIDE

	Millimoles of compound		Per cent recovery
	Theoretical[a]	Experimental	
Glucose in 200 mg of polysaccharide	1.23	1.16	94
Glycerol	1.16	1.03	89
Methylglyoxal	1.05	0.95	90

[a] Assuming that each molecule of polysaccharide contains 22 glucose units and will yield 22 equivalents of glycerol and 20 units of methylglyoxal.

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N. B. MADSEN[2]

Microbiology Research Institute
Research Branch,
Canada Department of Agriculture
Ottawa, Canada
Received September 5, 1962

[2] Present address: Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada.

An Automatic Determination of Protein-Bound Hexose[1]

In recent years great interest has developed in the structure of glycoproteins, in particular in the carbohydrate-protein bond. For such investigations large numbers of samples need to be examined for the presence of glycopeptides which result, for example, from the action of proteolytic enzymes on the glycoprotein. To make such routine analyses practical the orcinol method for hexoses of Lustig and Langer (1) as described by Winzler (2) has been adapted to the Auto Analyzer.[2]

The flow diagram of the method is shown in Fig. 1. The sampling

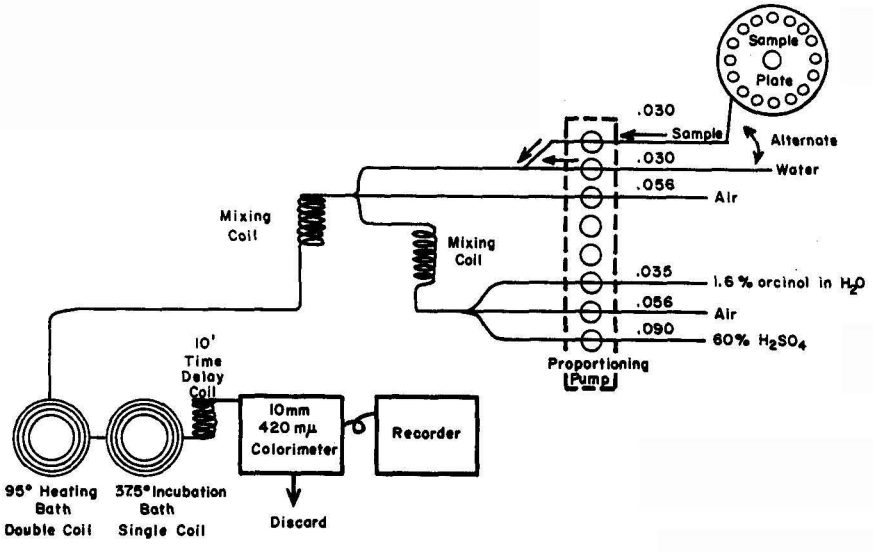

FIG. 1. Flow diagram of the method.

module is run at 40 per hour, alternating samples and cups of water. All tubing after the manifold is acid flex tubing. A double coil heating bath is used, followed by a 37° incubation bath and a time delay coil of approximately 10 min. The colorimeter uses a 10-mm flow cell and a 420-mμ filter. The reagents, 1.6% orcinol in distilled water and 60% sulfuric acid (by volume), are made up fresh daily as needed.

This method gives a satisfactory dose-response curve in the concentration range of 3 to 60 μg mannose/ml sample. A typical standard curve, using mannose as the standard hexose, is shown in Fig. 2. Other hexoses

[1] This investigation was supported by grant No. RG 7275 of the U. S. Public Health Service and grant No. G 9746 of the National Science Foundation.

[2] Technicon Instruments Corp., Chauncey, New York.

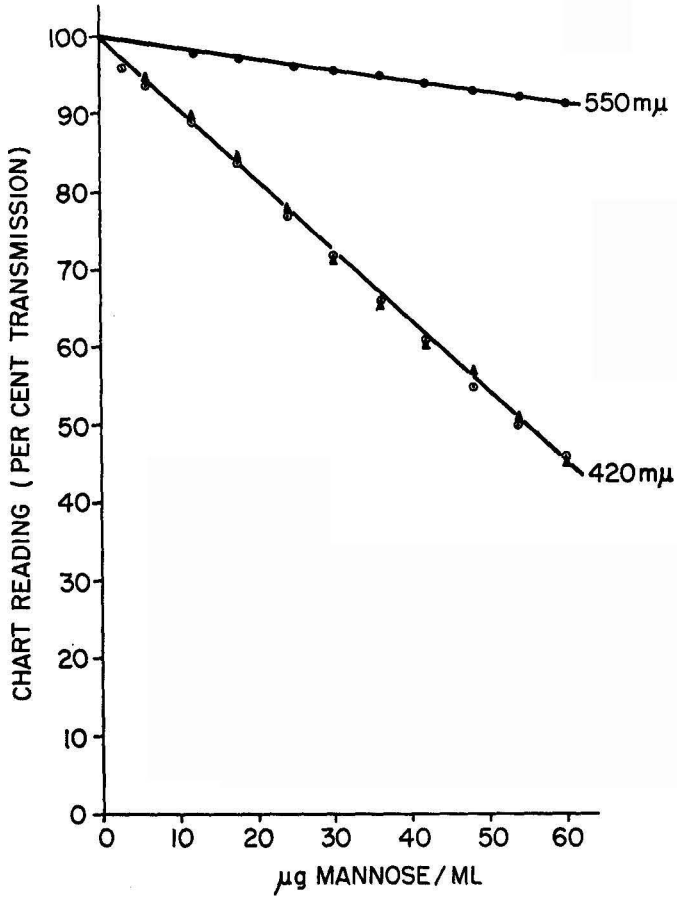

FIG. 2. Typical standard curves with mannose as the standard hexose.

also give linear responses but a different color yield is obtained. Thus the response in per cent of that obtained with mannose is: for glucose 78; for galactose 89; and for fructose 59. At the 420 mμ maximum, used to estimate the reaction, methylpentoses are detected: fucose gives 130% the response given by mannose, and rhamnose 142%, both on an equal weight basis. The other sugars present in certain glycoproteins, hexosamines and sialic acid derivatives, do not interfere. Ribose does give a color with a yield 178% that of mannose by weight. 2-Deoxyglucose gives a color yield of 13%. Of these interferences the only serious one is that of methylpentoses, since these do occur in glycoproteins. This can be overcome by using a 550 filter in the colorimeter. However, at this

wavelength, the method is considerably less sensitive (Fig. 2), and increasing the mannose concentrations results in turbid solutions.

The responses of the sugars enumerated are not significantly different from that of the manual procedure (2), even though the heating times differ somewhat between the two methods. As in the manual method, the protein does not interfere. No difference in response to mannose is seen in the presence or absence of β-lactoglobulin and recoveries of mannose added to ovalbumin are quantitative. The analysis for mannose in ovalbumin by the automated methods gives consistently lower results than that obtained in the manual procedure (3).

The procedure is highly reproducible, as is indicated by Fig. 2, in which the standard curve is drawn through two sets of points obtained on two independent occasions. The method has proven especially useful in the analysis of column fractions for glycopeptides. In such instances the water tube between samples can be omitted and satisfactory plots of elution patterns can be obtained at the full speed of 40 samples per hour.

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J. JUDD
W. CLOUSE
J. FORD
J. VAN EYS[3]
L. W. CUNNINGHAM

Department of Biochemistry
Vanderbilt University
Nashville, Tennessee
Received September 7, 1962

[3] Investigator, Howard Hughes Medical Institute.

Reaction Product of Acetoacetyl Coenzyme A and Hydroxylamine[1]

Acyl derivatives of coenzyme A (CoA), which are intermediates in the metabolism of fatty acids, have been conveniently identified as the products of their reaction with hydroxylamine. In this reaction, while the esters of saturated, α,β-unsaturated, and β-hydroxy acids are well known to yield the corresponding hydroxamic acids, the product with acetoacetyl

coenzyme A has not been unequivocally identified. This product is referred to as acetoacetyl hydroxamate by Green (1) and Hele *et al.* (2), and as methyl isoxazolone by Stadtman (3). Mahler, without specifying the reaction product, stated that β-ketoacyl derivatives of CoA did not form hydroxamates (4). A survey of literature did not reveal any experimental evidence establishing the identity of this compound. While consideration of organic reaction mechanisms would indicate isoxazolones to be the most likely product, it remained to be conclusively shown that hydroxylamine reacts exclusively with the keto group of the β-ketoacyl CoA forming the corresponding oxime, which then undergoes cyclization. The work reported here offers evidence in support of the suggested formation of methyl isoxazolone in the reaction between acetoacetyl thiol esters and hydroxylamine.

Methyl isoxazolone was prepared according to the procedure described by Hantzsch (5) with minor modifications. Distilled ethyl acetoacetate (3.4 mmoles) was added to 3 ml of an aqueous solution containing 4.4 mmoles each of $NH_2OH \cdot HCl$ and NaOH. The reaction mixture was kept in a water bath at 37°C for 18 hr and was then carefully acidified with concentrated HCl. The yellow, crystalline, needle-like compound formed was purified by repeated crystallization from hot water. The melting point of the pure product was found to be 169–171°C (reported, 169–170°C).

Acetoacetylthioglycolic acid, a model compound of acetoacetyl CoA, was prepared as follows: 2.3 gm of thioglycolic acid, neutralized to pH 7.5 with 7 *N* NaOH, was cooled in crushed ice and 2.2 gm of distilled, colorless, diketene was added to it dropwise with constant stirring. The reaction was allowed to proceed for 30 min in the cold with stirring until the top layer of diketene disappeared. More diketene was added, if necessary, until all the thioglycolic acid had reacted as indicated by the sodium nitroprusside test. $NH_2OH \cdot HCl$ solution (2.3 gm), adjusted to pH 10.5, was then added to the acetoacetylthioglycolic acid formed and the mixture was acidified as described earlier. A yellow crystalline substance separated which, on recrystallization, gave a product with melting point 168–170°C, suggesting that the compound was methyl isoxazolone. Determination of the ultraviolet absorption spectrum revealed this compound to have the same absorption peak (288–290 mμ) as methyl isoxazolone, showing the two compounds to be identical.

In order to check its chromatographic behavior, C^{14}-labeled methyl isoxazolone was prepared from ethyl acetoacetate-3-C^{14}. Identity of this

[1] Supported by the National Institutes of Health, U. S. Public Health Service research grant, A-3504.

compound was established from its melting point, 168–170°C, and its absorption spectrum, which was identical to that of the authentic methyl isoxazolone. When the labeled methyl isoxazolone was chromatographed on filter paper using a solvent system consisting of methylene chloride, butanol, acetic acid, and water[2] and the chromatogram scanned for radioactivity with an actigraph, a sharp peak having an R_f of 0.50 ± 0.03 was obtained.

A similar radioactive peak (R_f 0.50 ± 0.03) had been found invariably in the chromatograms of the extracts of hydroxamates formed in the mammary slices of lactating rabbits and goats incubated with C^{14}-labeled acetate, β-hydroxybutyrate, and butyrate. This peak did not correspond to any of the known hydroxamates. The attempts made for the identification of this peak had, in fact, resulted in the present investigation. The region corresponding to this peak was eluted from several chromatograms with ethanol, pooled, and concentrated. This was cochromatographed with authentic C^{14}-labeled methyl isoxazolone using two different solvent systems, butanol saturated with water, and benzene:formic acid:water (1:1:1). Only one radioactive peak (R_f 0.58 and 0.0, respectively) was obtained with each of the solvent mixture. This shows that acetoacetyl CoA of the tissue had formed methyl isoxazolone under the same conditions in which the nonketo thiol esters formed hydroxamic acids.

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REMEDIOS M. AVENA[3]
SOMA KUMAR

Chemistry Department
Georgetown University
Washington, D. C.
Received August 28, 1962

[2] Unpublished method for the separation of short chain acyl hydroxamates.

[3] This work is taken from a thesis submitted to Georgetown University in partial fulfillment of the degree of Doctor of Philosophy.

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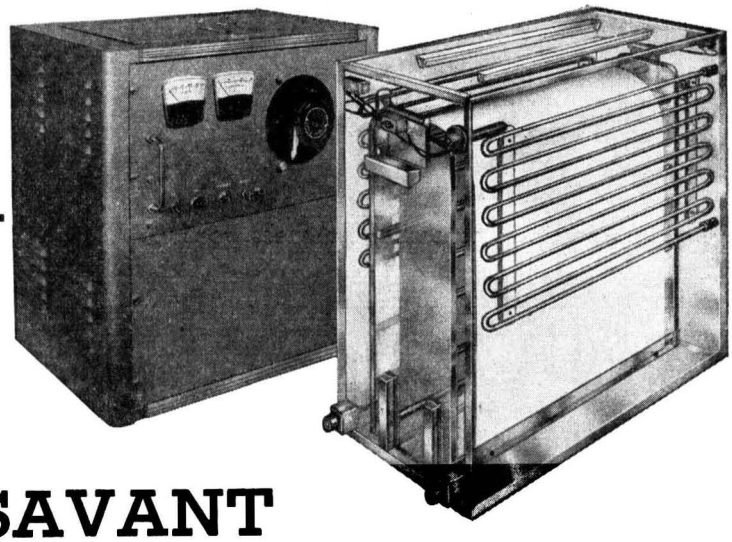

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