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Application of the Method of Good and Bessman for Estimating Glucosamine and Galactosamine in Tissue Hydrolyzates

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The methods of estimation of glucosamine (Glu-NH_2) and galactosamine (Gal-NH_2) elaborated to date require prior separations of both amines by means of ion-exchange chromatography—as in the methods of Gardell (1) and Pearson (2) or a separation of the amino sugar derivatives by distillation—as in the method of Cessi and Cessi (3); or they require yeast, as in the method of Pogell and Koenig (4).

Colorimetric methods of the determination of amino sugars in a mixture of both are based on the difference in color intensity and on the difference in the absorption maxima of the colored products formed during the reaction of Glu-NH_2 and Gal-NH_2 with *p*-dimethylaminobenzaldehyde (DAB) after previous acetylation in alkaline media (5, 6). These methods, when applied to the determination of the two amino sugars in biological material, give results charged with a great error, mainly because of the interference with neutral sugars, amino acids, and some salts introduced during isolation procedures.

The method of Good and Bessman (6), which is known to be easy and to give accurate results, was found to be useless in the estimation of the above-mentioned hexosamines in tissue hydrolyzates purified by the method of Spiro (7) or Boas (8). In the hydrolyzates purified by these two methods, DAB-reactive chromogens did not appear following previous acetylation with acetic acid anhydride in borate buffer, although a high content of hexosamine was found as estimated by the method of Blix (9).

To elucidate the cause of the absence of chromogen, the influence of lysine, hydroxyproline, glycine, and a mixture of them on the determination of Glu-NH_2 and Gal-NH_2 by the method of Good and Bessman has been investigated. The results obtained are presented in Table 1.

In order to purify the hydrolyzates from the excess of amino acids, the method of Pogell and Koenig was used (10). The principle of this

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TABLE 1
Influence of Some Amino Acids on Determination of Glucosamine and Galactosamine by the Method of Good and Bessman

Amino acid	μg added			μg estimated	
	Amino acid	Glucosamine-HCl	Galactosamine-HCl	Glucosamine-HCl	Galactosamine-HCl
—	—	80	20	78.0	21.4
Lysine-HCl	1000	80	20	78.3	21.5
	2000	80	20	66.2	26.6
	3000	80	20	15.6	41.4
L-Hydroxyproline	3000	80	20	13.8	45.0
Glycine	3000	80	20	Neg. values	44.8
Mixt. of amino acids[a]	3000	80	20	5.9	42.6

[a] The amino acids were mixed in 1:1:1 proportion.
D-Glucosamine-HCl was the product of L. Light & Co., England.
D-Galactosamine-HCl was the product of Mann Research Laboratories, New York.
L-Lysine monochlorohydride was obtained from "Toscat," England
L-Hydroxyproline was obtained from L. Light & Co., England.
Glycine was the product of "Labor," Wrocław, Poland.

is purification of amino sugars by means of two ion-exchange columns—one containing Dowex 50X12 in the form of H^+, the second containing Dowex 50X8 in the form of NH_4^+. Amino sugars from the second column were eluted with a 0.5 *M* solution of ammonium acetate. It was found, however, that even if the excess of amino acids was removed, the DAB-reactive chromogens did not appear in the purified hydrolyzates after prior acetylation with acetic acid anhydride in borate buffer. Therefore it seemed justified to investigate the influence of ammonium acetate and ammonium chloride on the estimation of Glu-NH_2 and Gal-NH_2 by the

TABLE 2
Influence of Some Ammonium Salts on Determination of Glucosamine and Galactosamine by the Method of Good and Bessman

Ammonium salt	μg added			μg estimated	
	Salt	Glucosamine-HCl	Galactosamine-HCl	Glucosamine-HCl	Galactosamine HCl
—	—	80	20	78.8	19.0
CH_3COONH_4	1000	80	20	78.8	18.9
	2000	80	20	64.0	20.5
	3000	80	20	60.8	20.5
NH_4Cl	500	80	20	78.0	19.5
	1000	80	20	62.8	23.0
	2000	80	20	33.6	30.2
	3000	80	20	25.6	28.0

method of Good and Bessman. The results are presented in Table 2.

To remove the interfering salts, a third column, containing Dowex 50X4 in the form of H^+, was used, amino sugars from this column being eluted with a 0.5 *M* aqueous solution of NH_3 (11). Ammonia was removed by evaporation *in vacuo*. Thus it appeared that removing the excess of amino acids and salts permits a precise determination of Glu-NH_2 and Gal-NH_2 by the method of Good and Bessman.

MATERIALS AND METHODS

Preparation of the Ion-Exchange Columns

The columns and the resin in the form of NH_4^+ and H^+ were prepared based on the method described by Pogell and Koenig (10), except that, instead of Dowex 50X12, Dowex 50X4 was used.

Hydrolysis

Tissues were hydrolyzed with HCl at 100°C. The details are given in Table 3.

TABLE 3
Conditions of Hydrolysis of Tissues at 100°C

Tissue	HCl, *N*	Hydrolysis time, hr
Bovine cornea	4	16
Bovine vitreous	4	6
Rat liver homogenate[a]	4	7
Human placenta[b]	4	12
Pig serum	3	7

[a] Before homogenization the rat liver was perfused with cold Krebs-Ringer solution at pH 7.4.

[b] Before hydrolysis, the placenta was perfused with cold 0.25 *M* sucrose solution containing sodium Versenate.

Purification of Tissue Hydrolyzates

The hydrolyzate was evaporated *in vacuo* at about 40°C until it was free of hydrogen chloride. The samples containing 1–2 gm of the hydrolyzed tissue dissolved in water were placed on the column packed with 10 ml of Dowex 50X4 in H^+ form. The column was washed with 50 ml of water at pH 4.5 in order to remove neutral sugars. Amino sugars were eluted with 22 ml of 0.75 *N* HCl; the greater part of the amino acids remained in the column. The eluate was evaporated *in vacuo* until it was free of hydrogen chloride. The dried remainder was dissolved in small amounts of water and quantitatively placed on the column packed with 7 ml of Dowex 50X8 in the form of NH_4^+ for further separation from

amino acids. The column was washed with 25 ml of 0.2 *M* ammonium acetate at pH 5.5. The amino sugars were eluted with 20 ml of 0.5 *M* ammonium acetate at pH 6.9 directly into the next column containing 10 ml of Dowex 50X4 in the acid form of H^+. After the whole eluate was introduced, the column was washed with 20 ml of water to remove acetic acid completely. Amino sugars were eluted with 30 ml of a 0.5 *M* aqueous solution of ammonia, the first portion of 10 ml of eluate containing no hexosamine being removed off. Ammonia was removed instantly by evaporating the eluates *in vacuo*.

RESULTS

The procedure described appears to be adequate for obtaining satisfactorily enough pure amino sugars from tissue hydrolyzates for exact quantitative determination of Glu-NH_2 and Gal-NH_2 by the method of Good and Bessman.

In order to examine the recovery rate of amino sugars during their purification on the ion-exchange columns, mixtures containing known amounts of both sugars were purified. The results are presented in Table 4.

TABLE 4
Recovery of Glucosamine and Galactosamine after Ion-Exchange Purification

μg applied		μg recovered		% recovery	
Glucosamine	Galactos-amine	Glucosamine	Galactos-amine	Gluctosamine	Galactosamine
800	200	775	205	97.0	102.5
700	300	680	310	97.1	103.3
600	400	592	381	98.7	95.2
500	500	471	506	94.2	101.2

The differences between the amounts of sugars which underwent the purification procedure and those analyzed by the method of Good and Bessman were small and are within the limits of error of the method applied.

After having purified the tissue hydrolyzates by the procedure described, the content of Glu-NH_2 and Gal-NH_2 was estimated in the following materials: in bovine cornea and vitreous, in rat liver homogenates, in human placenta, and in pig serum. The results are presented in Table 5.

Though the material is too small for statistical analysis, the results obtained indicate the usefulness of the method of Good and Bessman for the estimation of Glu-NH_2 and Gal-NH_2 in tissue hydrolyzates after their prior purification by means of the procedure described.

TABLE 5
The Amount of Glucosamine and Galactosamine in Tissues
(mean values for 6 determinations)

Tissue	μM per 1 gm of tissue		Molar ratio glucosamine
	Glucosamine	Galactosamine	Galactosamine
Bovine cornea	12.96 (± 0.16)	8.13 (± 0.29)	1.6
Bovine vitreous[a]	0.95 (± 0.10)	0.12 (± 0.03)	8.3
Rat liver homogenate	1.28 (± 0.21)	0.39 (± 0.06)	3.3
Human placenta	4.91 (± 0.52)	1.55 (± 0.20)	3.2
Pig serum[a]	5.04 (± 0.78)	0.54 (± 0.19)	9.1

[a] Data per 1 ml.

SUMMARY

To apply the method of Good and Bessman to the estimation of glucosamine and galactosamine in tissue hydrolyzates, a purification procedure of amino sugars freed of interfering substances was elaborated.

This was achieved by means of Dowex 50X4 (H^+) and Dowex 50X8 (NH_4^+) columns. The purified samples freed of amino acids were desalted on a Dowex 50X4 (H^+) column and the amino sugars were eluted with ammonia; the ammonia solution was instantly evaporated *in vacuo*. With this procedure the contents of glucosamine and galactosamine in some biological materials (as in bovine cornea and vitreous, in human placenta, in rat liver homogenates, and in pig serum) was estimated.

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ANALYTICAL BIOCHEMISTRY 19, 6–13 (1967)

A Monochromatic Light System for the Analytical Ultracentrifuge[1]

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Schachman *et al.* (1) and Cummings (2) have described the incorporation of monochromators into the ultraviolet optical system of the Beckman-Spinco Model E ultracentrifuge. In the system of Schachman *et al.*, a monochromator from a Beckman DU spectrophotometer was used in a carriage placed beneath the ultracentrifuge, which was raised approximately 10 cm and supported on metal skids. The light from the exit slit was reflected by an adjustable 45° mirror into the ultracentrifuge chamber. This system may be operated with either medium-pressure mercury arc lamps or xenon lamps and has been coupled to a split-beam scanning recording system (3) with a significant increase in accuracy in the measurement of concentrations. In the system of Cummings, a Farrand grating monochromator was used in the range of 260 to 380 mμ in conjunction with a 450 W xenon light source and conventional photographic techniques. Schachman has recently extended the useful range of his system to 230 mμ (4).

In this communication we should like to report the development for the ultracentrifuge of a monochromatic light system which incorporates a prism monochromator and a xenon-mercury light source. With Kodak commercial film, the system is readily usable in the range 260 to 700 mμ. By ultilizing Kodak III-O film, which is more sensitive in the far-ultraviolet, the effective range may be extended to 235 mμ for velocity sedimentation work. The light system is now available from Schoeffel Instrument Co. and may be easily incorporated into the standard ultraviolet system supplied by Spinco.

[1] Supported by research grants FR-00107 and GM-14583 from the National Institutes of Health.

COMPONENTS OF THE OPTICAL SYSTEM

Figure 1 is a diagram of the optical system. The light source housing, which contains a fan for air cooling, accommodates a number of different types of lamps, including xenon, mercury, and xenon-mercury. We have found the 250 W xenon-mercury lamp manufactured by Hanovia Division of Englehard, Inc., most satisfactory. Also contained within the lamp housing, directly behind the lamp itself, is a spherical reflector which enables more of the radiated light to be utilized. The output from the lamp is focused by the condensing lens on the entrance

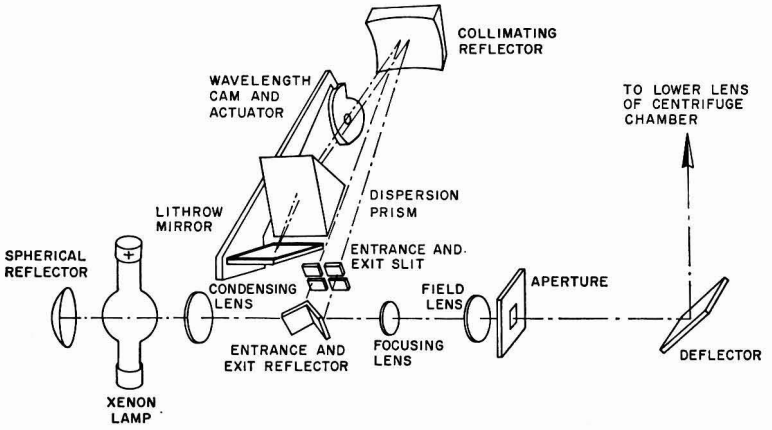

FIG. 1. Diagram of the monochromatic light system. A cooling fan, not shown, is contained in the light source housing. The dimensions of the monochromator itself are $7 \times 2\frac{1}{4} \times 2$ in.

slit of the monochromator. The lens system composed of the focusing lens and field lens forms an image of the exit slit of the monochromator at the focal point of the lower collimating lens contained within the ultracentrifuge chamber. Only insignificant errors are introduced by not varying the focusing of the light source image to account for the variance with wavelength of the focal length of the lower collimating lens (1). Deflection of the light beam into the chamber is accomplished by the 45° front surfaced mirror, which is adjustable by three screws. The exit slit of the monochromator, the image of which serves to illuminate the ultracentrifuge cell, appears on a radius from the center of rotation. Because of this geometry, any dispersion within the illuminating slit occurs at right angles to the direction of sedimentation. The entire cell is thus illuminated with light of constant spectral quality, and hence the effective absorption coefficient of molecules within the centrifuge cell will not vary with distance from the center of rotation.

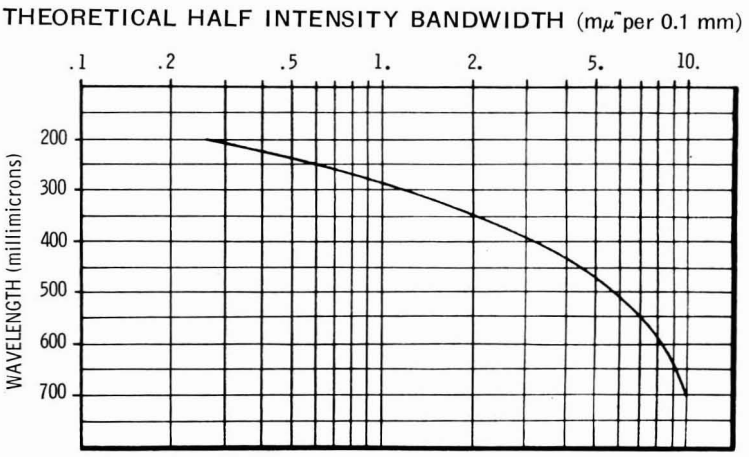

FIG. 2. Dispersion data for Schoeffel QPM 3 quartz prism monochromator.

The dispersion characteristics of this quartz prism monochromator are illustrated in Figure 2. In the range 260 to 700 mμ, the monochromator may be operated with a slit width of 1 mm or less when photographs are taken using Kodak commercial film and 10 sec exposures. This produces

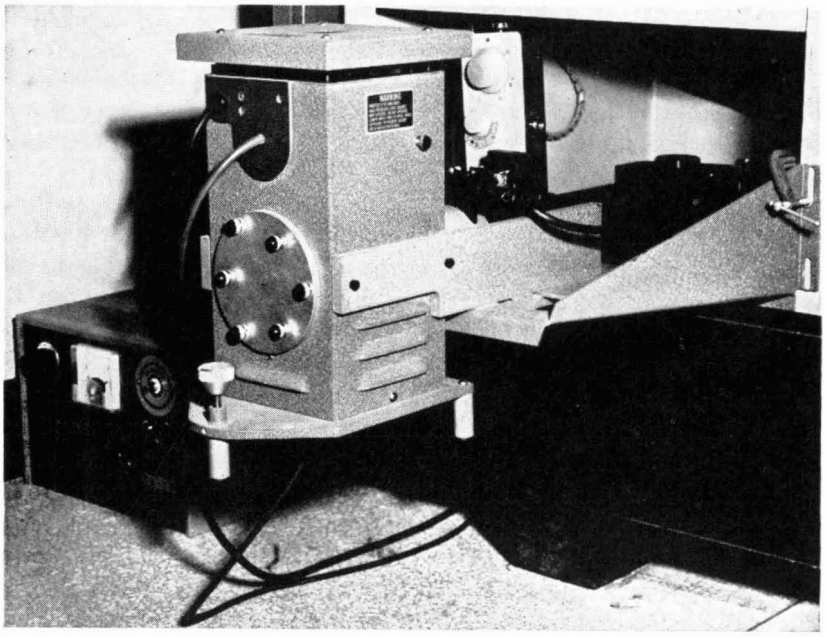

FIG. 3. Photograph of the optical system in place in the ultracentrifuge. The power supply for the light source is shown beside the centrifuge.

a 10 cm deflection when a densitometer tracing of the photograph is made with a Beckman RB Analytrol. When Kodak III-O film is used, 9 cm deflections are obtained with photographs taken with 235 mμ light, a monochromator slit width of 1.8 mm and 30 sec exposures. It is always possible to work with half-band intensities of ±10 mμ or less.

Figure 3 is a photograph of the optical system in place in the ultracentrifuge. All components are rigidly affixed to a supporting frame that forms an optical bench attached to the ultracentrifuge chamber housing. A shutter, which is raised by the automatic photography system, prevents illumination of the monochromator when photographs are not being taken. Although the supporting frame projects 16 in. in front of the ultracentrifuge, this has posed no difficulty with respect to accessibility to the centrifuge chamber, and the entire system may be shielded by a protecting superstructure if desired. The system is completely compatible with the existing ultraviolet optical system, and the only modification required is the removal of the standard gas filters. If it is desired to use the gas filter system from time to time, the tube leading from the monochromator to the reflecting mirror may be easily removed by loosening four set pins. The gas filters are then replaced and, since the standard light source has not been disturbed, the system is ready for use.

ALIGNMENT OF OPTICAL SYSTEM

The vertical position of the supporting frame is so chosen that the image of the exit slit formed by the focusing lens and field lens system is placed at the focal length of the lower collimating lens. With the use of a half-silvered quartz window in the centrifuge cell, the 45° mirror used to reflect light into the chamber is adjusted until the light reflected from the window coincides with the originating light beam. This is similar to the procedure recommended by Gropper (5) for the alignment of the schlieren optical system. Once the optical axis has been determined in this fashion, the other components supplied with the ultracentrifuge are aligned as detailed by Schachman *et al.* (1). Care must be taken that the light beam traverses the center of the camera lens, otherwise difficulties will be encountered when its position is changed with wavelength.

RESULTS AND DISCUSSION

Figure 4 contains a number of densitometer tracings of photographs taken at several representative wavelengths. These tracings are unaltered except that their relative heights have been varied in order to place them all in one figure. For all wavelengths except 235 mμ, the deflections are 10 cm; 9 cm deflections are obtained at 235 mμ under the

conditions detailed in the figure. The tracings illustrate the excellent uniformity of illumination that may be obtained at all wavelengths. By careful maintenance of cleanliness of the optical components and the use of standardly supplied slit masks over the lenses in the ultracentrifuge chamber plus the addition of a slit mask to the right of the

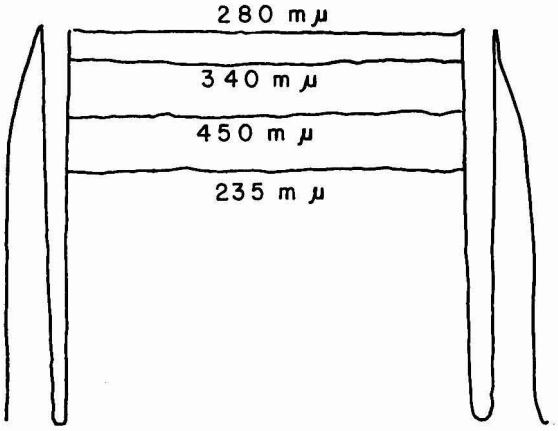

FIG. 4. Densitometer tracings of photographs taken at representative wavelengths using an empty 12 mm cell and rotor speed 20, 410 rpm. At 450 mμ the slit was 0.25 mm, 13 sec exposure; 340 mμ, 0.5 mm slit, 16 sec exposure; 280 mμ, 1.0 slit, 10 sec exposure; 235 mμ, 1.8 mm slit, 30 sec exposure. The pen height deflections of the tracings have been shifted to present them in one figure. They were all 10 cm except for that at 235 mμ, which was 9 cm. The photograph at 235 mμ was taken with Kodak III-O film, the others with Kodak commercial.

camera lens at the barracade supporting the optical dust tube, stray light effects were eliminated. This is indicated by the absence of spurious bands throughout the cell, the sharpness of the image of the knife edges in the counterbalance, and the total lack of darkening of the photograph corresponding to the region between the cell and the counterbalance spaces.

In order to determine whether there was a linear relationship between densitometer deflection and optical density, solutions of differing optical density at 265 mμ were placed successively in a 12 mm valve-type synthetic boundary cell and photographs taken at 20,000 rpm. Solutions of adenosine monophosphate and tryptophan were used in order to demonstrate that the absorption spectrum of the substance employed did not influence the result and only the absorbance at 265 mμ was important. If the light employed in the ultracentrifuge was not monochromatic we should expect the results with the two compounds to differ. In Figure 5 the net densitometer deflection, i.e., the difference between the

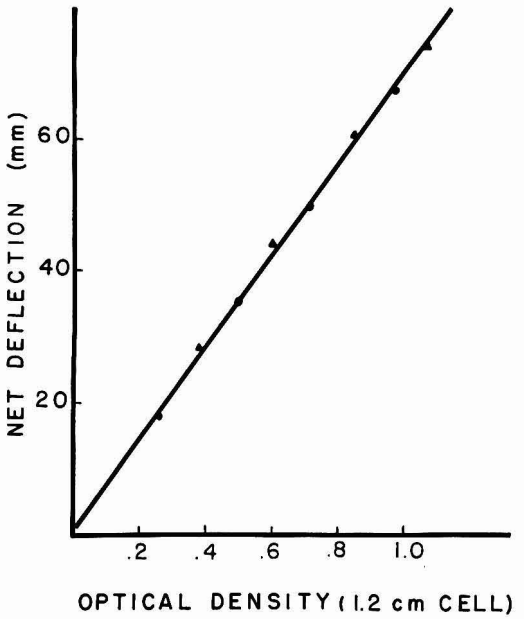

FIG. 5. Dependence of net densitometer deflection upon optical density of solutions differing in their absorption spectra. On the abscissa are the optical densities at 265 mμ measured on a Cary 15 spectrophotometer of the adenosine monophosphate (●) and tryptophan (▲) solutions.

values for solvent and particular solution, is plotted as a function of the optical density. The straight line is the least-squares line for the data and we note no significant difference between the adenosine monophosphate and tryptophan solutions.

Figure 6 depicts one of the most important advantages of the system—the ability to work routinely at 235 mμ. At this wavelength the peptide bonds of all proteins absorb strongly, and as little as 20 μg/ml of protein may be easily and accurately followed during conventional velocity

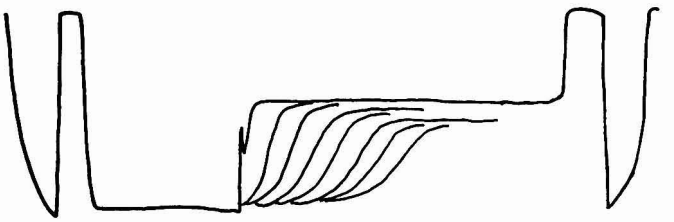

FIG. 6. Densitometer tracings of a sedimentation velocity run of a three-times crystallized preparation of phosphoglyceric acid dehydrogenase. Wavelength 235 mμ, 30 sec exposures at 16 min intervals using Kodak III-O film. A 30 mm Epon aluminum centerpiece was used, rotor speed 50, 740 rpm.

sedimentation. Figure 6 presents the densitometer tracings from a velocity centrifuge run on a three times crystallized preparation of phosphoglyceric acid dehydrogenase (6). The concentration of the enzyme was 45 μg/ml. By working with these dilute solutions, significant saving of precious material may be made. In addition, all measurements can be performed closer to the ideal state of infinite dilution. Attempts are now being made to extend the working range of the system further into the ultraviolet, with concomitant increased sensitivity for protein solutions.

There are many occasions in which low molecular weight colored ligands interact with macromolecules, and the monochromatic light system may be effectively utilized to study such interactions (1, 7). Figure 7 illustrates an experiment using a diazotized lactose hapten,

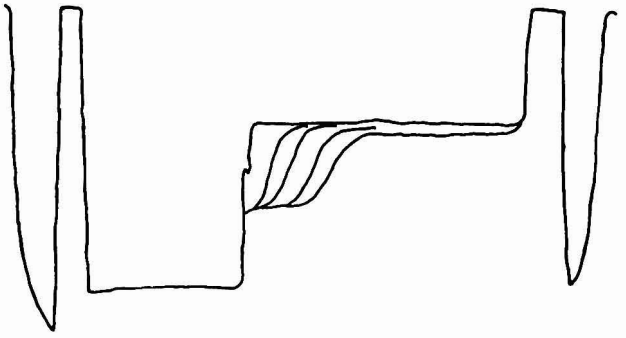

FIG. 7. Densitometer tracings of photographs taken at 450 mμ during sedimentation of specific γ-globulin antibody in the presence of its hapten antigen. γ-Globulin concentration $1.87 \times 10^{-6} M$, lactose hapten concentration $7.15 \times 10^{-6} M$. By estimation from the tracings, $3.74 \times 10^{-6} M$ antigen was bound, indicating two binding sites per mole of antibody.

having an absorption maximum at 450 mμ, and its specific antibody (8). From such experiments, the number of binding sites and binding constants may be estimated. Lamers *et al.* (3) have discussed and illustrated other ways in which monochromatic light may be utilized.

The advantages of a variable monochromatic light system are manifold and include: (*1*) increased accuracy of concentration measurement, (*2*) increased sensitivity for protein solutions, and (*3*) the ability to detect many colored ligands that interact with macromolecules. The system described here obtains these advantages with minimal modification of the existing system and hence at minimal cost.

SUMMARY

A monochromatic light system employing a xenon-mercury light source and a Schoeffel quartz prism monochromator is described for the

absorption system of the Spinco analytical ultracentrifuge. By using conventional photographic techniques, the system may be employed from 235 to 700 mμ. Applications of the system are illustrated and discussed.

ACKNOWLEDGMENT

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An Improved Fluorometric Procedure for the Estimation of Magnesium in Biological Materials[1]

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A sensitive fluorometric method for the estimation of magnesium in serum and urine was devised by Schachter (1). This method was based on the observation that 8-hydroxyquinoline combined with magnesium to form an alcohol-soluble chelate which exhibited a characteristic fluorescence. Although it was reported (1) that calcium ions were without effect on the fluorescence of this magnesium complex, at least up to a final concentration of 0.2 meq/liter of calcium, we observed that, when the sera of rats contained abnormally high calcium levels, (e.g., >8 meq/liter), serum magnesium values seemed unusually high; when serum phosphate levels were elevated, magnesium values appeared too low. An evaluation of the method showed that calcium and phosphate ions have a marked effect on the fluorescence of the magnesium-quinoline chelate.

This report describes the modifications employed to eliminate the interference of both calcium and phosphate ions. Specificity has been achieved by complexing calcium with oxalate and adsorbing phosphate on an anion exchange resin. The results with this procedure compare favorably with those obtained by atomic absorption (2).

MATERIALS AND METHODS

Instrumentation

Turner Fluorometer #111 was used for measurement of fluorescence.

Turner filter #47B (wavelength 436 mμ) was used for activation and #2A-12 (wavelength 530 mμ) for emission.

Micro ion-exchange column (Fig. 1).

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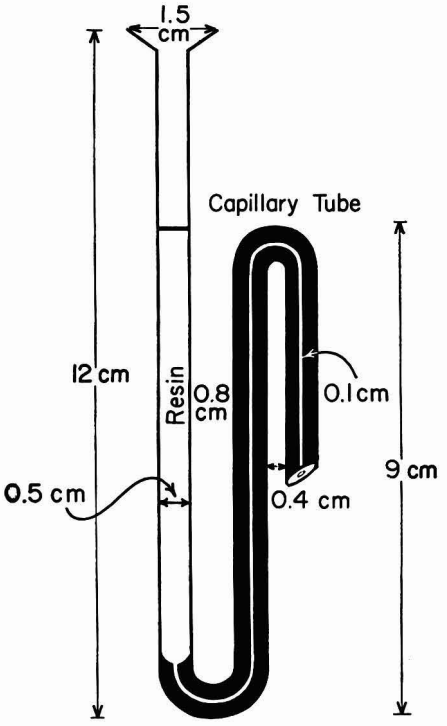

FIG. 1. Micro ion-exchange column. Inside diameter of column = 0.5 cm; inside diameter of capillary = 0.1 cm. The attached capillary maintains the level of liquid in the column at the top of the resin. The volume of the resin bed is approximately 1.6 cc and that of column plus funnel approximately 2.0 cc.

Reagents

All chemicals used were of reagent grade and deionized water was used to make up all solutions.

(1) *Magnesium Stock Solution,* 1 meq/liter: 1.216 gm of magnesium ribbon was dried in a vacuum desiccator, dissolved in 17 ml concentrated HCl and made up to 100 ml with water at 20°C.

(2) *Modified Fluorescent Reagents:*

(*a*) Reagent A—

2 vol 5% 8-hydroxyquinoline in absolute alcohol,
2 vol 2 *M* acetate buffer, pH 3.5,
1 vol deionized water,
30 vol absolute alcohol.

(*b*) Reagent B—a 2 *M* acetate buffer of pH 6.5 was used in place of the 2 *M* acetate buffer at pH 3.5. The compositions of reagents A and B are

essentially the same as those used by Schachter (1) with the exception of the water content, which was reduced from 5 to 1 vol since more aqueous sample is used in the present procedure. The proportions, however, of the final solutions are the same as in the Schachter procedure.

(3) *Potassium Oxalate Solutions:*

(*a*) For urine—10 meq/liter.
(*b*) For serum, bones and feces—125 meq/liter.
(*c*) For synthetic mixtures containing large amounts of calcium—1.5 meq/liter.

(4) *Anion-Exchange Resin:* Amberlite IRA-400 in the acetate form was used. The bulk resin was washed three times with an equal volume of 1 *N* HCl, then with deionized water until the effluent was neutral; washed three times with an equal volume of 1 *N* NaOH, and again with deionized water until the effluent was neutral. The resin was then treated with 1 *N* sodium acetate until the pH of the effluent was the same as that of the sodium acetate, and once more washed with deionized water until the effluent was neutral. Approximately 1.6 cc of resin was put into the micro ion-exchange column which had a small piece of glass wool at the junction of tube and capillary. As many as 50 columns may be used at one time. When the exchange capacity of the resin was reached, it was either discarded or regenerated according to the following procedure. Approximately 10 ml of 1 *N* NaOH was allowed to pass through the column; 5.0 ml of deionized water was then added to wash out excess NaOH; 10 ml of 1 *N* sodium acetate was added then to convert the resin to the acetate form; and finally the resin was washed with water until neutral. At least six serum samples (0.5 ml) could be passed through the resin column before it was necessary to replace or regenerate the resin.

Procedures For Biological Materials

(a) *Serum:* One drop of potassium oxalate (125 meq/liter) is added to 0.2 or 0.5 ml of serum in a test tube (10 × 75 mm), the solution is mixed, transferred with successive washings to the micro column, using a Pasteur pipet, until 5.0 ml of effluent is collected in a 5.0 ml volumetric flask. To one 0.5 ml aliquot of the effluent is added 3.5 ml of modified Schacter's reagent A and to another 0.5 ml fraction is added 3.5 ml of modified Schacter's reagent B. Addition of the alcoholic reagent precipitates the serum protein, which is centrifuged; the clear supernatant is poured into glass cuvets and its fluorescence measured in the fluorometer. The readings of a reagent blank, prepared by passing 0.2 or 0.5

ml of water through a column and treated in an identical fashion as the samples, are subtracted from those of the samples or standards. The difference in the readings at pH 6.5 and 3.5 is proportional to the concentration of magnesium ions. A magnesium reference standard of approximately the same concentration as in serum is carried through the same procedure and the unknowns are compared with it.

(b) *Rat Urine:* For normal rat urine, i.e., animals fed diets containing 0.8% calcium or less, two drops of 10 meq/liter potassium oxalate is added to 0.5 ml of urine (diluted rat urine, e.g., one part urine and two parts water) mixed thoroughly and passed through the resin in a similar fashion as serum. No nonspecific fluorescence was found in diluted rat urine or human urine that had been passed through the ion-exchange column.

(c) *Bone:* Bone (single femur) was stripped of soft tissue, the epiphyses pried off, and marrow flushed out with water; defatted with $CHCl_3/CH_3OH$ (2/1); and dried and ashed in a muffle furnace at 650°C. The ash was dissolved in a minimum of concentrated HCl and made up to 50 ml with deionized water. To 1 ml of bone solution was added 1.5 ml of potassium oxalate (125 meq/liter). The solution was allowed to stand 15 min and centrifuged at 1500 rpm for 10 min; 0.5 ml of supernate was added to a microcolumn and treated in a similar fashion to serum.

(d) *Feces:* Rat feces (four day collection) were ashed in a muffle furnace at 650°C, dissolved in a minimal amount of HCl, and made up to 50 ml. To 0.3 ml of the solution was added 1.2 ml of 125 meq/liter potassium oxalate and the solution diluted to 5.0 ml, let stand 15 min, and centrifuged as above; 0.5 ml of supernate was taken for magnesium determination as above. Duplicate determinations were carried out on all unkowns and standards. Analysis of variance was done on the data when pertinent (4).

EXPERIMENTAL AND RESULTS

A. *Effect of Calcium and Phosphate Ions on Fluorescence of Magnesium-8-hydroxyquinoline Complex*

It may be seen (Fig. 2) that, at the level of Mg^{++} found in serum (e.g., about 2 meq/liter), calcium concentrations above 5 meq/liter enhance the fluorescence while phosphate ions even at a level of 2.5 meq depress the fluorescence. It is fortuitous that the serum level of calcium in man is usually about 5 meq/liter and the level of phosphate the order of 2–3 meq/liter and consequently the normal serum concentrations of calcium and phosphate have only a minor effect on the serum levels of magnesium. However, the rat normally has a high serum phosphate

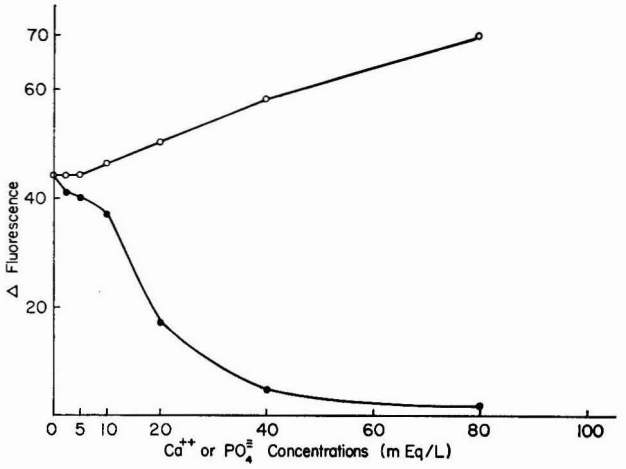

FIG. 2. Effect of increasing concentrations of calcium (○) or phosphate (●) ions on fluorescence of magnesium-quinoline complex. The magnesium concentration was kept constant at 2 meq/liter. "Δ Fluorescence" refers to the difference in dial readings at pH 6.5 and 3.5.

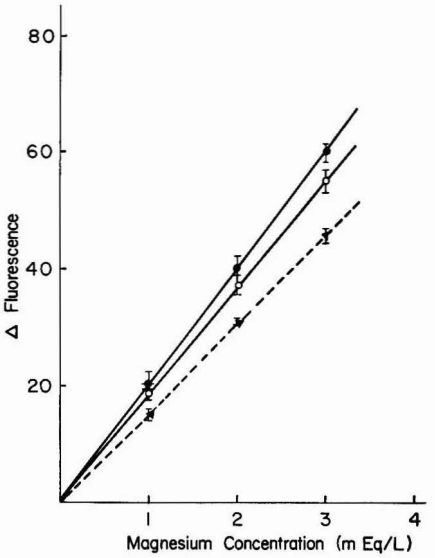

FIG. 3. Effect of simulated human and rat serum calcium and phosphate ion concentrations on fluorescence of varying levels of magnesium-quinoline chelate concentrations: (●) magnesium alone; (○) magnesium plus $[Ca^{++}] = 5.0$ meq/liter and $[PO_4^{---}] = 3.7$ meq/liter; (▲) magnesium plus $[Ca^{++}] = 5.0$ meq/liter and $[PO_4^{---}] = 7.7$ meq/liter.

(8–11 meq/liter) and it is obvious (Fig. 2) that, unless phosphate is removed, erroneous magnesium values will be obtained.

In Figure 3 may be seen the effects of concentrations of normal rat and human serum calcium and phosphate on different levels of magnesium concentration. It is apparent the major aberration results from the presence of phosphate, which in the case of the rat may cause a drop of approximately 25% and in man about 8.0% from the true value of magnesium.

B. *Studies on Separation of Calcium and Phosphate from Magnesium*

The association constants of magnesium oxalate and calcium oxalate are very similar, e.g., log K_a for magnesium is 3.43, and for calcium about 3.00. However, the association constant for magnesium 8-hydroxyquinoline is very much greater than that of calcium 8-hydroxyquinoline, e.g., log K_a for magnesium is 4.73 while that of calcium is 3.27 (3). Since the association constants of calcium oxalate and calcium 8-hydroxyquinoline are similar, e.g., 3.00 versus 3.27, it was felt that 8-hydroxyquinoline when added to oxalate solutions of these cations would preferentially replace oxalate in the case of magnesium, but have very little effect on calcium oxalate. Consequently, the use of oxalate to preferentially tie up calcium seemed to offer a likely way of preventing the enhancement of fluorescence of the magnesium-8-hydroxyquinoline complex by calcium ions. Further, the use of an ion exchange for the removal of phosphate seemed to afford a simple method for removal of phosphate and any other similar anions that might affect the fluorescence of the magnesium complex. In Table 1 may be seen the results of the addition of varying amounts of potassium oxalate for complexing calcium as well as the use of anion-exchange resin for removing phosphate. Excellent recoveries were obtained in all tests except solutions that contained abnormally large amounts of calcium (Nos. 3, 7, and 9). It is possible that some of the magnesium might have been coprecipitated with the calcium oxalate since a large amount of oxalate was added. Potassium, even at a level of 400 meq/liter, was without effect when added as the oxalate. The difference between recoveries of 94.2 and 100% is only one fluorescent unit, which approaches the optimal performance of the fluorometer. A comparison between samples 1 and 10 shows that the presence of oxalate (0.15 meq) has no effect on the fluorescence of the magnesium-quinoline complex.

Comparison between Present Procedure and Original Schachter Method. The Schachter method gives good recoveries for urine and sera provided the levels of calcium or phosphate are not greatly elevated (Table 2). However, when large amounts of phosphate are present very

TABLE 1

Elimination of Fluorescent Interference Caused by Calcium and Phosphate Ions

3.0 ml of test solution was used in all instances. Varying volumes of a 1.5 meq/liter solution of potassium oxalate were added to give the concentrations of oxalate noted below and the solutions were made to 5.0 ml. (Magnesium concentration now 1.2 meq/liter.) Solutions were allowed to stand 15 min, then centrifuged at 1300 rpm for 10 min and 0.5 ml of supernatant was added to a resin column (0.9 × 10 cm) which contained about 4 cc of resin. Deionized water was added stepwise until 10 ml was collected. The effluents were negative for Ca^{++} and PO_4^{---}; 0.5 ml was used for the determination of magnesium. All determinations were done in duplicate.

No.	Sample containing				Δ Fluorescence[a]	% recovery
	$g^{++}M$ meq/liter	Ca^{++} meq/liter	PO_4^{---}, meq/liter	$Oxalate^{---}$, meq		
1	2	0	0	0.15	18	100
2	2	0	500	0.15	19	105.8
3	2	500	0	1.80	16	89.2
4	2	5	10	0.30	18	100
5	2	50	50	0.30	18	100
6	2	50	250	0.30	18	100
7	2	250	50	0.90	17	94.2
8	2	50	500	0.30	19	105.8
9	2	500	50	1.80	17	94.2
10	2	0	0	0	18	100.0

[a] "Δ Fluorescence" refers to the difference in intensities of fluorescent readings between pH 6.5 and 3.5 after reagent blank readings were subtracted at each pH.

TABLE 2

Comparison between Original and Present Method for Magnesium Determination

Sample		Mg^{++}, meq/liter				% recovery
		Found	Added	Calculated	Obtained	
(A) *Without removing calcium and phosphate* (original method of Schachter's)						
Serum	A	1.13	1.00	2.13	2.24	105.2
	B	1.23	1.00	2.23	2.38	106.7
Urine	A	5.50	1.50	7.00	7.13	101.9
	B	5.00	1.50	6.50	6.38	98.2
Bone	A	1.68	3.00	4.68	2.32	49.6
	B	1.84	3.00	4.84	2.48	51.2
Feces	A	6.00	3.00	9.00	6.30	70.0
	B	5.00	3.00	8.00	5.30	66.3
(B) *With removing calcium and phosphate* (present procedure)						
Serum	A	1.83	1.00	2.83	2.94	103.9
	B	2.06	1.00	3.06	3.06	100.0
Urine	A	4.76	1.50	6.26	6.39	102.1
	B	4.08	1.50	5.58	5.55	99.5
Bone	A	3.25	3.00	6.25	6.50	103.8
	B	3.80	3.00	6.80	7.00	102.9
Feces	A	32.33	5.00	37.33	38.00	101.8
	B	25.33	5.00	30.33	30.66	100.9

poor recoveries are obtained. The removal of phosphate ions by the resin and the chelation of calcium ions with oxalate results in excellent recoveries using the present procedure (Table 2). Analysis of variance showed that the per cent recovery by Schacter's method varied significantly from 100% in the case of bone or feces ($p < 0.001$) while with the present procedure recoveries were not significantly different from 100%.

Comparison of Magnesium Determinations by Present Procedure and the Method of Atomic Absorption. The possibility existed that, although good recoveries were obtained using the present procedure, the absolute amounts of magnesium obtained by this method might differ from those obtained by a more specific procedure such as atomic absorption. Consequently, the results obtained by the present procedure were

TABLE 3
Comparison between Fluorometry and Atomic Absorption

	Sample composition			Atomic absorption		Present procedure	
Samples	Ca^{++}, meq/liter	PO^{t---}, meq/liter	Mg^{++}, meq/liter	Mg^{++}, meq/liter	% recovery	Mg^{++}, meq/liter	% recovery
Synthetic mixture							
1	100	100	0.50	0.52	104	0.55	110
2	5	10	2.00	2.00	100	2.06	103
3	400	300	5.00	5.05	101	4.52	90
4	500	300	50.0	54.0	108	50.8	102
5	500	400	250.0	261.0	104	250.0	100
				Av.	103 (3.1)[a]	Av.	101 (7.2)[a]
Feces							
A	370	220		36.6		39.5	
B	310	240		209.		218.	
C	310	180		3.8		4.3	
D	100	100		113.		112.	
Bones							
A	120	120		3.31		3.05	
B	135	128		4.18		3.95	
C	140	124		4.53		4.20	
D	100	100		4.36		3.75	
Urines							
A	2.0	31.0		4.88		4.20	
B	2.7	2.0		24.4		24.0	
C	6.0	4.0		69.7		70.0	
D	2.0	25.0		0.41		0.50	

[a] Numbers in parentheses refer to standard deviation.

compared with those obtained with atomic absorption using a Perkin-Elmer atomic absorptionmeter. The latter determinations were kindly done by Dr. Ann Briscoe, Bellevue Hospital, First Medical Division, College of Physicians and Surgeons, Columbia University. The average recoveries by the fluorometric procedure are equal to those by atomic absorption. Comparison between the two methods (Table 3) shows a high degree of correspondence with the analysis of variance showing no significant differences between the two methods.

Interference from Other Cations. Iron and zinc also complex with 8-hydroxyquinoline; however, iron concentrations (either ferrous or ferric) of as much as 1.0 meq/liter were without effect on the determination of magnesium by the present procedure. The iron concentration in serum, urine, feces, and diets used in these experiments or in man is usually in the order of 0.1 meq/liter.

Zinc in concentrations of 0.1 meq/liter enhances the fluorescence of magnesium about 5% and a fivefold increase in zinc concentrations only raises the apparent magnesium concentration 10%. The normal values for serum zinc is about 0.1 meq/liter and the values in feces and urine much less. Thus, the interference from zinc should not impair the usefulness of this procedure for the determination of magnesium in biological materials, with the minor exceptions of some plant materials, which may have high concentrations of zinc.

SUMMARY

An improved, highly specific method is described for the fluorometric estimation of magnesium in biological materials. Interfering substances such as phosphate ions, which depress fluorescence, and calcium ions, which enhance fluorescence, have been eliminated. This method is reliable and reproducible over a wide range of calcium and phosphate levels and is convenient for routine analyses.

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Computation of Density Distributions in Solutions at Sedimentation Equilibrium

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In the separation of biological particles according to rate of sedimentation through preformed density gradients of sucrose solutions it has been customary to regard the density distribution as unchanging during the period of centrifugation. This assumption has generally been justified: relatively long periods of centrifugation at speeds up to 40,000 rpm have been used repeatedly in these band-velocity or rate-zonal separations. As high rotor speeds become available, and slower sedimenting particles become the target for separation, the stability of the sucrose gradient will eventually become of concern. The theoretical treatment of the transient redistribution is formidable. As a first step in the problem, we undertook the simple task of computing equilibrium density distributions. The tendency for a gradient to redistribute should be greater the farther the gradient is from its equilibrium distribution.

I. SOME COMPUTED EQUILIBRIUM DENSITY DISTRIBUTIONS AND SOME NONEQUILIBRIUM GRADIENTS

Equilibrium Density Distributions of Sucrose Solutions

Figure 1 shows density and concentration distributions for sucrose solutions at sedimentation equilibrium, corresponding to four different rates of rotation in swinging bucket rotors. The radii used in the computation correspond to those of the SW 25.1, SW 39, SW 50, and SW 65 rotors. The distributions were computed by the graphic method described in Part II. It was assumed that the centrifuge tubes were full, and the hemispherical shape of the tube bottom was neglected. The additional gradient produced by compression is not included because it does not stabilize against convection resulting from localized concentration buildup (1). The parameter z_e is the mean sucrose concentration in weight per cent. It would be the starting concentration if the solution were initially

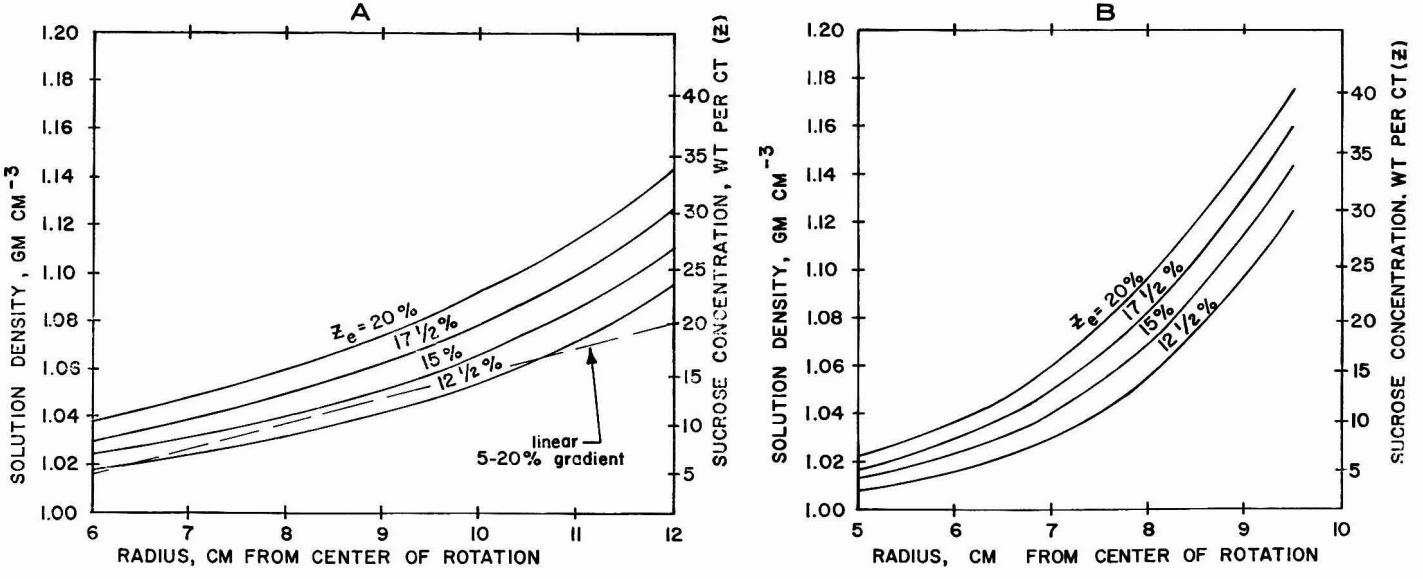

FIG. 1. The density/concentration at sedimentation equilibrium for sucrose solutions at 25°C, computed by the graphic method of Part II, for full cylinders (the parameter z_e refers to the mean concentration in weight per cent): (A) 25,000 rpm (SW 25.1 rotor), (B) 40,000 rpm (SW 39 rotor), (C) 50,000 rpm (SW 50 rotor), (D) 65,000 rpm (SW 65 rotor).

C
SOLUTION DENSITY GM CM-3
SUCROSE CONCENTRATION, WT. PER CT (%)
RADIUS, CM FROM CENTER OF ROTATION
%e = 20%
17 1/2 %
15%
12 1/2 %
D
SOLUTION DENSITY , GM CM-3
SUCROSE CONCENTRATION, WT. PER CT (%)
RADIUS, CM FROM CENTER OF ROTATION
TOP
BOT
%e =32.7 %
%e = 20%
17 1/2 %
15%
12 1/2 %

uniformly distributed, or the average of top and bottom concentrations if a linear gradient were the starting condition.

At 25,000 rpm the equilibrium density distribution is not greatly different from commonly used preformed gradients. For example, compare

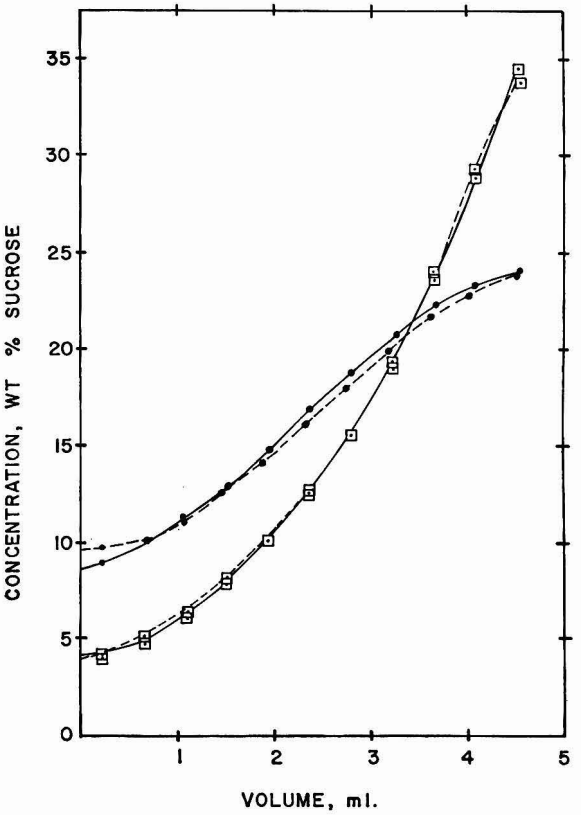

FIG. 2. Sedimentation of a nonequilibrium sucrose gradient: (●) 2 tubes of gradient formed by 1 ml layers of 5, 10, 15, 20, 25% sucrose, diffused 20 hr, room temperature; (□) 2 tubes of gradient formed as above, centrifuged 64 hr, 65 krpm, 25°C.

the equilibrium distribution at $z_e = 12.5\%$ with a linear 5–20% gradient. Thus one would expect to be able to centrifuge a sucrose gradient indefinitely without appreciable gradient shift.

As the rate of rotation is increased, the equilibrium distributions become more curved, and the sucrose tends to accumulate toward the bottom of the container. At 65,000 rpm nearly all of the sucrose would eventually be found in the bottom half of the tube.

Experimental Sedimentation of a Nonequilibrium Sucrose Gradient

One implication of these results is that preformed linear sucrose gradients will tend to sediment at rates of rotation higher than about 25,000 rpm. It is generally assumed in rate separations of macromolecules through sucrose gradients that the density distribution of sucrose solution

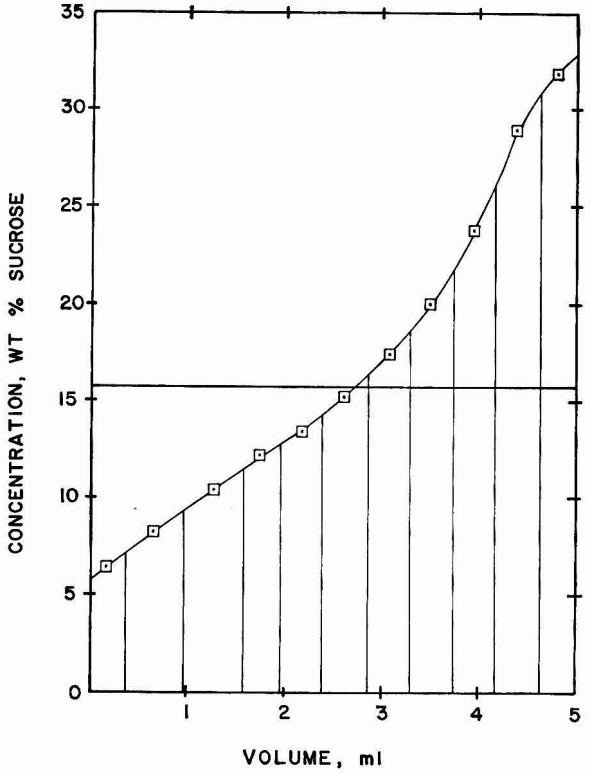

FIG. 3. Sedimentation of an originally homogeneous sucrose solution: (—) tube with homogeneous sucrose solution, nominal 15.5%, (□) tube as above after centrifugation for 64 hr, 65 krpm, 25°C.

does not change during centrifugation. Sedimentation coefficients associated with zone positions are inferred on this basis. Therefore it is of interest to know something about the rate of shift of the gradient itself.

Figures 2 and 3 show the amount of sedimentation observed in sucrose solutions of about 15% mean concentration after centrifugation for 64 hr at 65,000 rpm in the SW 65 rotor. Two of the three tubes in the swinging-bucket rotor contained a gradient at the start, while the third contained an equal amount of sucrose uniformly distributed. (The amount of redistribution is, as expected, greater for the tube initially containing uni-

formly distributed solute.) In either case a considerable amount of additional sedimentation would be required before the equilibrium distribution (Fig. 1D) would be closely approached.

The amount of centrifugation in the above experiment is about what would be required to sediment macro species having sedimentation coefficients in the 1 S region to near the bottom of the tube. The conclusion to be drawn is that separation of materials according to rate should be possible with this degree of gradient shift, but that caution should be exercised in inferring sedimentation coefficient from band position.

Equilibrium Density Distribution of Sodium Chloride Solutions at 65,000 rpm

If one wishes to avoid altogether the possibility of gradient shift, one could do the rate separation in a gradient preformed to the equilibrium shape. The equilibrium distribution for sucrose at 65,000 rpm is not particularly well suited for a rate separation, since there is virtually no stabilizing gradient in the upper half of the tube. A substitute solute for sucrose is needed which has a lower molecular weight and still is able to give solutions of appreciably higher density than water. Sodium chloride is an example of such a material. Its computed equilibrium density dis-

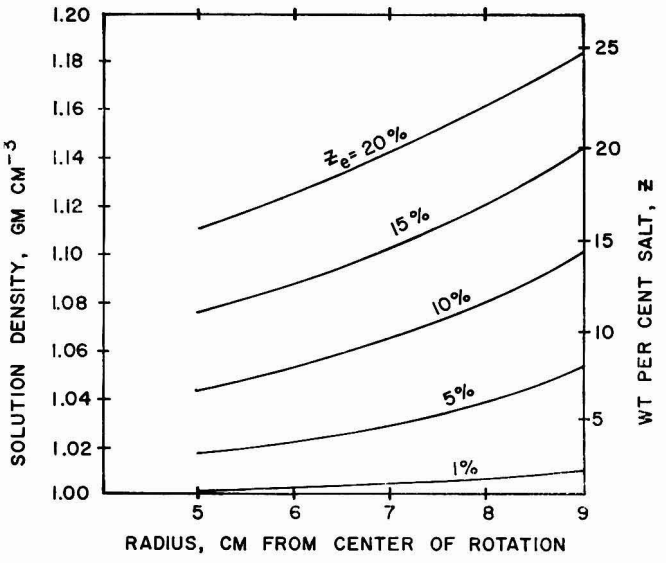

FIG. 4. Density/concentration distributions at sedimentation equilibrium for sodium chloride solutions at 25°C and 65,000 rpm (SW 65 rotor), computed by the graphic method of Part II for full cylinders. The parameter z_e refers to the mean concentration in weight per cent.

tributions at 65,000 rpm (Fig. 4)—in the SW 65 rotor—are seen to be similar to the sucrose distributions at 25,000 rpm (Fig. 1A). A density gradient of salt preformed to the shape of Figure 4 should be suitable for rate separations of slowly sedimenting components which are not adversely affected by high salt concentrations. (See section in Part II on "Effect of Pressure on Solute Distribution.")

II. A GRAPHIC METHOD FOR OBTAINING EQUILIBRIUM DENSITY DISTRIBUTIONS

Description of Problem

We employ the theory and terminology as presented by Ifft *et al.* (2). In any binary solution at sedimentation equilibrium the compositional density gradient is given by:

$$\frac{d\rho}{dr} = \frac{\omega^2 r}{\beta(\rho)} \tag{1}$$

where

$$\beta(\rho) = \frac{d \ln a}{d\rho} \frac{RT}{(1 - \bar{v}\rho)M} \tag{2}$$

In order to obtain a density distribution, equation (1) is rearranged to separate variables, and integrated:

$$\int_{\rho_0}^{\rho} \beta(\zeta) d\zeta = \frac{\omega^2}{2} (r^2 - r_0^2) \tag{3}$$

where ζ represents the variable of integration. The left-hand member of equation (3) will be designated by:

$$I(\rho) - I(\rho_0) = \int_{\rho_0}^{\rho} \beta(\zeta) d\zeta \tag{4}$$

Values of $I(\rho)$ are determined from the solution properties at a particular temperature. It is an empirical quantity. If some particular density ρ_0 can be associated with a particular radius r_0, equation (3) can be solved to give a radius for every known value of $I(\rho)$; i.e., a density distribution is established.

After the density-radius relation is determined for the entire container, from meniscus radius r_a to bottom radius r_b, the mean density, ρ_e, corresponding to the total amount of solute, can be computed by applying conservation of mass. For cylinders, neglecting the rounded bottom, this is:

$$\rho_e = \frac{\int_{r_a}^{r_b} \rho \, dr}{r_b - r_a} \tag{5}$$

and for sector cells:

$$\rho_e = \frac{2\int_{r_a}^{r_b} \rho r\, dr}{(r_b^2 - r_a^2)} \tag{6}$$

In general there are two types of problems of interest. We describe these problems with reference to the foregoing equations, which implies the use of numerical techniques. Ifft, Voet, and Vinograd (2) and Ludlum and Warner (3) describe two such numerical techniques. In this report, however, the equations are solved by a graphic technique described in the following section.

Problem Type A. In this problem we are given a definite density value ρ_0 which must occur at a particular radius r_0. For example, we might specify that the density should not exceed some maximum value, such as that corresponding to the saturated solution, at the tube bottom; or we might specify that a particular density occur in the middle of the cell. Once having obtained the solution properties and computed $I(\rho)$ (eq. 4), we could employ equation (3) to obtain the density-radius relation from r_a to r_b, using the point (r_0, ρ_0) as an "anchor." Application of the conservation of mass equations (5) and (6) will then give the mean density ρ_e, which tells us how much solute is needed to get the distribution just determined.

Problem Type B. In the second type of problem we are given a volume and concentration for the solution and asked to compute how this amount of solute will distribute itself at equilibrium. This type of problem requires a trial-and-error approach because we need a distribution before we can compute the corresponding value for ρ_e by equations (5) and (6).

Graphic Technique for Rapidly Obtaining Density Distributions, Illustrated on Sucrose Solutions

Figure 5 shows the $I(\rho)$ vs. ρ plot obtained for sucrose solutions at 25°C (see eq. 4). The computations involved in obtaining this particular plot are described in the following section. Above the $I(\rho)$ plot is a facsimile of a transparent overlay which is a radius scale for use at 40,000 rpm, plotted to the same scale as $I(\rho)$. The basis for constructing this overlay is equation (3). A convenient radius is picked, for example 7 cm, as an origin. Horizontal lines are then drawn at intervals according to the values of $(\omega^2/2)(r^2 - 7^2)$ for conveniently spaced values of r to cover the radii of interest. Thus one inch might represent an increment of 2×10^9 cm^2 sec^{-2} in both the quantities $I(\rho)$ and $(\omega^2/2)(r^2 - r^2_0)$.

In order to obtain a density distribution, the overlay is laid down on top of the $I(\rho)$ plot. The solution density associated with each radius is

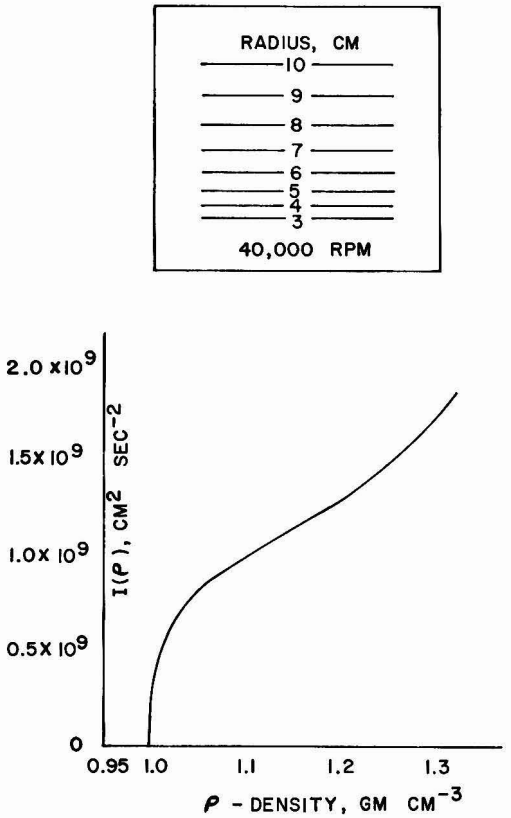

FIG. 5. *At bottom,* working plot of $I(\rho) = \int_{0.998}^{\rho} \rho\beta(\zeta)\, d\zeta$ for sucrose solutions at 25°C. *At top,* facsimile of the radius scale overlay for 40,000 rpm.

read from the bottom axis, corresponding to the intersection of the radius lines with the $I(\rho)$ curve. The advantage of the overlay is that it can be shifted upward or downward, each shift resulting in a new distribution corresponding to more or less solute, respectively.

In solving a type A problem (previous section), the overlay is laid down so that the specified density and radius coincide. Then the rest of the distribution can be read off. The mean density is then obtained by graphic, or numerical, integration of this distribution (eqs. 5 and 6).

In a type B problem, we have found it convenient to determine several density distributions by arbitrarily positioning the overlay several times. The mean density for each position of the overlay is computed, and the final position for the overlay is selected by interpolation among the previously selected positions.

TABLE 1
Equilibrium Density Distributions (for 40,000 rpm) of Sucrose Solutions at 25°C

r	ρ						
5.0	1.001	1.005	1.008	1.013	1.017	1.022	1.027
5.5	1.002	1.007	1.011	1.017	1.022	1.028	1.034
6.0	1.004	1.010	1.016	1.023	1.029	1.036	1.043
6.5	1.007	1.014	1.022	1.030	1.038	1.046	1.055
7.0	1.010	1.020	1.030	1.040	1.050	1.060	1.070
7.5	1.015	1.028	1.040	1.053	1.064	1.076	1.087
8.0	1.022	1.039	1.056	1.070	1.083	1.097	1.109
8.5	1.033	1.055	1.074	1.091	1.105	1.120	1.134
9.0	1.047	1.074	1.096	1.115	1.131	1.146	1.160
9.5	1.066	1.098	1.123	1.143	1.159	1.174	1.186
Trapezoid ρ_e	1.0193	1.0332	1.0456	1.0574	1.0678	1.0786	1.0887
Trapezoid z_e	5.68	9.12	12.12	14.93	17.35	19.82	22.10

In Table 1 are shown seven such density distributions and the ρ_e value obtained by numerical integration (trapezoidal rule). The average concentration z_e, corresponding to ρ_e, is obtained directly from the density-concentration data for the solutions under study.

Equilibrium Distribution Parameter $\beta(\rho)$ for Sucrose and Sodium Chloride Solutions

Figure 6 shows a plot of the distribution parameter $\beta(\rho)$ for sucrose solutions at 25°C. Results are shown from the work of Ifft *et al.* (2) and Trautman (4), as well as points computed for this study.

In this study, thermodynamic activity data were obtained from the compilation of Robinson and Stokes (5). For sucrose, a nonelectrolyte:

$$a = \gamma m \tag{7}$$

so that:

$$\frac{d \ln a}{d\rho} = \frac{d \ln a}{dm}\frac{dm}{d\rho} = \left(\frac{1}{m} + \frac{1}{\gamma}\frac{d\gamma}{dm}\right)\frac{dm}{d\rho} \tag{8}$$

The thermodynamic activity data indicate that for concentrations up to 0.5 molal the activity coefficient varies linearly with concentration:

$$\gamma = 1 + 0.17m \tag{9}$$

whence:

$$\frac{d \ln a}{dm} = \frac{1}{m} + \frac{0.17}{1 + 0.17m} \tag{10}$$

In the higher concentration region, the derivatives were approximated by taking differences.

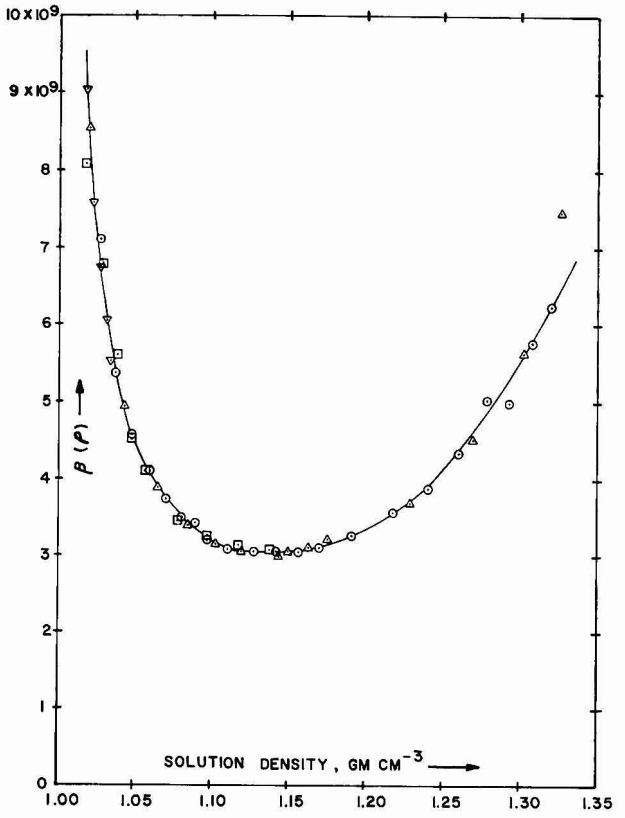

FIG. 6. Equilibrium distribution parameter $\beta(\rho)$ for the sucrose/water system at 25°C: (△) Trautman (4), (□) Ifft *et al.* (2), (▽) this report, low concentration region, (○) this report, major concentration region.

Values of $(1 - \bar{v}\rho)$ were obtained by applying the expression:

$$(1 - \bar{v}\rho) = \frac{1 - z}{\rho} \frac{d\rho}{dz} \tag{11}$$

in finite difference form to density-concentration tables (6).

Tables 2 and 3 present tabulated values for $\beta(\rho)$, and Table 4 gives the values of $I(\rho)$ obtained by numerical integration (Simpson's Rule) of the graphically interpolated $\beta(\rho)$ function, using a starting density of 0.998, which corresponds to a sucrose concentration of about 0.024 wt %. The starting density cannot be the density of water because $\beta(\rho)$ becomes unbounded as concentration approaches zero. The working plot $I(\rho)$ vs. ρ was made to a larger scale than shown in Figure 5.

TABLE 2
Values for $\beta(\rho)$ for Sucrose Solutions at 25°C (Low Concentration Region)

Weight fraction	Density, gm cm^{-3}	$\beta(\rho)$
0	0.99706	
0.01	1.00091	49.54×10^9
0.02	1.00479	25.03×10^9
0.03	1.00869	16.87×10^9
0.04	1.01263	12.80×10^9
0.05	1.01659	10.36×10^9
0.06	1.02058	9.028×10^9
0.07	1.02460	7.592×10^9
0.08	1.02865	6.735×10^9
0.09	1.03272	6.072×10^9
0.10	1.03683	5.561×10^9

Tables 5 and 6 present the $\beta(\rho)$ and $I(\rho)$ values used to compute the equilibrium density distributions for sodium chloride solutions at 25°C and 65,000 rpm shown in Figure 4. The necessary concentration-density data were obtained from *International Critical Tables,* and the thermo-

TABLE 3
Values for $\beta(\rho)$ for Sucrose at 25°C (Full Concentration Range)

Molality	Weight fraction	ρ, gm cm^{-3}	$\beta(\rho)$
0			
0.05	0.0169	1.00340	29.89×10^9
0.15	0.0487	1.01607	10.80×10^9
0.25	0.0788	1.02816	7.121×10^9
0.35	0.1070	1.03971	5.427×10^9
0.45	0.1335	1.05073	4.597×10^9
0.55	0.1584	1.06130	4.103×10^9
0.65	0.1820	1.07150	3.738×10^9
0.75	0.2043	1.08130	3.478×10^9
0.85	0.2254	1.09070	3.428×10^9
0.95	0.2454	1.09976	3.165×10^9
1.1	0.2735	1.11272	3.088×10^9
1.3	0.3080	1.12901	3.059×10^9
1.5	0.3393	1.14412	3.042×10^9
1.7	0.3679	1.15824	3.042×10^9
1.9	0.3941	1.17145	3.099×10^9
2.25	0.4351	1.19260	3.259×10^9
2.75	0.4848	1.21907	3.571×10^9
3.25	0.5266	1.24206	3.937×10^9
3.75	0.5621	1.26209	4.330×10^9
4.25	0.5926	1.27970	5.010×10^9
4.75	0.6192	1.29535	4.987×10^9
5.25	0.6425	1.30927	5.753×10^9
5.75	0.6631	1.32176	6.227×10^9

TABLE 4

Values for $I(\rho) = \int_{0.998}^{\rho} \beta(\zeta)\, d\zeta$ for Sucrose Solutions at 25°C

ρ, gm cm^{-3}	$I(\rho)$	ρ, gm cm^{-3}	$I(\rho)$
0.998	0	1.070	0.878×10^9
1.000	0.224×10^9	1.080	0.926×10^9
1.002	0.323×10^9	1.090	0.960×10^9
1.004	0.388×10^9	1.100	0.988×10^9
1.006	0.438×10^9	1.12	1.050×10^9
1.008	0.477×10^9	1.14	1.111×10^9
1.010	0.510×10^9	1.16	1.172×10^9
1.012	0.538×10^9	1.18	1.234×10^9
1.014	0.563×10^9	1.20	1.298×10^9
1.016	0.586×10^9	1.22	1.367×10^9
1.018	0.606×10^9	1.24	1.441×10^9
1.020	0.624×10^9	1.26	1.523×10^9
1.030	0.699×10^9	1.28	1.615×10^9
1.040	0.757×10^9	1.30	1.718×10^9
1.050	0.806×10^9	1.32	1.834×10^9
1.060	0.850×10^9		

TABLE 5

Values for $\beta(\rho)$ and Pressure Coefficient for Sodium Chloride Solutions at 25°C

z, weight fraction	ρ, gm cm^{-3}	$\beta(\rho)$	φ
0	0.99706		
0.01	1.00409	161.0×10^9	
0.02	1.01112		18.37×10^{-5}
0.03	1.01819	56.82×10^9	
0.04	1.02530		16.83×10^{-5}
0.05	1.03245	36.54×10^9	
0.06	1.03963		16.13×10^{-5}
0.07	1.04685	28.15×10^9	
0.08	1.05412		15.54×10^{-5}
0.09	1.06143	23.67×10^9	
0.10	1.06879		15.02×10^{-5}
0.11	1.07619	21.13×10^9	
0.12	1.08365		14.61×10^{-5}
0.13	1.09115	19.51×10^9	
0.14	1.09872		14.29×10^{-5}
0.15	1.10634	18.50×10^9	
0.16	1.11401		14.04×10^{-5}
0.17	1.12174	17.88×10^9	
0.18	1.12954		13.85×10^{-5}
0.19	1.13740	17.53×10^9	
0.20	1.14533		13.64×10^{-5}
0.21	1.15333	17.38×10^9	
0.22	1.16140		13.47×10^{-5}
0.23	1.16954	17.39×10^9	
0.24	1.17776		13.32×10^{-5}
0.25	1.18605	17.39×10^9	
0.26	1.19443		

TABLE 6

Values for $I(\rho) = \int_{0.998}^{\rho} \beta(\zeta)\, d\zeta$ for Sodium Chloride Solutions at 25°C

ρ, gm cm^{-3}	$I(\rho)$	ρ, gm cm^{-3}	$I(\rho)$
0.998	0	1.080	5.436×10^9
1.000	1.2907×10^9	1.090	5.637×10^9
1.002	1.8781×10^9	1.100	5.829×10^9
1.004	2.2627×10^9	1.110	6.015×10^9
1.006	2.5495×10^9	1.120	6.196×10^9
1.008	2.7780×10^9	1.130	6.374×10^9
1.012	3.141×10^9	1.140	6.550×10^9
1.016	3.423×10^9	1.150	6.724×10^9
1.020	3.653×10^9	1.160	6.898×10^9
1.030	4.103×10^9	1.170	7.072×10^9
1.040	4.449×10^9	1.180	7.246×10^9
1.050	4.739×10^9	1.190	7.420×10^9
1.060	4.993×10^9	1.200	7.594×10^9
1.070	5.223×10^9		

dynamic activity data from Robinson and Stokes (5). At low concentration an analytical approximation for $\beta(\rho)$ was found:

$$\beta(\rho) = \frac{10^9}{0.8835(\rho - 0.99706)} \tag{12}$$

so that values of $I(\rho)$ were computed analytically over the range $0.998 \leqslant \rho < 1.01$ gm cm^{-3}. Above $\rho = 1.01$, integration was by Simpson's Rule, using graphically interpolated values of $\beta(\rho)$.

Effect of Pressure on Solute Distribution

Figure 7 shows a comparison between observed and computed NaCl solution density distributions in the SW65 rotor. The experimental points (circles) represent averages obtained on three tubes containing 5 ml of solution which had been layered into the tubes in concentrations of 6, 8, 10, 12, and 14 wt % and centrifuged for 69 hr. The tubes were punctured and 1 ml samples collected for refractive index measurement. The smooth curve represents the density distribution calculated using the β values from Table 5 and the measured rate of rotation, 64,455 rpm.

The difference between computed and observed gradients is greater than can be accounted for by uncertainty in the measurement. (We demonstrated, for example, that preformed gradients, placed in the rotor, accelerated to 65,000 rpm, brought to a stop without braking, removed from the rotor, and recovered from the tubes, had the same density distribution as identical tubes preformed at the same time but allowed

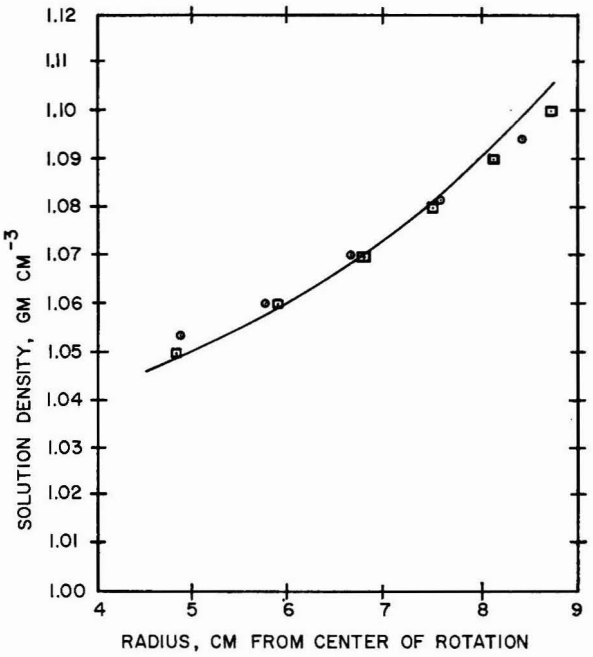

FIG. 7. Comparison between computed and observed density distribution for 10% NaCl solutions at 25°C. The circled points represent densities of 1 ml aliquots withdrawn from the centrifuge tubes of the SW 65 rotor after centrifuging a step-preformed gradient for 69 hr at 64,455 rpm. The solid line represents the density distribution computed for the same conditions. The square points represent a distribution computed taking into account the pressure effect.

to stand during the interim. Also it was shown that identical results are obtained, whether the gradient is removed from the bottom or top of the tube.) The difference appears to be attributable to a pressure effect on the solute distribution. The square points represent a density distribution computed from the original estimate by applying a first-order correction term derived by Hearst, Ifft, and Vinograd (7). The correction has the following form:

$$\frac{d\rho}{dr} = \frac{\omega^2 r}{\beta}(1 - \varphi P) \tag{12}$$

where P represents pressure and $\omega^2 r/\beta$ the gradient computed without pressure effect. Table 5 lists values of φ for NaCl solutions, computed using the compressibility data of Pohl (8). At the high pressures encountered near the tube bottom (at this rate of rotation about 1400 atm), the first-order correction term $1 - \varphi P$ can approach 0.75, enough to result in a measurable shift in solute distribution.

The proper technique for incorporating the pressure correction in the gradient calculations would require iteration, first establishing the pressure distribution, adding the pressure correction, adjusting the distribution to give the same total salt content, recomputing the pressure distribution, etc. Since the quantity φ is itself an approximation to the pressure effect, this extended technique is probably not warranted.

The distributions of Figure 4 are not corrected for pressure effect.

Sources of Data of the Equilibrium Distribution Parameter

Ifft, Voet, and Vinograd (2) list β values for sucrose, KBr, RbBr, and CsCl. Trautman (9) gives values for the reciprocal parameter, density gradient per unit force field strength, for CsCl. Trautman (4, p. 263) expands the compilation to include CsCl, KBr, RbCl, RbBr, urea, and sucrose. Hu, Bock, and Halvorson (10) list the same parameter for fourteen salts in both D_2O and H_2O. Ludlum and Warner (3) show a plot of $\beta(\rho)$ for Cs_2SO_4. Vinograd and Hearst (11, p. 417) repeat the compilation of Ifft *et al.* (2).

SUMMARY

In Part I of this paper we show the density distributions which would result from solute redistribution when sucrose solutions are centrifuged to equilibrium in swinging-bucket rotors at rates of rotation up to 65,000 rpm. The distributions were computed by a graphic technique described in Part II.

The equilibrium distributions at 25,000 rpm are not greatly different from the typical preformed distributions commonly used to support band velocity (rate-zonal) separations. As the rate of rotation is increased the equilibrium distributions depart from linearity. At 65,000 rpm most of the sucrose would eventually be in the bottom half of the tube.

The computed equilibrium density distribution is shown for sodium chloride at 65,000 rpm. It is pointed out that a salt gradient preformed to this shape should have a stabilizing effect similar to the commonly used sucrose gradients, while at the same time being nonsedimenting.

The graphic computational technique described in Part II makes possible the rapid determination of a density distribution once the solution properties have been computed and plotted.

LIST OF SYMBOLS

a thermodynamic activity

I integral density distribution parameter, gm cm^{-1} sec^{-2} [see equation (4)]

M molecular weight of solute, gm $mole^{-1}$

m solute concentration, molal scale
R thermodynamic conversion factor, 8.314×10^7 erg. $°C^{-1}$ $mole^{-1}$
r distance from center of rotation, cm
$\bar{v}$ partial specific volume of solute, cm^3 gm^{-1}
z solute concentration, weight fraction

Subscripts

a meniscus
b tube bottom
e mean

Greek

β equilibrium density distribution parameter, cm^2 sec^{-2} [see equations (1) and (2)]
γ activity coefficient, molal scale
ρ solution density, gm cm^{-3}
φ pressure coefficient, 1/P, [see equation (12)]
ω angular velocity, rad sec^{-1}

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ANALYTICAL BIOCHEMISTRY 19, 40–45 (1967)

Diagnosis of Homocystinuria by Gas Chromatography[1]

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Homocystinuria (1–3) is an inborn error of methionine metabolism associated with ectopia lentis, mental retardation, arterial and venous thromboses, and skeletal abnormalities similar to those associated with the Marfan syndrome (4). The disease is associated with a deficiency of cystathionine synthase (5), which results in an elevated urinary excretion of homocystine. Homocystinuria may be relatively common since it has been reported second in incidence only to phenylketonuria in a survey of 2290 mentally defective individuals studied in Northern Ireland (6). It has also been estimated that 5% of cases of nontraumatic ectopia lentis have homocystinuria (7) and it is likely that a similar proportion of patients with the Marfan syndrome have homocystinuria (4). The early detection of patients with homocystinuria is important because of the possibility that dietary restriction of methionine or supplementation with cysteine may prove beneficial in preventing mental retardation and the vascular thromboses.

At the present time, there is no simple method for the determination of urinary homocystine. In our hospital, as in others, urine specimens from suspected cases are screened for sulfur-containing amino acids by the cyanide nitroprusside test (8). When this test is positive, the presence of homocystine must be confirmed by more specific analytical methods. These include: (*1*) a variation of the method of Spackman, Stein, and Moore (9) using an automatic amino acid analyzer, (*2*) a somewhat shorter quantitative method involving oxidation of homocystine followed by ion-exchange chromatography of homocysteic acid (10), and (*3*) thin-layer chromatography of the dinitrophenyl derivative (11). We recently developed for the rapid determination of urinary homocystine by gas chromatography a simple method based upon the *N*-trifluoroacetyl methyl ester derivative.

[1] This study was aided by grants from the National Institutes of Health (AM-08391-50 and HDO 1326-01).

EXPERIMENTAL

Gas chromatographic determinations were carried out with a Barber-Colman model 10 instrument as described previously (18). DL-homocystine (Sigma Chemical Co.) is converted to the *N*-trifluoroacetyl methyl ester by dissolving 1 ml anhydrous trifluoroacetic anhydride and 0.5 ml trifluoroacetic acid and allowing to stand at room temperature for 20 min. After evaporation of the excess reagents to dryness under air, the oily residue is treated with an ethereal solution of distilled diazomethane for 15 min or until the evolution of nitrogen ceases while the yellow color persists. The excess diazomethane is then evaporated under a stream of air and the derivative is chromatographed in methanol. The reaction sequence for homocystine is shown in Figure 1. The derivative was found

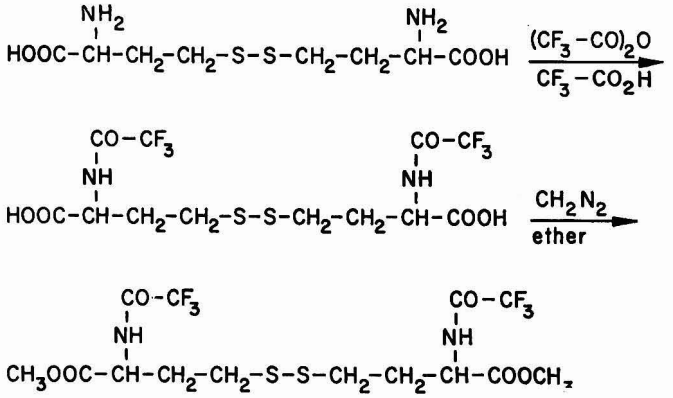

FIG. 1. Reaction sequence for the formation of the *N*-trifluoroacetyl methyl ester of homocystine.

to have a symmetrical single peak on one nonpolar and three polar columns. Retention times relative to methyl veratrate are shown in Table 1.

Elementary analyses (Table 1) were satisfactory and inspection of infrared spectra after passing through an SE-30 column revealed that no decomposition had taken place.

When normal urine is evaporated to dryness and treated by this method, chromatography results in a very large number of peaks and attempts to identify added homocystine were unsuccessful. A method of extraction was worked out as follows: 10 ml of urine is passed through a cation-exchange resin column (Dowex 50W-X4, 20-50 mesh, in the hydrogen form) and the column washed with 30 ml of distilled water. The amino acids are then eluted with 20 ml of concentrated NH_4OH. The aqueous solution is then evaporated to dryness in a flask evaporator *in vacuo*.

TABLE 1

Relative Retention Time of Homocystine Derivative on Polar and Nonpolar Columns

Compound	SE-30[1]	EGA[2]	XE-60[3]	QF-1[4]
Methyl veratrate	1.0[a]	1.0[b]	1.0[c]	1.0[d]
Homocystine derivative[5]	0.19	0.71	1.30	0.61

[1] 7% coated on 100–120 mesh Chromsorb W. *Conditions:* 140° column temperature. Argon inlet pressure 6 psi, outlet pressure atmospheric. Flow rate 43 ml/min.

[a] Approximate retention time of methyl veratrate 22 min.

[2] 5% coated on 100–120 mesh Chromsorb W. *Conditions:* 140° column temperature. Argon inlet pressure 10 psi, outlet pressure atmospheric. Flow rate 67 ml/min.

[b] Approximate retention time methyl veratrate 13 min.

[3] 3% coated on 80–100 mesh Chromport XXX, *Conditions:* 150° column temperature. Argon inlet pressure 8 psi, outlet pressure atmospheric. Flow rate 46 ml/min.

[c] Approximate retention time of methyl veratrate 7 min.

[4] 5% coated on 80–100 mesh Gas Chrom P. *Conditions:* 140° column temperature. Argon inlet pressure 5 psi, outlet pressure atmospheric. Flow rate 46 ml/min.

[d] Approximate retention time of methyl veratrate 14 min.

[5] A White crystalline solid (m.p. 96–98°C). *Theoretical* C 34.42%, Found 34.69%. Theoretical H 3.71%, found 3.19%. Theoretical N 5.73%, found 6.30%. (Microanalysis performed by Clark Microanalytical Laboratories, Urbana, Illinois.)

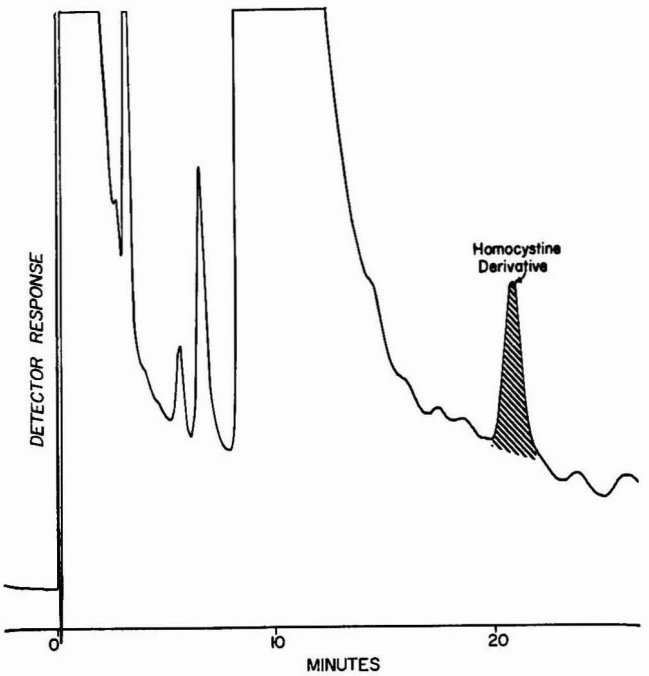

FIG. 2. Gas chromatograph of urine (85 μg creatinine equivalent) of a patient with homocystinuria, treated as described in the text, on a 5% QF-1 column at 140°C. The other column conditions were as described in Table 1. This peak corresponds to 150 μg/mg creatinine.

Recoveries of homocystine added to urine in amounts ranging from 100 to 500 μg were 90–100%.

Homocystine is not detectable in the urine of normal children. In three cases of patients with ectopia lentis, mental retardation, and skeletal features suggesting the Marfan syndrome it was detected in abnormal amounts.

Samples of urine were treated as described above and then chromatographed. Figure 2 shows the appearance of a chromatograph of a specimen

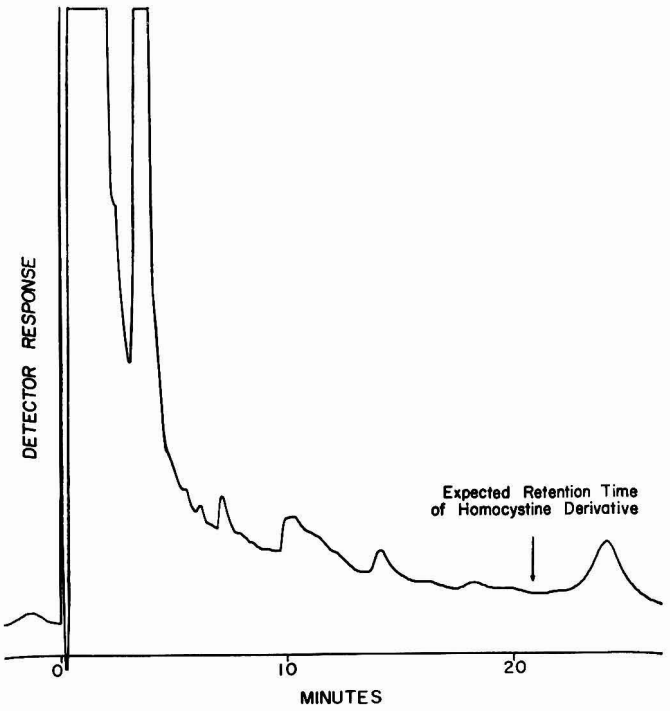

FIG. 3. Gas chromatograph of urine (550 μg creatinine equivalent) of normal control. Treatment and conditions as in Figure 2.

from a patient with homocystinuria. Note the large peak corresponding to the exact retention time of homocystine. The peak was trapped in a Teflon tube and rechromatographed on the other three columns (EGA, XE-60, SE-30). On each column, a single peak with the same width at half-height was found at the exact retention time of the homocystine derivative. Figure 3 shows a chromatograph of a urine specimen from a normal control treated in the same way. In this case no homocystine was detected at all, although six times as much extract (creatinine equivalent) was

TABLE 2
Urinary Excretion of Homocystine in Three Patients with Homocystinuria

Patient	μg Homocystine/ mg creatinine	mg Homocystine/ 24 hr
J. C.	150	—
D. B.	390	—
	130	68
	200	97
R. B.	880	
	20	24
	180	100
Normals	None <1.0	None <1.5
Homocystinurics (3)	—	7–80

injected. The excretion of homocystine was measured in several other samples and the results are recorded in Table 2.

DISCUSSION

All the methods so far described for the gas chromatography of amino acids involve the preparation of volatile derivatives (12). Several groups of investigators (13–17) have studied the *N*-trifluoroacetylated amino acid methyl esters with the objective of developing a rapid method for determining a large number of amino acids in one analysis, but in these there has not been complete success, and none of the authors has described analytical techniques for the gas chromatography of urinary acids. Our method of derivative preparation is a modification of that of Wagner and Winkler (15) and Cruickshank and Sheehan (17) which permits derivative formation in about 45 min. With the large amounts of homocystine in these patients, volumes as small as 5 ml of urine are adequate for study, and altogether a complete analysis can be carried out in about 2–3 hr.

SUMMARY

A short gas chromatographic technique for the determination of urinary homocystine has been developed which should be applicable to other amino acids. This technique involves the formation of the *N*-trifluoroacetyl amino acid methyl ester following separation of the amino acids by ion-exchange chromatography, and has been successfully applied to the confirmation of three suspected cases of homocystinuria.

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ANALYTICAL BIOCHEMISTRY 19, 46–49 (1967)

Detection of *N*-Methylated Ethanolamines in Phosphatide Hydrolyzates by Thin-Layer Chromatography

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In the biosynthesis of lecithin (1–7) *N*-methylated phosphatidyl ethanolamines are obtained as intermediates. Detection via paper or thin-layer chromatograms of phosphatidyl *N*-methylethanolamine (MMPE) and phosphatidyl *N,N*-dimethylethanolamine (DMPE) in the presence of phosphatidyl ethanolamine (PE) is difficult owing to inadequate separation (4, 7, 8). Column separations via silicic acid and diethylaminoethylcellulose (9) are very time-consuming. Paper and thin-layer chromatographic separations of the water-soluble amino components after hydrolysis have also been performed (9–11). Ethanolamine (E) and *N,N*-dimethylethanolamine (DME) were, however, found to have identical R_f values on the thin-layer plates (11). Furthermore, the formation of $ClCH_2CH_2N(CH_3)_3)Cl$ (12) in the hydrolysis with 6 *N* hydrochloric acid is possible. With the method described below E, *N*-monomethylethanolamine (MME), and DME can be detected in the presence of each other without losses and without formation of artifacts.

EXPERIMENTAL

Ten grams of soybean lecithin (13% cephalin) (14) or 18 gm of egg yolk (10% phosphatides containing 15% cephalin) (15) was hydrolyzed by swirling and refluxing with 200 ml of 2 *N* sulfuric acid for ca. 3 hr. After cooling down, powdered barium carbonate was carefully added while stirring until neutral reaction. The precipitate was filtered by suction and subsequently washed with alcohol. The filtrate was acidified with a few drops of concentrated hydrochloric acid and then concentrated in vacuum at 50°C to ca. 1 ml. After rinsing into the steam distillation apparatus (13), the solution was made alkaline by adding pellets of sodium hydroxide. The steam distillate was collected in a receiver containing 100 ml of 0.1 *N* hydrochloric acid. Approximately 300 ml of the distillate was evaporated to dryness at max. 50°C in vacuum and dis-

solved in a mixture of 3 ml of water and 2 ml of alcohol (volume of solution: 4.9 ml).

Plates coated with silica gel G were used in the TLC method. A spray solution prepared from 100 ml of 0.5 *N* potassium permanganate and 0.6 gm of caustic soda was used for staining. After heating the plates to 60°C in the drying cabinet E, MME, and DME appeared as yellow spots on a red-violet background.

RESULTS

Sensitivity of the Method

First we tested the limit of the thin-layer chromatographic detection. After chromatographing equal parts by weight (20 μg) of E, MME, and DME on silica gel G, using the solvent 96% ethanol/7% ammonia (1/2) (11), yellow spots were obtained decreasing in their color intensity and size in the sequence of E, MME, DME. Since the boiling points of these compounds decrease in the same order (E 170°C, MME 159.6°, DME 133.8°), we suspected evaporation losses during chromatography or on drying the plate. The evaporation losses may be eliminated by chromatographing—instead of the volatile amines—their nonvolatile salts. We found that the mixture of E, MME, DME, and choline could readily be separated on silica gel G, using the solvent methanol/concentrated hydrochloric acid 95/5. Detectable losses of material did not occur during hydrolysis, steam distillation, and chromatography. As little as 1 μg of MME[1] and DME[1] can still be detected in the presence of 1000 μg of E.[1]

Phosphatides in Soybean and Egg Yolk

Figure 1 shows clearly that there are no detectable amounts of MME and DME present in soybean phosphatides and fresh egg yolk. By taking into consideration the detection limit of 1 μg of MME and DME, the molecular weights as dioleoyl derivatives of PE (MW 743), MMPE (757), and DMPE (771), as well as the known amount of hydrolyzates applied, the maximum possible contents of MMPE and DMPE can be calculated. For egg yolk, the MMPE and DMPE contents were found to be less than 0.003% and 0.002%, respectively, while soybean phosphatides contained less than 0.007% each of MMPE and DMPE.

The thin-layer chromatogram (Fig. 1) shows underneath the position

[1] E ex Riedel de Haen; MME and DME were prepared by methylating E with paraformaldehyde/hydrogen (Raney Ni) according to Maxwell (17) and by subsequent distillative fractionation. The substances were found to be pure by thin-layer chromatography.

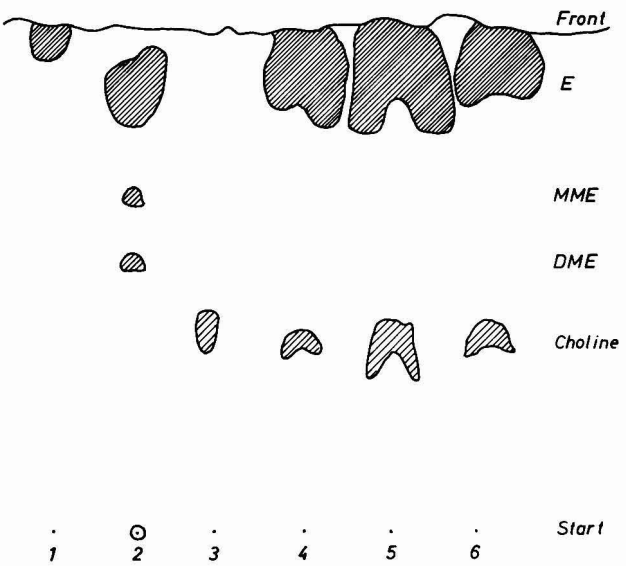

FIGURE 1

corresponding to DME faint spots of choline, which was identified in a duplicate determination by spraying successively with the following solutions (16):

1. 1% sodium hydroxide solution.
2. 1% potassium hexacyanoferrate(II) solution.
3. 0.5% cobalt(II) chloride·$6H_2O$ solution.

SUMMARY

A method was developed for the thin-layer chromatographic detection of small amounts of *N*-monomethylethanolamine (MME) and *N*,*N*-dimethylethanolamine (DME) in the presence of large amounts of ethanolamine. The method was used in the investigation of soybean phosphatides and egg yolk for the presence of *N*-methylated cephalins. In both natural products neither MME nor DME was found.

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ANALYTICAL BIOCHEMISTRY 19, 50–55 (1967)

Preliminary Experiments on the Application of Zonal Ultracentrifugation to Studies of Pesticide Distribution in Subcellular Components of Mammalian Tissues

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Density gradient ultracentrifugation, which separates particles on the basis of sedimentation rates and density, has been adapted by Anderson (1) and developed as the zonal ultracentrifuge. This instrument has been useful in solving several biomedical problems. Its capabilities have now been extended to investigation of the distribution of pesticide residues in tissues.

It has been reported that esterase activity is reduced in both liver and brain following acute poisoning with carbamate insecticides. In general, studies on the distribution of carbamate insecticides in mammals have been limited to characterization of degradation products (2–4). However, investigators have shown, through the use of enzyme inhibition studies following acute poisoning, the probable distribution of antiesterase carbamates in certain mammalian tissues (5, 6). Subcellular distribution of carbamate inhibitors has not been investigated.

With the aid of differential and zonal ultracentrifugation to separate and isolate large quantities of subcellular compounds very nearly free of cross contamination, an investigation was made of the distribution of an anticholinesterase carbamate pesticide, Banol (Upjohn Co.), following acute poisoning.

METHODS AND MATERIALS

Carbonyl-^{14}C labeled Banol (3,4-dimethyl-6-chlorophenyl *N*-methylcarbamate) was administered intraperitoneally to male Osborne-Mendel rats, each weighing about 250 gm, at 5 mg/kg (approximately the LD_{50}).

[1] Present address: U. S. Patent Office, Washington, D. C.

The amounts administered, in terms of radioactive material, were 5.35 μc (specific activity 0.539 mc/mm) for differential ultracentrifugation and 13.43 μc (specific activity 5.75 mc/mm) for zonal ultracentrifugation. Tissues from normal rats were evaluated for esterase activity. Rats were killed 50–60 min after treatment; brain and liver were removed and cooled at 4°C. Liver was homogenized in 4 times its volume and brain in 10 times its volume of 0.1 *M* tris(hydroxymethyl)aminomethane-HCl (tris-HCl) buffer (0.02 *M* Mg), pH 7.8, for differential centrifugation. Liver was also treated with 4 times its volume of 0.25 *M* sucrose for zonal ultracentrifugation. Nuclei and mitochondria were removed by low-speed centrifugation. The supernatant fraction was treated with 1.25% deoxycholate (DOC) prior to zonal centrifugation, which separated the microsomal subunits.

Centrifugation

Differential centrifugation (International Centrifuge PR-2) was used to isolate nuclei (4000 *g*, 15 min) and mitochondria (14,500 *g*, 15 min). Microsomes were separated (100,000 *g*, 2 hr) by a Beckman-Spinco ultracentrifuge model L, #40 rotor. The B-IV rotor, loaded as described by Anderson (1), was used for zonal ultracentrifugation of DOC-treated microsomes with a linear, buffered, sucrose density gradient ranging from 15 to 35% with a 40% cushion and containing 0.05 *M* tris-HCl, 0.0015 *M* $MgCl_2$, and 0.010 *M* KCl at pH 7.6. The centrifuge was operated at 40,000 rpm for 3 hr and the 1600 ml volume was analyzed continuously at 260 mμ in a Gilford 220 absorbance indicator, modified to hold the Oak Ridge quartz flow cells (7, 8); 40 ml fractions were collected for further analysis.

Analysis of Radioactivity

A Packard scintillation spectrometer (model 314-EX) operated at 4°C was used to measure radioactivity. All counts were corrected to 100% efficiency values based on an internal standard. For direct counting of aqueous samples the scintillation mixture consisted of a dioxane solution of 0.7% (w/v) PPO (2,5-diphenyloxazole), 0.03% (w/v) POPOP (1,4-bis-2-(5-phenyloxazaole)benzene), and 10% (w/v) naphthalene. Solid and liquid samples were air-dried in cellulose bags and combusted in the presence of oxygen in a Schöniger apparatus (9). The $^{14}CO_2$ produced was absorbed in a toluene scintillation solution containing 20% phenylethylamine, 20% methyl alcohol, 0.5% (w/v) PPO, and 0.01% (w/v) POPOP. A portion of each fraction was assayed in triplicate and, where possible, both direct counting and combustion techniques were compared.

Enzyme Analysis

Liver aliphatic esterase activity in the fractions obtained by the zonal ultracentrifuge from untreated animals was measured with an automatic recording titrator (Radiometer TTT-1) set to maintain pH 7.4 stat conditions at 37°C with 0.01 *M* KOH. The substrates used in these experiments were glycerol triacetate and glycerol tributyrate emulsified with the aid of 1% Triton X-100; the esters were initially present in concentrations of 1×10^{-2} *M* in a total volume of 3.2 ml. Enzyme activity was assayed for 1–3 min in the presence of various concentrations of sucrose, as the zonal fractions were not separated from the density gradient media.

RESULTS

Distribution of Carbonyl-^{14}C-Banol in Rat Brain and Liver

Distribution studies of radioactivity found in brain and liver were divided into (*a*) those utilizing a Banol sample of low specific activity (0.539 mc/mm) and differential centrifugation to separate the (gross) components of cells, and (*b*) those utilizing a Banol sample of higher specific activity (5.75 mc/mm) and zonal ultracentrifugation to study DOC-treated liver microsomes.

The distribution of radioactivity in brain and liver is presented in Table 1. Radioactivity recovered in the four fractions differentially

TABLE 1
Subcellular Distribution of Carbonyl-^{14} C-Banol in Rat Brain and Liver

Cell fraction	Per cent recovered ^{14}C activity		
	Brain	Liver	
Nuclei	25	66	49[a]
Mitochondria	5	14	
Microsome	2	9	51[a]
Soluble	68	11	

[a] Combined nuclei-mitochondria and microsome-soluble fractions obtained prior to zonal centrifugation.

separated from the whole brain was concentrated primarily in the soluble (68%) and nuclei (25%) fractions. No appreciable radioactivity was found in the mitochondria (5%) or microsome (2%) fractions. Radioactivity recovered in the four fractions separated from the whole liver was concentrated in the nuclei (66%) with lesser amounts in the other fractions (mitochondria, 14%; microsomes, 9%; and solubles, 11%).

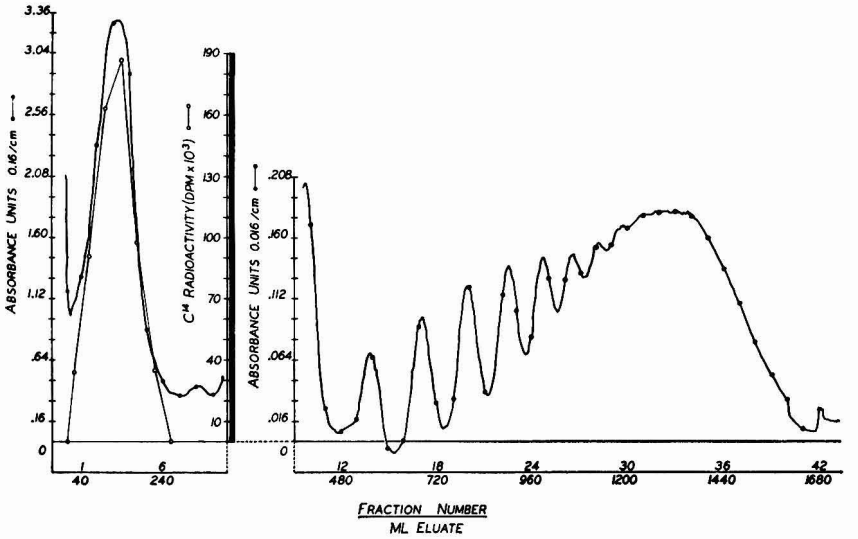

FIG. 1. Spectrometric (●) and radiometric (○) assay of DOC-treated rat liver microsomes separated by zonal ultracentrifugation.

Prior to zonal centrifugation of DOC-treated microsomes, the nuclei and mitochondria were separated from the microsomes and soluble fraction of the liver by differential centrifugation. The combined nuclei-mitochondria fraction contained 49% of the recovered radioactivity; the microsome-soluble fraction contained 51%.

Results of spectrophotometric and radiometric analyses of the DOC-treated microsome-soluble fraction separated by zonal ultracentrifugation are presented in Figure 1. This separation yielded a soluble band and 9

TABLE 2
Aliphatic Esterase Activity of Untreated Rat Liver Submicrosomal Fractions

Zonal ultracentrifuge fractions (40 ml)	Enzyme activity,[a] μ moles substrate hydrolyzed/min	
	Triacetin	Tributyrin
1	0	0.320
2	0.852	0.639
3	0.852	1.118
4	0.063	1.385
5	0.063	1.011
6	0.063	0.125
7	0.025	0.063
10, 14, 16, 19, 21, 22, 33, 35, 40	0	0

[a] Assay conditions include: 2.8 ml of the 40 ml fraction; 0.4 ml substrate (0.01 *M*).

(possibly 12) ribosome and polysome bands. Analysis of ^{14}C activity in the zonal bands showed that the first 6 fractions comprising the soluble band contained all of the radioactivity. No ^{14}C activity was evident in any of the ribosome and polysome fractions.

Aliphatic Esterase Activity in Liver Microsomal Subunits

Aliphatic esterase activities of normal rat liver submicrosomal fractions obtained with the zonal centrifuge are shown in Table 2. Enzyme activity, expressed as μmoles substrate hydrolyzed/min, was noted in the first seven fractions comprising the soluble band. No aliphatic esterase activity was observed in nine representative fractions of the ribosome and polysome peaks.

DISCUSSION

With the aid of differential and zonal centrifugation techniques the distribution of an anticholinesterase pesticide was studied. ^{14}C radioactivity was present in nuclei of both brain and liver. The nuclei fraction was separated from other components of the cell by differential centrifugation and was not treated further. It is possible that the contamination of the nuclei fraction with whole cells may have resulted in the high levels of radioactivity. In further investigations of this observation, low-speed zonal centrifugation will have to be used to isolate quantities of nuclei from liver and brain cells of treated animals.

All ^{14}C radioactivity in liver microsomal subunits (ribosomes and polysomes) obtained by zonal ultracentrifugation was present in the solubilized membrane (endoplasmic reticulum) fraction. Coinciding with the radioactivity in this fraction was the aliphatic esterase activity. Neither radioactivity nor esterase activity was observed in any polysome fraction. Some loss in enzyme activity was evident when the activity of the DOC-treated microsomes (0.25 *M* sucrose), prior to zonal ultracentrifugation, was compared with the activity obtained following the zonal assay. No attempt was made to remove the proteinaceous material from the sucrose gradient, which ranged from 15 to 35% sucrose; the high sucrose content may have had some effect in reducing esterase enzyme activity. As no polysome band showed any enzyme activity, it is possible that all esterase activity was solubilized and migrated with the solubilized reticulum band.

SUMMARY

Differential and zonal ultracentrifugations were used in preliminary studies of the distribution of cholinergic carbamate insecticides in subcellular regions of rat brain and liver. Following acute intoxication with

carbonyl-^{14}C labeled Banol, radioactivity was observed to be present in various subcellular fractions. Radiometric and enzymic assay of microsomal subunits indicated that an esterase enzyme and carbamate migrated with the solubilized endoplasmic reticulum. The zonal ultracentrifuge proved valuable in separating and isolating large quantities of biological material and should continue to be useful in studies of distribution of pesticide residues in animal (and possibly plant) tissues.

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ANALYTICAL BIOCHEMISTRY 19, 56–60 (1967)

Use of the AutoAnalyzer for Determining the Blue Value of the Amylose-Iodine Complex and Total Carbohydrate by Phenol-Sulfuric Acid[1]

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Measurement of the blue color of the amylose-iodine complex in aqueous solution (blue value) has been widely used to determine the amount of amylose present in a starch sample (1, 2) and has been employed in measuring changes in the amylose molecule due to hydrolysis by amylases (3). We have used the measurement of the blue value extensively in α-amylolysis studies of amylose and starch.

The measurement of total carbohydrate by the phenol-sulfuric acid procedure (4) has also been widely employed in our enzymic work and in the analysis of various fractions from the chromatographic separation of maltodextrins on paper and on charcoal and Sephadex columns. Although procedures have been published for the automated analysis of total carbohydrate by acid hydrolysis with subsequent reducing value measurement (5) and by the anthrone procedure (6), we have preferred the phenol-sulfuric acid procedure because of its simplicity, greater sensitivity for low concentrations of carbohydrate (10–180 μg/ml), and high solubility of phenol in water as compared with the very low solubility of anthrone in water.

The use of the Technicon AutoAnalyzer has been shown to increase the reliability of an analytical determination by reducing the systematic and indeterminate errors; it also permits relative ease of analysis and greatly increases the number of samples that may be analyzed. Thus, we have adapted the blue value procedure of McCready and Hassid (2) and the phenol-sulfuric acid procedure (4) to the Technicon AutoAnalyzer and present these adaptations here.

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METHODS

Blue Value. Stock iodine solution (1.0 ml) containing 2.0 mg of iodine and 20.0 mg of potassium iodide per milliliter was diluted to 100 ml; 0.05 ml Brij-35 (obtained from Technicon, Chauncey, New York) and 1.0 ml concentrated hydrochloric acid were added per 100 ml of solution. The working iodine solution was prepared fresh for every set of analyses.

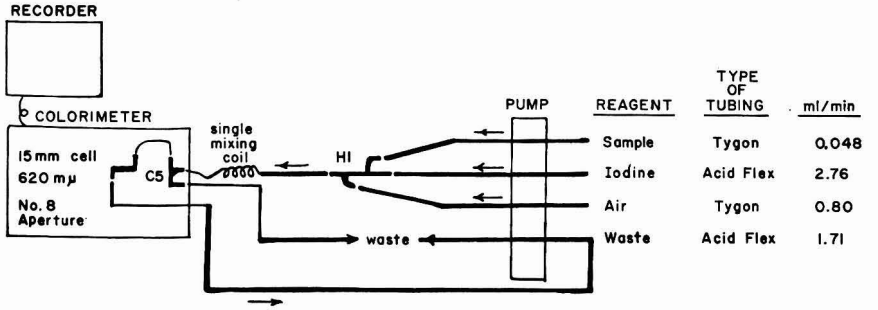

FIG. 1. AutoAnalyzer manifold diagram for determination of the blue value of amylose-iodine complex.

Figure 1 shows the AutoAnalyzer manifold diagram for the blue value analysis. A 620 mμ interference filter, a No. 8 aperture in the reference well, and a 15 mm tubular flow-through cell were employed. Sixty samples could be analyzed per hour. The upper limit for the concentration of undegraded amylose was 1.5 mg/ml.

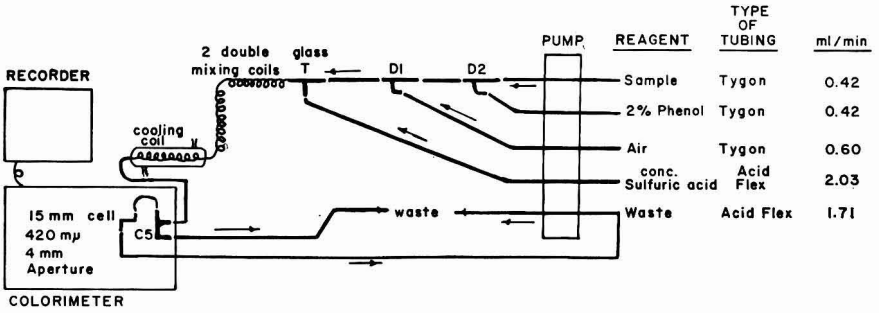

FIG. 2. AutoAnalyzer manifold diagram for determination of total carbohydrates by phenol-sulfuric acvid.

Phenol-Sulfuric Acid. (A) 2.0% phenol in water; (B) analytical grade sulfuric acid. Figure 2 shows the AutoAnalyzer manifold diagram for determining total carbohydrate. A 420 mμ interference filter and a 15 mm flow-through tubular cell were used. A special aperture plate was

made by drilling a 4 mm diameter hole in a No. 0 aperture. The upper limit for carbohydrate concentration was 180 μg/ml. Forty samples could analyzed per hour.

RESULTS AND DISCUSSION

Figure 3 shows a typical analysis of the drop in the iodine-amylose color during the α-amylolysis of amylose by porcine pancreatic α-amylase. Each point on the curve represents the average of triplicate determinations of individual aliquots.

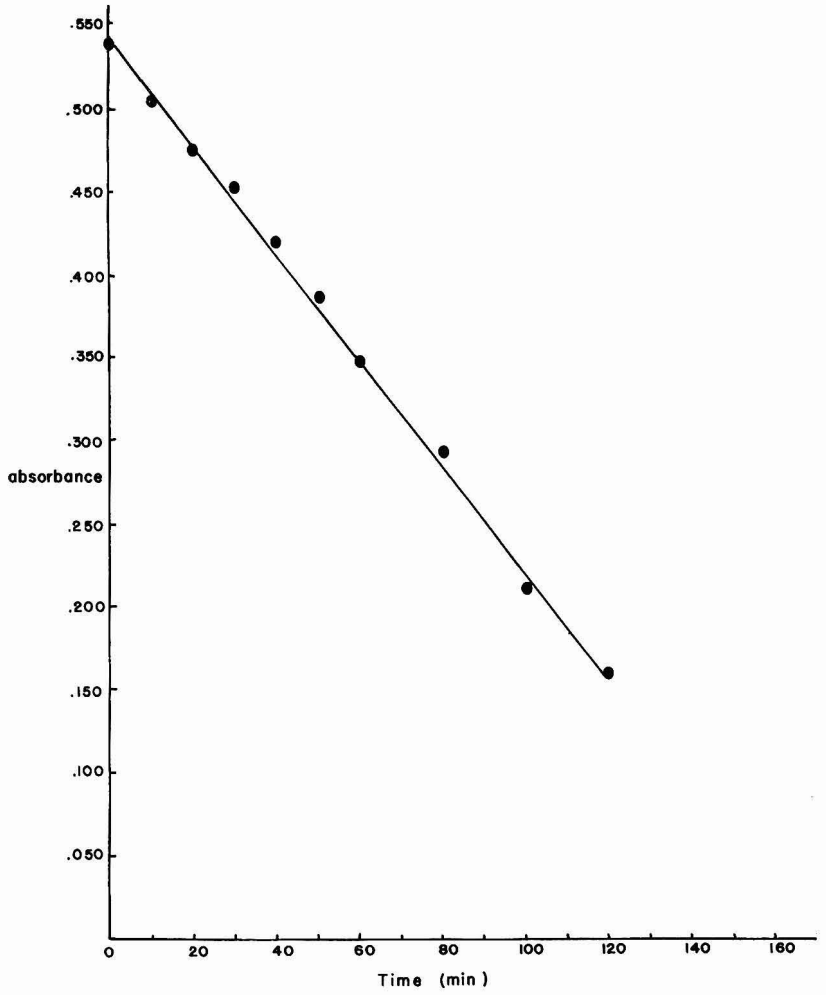

FIG. 3. Measurement of change in blue value during α-amylolysis of amylose.

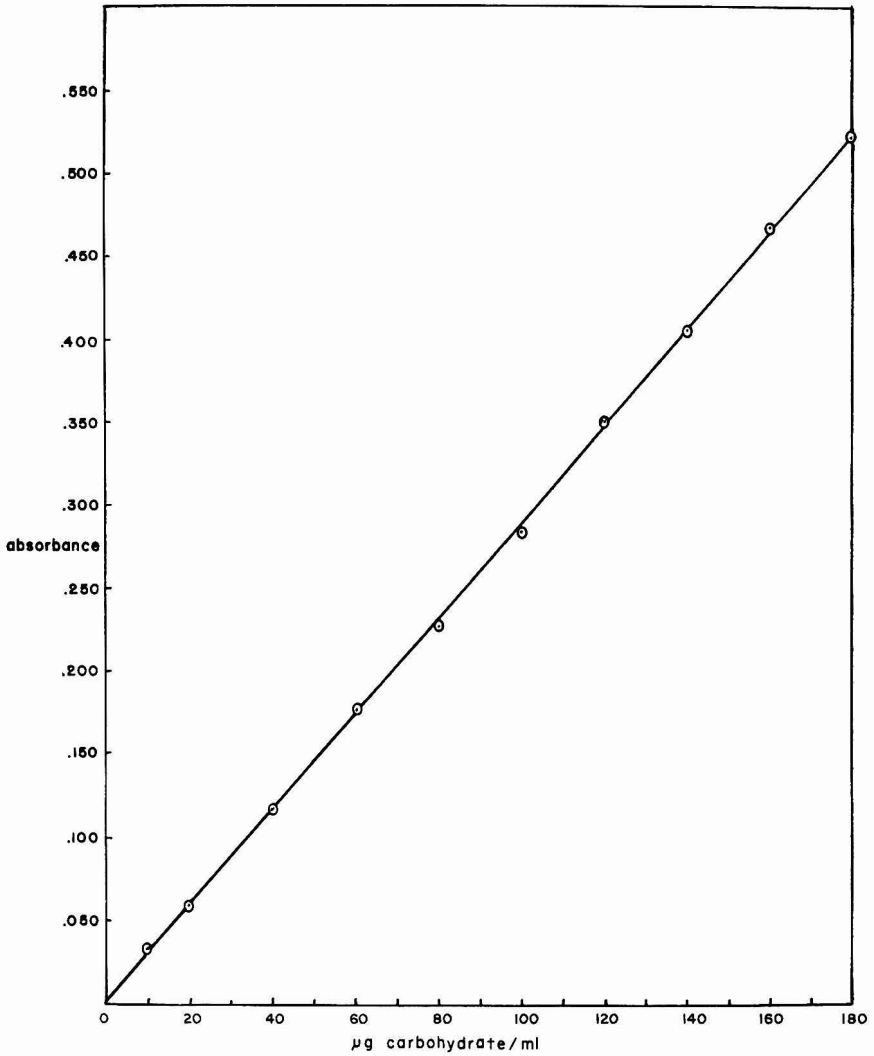

FIG. 4. Standard curve for analysis of total carbohydrate using phenol-sulfuric acid. The standard was a vacuum-dried sample of commercial maltose.

Figure 4 is a typical standard curve for the phenol-sulfuric acid determination of total carbohydrate. The standard used to obtain this curve was a vacuum-dried sample of maltose. Each point represents the average of triplicate determinations. By using the AutoAnalyzer, the precision was ten parts in a thousand. It has been debated whether this analysis is dependent on the structure of the carbohydrate, especially

when polysaccharides were involved. We have obtained standard curves using six different α-linked glucans: maltose, amylose, dextran, cyclohexaamylose, cycloheptaamylose, and cyclooctaamylose. Each of these carbohydrates gave linear plots in the range of 10–180 μg/ml; the slopes had a standard deviation from the mean between 1 and 3%. Thus, the structure of these six α-glucans did not have an effect on the analysis; the absorbance obtained was proportional to an equivalent weight of glucose ($C_6H_{12}O_6$) in each glucan sample.

SUMMARY

The blue value determination of the amylose-iodine complex and the phenol-sulfuric acid procedure for the determination of total carbohydrate were each adapted to the Technicon AutoAnalyzer for automated analyses. Linear relationships between absorbance and amount of material were obtained for each method. It was found that the phenol-sulfuric acid procedure gave identical standard plots for six different α-glucans.

ACKNOWLEDGMENT

We wish to express our gratitude to Rosalie Ackerman for technical assistance.

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ANALYTICAL BIOCHEMISTRY 19, 61–71 (1967)

A Modified Starch for Use in Amylase Assays[1]

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Since the hydrolysis of starch by various amylases results in the formation of maltose, oligosaccharides, and glucose, the assay of these enzymes has frequently been performed with one of a variety of methods that measures the appearance of reducing sugars (1–4). Unfortunately, in the more sensitive methods, which use alkaline copper or iron oxidants, blank values become significantly elevated due to the presence of terminal reducing glucosyl residues in the starch substrate. Furthermore, when attempting to use the reducing sugar procedure recently described by Dygert *et al.* (5), in which the chelating agent neocuproine (2,9-dimethyl-1,10-phenanthroline) is employed in only limited amounts to minimize the reagent blank value, an additional problem is encountered. When starch is added for assay of amylase, the copper-neocuproine reagent is essentially depleted by reaction with the inherent reducing groups of the substrate, and little or no additional response with the subsequently liberated reducing glucosyl residues is observed. It seemed desirable, therefore, to have a starch available that could not react in these reducing sugar methods but that would, nonetheless, retain full activity as a substrate. The work reported in this paper describes the treatment of starch by sodium borohydride in a simple, reproducible procedure for the preparation of an amylase substrate that possesses essentially no interfering reducing power and is suitable for use with the various reducing sugar methods.

[1] This work was supported by Research Grant AM 09194-02 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service. A preliminary report of this study was presented at the 152nd Meeting of the American Chemical Society, September 1966, New York, N. Y.

[2] Paper of the Journal Series, New Jersey Agricultural Experiment Station.

METHODS

Preparation of $NaBH_4$-Treated Starch Solutions

A 5% solution was prepared by gently boiling a suspension of 2.5 gm of a soluble starch in 10 ml of water until solubilized (15–30 sec) and then diluted to 45 ml. The clarity of the resulting solution varied from water clear to cloudy white, depending upon the source of the material. The solution was cooled in an ice bath, and then a previously cooled solution of 75 mg of sodium borohydride (Metal Hydrides, Beverly, Mass.) in 5 ml water was gradually added while magnetically mixing with a Teflon-coated stirring bar. The frothing which occurred at this stage decreased upon standing. The reaction mixture was next brought to 25°C by equilibrating in a water bath and was then incubated without stirring. Although the reduction was complete in less than an hour at this temperature, it was convenient to store the reaction mixture in the refrigerator or at room temperature overnight or longer. Since it showed little tendency to retrograde, support microbial growth, or lose activity as a substrate, this solution was used as the stock starch solution until it was exhausted.

For daily use, 10 ml of the solution was removed and 0.2 ml of acetone was added dropwise, with shaking, to destroy excess sodium borohydride. A precipitate that formed when acetone was added too rapidly dissolved upon vigorous shaking. After standing 20 min at room temperature, the solution was brought to pH 7.0 with 1 *N* acetic acid and quantitatively transferred to a volumetric flask. The volume was adjusted to 50 ml with buffer appropriate for the enzyme to be assayed. Although retrogradation of this solution occurred when stored in the cold, its original clarity could be quickly restored by gentle heating of the neutralized (pH 7.0) solution.

Preparation of Untreated Starch Solutions

The various starch powders were purchased from local suppliers, except for the Noredux Standard starch, which was obtained from B. Siegfried & Co., Zofingen, Switzerland. One gram of untreated starch was suspended in 20 ml of distilled water and boiled gently until solubilized. The volume was brought to 100.0 ml with buffer or distilled water. Varying amounts of this solution containing starch at a concentration of 10 mg/ml were assayed for reducing power.

Enzyme Solutions

Crystalline *hog pancreatic α-amylase* suspension obtained from Worthington Biochemicals was centrifuged to remove the supernatant liquid

and resuspended in a 20 mM sodium phosphate, pH 7.0, solution containing a mixture of proteolytic enzyme inhibitors (6) at the following concentrations: bromoacetate, 10^{-4} M; iodoacetamide, 10^{-4} M; and diisopropylfluorophosphate, 10^{-4} M. Stock solutions prepared by dissolving 5 mg of the enzyme in 5 ml of the inhibitor solution and stored in the refrigerator were stable for weeks. Solutions for daily use were diluted from 0.1 ml of the stock solution with either 50 mM tris-HCl or 20 mM phosphate buffer, pH 7.0, containing 6 mM NaCl. Protein concentration was determined from absorbance measurements at 280 mμ, using an $E^{1\%}_{1\,cm}$ value of 26 (7).

Crystalline *human salivary amylase* ($E^{1\%}_{1\,cm} = 26.0$) was prepared from pooled saliva, as described by Fisher (8), and stored as a suspension in 20 mM sodium glycerophosphate, pH 6.9, containing the proteolytic enzyme inhibitors. Working solutions of enzyme were prepared daily by carefully adding 0.1 N sodium hydroxide to a portion of the enzyme suspension and adjusting the solution to a concentration of about 1 mg/ml with the glycerophosphate buffer.

Crystalline *fungal α-amylase* was prepared (9) from a crude extract kindly furnished by Takamine Laboratories, Clifton, New Jersey. The crystals were dissolved in 50 mM sodium acetate, pH 5.3, dialyzed against deionized water, and lyophilized. Stock solutions and dilutions were prepared in the acetate buffer; concentration was based upon $E^{1\%}_{1\,cm}$ equal to 22.1 (9).

Bacillus subtilis α-amylase, obtained as a crystalline powder from Enzyme Development Corporation, New York, N. Y., was dissolved (10) by addition of dilute sodium hydroxide and diluted in 20 mM sodium glycerophosphate pH 5.9. Concentration was determined (10) from the $E^{1\%}_{1\,cm}$ value of 25.0.

Solutions of *sweet potato β-amylase* ($E^{1\%}_{1\,cm} = 12.4$) were prepared daily from the crystalline suspension obtained from Worthington Biochemicals by adding an aliquot of the suspension to 0.1 M sodium acetate at pH 4.8 (11).

Assay Procedures

Amylase activity was measured by incubating an aliquot of enzyme with a starch solution, using a final volume of 2.0 ml contained in calibrated sugar tubes graduated at the 25 ml mark. Following a 10 min temperature equilibration period, the reaction was started by adding 1.0 ml of a 1.0% starch solution to 1.0 ml of the enzyme solution. After incubation for 5 min at 30°C, the reaction was terminated by the addition of the appropriate quantity of reagent as designated in each of the reducing sugar procedures used.

Reducing Sugar Methods

(*a*) When the neocuproine procedure described by Dygert *et al.* (5) was used, 4 ml of the copper and neocuproine reagents were added. This quantity was sufficient for the measurement of up to 100 μg of glucose. Tubes were heated in a boiling water bath for 12 min and the color developed was read in a Klett-Summerson colorimeter, using a 42 filter, or at 450 mμ in a Beckman DU spectrophotometer. At these wavelengths, absorbance and colorimeter readings were interconvertible by multiplying Klett units by the theoretical factor 0.002, but the 45 filter gave Klett values considerably lower than expected.

(*b*) The Somogyi method which utilizes a copper tartrate oxidant was used as modified by Nelson (1). Absorbance was determined at 660 mμ in the spectrophotometer.

(*c*) The ferricyanide method was used as originally described by Hoffman (4) except that readings were made with the Beckman DU spectrophotometer at 420 mμ.

In both the neocuproine and Somogyi methods, tubes were diluted to the 25 ml graduation with deionized water from a squeeze bottle and then carefully adjusted to the line, using a capillary pipet. This procedure permitted dilutions (5) to be made with an accuracy of between 0.2 and 0.3%. Each tube was covered with Parafilm and mixed by inverting several times. Measurements were always made at the time recommended for each procedure and usually completed within an hour.

RESULTS

A comparison of the reducing power of five different commercially available soluble starches, before and after sodium borohydride treatment, is presented in Table 1. Prior to treatment, the Noredux Standard starch gave the lowest reducing value. On the other hand, the Baker and Adamson material required a 5- to 10-fold dilution to be within the limits of the assay, indicating either a low degree of polymerization, in view of its transparent solutions, or contamination with very low molecular weight fragments. Nonetheless, following $NaBH_4$ treatment, the reducing power of all the starch preparations was diminished to an extremely low level when assayed by each of the three reducing sugar procedures.

The rate of reduction by $NaBH_4$ of two soluble starches differing markedly in reducing power was measured. The reaction of 2.5 gm of starch (Baker and Adamson, or Merck soluble) in 50 ml of water with 75 mg of $NaBH_4$ was initiated in the cold and rapidly brought to 25°C. At timed intervals, 5.0 ml samples were withdrawn and added to

tubes containing 0.1 ml of acetone and 0.18 ml of 1.0 N acetic acid. After diluting to 25 ml, 1.0 ml was assayed by the neocuproine method. Under these conditions, the Baker and Adamson starch (see Table 1) reacted with $NaBH_4$ at a rate that did not differ significantly from the less strongly reducing Merck starch. The reaction was shown to proceed rapidly in a pseudo-first-order fashion with a rate constant of 0.18 min^{-1}

TABLE 1
Comparison of the Reducing Power of Various Starches before and after Sodium Borohydride Treatment

Starch (10 mg) and treatment	Method of measurement: Neocuproine A_{450}	Neocuproine Glucose equiv., μg	Nelson A_{660}	Nelson Glucose equiv., μg	Ferricyanide ΔA_{420}	Ferricyanide Glucose equiv., μg
Mallinckrodt						
None	0.427	48.7	0.239	43.0	0.315	90
Reduced	0.012	1.4	0.020	3.6	0.045	13
Merck, Lintner						
None	0.510	58.1	0.260	46.8	0.349	100
Reduced	0.008	0.9	0.026	4.7	0.040	12
Merck, soluble						
None	0.552	63.9	0.274	49.3	0.405	119
Reduced	0.016	1.8	0.012	2.2	0.045	12
Noredux Standard						
None	0.265	30.2	0.113	20.3	0.183	54
Reduced	0.015	1.7	0.010	1.8	0.040	12
Baker & Adamson						
None[a]	3.20	364.8	1.33	239.4	2.12	560
Reduced	0.016	1.8	0.013	2.3	0.055	15

[a] Based upon measurements made on 1–3 mg of starch.

and to be essentially complete within 1 hr. For maximum and complete reaction, it was best to permit reduction to occur at the pH developed in the $NaBH_4$ solution without added buffer, i.e., at pH 9.5. To terminate the reaction, an excess of acetone was added to destroy the borohydride, followed by neutralization with acetic acid. The sodium borate formed at this point did not adversely affect enzymic activity[3] and was not removed.

To test the effect of added starch upon the linearity of response in

[3] Although sodium borate did not interfere in the assay systems employed in these studies, it should not be assumed that borate will be without effect under other assay conditions.

the neocuproine reducing sugar assay, a calibration curve was prepared in which either the untreated or the modified starch was added to standard solutions containing 10 to 100 μg of glucose. As illustrated in Figure 1, the addition of 10 mg of untreated starch suppressed response and markedly compressed the usable range of the assay; i.e., 10 mg of Merck soluble starch, containing the equivalent of 60 μg of reducing

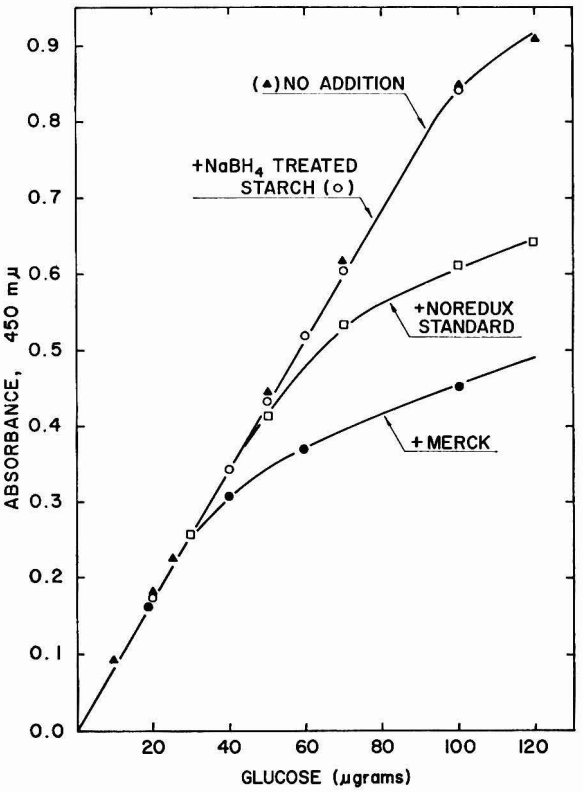

FIG. 1. Effect of 10 mg of untreated or $NaBH_4$-treated starch on the calibration curve for the neocuproine assay of glucose.

glucosyl units, decreased the usual range of assay from its upper limit of 100 μg to approximately 40 μg. This decrease, caused by insufficient copper-neocuproine reagent, arose from depletion of the reagent by its reaction with the reducing groups inherent in the untreated starch. Furthermore, when less reagent was used to determine lower levels of glucose, it was completely consumed by reaction with the starch, leaving no reagent for measurement of subsequently liberated reducing groups.

On the other hand, modified starch exhibited no adverse influence

on either the range or linearity of the assay at even the lowest level of reagent employed (1.0 ml). By the borohydride treatment, therefore, starch is rendered suitable for use as a substrate in a sensitive reducing sugar assay and, as a result, may be employed in kinetic studies of the early phases of the amylase reaction, as illustrated in Figure 2.

The suitability of the various modified starches as substrates for porcine pancreatic α-amylase is shown in Table 2. Each of the modified starches was hydrolyzed at a rate essentially equivalent to that

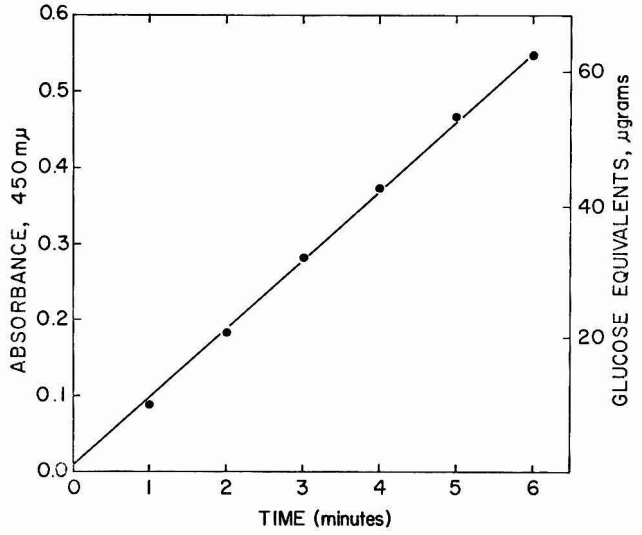

FIG. 2. Time-dependent release of reducing groups by a low level of enzyme using a modified starch substrate (pancreatic amylase, 25 mμg; $NaBH_4$-treated Merck, soluble starch, 10 mg). The neocuproine procedure was used. Absorbance not corrected for starch blank to demonstrate its small contribution to experimental values.

observed with the respective untreated starch. It is significant to note that the modified Baker and Adamson starch proved to be as effective as the other starches, although the untreated material was unusable in the standard procedure because of its high reducing value.

A severe defect of the sensitive methods for the assay of reducing sugars is that of range restriction due to high blank values and reagent exhaustion. That the modified starch eliminates this limitation is evident from the above and also from a comparison of the rates at which reducing sugar was released upon taka-amylase hydrolysis of the modified and untreated Merck starch (Fig. 3). With the untreated starch as substrate, the rate of hydrolysis per milligram protein appeared to

TABLE 2
Comparison of the Activity of Porcine Pancreatic α-Amylase towards Untreated and Reduced Starches

Starch and treatment	Method of measurement[a]			
	Neocuproine		Nelson	Ferricyanide
	(1)[c]	(2)[d]	(3)[b,c]	(4)[c]
Mallinckrodt				
None	0.315	0.143	0.194	0.243
Reduced	0.288	0.140	0.198	0.231
Merck, Lintner				
None	0.277	0.129	0.179	0.243
Reduced	0.289	0.115	0.165	0.233
Merck, soluble				
None	0.252	0.159	0.191	0.225
Reduced	0.250	0.147	0.181	0.227
Noredux Standard				
None	0.288	0.124	0.193	0.231
Reduced	0.300	0.149	0.191	0.233
Baker & Adamson				
None[e]	0.263	0.186	0.173	0.204
Reduced	0.253	0.124	0.157	0.209

[a] Enzyme activity expressed in terms of the absorbance change with respect to the corresponding starch blank following a 3 min incubation: neocuproine, ΔA_{450}; Nelson's, ΔA_{660}; ferricyanide, ΔA_{420} (absorbance decrease).

[b] Assays were performed in phosphate buffer to avoid interference by tris.

[c] 50 mμg of enzyme (age of preparation, 2 weeks) was incubated at 30°C for 3 min as described in the text.

[d] 100 mμg of enzyme (age of preparation, 1 year) was incubated at 30°C for 3 min.

[e] Because of high blank values, the assay system was modified as follows: 10 times the respective quantity of enzyme was added and a 0.2 ml sample was removed for assay.

decrease as the quantity of enzyme added was increased beyond 200 mμg, but the anticipated linear response with enzyme concentration was observed when the sodium borohydride treated starch was used.

The general applicability of the modified starch as a substrate for a variety of amylases can be seen upon examination of the data presented in Table 3. Each of the amylases tested hydrolyzed the treated Merck starch at a rate that was equivalent to or exceeded that of the untreated starch.

The stability of the starch reaction mixture deserves to be noted. Ordinary solutions of starch retrograde quite rapidly at neutral pH, as evidenced by increasing cloudiness and subsequent precipitation. However, the reaction mixture described in this paper did not change in ap-

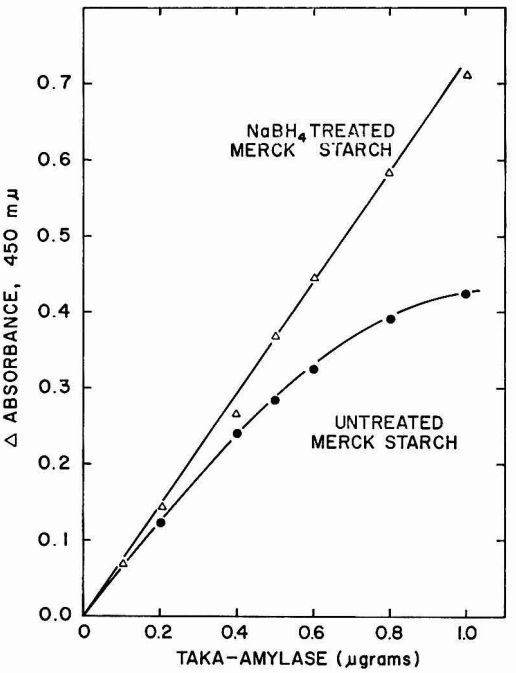

FIG. 3. Hydrolysis of treated and untreated starch (10 mg) by different amounts of taka-amylase as measured with the neocuproine method.

pearance for 10 days, at which time a small amount of precipitate was occasionally noted. It was possible to remove this precipitate by filtration and use the solution without error. Furthermore, microbial growth was never observed in this solution.

TABLE 3
Comparison of the Activity of Various Amylases toward Sodium Borohydride Treated and Untreated Starch

Amylase	Type	Enzyme,[a] mμg	Starch[b] Activity[c] Reduced	Starch[b] Activity[c] Untreated
B. subtilis	Alpha	50	0.248	0.222
A. oryzae	Alpha	400	0.263	0.243
Human salivary	Alpha	25	0.165	0.150
Sweet potato	Beta	50	0.182	0.191
Hog pancreatic	Alpha	50	0.252	0.250

[a] Quantity of enzyme per tube as described in text for standard assay procedure.
[b] Each tube contained 10 mg of starch (Merck, soluble).
[c] Increase in absorbance above starch blanks as determined with the neocuproine method. Incubation with enzyme was for 5 min at 30°C.

DISCUSSION

The interference by starch in reducing sugar assays for amylolytic enzymes increases enormously with increasing sensitivity of the method employed. Comparison of the data in Tables 1 and 2 reveals the extremely high contribution of various starch blanks to the total reducing value obtained in the presence of enzyme. To eliminate this interference, it became necessary to prepare a starch devoid of its native reducing power.

At least two possible approaches existed to the preparation of such a starch. One of these, mild oxidation of the terminal reducing glucosyl residues to the corresponding carboxylic acid, was considered unsuitable because the formation of an ionic substrate might induce undesirable electrostatic interactions with the enzyme. The alternative possibility, that of reduction of the glucosyl groups to the corresponding alcohol, glucitol, appeared more desirable. Since the specificity pattern displayed by amylases results from action directed toward either the nonreducing ends or the internal glucosidic linkages (12), little or no alteration in substrate properties was anticipated of a starch modified at the reducing ends.

The findings presented in this paper demonstrate the effectiveness of the reduction technique in preparing a unique starch substrate for the amylolytic enzymes. Since the modified substrate is devoid of reducing power and does not react in the neocuproine, Nelson, or ferricyanide methods, these sensitive procedures can be used throughout their entire range. Furthermore, the modified starch decreases the value of the blank to a level that virtually eliminates it as a potential source of error. In addition, the absence of reducing power in the substrate will (*a*) allow the measurement of lower levels of amylase activity, especially in physiological fluids, in clinical procedures and in kinetic experiments, (*b*) be an aid in the determination of K_m values for starch-degrading enzymes, (*c*) permit operation at substrate concentrations well beyond saturation levels, and (*d*) provide generally greater statistical confidence in the results of amylase assays performed with sensitive reducing sugar methods.

SUMMARY

When sensitive reducing sugar methods are employed for the assay of starch-hydrolyzing enzymes, the terminal reducing glucosyl residues in the starch react to produce extremely high blank values, causing both restriction in the usable range of the assay and increased variation of the results. To eliminate this problem, the interfering glucosyl groups were converted to glucitol residues by treating the starch with sodium

borohydride. The treated starch no longer interferes in the Somogyi, neocuproine, or ferricyanide reducing sugar determinations and retains full activity as a substrate for a variety of amylases. Results obtained with several commercially available starch samples are presented.

ACKNOWLEDGMENT

The author wishes to thank Dr. John A. Thoma for providing the details of the neocuproine procedure prior to publication. The author is also greatly indebted to Mrs. Carol B. Jones for her excellent technical assistance throughout these studies.

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A Reevaluation of the Roe Procedure for the Determination of Fructose

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The chemical assay frequently used for the determination of fructose employs resorcinol in a concentrated acid solution. This assay, first adapted for quantitative determinations by Roe (1), is based upon the estimation of a chromophoric substance formed by the reaction of resorcinol and hydroxymethylfurfural. The mechanism of this reaction is not understood, and the reaction is not specific for fructose. A chromophore is produced by any sugar or phosphorylated sugar which is capable of being converted to hydroxymethylfurfural under the conditions of the assay system.

Conditions were sought which would increase the sensitivity of the assay in the quantitative determination of fructose. A minimal contribution by glucose to the formation of chromophoric substances was a second requirement of the chemical assay.

Several parameters of the assay system were examined for their effect on chromophore formation with either glucose or fructose as a source of hydroxymethylfurfural. This paper describes the results of these experiments and outlines conditions which will allow a quantitative estimation of 0.02 to 0.25 μmole of fructose. This represents approximately an 8-fold increase in sensitivity over the modified Roe procedure (2).

METHODS

All chemicals used were A.R. grade. The determinations were carried out in 15 $\times$ 150 mm Pyrex test tubes with a total reaction volume of 5.0 ml. After reacting the hexose with resorcinol in strong HCl for a specified time and temperature, the reaction mixture was cooled and the absorbance was measured either with a Zeiss PMQ II spectrophotometer at 420 mμ or a Klett-Summerson photoelectric colorimeter using a

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number 42 filter.[2] A linear relationship between absorbance and fructose concentration was obtained with both instruments.

The influence of the quantity of each component in the reaction mixture, the reaction time, and the temperature upon the quantity of chromophore formed are the subject of this paper.

RESULTS AND DISCUSSION

Preliminary experiments were conducted to determine the influence of reaction period, reaction temperature, solvent for resorcinol, resorcinol concentration, concentration of acid, and cooling rate upon the absorb-

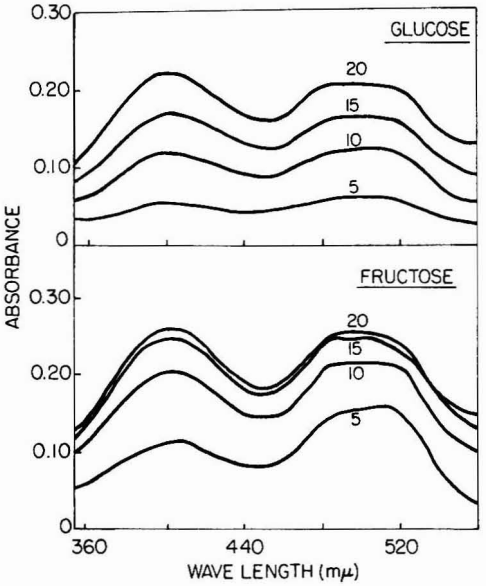

FIG. 1. Absorption spectra of chromophore formed from fructose and glucose. Absorption spectra of the reaction mixture containing either 10 μmoles glucose (upper figure) or 0.2 μmole fructose (lower figure) after incubating at 75°C for time intervals of 5, 10, 15, or 20 min. Each reaction mixture contained 9 μmoles resorcinol (0.1%) in 1 ml absolute ethanol, 3 ml 12.2 *N* HCl, and the glucose in 1 ml H_2O. Absorbance was determined at 420 mμ in 1 cm cuvets after cooling the sample to room temperature.

ance at 420 mμ due to the chromophoric substances formed. Figure 1 shows the absorption spectra when either 10 μmoles of glucose or 0.2 μmole of fructose is reacted in acidic resorcinol solution for 5, 10, 15, or 20 min. Although the spectra obtained after heating at 75°C for 20 min

[2] The No. 42 filter has an approximate spectral range of 400 to 465 mμ (Klett-Summerson Photoelectric Colorimeter Clinical Manual).

are not identical, both spectra have broad maxima in the region of 380 to 420 mμ and 480 to 520 mμ. The absorbance, using a No. 42 filter in a Klett-Summerson colorimeter, is a linear function of either fructose or glucose concentration from 0 to 400 Klett units. A No. 40 or 54 filter did not give a linear relationship under all conditions. A No. 42 filter was employed for the remainder of the investigation.

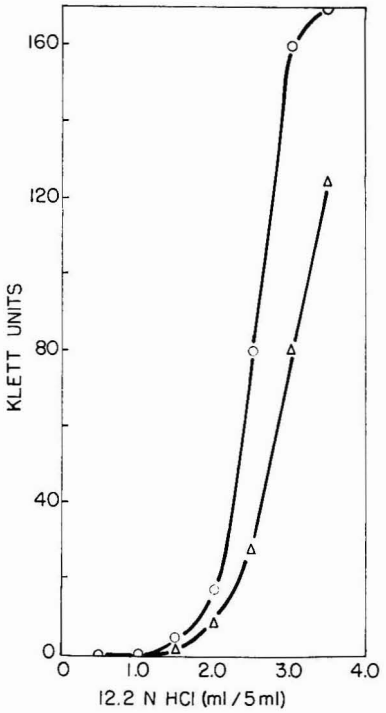

FIG. 2. Effect of acid concentration on absorbance: (○) fructose or (△) glucose was incubated for 15 min under the conditions described in Figure 1 in the presence of various concentrations of HCl as indicated in the figure. The total reaction volume was brought to 5.0 ml by addition of water when necessary. Absorbance was measured in a Klett-Summerson photoelectric colorimeter with a No. 42 filter.

Absolute ethanol was used as the solvent for resorcinol instead of 0.2 *M* acetic acid because resorcinol in aqueous solutions markedly decreases the absorbance. This change in solvent further increased the sensitivity of the method by 70%.

Figure 2 shows the influence of acid concentration upon absorbance. The modified Roe procedure (2) uses acid equivalent to 2.5 ml of 12.2 *N* HCl in this assay system. This figure shows that 3.0 ml of 12.2 *N* HCl yields optimum absorbance with fructose, and that this approximately

doubles the quantity of chromophoric material absorbing in the 420 mμ region as compared to the modified Roe procedure.

In view of these changes, the optimal reaction time and reaction temperature were also reexamined. Figure 3 shows the results of these experiments using a reaction temperature of 75°C, 3.0 ml of 12.2 *N* HCl, and resorcinol in absolute ethanol in 5.0 ml reaction volume. This figure shows that absorbance is a linear function of time during the interval

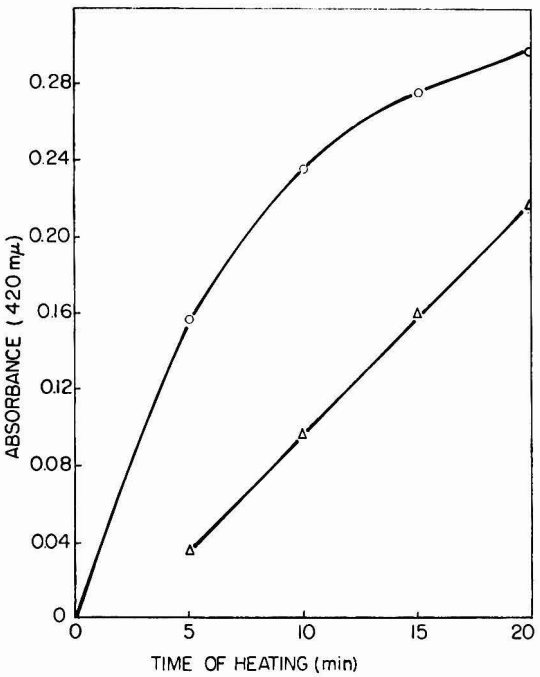

FIG. 3. Influence of reaction time on absorbance: (○) fructose or (△) glucose incubated as described in Figure 1.

5 to 20 min when glucose was assayed. However, unlike glucose, fructose reacts rapidly under these conditions. A 5 min reaction period was chosen for further experimentation. Experiments with reaction temperatures of 73° or 80°C gave similar results and showed the sensitivity of the assay increased with an increase in temperature. Experiments to establish the reaction temperature which would yield maximum sensitivity and a minimum contribution by glucose were performed. Figure 4 shows a linear increase in absorbance with increase in temperature in reactions containing fructose and a nonlinear increase in absorbance in reactions containing glucose. Figure 5 shows the influence of temperature

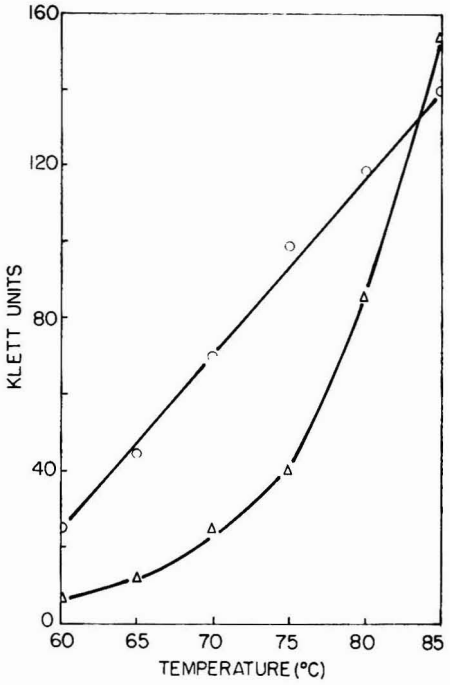

FIG. 4. Influence of reaction temperature on absorbance: (○) fructose or (△) glucose incubated for 5 min under the conditions described in Figure 1.

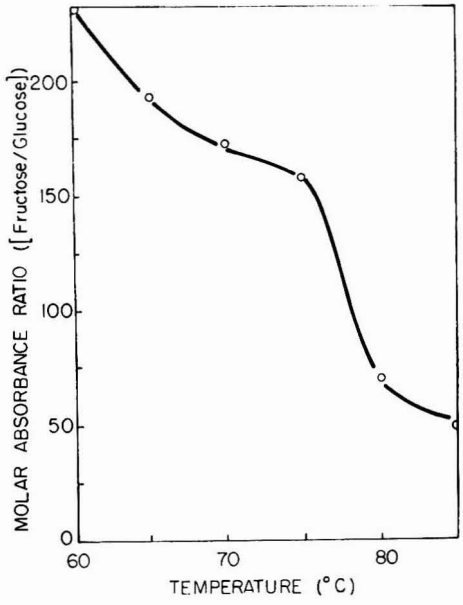

FIG. 5. Influence of reaction temperature on ratio of quantities of chromophore formed from fructose and glucose. See Figure 4 for conditions.

upon the ratio of absorbancies over a range of 60° to 85°C. This figure shows the ratio of absorbancies decreases slowly between 65° and 76°C followed by a sharp decrease in the ratio between 76° and 85°C. A temperature of 76°C appears to be optimum for the determination of fructose in reactions containing both glucose and fructose. This temperature results in a sensitive assay and at the same time holds the contribution by glucose to less than 1% when considered on an equal molar basis.

It was also found that rapid cooling of the reaction mixture in a 0°C bath following the 5 min heating period was necessary in order to obtain reproducible results and a maximum sensitivity.

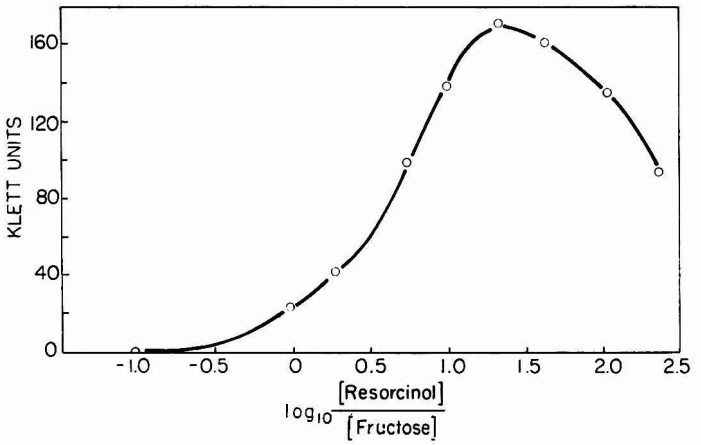

FIG. 6. Influence on absorbance of ratio of resorcinol to fructose. The reaction was carried out at 77°C under the conditions described in Figure 1, with resorcinol as the independent variable.

Roe (1) indicates that the concentration of resorcinol is unimportant when in sufficient excess. However, it seemed necessary to investigate the influence of resorcinol concentration in our modified conditions. The assays were performed using various ratios of fructose and resorcinol in absolute ethanol. The conditions of 3.0 ml 12.2 *N* HCl and reaction temperature of 77°C for 5 min followed by cooling at 0° were used. The various concentrations of resorcinol used were added to the reaction mixture in 1.0 ml of absolute ethanol. Figure 6 shows the influence of the ratio of resorcinol/fructose on the absorbance obtained. This figure shows that 22- to 60-fold excess of resorcinol is required for maximum absorbance while greater or lesser molar ratios give lower absorbancies. The optimal concentration of resorcinol was found to be 4.5 μmoles in reaction mixtures containing 0.02 to 0.25 μmole of fructose.

A reevaluation of the reaction time showed that chromophore formation from fructose was linear for the first 4 to 6 min and that there was little additional chromophore formation between 8 and 10 min. *The final conditions established for the assay are as follows:* 1.0 ml of sample

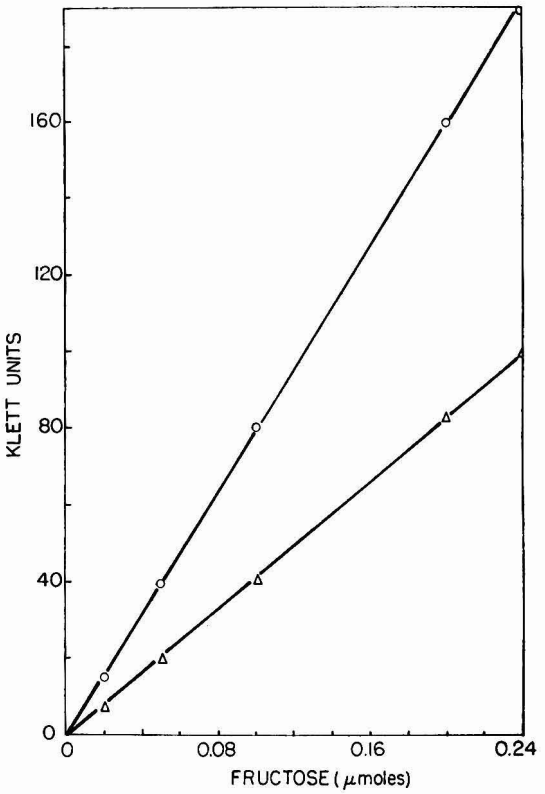

FIG. 7. Relationship between fructose concentration and absorbance measured with a Klett-Summerson photoelectric colorimeter with a No. 42 filter (○) or with a No. 54 filter (△). The reaction mixture containing 36.5 μmoles HCl, 4.5 μmoles resorcinol, and 1.0 ml ethanol in a total volume of 5.0 ml was incubated at 77° for 8 min. Following incubation the reaction mixture was cooled rapidly in a 0° bath. Absorbance was measured after the reaction mixture reached room temperature.

containing 0.02 to 0.25 μmole of fructose, 3.0 ml of 12.2 *N* HCl, and 1.0 ml of 0.05% resorcinol in absolute ethanol are incubated at 77°C for 8 min and cooled immediately at 0°. Figure 7 shows the linear relationship obtained between absorbance and fructose concentration over a range of 0.02 to 0.25 μmole of fructose. When higher concentrations

of fructose are being determined the concentration of resorcinol must be increased accordingly.

SUMMARY

In an examination of the Roe procedure for determining fructose the following were established:

1. The sensitivity of the assay can be increased 2-fold by increasing the HCl concentration from 6.1 to 7.3 *N*.

2. A reaction temperature of 77°C is optimum for maintaining sensitivity and at the same time holding the contribution by glucose to less than 1% on a molar basis when the reaction mixture contains both glucose and fructose.

3. The ratio of resorcinol to fructose influences the absorbance observed. A broad peak is observed with a maximum at a molar ratio of 45 to 1.

4. Conditions were established which allowed a quantitative determination of fructose in the range of 0.02 to 0.25 μmole.

ACKNOWLEDGMENT

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Colorimetric Differentiation of Hexosamines

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The Elson-Morgan reaction commonly used in the colorimetric determination of glucosamine and galactosamine is based on their reaction with acetylacetone in alkaline solutions at 100°C. The reaction products are chromogens which give a color with *p*-dimethylaminobenzaldehyde in HCl (1, 2). Subsequent modifications have improved the sensitivity and reproducibility of the method. Less successful, however, were modifications made to differentiate glucosamine from galactosamine.

A method was published in 1963 (3) for the analysis of galactosamine, glucosamine, and mannosamine involving the separation of a volatile fraction among the products of condensation of acetylacetone and amino sugars. This method required, however, a distillation step that was inconvenient in our work, where a large number of samples, effluent volumes of an ion-exchange column, had to be analyzed.

The method proposed is simple and is based on the differences in reactivity of galactosamine, glucosamine, and mannosamine with an alkaline solution of acetylacetone at 0°C. This procedure, in conjunction with another method in which the three hexosamines gave about the same color intensity, was used to establish colorimetric ratios useful as criteria of purity and identification.

Several reports were responsible for suggesting the conditions which led to the development of the methods presented for hexosamine analysis (3–8).

EXPERIMENTAL

Materials. Dimethylaminobenzaldehyde (reagent grade) and 2,4-pentanedione (acetylacetone) were obtained from Eastman Kodak. Glucosamine HCl, galactosamine HCl, and mannosamine HCl were obtained from Sigma Chemical Co. and were recrystallized in H_2O-ethanol.

Reagents. The alkaline acetylacetone reagent was a solution of 3% (v/v) acetylacetone in 0.70 *M* Na_2CO_3. The pH of this solution was

adjusted to 9.8 with solid Na_2CO_3 and the solution used within 1 hr after preparation (pH adjustments occasionally were necessary whenever fresh distilled acetylacetone was not used). Mixing 1 ml of alkaline acetylacetone reagent with 0.5 ml of 0.3 *M* HCl should give pH 9.5. This mixture, when then heated for 1 hr at 80°C, should give pH 9.4.

The Ehrlich reagent was a solution of 3.2 gm of *p*-dimethylaminobenzaldehyde in 60 ml of concentrated HCl and 60 ml of 95% ethanol.

METHODS

Hexosamine Method. 0.5 ml of the sample in 0.3 *M* HCl containing less than 200 μg hexosamine HCl, was delivered into tubes refrigerated in ice; 1 ml of alkaline acetylacetone reagent was added to each tube. These tubes were glass-stoppered, submerged in crushed ice, and incubated in a refrigerator at 0°C. After 18 hr, 10 ml of 90% ethanol was added while under refrigeration. This was followed by the addition of 1 ml of the Ehrlich reagent. The mixtures were shaken after each addition and incubated at 60° for 30 min. After the incubation period, the solutions were cooled in a H_2O bath (20–25°C) for 1 hr. Absorbance measurements were made at 525 mμ.

Total Hexosamine Method. To 0.5 ml sample, in 0.3 *M* HCl containing less than 100 μg of hexosamine HCl, was added 1 ml of alkaline acetylacetone reagent. This solution was heated in glass-stoppered tubes for 1 hr at 80°C. After cooling, in a H_2O bath (20–25°C) for 5 min, 10 ml of 90% ethanol and 1 ml Ehrlich reagent were added, with shaking after each addition. Absorbance measurements were made at 535 mμ after 1 hr.

All absorbance measurements were made in a Gilford spectrophotometer model 2000 with 1 cm square cells.

RESULTS AND DISCUSSION

The results are shown in Table 1. Coefficients of variation for each hexosamine were calculated from the results of six determinations. At the

TABLE 1
Analysis

Sample (100 μg)	Absorbance		Ratio[a]
	Hexosamine, 525 mμ	Total hexosamine, 535 mμ	
Mannosamine HCl	0.940	0.613	0.65
Galactosamine HCl	0.370	0.592	1.60
Glucosamine HCl	0.010	0.600	60.00

[a] Ratio of total hexosamine to hexosamine.

concentration of 100 μg of hexosamine HCl, the relative standard deviation, 100(σ/mean), found was 1.8 to 2.3% in both methods and for the amino sugars analyzed. Based on the concentration of hexosamine HCl (chromogens transformed) in the color solution, the molar absorbance coefficients (mole^{-1} liter cm^{-1}) for mannosamine, galactosamine, and glucosamine[1] were 25300, 9350, and 269 (*Hexosamine* method). Thus, the color intensity produced by glucosamine was about 3% of the color intensity produced by galactosamine and about 1% of the color produced by mannosamine. With the *Total Hexosamine* method, the molar absorbance coefficients of the color solution produced by mannosamine, galactosamine, and glucosamine were 16500, 15950, and 16150, respectively. On the basis of these results, the direct determination of galactosamine or mannosamine in mixtures containing glucosamine certainly is feasible.

Color Formation and Specificity. We have studied the effect of temperature on the Elson and Morgan reaction by changing this variable on reaction mixtures containing the hexosamine in the alkaline acetylacetone reagent incubated for 1 hr. At 80°C, galactosamine, glucosamine, and mannosamine produced about the same color intensity. Lower tempera-

TABLE 2
Effect of Time of Incubation

Components[a]	Absorbance at 525 mμ			
	Time, hr			
	18	42	64	138
Glucosamine	0.010	0.060	0.075	0.225
Galactosamine	0.375	0.600	0.675	0.680
Mannosamine	0.940	1.150	1.050	1.250

[a] 100 μg of amino sugar HCl incubated in the alkaline acetylacetone reagent at 0°C for different periods of time. After addition of the Ehrlich reagent, mixtures were incubated at 60° for 30 min (*Hexosamine* method).

tures yield absorbancies of different intensities. At 25°, the difference of absorbance produced between the hexosamines was large enough for their differentiation, but the reaction was less specific. Better results were obtained when the reaction mixture was incubated for 18 hr at 0°. These conditions were optimal for the direct analysis of glucosamine in solution

[1] The molar absorbance for glucosamine could not be determined by extrapolation from a standard curve measuring absorbance vs. concentration (500–2000 μg). Although a lineal function, the line intercept was 0.01 absorbance unit above the origin. At a lower concentration range (100–400 μg) the standard curve was lineal through the origin. From several measurements the molar absorbance of the color produced by glucosamine was 294 mole^{-1} liter cm^{-1}.

mixtures containing galactosamine or mannosamine (Table 2). The color was formed after addition of the Ehrlich reagent and was fully developed by heating the solution for 30 min at 60° (Table 3).

The color produced by galactosamine and glucosamine was stable for at least 7 hr. A decrease in color intensity at the rate of about 1%/hr was observed in the color produced by mannosamine. Absorbance measurements were in a linear relationship to the amount of hexosamine taken and were reproducible. A negative deviation of Beer's law was observed when the concentration of galactosamine HCl and mannosamine HCl were above 200 μg (*Hexosamine* method).

TABLE 3
Effect of Temperature on Development of Color

Components[a]	Absorbance at 525 mμ				
	Temperature, °C				
	40	50	60	70	80
Glucosamine	0.005	0.008	0.011	0.013	0.024
Galactosamine	0.214	0.300	0.390	0.495	0.570
Mannosamine	0.850	0.914	0.930	0.879	0.830

[a] 100 μg of amino sugar HCl incubated in the alkaline acetylacetone reagent at 0°C for 18 hr. After addition of the Ehrlich reagent, mixtures were incubated at different temperatures for 30 min (*Hexosamine* method).

Although of different intensities, the absorption spectra of the color produced by glucosamine, galactosamine, and mannosamine were the same, showing one peak at 525 mμ and a shoulder at 490–505 mμ (*Hexosamine* method).[2] Optimal pH for incubation of the hexosamine sample with the alkaline acetylacetone reagent was 9.5–9.6 and remained essentially the same after 18 hr incubation at 0°C. With the exception of mannosamine, an increase in color intensity was found when an electrolyte (NaCl or KCl) concentration was increased in contrast to a decrease in the color intensity observed when the method of *Total Hexosamine* was used (Table 4).

Mechanism. The reaction products (chromogens) were studied by paper chromatography. Reaction mixtures containing 2 mg of each hexosamine in 1 ml of the alkaline acetylacetone reagent adjusted to pH 9.5 were

[2] The absorption spectra was measured in a Cary recording spectrophotometer model 14. Cells of 2 cm light path length and an expanded scale attached to the spectrophotometer were used to measure the absorption spectra produced by 200 μg of glucosamine HCl. With an amount of glucosamine so that its color yield at 525 mμ was equivalent to the color intensity produced by galactosamine, the absorption spectra produced by glucosamine showed an additional peak at 405 mμ of about equal intensity to the absorption peak at 525 mμ.

incubated at 0°C for 18 hr. At the end of the incubation period the reaction mixture was lyophilized. The residue was dissolved in the minimal amount of cold H_2O, applied to Whatman 3MM and submitted to descending chromatography in *n*-butanol saturated with H_2O at 5° for 16 hr. On the dry paper, after spraying with the Ehrlich reagent, at least two spots were immediately detected. The fast moving component ($R_f = 0.92$) was violet, the slower component ($R_f = 0.54$) was pink. The color of these

TABLE 4
Effect of Salt

NaCl, mmole added	Methods					
	Hexosamine (absorbance at 525 mμ)[a]			Total hexosamine (absorbance at 535 mμ)[a]		
	1	2	3	1	2	3
0.0	0.010	0.380	0.932	0.592	0.585	0.615
0.5	0.013	0.395	0.907	0.545	0.553	0.592
1.0	0.017	0.406	0.898	0.527	0.525	0.587
1.5	0.021	0.430	0.844	0.515	0.512	0.578
2.0	0.020	0.430	0.844	0.500	0.506	0.572

[a] Absorbances produced by 100 μg hexosamine HCl. Numbers 1, 2, and 3 stand for glucosamine, galactosamine, and mannosamine, respectively. The relative standard deviation was less than 3%.

spots was very weak and barely detectible when glucosamine was used. As many as a total of 5 spots were formed after 24 hr. Spraying of the paper with a fresh solution of 4% $FeCl_3 \cdot 6H_2O$ in 50% ethanol-H_2O (v/v) showed the presence on the paper of one purple spot ($R_f = 0.54$) identical in mobility and color to the ketoimine derivative of glucosamine (Fig. 1) described by Cessi and Serafini-Cessi (3). The color of this chromatographic component was relatively intense in the products of the reaction produced by glucosamine and was almost absent in the products of the reaction produced by galactosamine and mannosamine.[3] Not even a trace of free hexosamine could be detected in any of the glucosamine, galactosamine, or mannosamine reaction products upon spraying the paper with the Elson and Morgan reagent (9).

In analogy to the ketoimine derivative of glucosamine, the ketoimine

[3] Additional pink spots detected in the reaction products by galactosamine and mannosamine were formed on the paper sprayed with $FeCl_3$ after 24 hr. The stability and color nature of these spots did not, however, correspond with the color properties expected for ketoimine derivatives (10). Their number and mobility were identical to the chromatographic components observed when the paper was sprayed with the Ehrlich reagent and was left standing for the same period of time. Apparently the color produced by $FeCl_3$ on these new lately developed components was generated by the chromogens, i.e., substituted pyrrole derivatives.

derivatives of galactosamine and mannosamine fail to give a color with the Ehrlich reagent, are rapidly hydrolyzed in diluted acid, and give a violet color when heated in the alkaline acetylacetone reagent followed by addition of the Ehrlich reagent (Elson-Morgan reaction). Their pres-

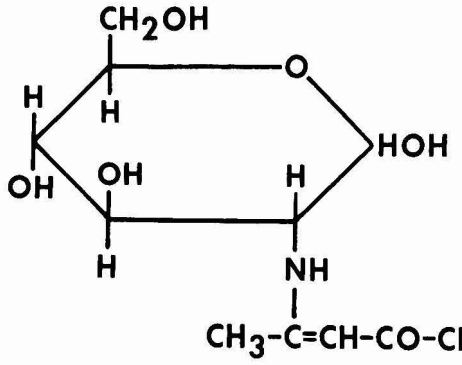

FIG. 1. Structure of the ketoimine derivative of glucosamine.

ence in the reaction medium was demonstrated only when the chromogens of the hexosamines were produced at 0°.

Apparently the hexosamine ketoimine derivatives are formed as final reaction products when the method of *Hexosamine* is used and presumably they are transient intermediates or are not formed under the conditions of

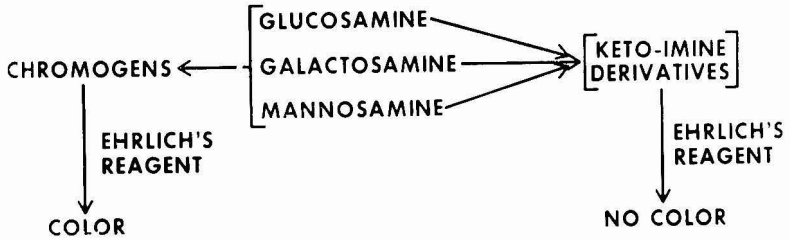

FIG. 2. Mechanism of color formation (*Hexosamine* method).

the *Total Hexosamine* method. Their relative concentration (ketoimine derivatives and chromogens) could therefore account for the difference in the color intensity observed within the hexosamines. A temporary working hypothesis for the mechanism of the reaction (*Hexosamine* method) is shown in Figure 2.[4]

Direct Analysis of Galactosamine and Glucosamine in Articular Carti-

[4] The reversibility of the reaction (formation of hexosamines from their ketoimine derivatives), and the formation of a weak chromogen as in the case of the glucosamine ketoimine derivative (3), should be taken into consideration in reaction mixtures incubated for long periods of time (Table 2).

lage. Mucopolysaccharides, mucoproteins, and cartilage preparations with less than 15% inorganic material estimated by ashing the sample to a residue and with no less than 200 μg of hexosamine were heated in 4 *M* HCl (2 ml) at 100°C for 14 hr in ampoules of 10 ml capacity sealed under N_2 gas.[5] After the incubation period, the vials were opened and the samples evaporated to dryness in a vacuum desiccator, containing KOH pellets, which was attached to an oil pump. Prior to their colorimetric analysis, the hexosamines present in the residue were purified according to the method of Boas (10) as described by Roseman and Daffner (12).

TABLE 5
Analysis of Human Articular Cartilage

Sample[a]	Galactosamine/glucosamine	
	Proposed method	Ion exchange (13)
1	1.38 1.38 1.37	1.40 1.43
2	0.70 0.72 0.70	0.72 0.70
3	2.44 2.43 2.40	2.50
4	1.16 1.16 1.15	1.17 1.18

[a] Normal (whole) articular cartilage sections. Age group from 67 to 82 years.

For this purpose, the residue was dissolved in 1 ml of H_2O and transferred to a glass column (1 $\times$ 25 cm) containing a suspension of 0.5 ml AG 50W-X8 (H^+)[6] ion-exchange resin. The column was washed with 10 ml of H_2O and the hexosamines were eluted with 0.5 *M* HCl. A total volume of 5 ml was collected directly into vials of 20 ml capacity (2.5 $\times$ 5 cm). This solution was evaporated to dryness, as described above, and the residues were dissolved in 0.30 *M* HCl to give solutions with hexosamine contents of 100 to 200 μg/0.5 ml. Aliquots from the same solutions were taken and analyzed by the *Hexosamine* and *Total Hexosamine* methods. The results obtained were in agreement with results obtained by using the ion-exchange column chromatography method of Gardell (13) (Table 5).

Stability of Hexosamines to Conditions Used in the Acid Hydrolysis. Reproducible results were obtained when the hexosamines were hydrolyzed under N_2 gas or in vacuum. (See Table 6.) Hydrolysis in 4 *M* HCl at

[5] Suitable conditions for the hydrolysis of other tissues should be investigated prior to the preparation of any routine hexosamine analysis. The ion-exchange method of Gardell (13) is adequate for this purpose. By using this method, we found that, whenever elution peaks in addition to those of glucosamine and galactosamine were obtained, they were usually identified as components of the partially hydrolyzed hexosamine containing polysaccharide. As would be expected on the application of a direct method of analysis to a solution mixture of two components, failure to hydrolyze the tissue completely will lead to erroneous results.

[6] Bio-Rad Laboratories, Richmond, California.

TABLE 6
Stability of Hexosamines to Conditions Used in the Acid Hydrolysis[a]

Method	Absorbance at 535 mμ	
	Before hydrolysis	After hydrolysis
1. Glucosamine	0.603	0.569
Galactosamine	0.595	0.565
Mannosamine	0.630	0.500
2. Glucosamine	0.610	0.308
Galactosamine	0.600	0.324
Mannosamine	0.620	0.050

[a] 1. Hydrolysis in 4 *M* HCl at 100°C for 14 hr under N_2 gas.
2. Hydrolysis in 6 *M* HCl at 110°C for 22 hr in vacuum.
Concentration of hexosamine in the acid solutions was 0.4%. For analysis, dilution of the acid solutions was made to a final acid concentration of 0.3 *M* HCl. Aliquots of 100 μg for each hexosamine were determined by using the *Total Hexosamine* method. The relative standard deviation of these determinations was less than 2%.

100°C for 14 hr under N_2 gas destroyed about 5% of the glucosamine and galactosamine. About 20% mannosamine was destroyed. Hydrolysis in 6 *M* HCl at 110° for 22 hr in vacuum destroyed about 47% of the glucosamine and galactosamine. About 92% mannosamine was destroyed.

SUMMARY

A colorimetric method is introduced to differentiate glucosamine, galactosamine, and mannosamine. Direct evaluation of galactosamine in mixture with glucosamine was possible since galactosamine was essentially the only component responsible for the color of the reaction; the influence of glucosamine on the intensity of the color was negligible. The proposed method has been found highly satisfactory for the analysis of hexosamines in preparations of mucopolysaccharides, mucoproteins, and articular cartilage.

The present report also includes observations on the reactivity of hexosamines with acetylacetone which has extended the usefulness of the Elson-Morgan reaction as a method of differentiating amino sugars. It also proposes the possible mechanism of the reaction.

ACKNOWLEDGMENTS

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ANALYTICAL BIOCHEMISTRY 19, 89–94 (1967)

Automated Determination of Ascorbic Acid

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Received October 28, 1966

Our laboratory has been involved for some time in the assay of luteinizing hormone (LH) by the ovarian ascorbic acid depletion method. During the course of these studies we have partially converted the method from a manual to an automated procedure with a resulting improvement in simplicity of operation, accuracy, and reproducibility. This report discusses the conversion of the biochemical determination to the Technicon AutoAnalyzer.

METHODS

The procedure is based on a modification of the Mindlin-Butler method (1), as described by Parlow (2), for the bioassay of LH using the immature rat. One ovary is excised, homogenized in 2.5% metaphosphoric acid (MPA) in an ice bath, and filtered. The filtrates are decanted into sampling tubes and placed in the sampler (Fig. 1). Tubes containing MPA are placed between ovarian extract tubes to provide a system wash and to allow for adequate separation of the peaks. The dye:buffer solution ratio was changed from 1:1 to 3:1.

The dye solution is prepared by adding 50 mg 2,6-dichloroindophenol to 200 ml water, heating to 95°C, filtering, and diluting to 1 liter. Sodium acetate (22.65 gm) is dissolved in 500 ml water and adjusted to pH 7.0 using a 6% acetic acid solution. Tween 80 is added (0.4% v/v) to the dye-buffer solution and the MPA solution used in the wash tubes to improve the bubble pattern; it is not added to the MPA used in the ovarian homogenization and extraction. The time delay between the mixing of equal parts of sample and dye-buffer and the reading of the resulting solution was changed from 25 to 50 sec. Both the extracts and the MPA solution in the wash tubes are sampled for 1 min in a one-to-one proportion to the dye-buffer solution. The absorption of the solution is determined with a Technicon filter (520-18-28 mμ).

Standard solutions of ascorbic acid are placed in the sampler (i.e., two aliquots of each of eight standard solutions) at the beginning of

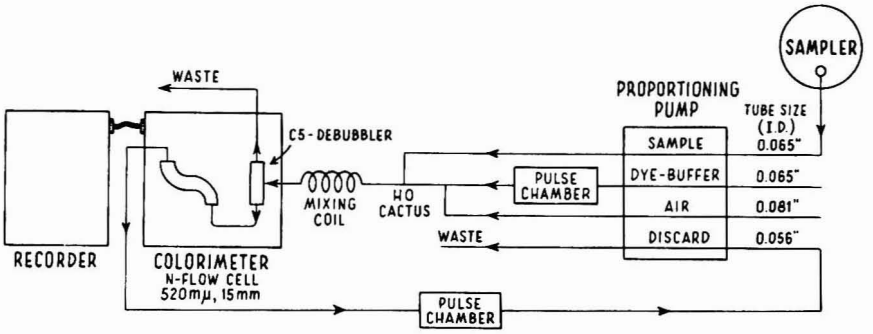

FIG. 1. Flow diagram utilizing Technicon modules for determination of ascorbic acid. All pumping and transmission lines are Tygon tubing. Pulse chambers are glass units with capacities of 2 ml.

the assay. Thereafter, one sample of a standard ascorbic acid solution is inserted after every ten ovarian extracts. This procedure allows the operator to check the instrument initially and periodically for precision. The randomly inserted standards should generate a curve identical to that obtained at the beginning of each assay.

RESULTS

The maximum absorption, as determined by a Cary recording spectrophotometer (model 11), of curves 1, 2, and 3 (Fig. 2) occurred at 516 mμ. The maximum absorbance of curve 4 was shifted toward the red

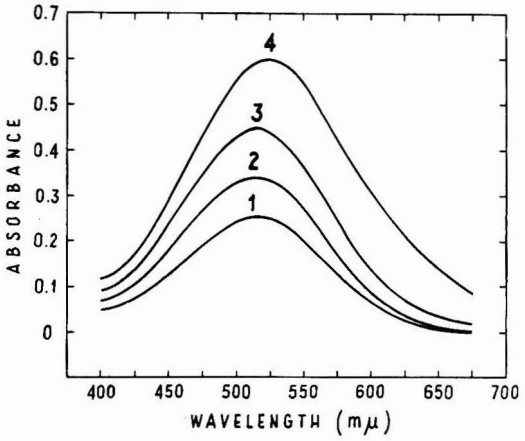

FIG. 2. Absorption spectra of solutions containing the 3:1 dye-buffer mixture and metaphosphoric acid. The curves represent four ratios of dye-buffer to MPA: (1) 1:2, (2) 1:1.5, (3) 1:1, (4) 1:0.5. Curves were obtained with a Cary recording spectrophotometer, model 11.

portion of the spectra by 8 mμ to a new maximum of 524 mμ. The loss of the red color and increase of blue in this solution were also evident visually. Addition of Tween 80 to the solutions forming the working dye solution caused a shift in the wavelength maximum to 535 mμ. This shift indicated that the maximum sensitivity region of the Technicon filter was now on the red (or ascending) side of the absorption curve rather than at the peak or on the blue (descending) side of the curve. The solution containing the working dye mixture was placed in the

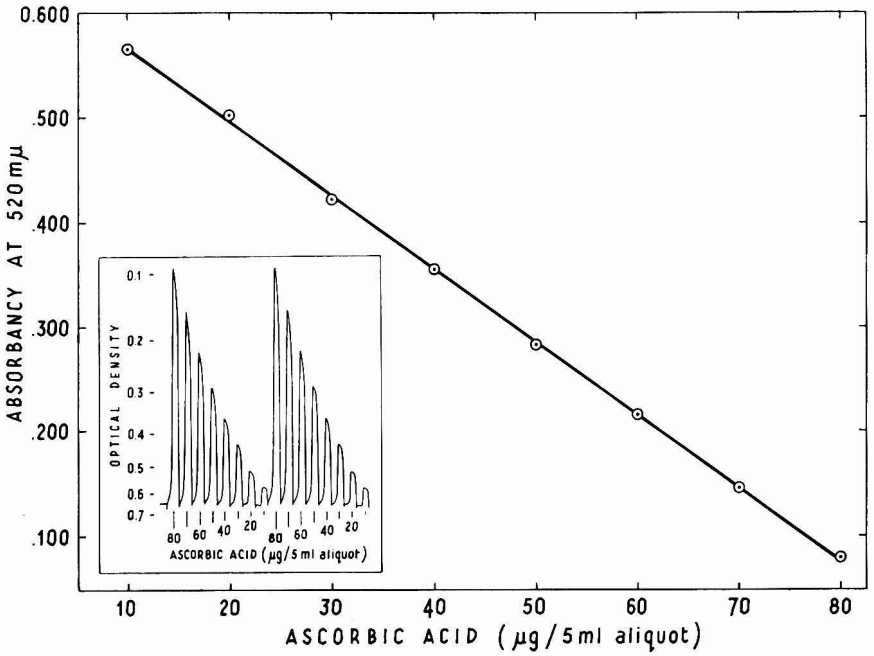

FIG. 3. Standard calibration curve for determination of ascorbic acid. Curve represents two groups of standards shown in the inset.

Cary spectrophotometer for 10 min to study the stability of this solution. At the end of this time, 96% of the original color was still present, with no change occurring prior to 1 min. The addition of Tween 80 did not affect this rate of color loss.

The calibration curve shown in Figure 3 is typical of those obtained routinely. This curve represents the two sets of standards shown in the inset. Transformation of the logarithmic (optical density) data in the inset of Figure 3 to an arithmetic scale results in a linear plot. The dye-buffer-MPA baseline is shown by the small line on the bottom left side of the inset after the 80-standard peak. However, as shown, during the

determinations this baseline is not present because there is a break between the sample-to-wash cycle, and therefore a small amount of the dye-buffer solution is not mixed with MPA. This results in a small amount of blue color passing through the colorimeter, and an over shift on the recorder. The inset also demonstrates the ability of the method to measure clearly a low concentration after a high concentration.

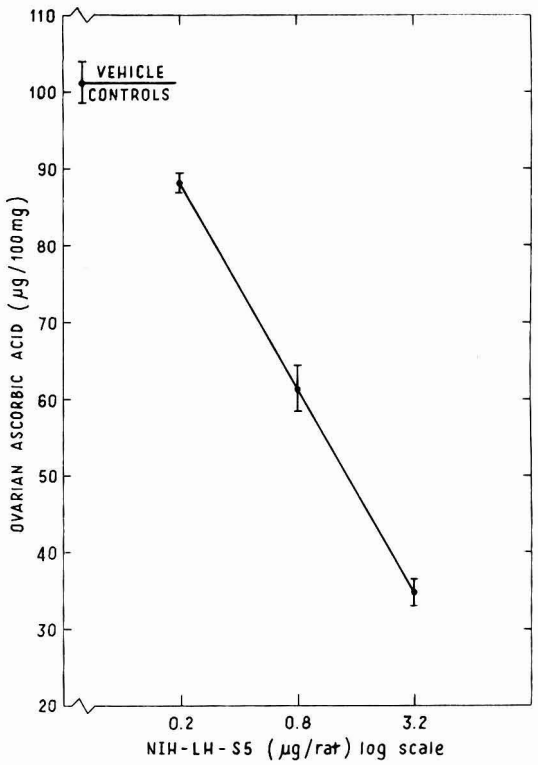

FIG. 4. Ascorbic acid content in ovaries from three groups of immature female rats receiving NIH-LH-S5. Each point represents mean response of 10 animals; brackets indicate standard error of mean.

An experiment was conducted with the standard solutions to verify the accuracy of the sample measurements. The eight standard solutions (used in Fig. 3) were anlyazed in ascending order: five samples of the lowest concentration, five of the next, proceeding in this manner through all of the solutions. In addition these same solutions were analyzed in random order. The slope $\pm$S.E. of the curve for the sequential analysis was 1.150 ± 0.0049 and for the random analysis 1.152 ± 0.0048, indicating that the two curves were essentially identical.

Likewise, the reproducibility of the method over a period of five weeks was accessed by comparing the slopes of curves obtained for sequentially and randomly inserted standards. Analysis of the data showed an occasional slight difference (ca. 1%) between the random or sequential determinations on a week-to-week basis. This may be due to minor variations in reagent preparation. However, it should be emphasized that no significant differences occurred in the slopes of the random and sequential curves within a given assay.

Although the above experiments indicated good reliability for the method when analyzing standard solutions, it remained to be determined if the same was true for ovarian extracts. Figure 4 illustrates the results of an assay of ascorbic acid in ovaries from immature female rats primed according to the method of Parlow (2), and treated with three dosages of NIH-LH-S5. When the data were plotted on a semilogarithmic paper a linear response resulted. In order to check for the possible interference of other materials in the color depletion reaction, samples of ovarian extracts were mixed with the working dye solution and placed in the Cary spectrophotometer to study the rate of color decay. These data indicated that the rate of decay was constant and gradual with no significant color loss occurring within the first minute.

DISCUSSION

The results indicate that this method is applicable to the determination of ovarian ascorbic acid. Experiments with standard solutions showed that the method does not bias the data in favor of random or sequential sampling, i.e., samples containing low concentrations of ascorbic acid are read accurately following samples with high concentrations. Although there were variations between the slopes of standard ascorbic acid solutions run during the course of several weeks, they were of the order of 1% or less and therefore probably insignificant with respect to the bioassay of luteinizing hormone, since animal variation would be at least as great.

Time decay studies showed that this colorimetric procedure is stable during the period (50 sec) required for mixing and reading of the sample and reagents. This time factor may need to be adjusted to a shorter period for materials with a higher RSH content in the filtrate. However, the results of the NIH-LH-S5 study did not indicate any abnormal variations which could be attributed to nonspecific interference. The variations occurring within groups appeared to be normal for this bioassay and were not dose related. These observations indicate that the method is satisfactory for use with biological extracts with good accuracy and reproducibility.

SUMMARY

A method for the determination of ovarian ascorbic acid based on a modification of the Mindlin-Butler method was developed using Technicon modules. The method is accurate and reproducible with standard ascorbic acid solutions and ovarian extracts.

ACKNOWLEDGMENT

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ANALYTICAL BIOCHEMISTRY 19, 95–97 (1967)

Cyanide and the Determination of Protein with the Folin Phenol Reagent

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Hydrogen cyanide is found in concentrations up to 0.135% in flax seedlings (1). Folin and Ciocalteu reported that cyanide produces with the phenol reagent a blue color that interferes in the measurement of protein (2). They found that the better grades of KCN produced less color than cruder preparations and that metal salts increased the intensity of the resulting blue color. While many substances are known to interfere with the determination of proteins by the phenol method and several modifications of the original method have been made (3–5), the cyanide effect appears to have escaped attention. Yet cyanide is readily oxidized in alkaline solutions (6).

Using a modification of the Lowry method (3), I found that the Folin phenol reagent provides a convenient, though nonspecific, method for measuring cyanide. In the range 8 to $20 \times 10^{-4} M$ KCN, absorbance is almost linearly proportional to the concentration (Fig. 1). The values obtained with three separate samples of KCN were practically identical. The phenol reagent does not react with thiocyanate and cyanate, either in the presence or in the absence of copper sulfate; by contrast cyanide produces approximately ten times as much color in the presence of this salt (Table 1). Concentrations below $2 \times 10^{-4} M$ cyanide produce no blue color with the phenol reagent. Since the absorbances due to protein and to cyanide are additive, protein determinations can be made in the presence of low, known cyanide concentrations (Table 2).

Miller's procedure (5) was used with minor changes; the alkaline reagent was conveniently prepared by mixing equal volumes of the two components, and a borate-NaOH buffer was used. Similar results were obtained with the unmodified method.

To 1 ml of sample add 1.0 ml of alkaline reagent (composed of equal volumes of 0.1% sodium potassium tartrate in 0.025 N Na_2CO_3 and 0.05% $CuSO_4 \cdot 5H_2O$) and 1.2 ml buffer (12.4 gm H_3BO_3 and 19.0 gm NaOH/500

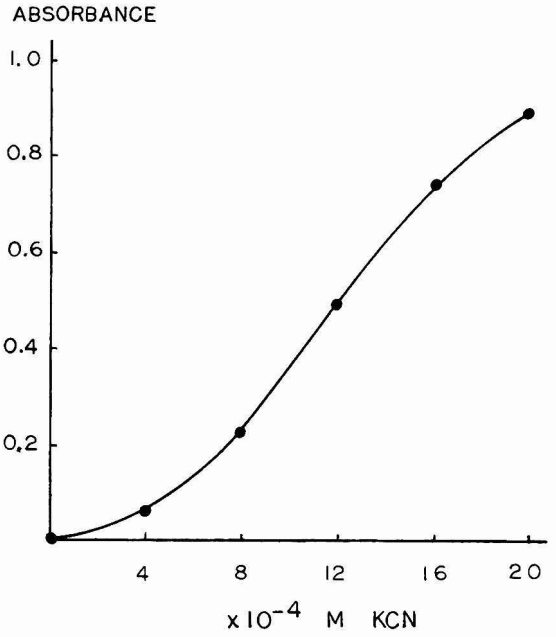

FIG. 1. Standard curve for KCN.

TABLE 1
Reaction of Cyanide, Cyanate, and Thiocyanate with the Folin Phenol Reagent in the Presence and Absence of Copper Sulfate

Compound	Absorbance	
	$+CuSO_4$	$-CuSO_4$
2×10^{-3} *M* KCN	1.00	0.07
2×10^{-3} *M* KCNO	0.00	0.00
2×10^{-3} *M* KCNS	0.00	0.00

TABLE 2
Analysis of Mixtures of Cyanide and Protein with the Folin Phenol Reagent

Egg albumen, mg	Absorbance			
	0	2	4	6×10^{-4} *M* KCN
0.0	0.00	0.02	0.08	0.20
0.025	0.10	0.12	0.19	0.31
0.05	0.20	0.22	0.29	0.40
0.10	0.37	0.40	0.47	0.56

ml). Wait 10 min. Add 3.0 ml of 1:10 diluted phenol reagent forcibly with mixing. Heat the samples for 10 min at 50°C, cool to room temperature, and determine the absorbance at 700 mμ. The final pH of the solutions should be between 9.5 and 10. The error within tests is approximately 3%; but, as it is much larger between tests, a set of standards should be run with each analysis.

ACKKNOWLEDGMENT

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ANALYTICAL BIOCHEMISTRY 19, 98–108 (1967)

Solubility Studies of Estrone in Organic Solvents Using Gas-Liquid Chromatography[1]

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Solubilities of estrone, 1,3,5(10)-estratrien-3-ol-17-one, in various organic solvents were needed in this laboratory as a prerequisite for chemical kinetic studies and, in a broader scope, to elucidate attractive and repulsive forces that control every phase of equilibrium (1). We also are interested in solubilities of estrone because of application to clinical analysis.

In spite of voluminous literature on the estrogens, systematic data on such solubility are practically nonexistent. In 1931, de Jongh and coworkers (2) published some solubility values for estrone in ethanol, chloroform, acetone, and benzene. In 1941, Doisy and his colleagues (3) determined the solubilities of the four major estrogens in ten organic solvents at 10° and 30°C by evaporation of the solvents and weighing of the solute, and by a colorimetric method for petroleum ether. Sjoblom (4) combines the data of de Jongh and Doisy in a table when mentioning the solubility of estrone in organic solvents in his fine treatise on its solubility in aqueous solutions of association colloids. There are also some approximations for the solubility of estrone in five organic solvents at their boiling points and either 15° or 25° in *The Merck Index* of 1960 (5). None of these values coordinates.

METHOD

The present study utilizes gas-liquid chromatography to determine the solubility of estrone in twelve organic solvents at several different temperatures, including 0°, 15°, 25°, 30°, and 40°C, depending upon boiling and freezing points. Its solubility in hexane at 30° was also determined. The solvents shown in Table 1 were chosen for their value for use in our future kinetic studies; therefore, hexane was not further

[1] Presented in part at the Second International Congress on Hormonal Steroids, Milan, Italy, May 23–28, 1966.

investigated. Gas chromatograms were run of each solvent to determine purity; solvents were kept over drying agents both before and after any necessary distillation. Estrone (Mann Research Laboratories, Inc., New York) had a m.p. of 255–260°C on a Fisher block, uncorrected. However, in spite of the 5° range, gas chromatography showed only one peak for estrone on several different columns of varying polarity.

Originally the intention was to form a derivative of estrone as its trimethylsilyl ether. With Gas Chrom Q (Applied Science Laboratories, State College, Pennsylvania) as a support, the chromatogram showed that estrone did not completely convert, although the free steroid peak

TABLE 1
Differential Heats of Solution of Estrone

Solvent	ΔH_{sat}, kcal/mole
p-Dioxane	1.5
Cyclohexane	2.0
Tetrahydrofuran	2.4
Acetone	5.0
Methanol	5.0
Methylene chloride	5.1
Absolute ethanol	5.1
Ethyl ether	5.5
Ethanol (95%)	5.6
Benzene	6.2
Chloroform	6.9
Toluene	7.3

did not show up when using other supports, only a smaller silyl ether peak. The unconverted free steroid had been absorbing on the support and not appearing on the chart. Rather than attempting variations of the method of preparing the silyl ether, it seemed advantageous to quantitate the free steroid itself.

Figure 1 shows the chromatograms of varying ratios of standard solutions of estrone and of cholesterol used for weekly "relative area vs. relative concentration" graphs. Cholesterol (the internal standard) is detected as the first peak with a retention time of 20 min, and estrone, the second, with a retention time of 27 min. Occasionally, estrone showed a small additional peak which followed the main one. It was considered an isomer that formed upon evaporation of the solvent, showing a tendency for interconversion, and was included in the total amount of estrone. The small peak just following the solvent front is a slight impurity in tetrahydrofuran which was not removed by distillation and is really less than 0.2%, but is accentuated by the evaporation of several

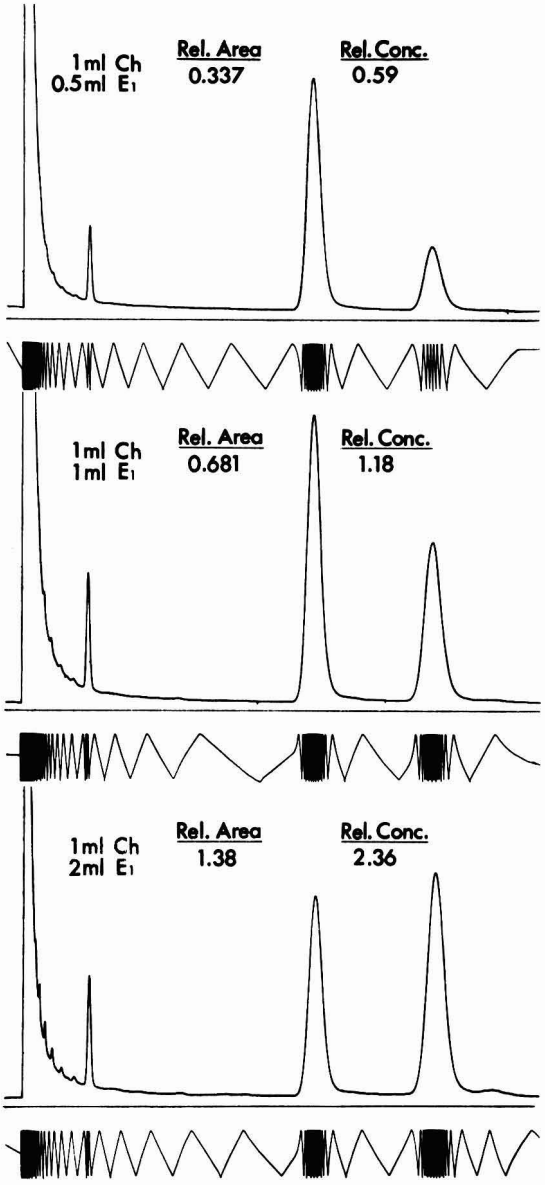

FIG. 1. Chromatograms of various ratios of cholesterol and estrone, as free steroids, on column of 3% XE-60 phase, on Gas Chrom Q, 80/100 mesh, 6 ft × 4 mm i.d., column temperature 227°, injector 265°, detector 265°C, inlet pressure of N_2 32 lb.

milliliters of each standard solution. Peak areas are measured with a disc integrator (Disc Instruments, Inc.) corrected for drift where relevant.

The glass column is 6 ft $\times$ 4 mm i.d, filled with 3% XE-60 phase on Gas Chrom Q, 80/100 mesh which was coated by Applied Science Laboratories. The glass wool plug is siliconized. A Barber-Colman, 5000 series, flame-ionization chromatograph was used. The attenuator was set at 5, the sensitivity at 1000. The 5 mV recorder speed was 20 in./hr.

Column temperature was 227°, injector 265°, detector 265°C. Inlet pressure of the nitrogen carrier gas was 32 lb. These conditions are varied slightly as the age of the column increases. The chromatograms in Figure 1 were made when the column was at the beginning of its third week; its life span for baseline separation is six weeks or more.

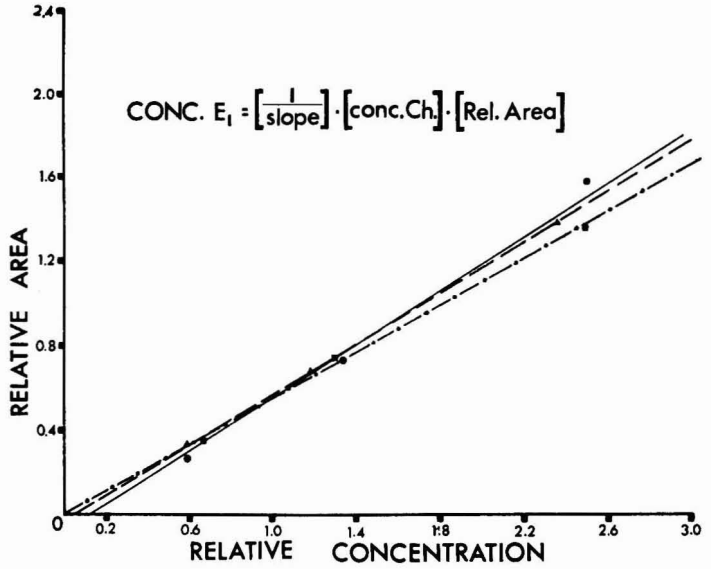

FIG. 2. Relative area vs. relative concentration graph for cholesterol and estrone, prepared from weekly chromatograms similar to those shown in Figure 1.

Figure 2 is a relative area vs. relative concentration graph, showing slightly different slopes for three different weeks. The chromatograms in Figure 1 were used for the top line on this graph. Figure 2 shows the linearity of the different ratios of cholesterol and etsrone used in Figure 1, and detector response as well as column behavior for the two steroids. The pertinent slope is applied each week in the following

equation to determine the concentration of estrone (E_1) when using cholesterol (Ch) as an internal standard:

$$E_1 \text{ concn.} = \left(\frac{E_1 \text{ area}}{\text{Ch area}}\right) (\text{Ch concn.}) \left(\frac{1}{\text{slope}}\right)$$

Solubility was approached from both undersaturation and supersaturation equilibrium (6). For undersaturation, the solvent with excess estrone was maintained for at least four days at the required temperature; it was shaken vigorously by hand at regular intervals. For supersaturation, the reaction tubes with solvent and excess solute were periodically shaken and held at least 10°C above the desired temperature for two days, then slowly dropped to this temperature and held there for two more days, so that more of the dissolved material crystallized out. Special long, narrow reaction tubes with ground-glass stoppers held 20 ml of solution and allowed good heat exchange. The saturated supernatants were removed with filter-pipets to prevent entrance of small crystals along with the sample.

Temperature was held constant. For 0°C, an ice bath in a large Dewar flask was used. For 40°, a Fisher Isotemp bath with oil kept the temperature ±0.01°C. For 15°, 25°, and 30°C, it was opposed by a Fisher Cloud and Pour apparatus cooling bath; again, the temperature was constant.

For quantitation, triplicate determinations (each of a different ratio of estrone and cholesterol) were calculated by gas chromatography for each solvent at each temperature. If the ratios appeared unfeasible, either dilution or larger ratios were used for three other runs. All solvents were evaporated under nitrogen in an oil bath at 50°C; then a measured amount of tetrahydrofuran was added as the solvent for gas chromatography. The average deviations for the γ/ml amounts for three chromatograms of three different ratios were usually less than 1%. The largest deviation was 5%, where there was the greatest solubility with the biggest dilution factors.

THEORY

Solubility may be defined as the capacity of two or more substances to form spontaneously, one with the other, without chemical reaction, a homogeneous molecular or colloidal dispersion (6). One would expect estrone and these organic solvents to form a homogeneous molecular dispersion.

Figure 3 shows a linear relation between log N_2 vs. $1/T$ (where N_2 is the mole fraction of solute, and T is in degrees Kelvin) which might indicate an ideal solution within the temperature range of interest, for absolute methanol and estrone. (This also appeared to be the case with

the other solvents.) Equilibrium seems to take a longer time from undersaturation, where it is approached from the crystalline rather than the molecular side, as it involves passage of molecules across a boundary between two phases (7). Therefore, a rising temperature could introduce a considerable error.

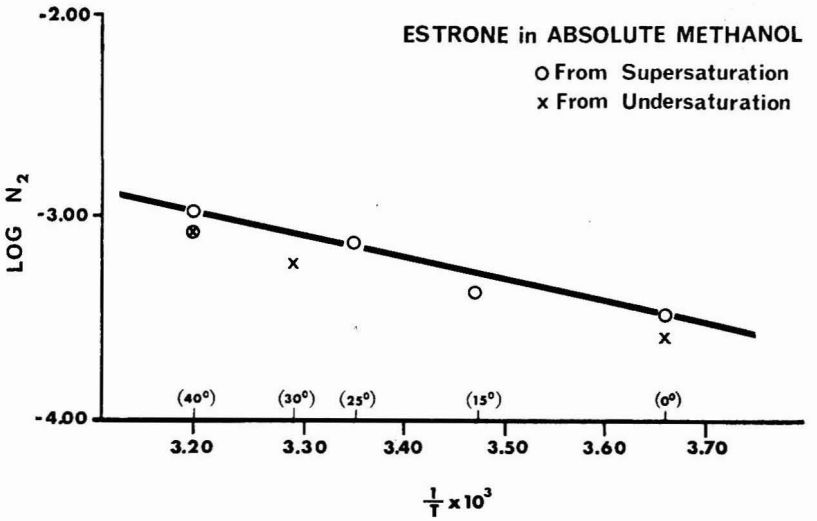

FIG. 3. Graph for log N_2 vs. $1/T$, from van't Hoff equation, for solubility of estrone in absolute methanol.

The van't Hoff equation (7) is used to calculate the enthalpy, ΔH_{sat}, which is the "differential heat of solution" at saturation for a given P and T, that is, the heat of solution of one mole of solute in the practically saturated solution. If ΔH is independent of T, which is usually true for nonelectrolytic solutions, and especially within a small range, then:

$$\frac{d \ln N_2}{dT} = \frac{\Delta H_{sat}}{RT^2}$$

where R is the gas constant in cal/mole. Upon rearrangement and integration:

$$\ln N_2 = \frac{-\Delta H_{sat}}{RT} + C$$

So plotting log N_2 vs. $1/T$:

$$\text{slope} = \frac{-\Delta H_{sat}}{2.303\text{R}}$$

Therefore:

$$\Delta H_{sat} = -(\text{slope})\ (2.303)\ (1.987)\ \text{cal/mole}$$

RESULTS

Table 1 shows ΔH_{sat} for all solvents. Values range from 1.5 kcal/mole for *p*-dioxane to 7.8 kcal/mole for benzene, showing that intermolecular forces and a large degree of at least temporary order must exist in these liquids (1). An example is ΔH_{sat} for estrone in absolute methanol, as calculated from Figure 3 with a value of 5.0 kcal/mole. These values are within the 2 to 10 kcal/mole limit usually found for the solubility of solids in liquids (6).

Table 2 compares the quantitation by gas-liquid chromatography to that by evaporation of solvent and weighing of the residue. The comparison is close, with gas chromatography probably much more

TABLE 2
Comparison of Methods of Quantitation of Estrone[a]

Solvent	Temp., °C	Gas chrom., γ/ml	Evapn., γ/ml
Tetrahydrofuran	30	48,336	47,458
p-Dioxane	30	19,200	19,180
Acetone	30	11,535	12,105
Chloroform	30	15,680	15,178
Methylene chloride	30	6,384	6,930
Absolute ethanol	30	3,516	3,666
95% ethanol	40	6,227	6,382
Methanol	30	4,041	3,761
Ethyl ether	30	2,127	2,308
Benzene	30	1,352	1,964
	25	1,071	1,522
Toluene	30	1,011	1,664
Cyclohexane	30	23	46
Hexane	30	4	19

[a] Taken from undersaturation, not necessary at equilibrium.

accurate due to mechanical difficulties involved in drying and weighing of residues. (The values in Table 2 were taken from an undersaturation approach, and do not necessarily represent equilibrium, as do those in Table 4).

Weighing errors may result in low values due to blowing off of small crystals if the nitrogen used in drying were too energetic a stream. Values may be low or high where there is only a small weight difference between residue and container (such as in the case of cyclohexane and hexane), or where several transfers occur with highly volatile solvents. High values may result because complete dryness is often difficult to evaluate. In the evaporation of 10 ml of solvent from solution (or 5 ml

where there is a high solubility), even constant weight may not mean that all solvent has evaporated.

Benzene and toluene consistently showed slightly higher values of solubility in several weighings of different aliquots, probably due to the estrone crystals not being entirely free of solvent. There is an affinity of aromatic nuclei when in close proximity, possibly a π–π interaction (8) of the electrons in the π molecular orbitals of these two compounds. There is also the possibility that benzene and toluene, with weakly basic characteristics (1), complex with the phenolic proton of estrone, since short-range, specific forces become more important as nonelectrolytic solutions become more concentrated (1).

Chloroform, with three electrophilic chlorine atoms, perhaps might be a charge-transfer complex, too. It became rose color with estrone in solution, most intense when coldest. This is strongly indicative of specific interaction with the solvent molecules (1). With *o*-allylphenol, chloroform is characterized as an acceptor of the "hydrogen-donating" type

TABLE 3
Comparison of Solubility of Estrone at 30°C with Doisy's Results

Solvent	(gm estrone/100 gm solvent) Doisy (evaporation)	Ruchelman (gas chromatography)
p-Dioxane	2.84	1.91
Acetone	1.78	1.58
Chloroform	0.53	1.07
95% ethanol	0.67	0.82
Methanol	0.63	0.68
Benzene	0.13	0.16
Toluene	0.10	0.12

(that is, with a hydroxylic substance as a donor) (9). The solution of estrone in *p*-dioxane was slightly yellow, although the solvent was previously clear; and the gas chromatogram showed no decomposition products for estrone. Doisy (3) also noted color in the tubes containing chloroform and dioxane.

Szent-Györgyi (8) mentions that estradiol, with its conjugated π pool, gives a charge-transfer with trinitrobenzene; also that cortisone transfers to I_2. Ketosteroids have keto groups in which the oxygen, with its lone pair of nonbonded electrons, may act as a strongly localized donor for coordination; so the color changes of these steroids and iodine vapor, in general, are probably due to charge transfers (8).

Table 3 compares the values of solubility of estrone in the solvents Doisy (3) used with those that overlapped the ones we investigated. Our

figures were taken from values obtained at 30°C, at equilibrium, using gas chromatography. Values are similar except for chloroform and *p*-dioxane. In chloroform, estrone floats at the top of the solvent and the interface would be small and allow less solubility unless shaken thoroughly. The solubility of estrone in *p*-dioxane is so large that evaporation of solvent and weighing of the powder might incur error. We had to evaporate two aliquots, as the first one turned yellow and oily and weighed almost twice as much as the value by gas chromatography. This was the only solvent that reacted with estrone in this manner upon evaporation.

Table 4 shows the solubility of estrone in all thirteen organic solvents at all temperatures which were investigated. At 40°, estrone was found to be most soluble in tetrahydrofuran and least soluble in cyclohexane,

TABLE 4
Solubility of Estrone in Organic Solvents at Various Temperatures

Solvent	Solubility in γ/ml at 0°C	15°C	25°C	30°C	40°C
Tetrahydrofuran	39,573	41,880	53,784	48,336	70,280
p-Dioxane	[a]	26,032	30,240	36,693	34,491
Acetone	6,723	9,728	14,599	17,075	20,244
Chloroform	4,731	7,397	12,199	15,680	19,368
Methylene chloride	3,273	4,492	6,549	8,304	[b]
Absolute ethanol	2,696	3,421	6,836	7,137	8,920
95% ethanol	2,271	4,600	6,420	6,650	8,636
Methanol	2,257	2,773	5,203	5,380	5,798
Ethyl ether	1,103	1,762	1,638	2,127	[b]
Benzene	[a]	859	1,071	1,352	2,029
Toluene	287	613	824	1,011	1,583
Cyclohexane	[a]	24	30	32	35
Hexane	[c]	[c]	[c]	4	[c]

[a] Solution freezes.
[b] Solution boils.
[c] Not determined.

by a factor of 2008 times. At 0°, it was most soluble also in tetrahydrofuran and least soluble in toluene, by a factor of 1038 times. (Cyclohexane froze at 0°, but extrapolation would put the solubility at 14 γ/ml, a factor of 2827 times.) The next best solvent for estrone was *p*-dioxane. These cyclic ethers have basic properties which probably affect this solubility. Doisy (3) reported an exceptionally large solubility of estriol in pyridine, also basic. In contrast, the solubility of estrone in ethyl ether was much lower, so that steric conformations must be important, too, although the estrone molecule is essentially flat.

Relatively low solubility in the alcohols may be due to their being associated solvents with indefinite, chain-like hydrogen bonding which might prevent interaction with estrone. Adding 5% water (by volume) to ethanol slightly decreases solubility, so that it is not likely that the water will interact with the aromatic nucleus of estrone. Somewhat higher solubilities in the chlorinated hydrocarbons may be due to O—H—Cl bridges (3); also, there is twice the solubility in chloroform as in methylene chloride at 30°C.

Phenol is less self-associated in benzene than in carbon tetrachloride (9), and this might be likely for estrone, too. In addition, there is a possibility for dimerization of the hydroxy group of one estrone molecule with the keto group of another in solvents of low dielectric constant, but its very low concentration in toluene, benzene, cyclohexane, and hexane probably precludes this. Also, Doisy (3) gives a very low solubility of estrone (8.9 γ/ml at 30°C) in petroleum ether (b.p. 89–92°C).

DISCUSSION

Investigators using cyclohexane, hexane, and petroleum ether (and benzene and toluene at low temperatures) to dissolve estrone for various analytical procedures should be aware that they might be measuring only the amount in solution and not the total content.

They should also be congnizant of the motility of the estrone molecule under varying conditions of separation.

In observing the order of solubility of estrone at 30°C, it will be noted that the two cyclic ethers are outstandingly good, followed by acetone, then the two chlorinated solvents, then the three alcohols (including aqueous ethanol), then the straight-chain ether, then the two aromatics, and, finally, there is almost an insolubility in the two saturated hydrocarbons. This would indicate that the solute is very much affected by the solvent, and probably not really an ideal solution in most of these solvents. However, there is no correlation between the solubility of estrone and the dielectric constants, the boiling points, or the freezing points of the solvents. A positive deviation from Raoult's Law indicates less solubility, and a negative, more solubility (1). The biggest factor that is influencing the solubility of estrone seems to be the phenolic portion, both because of its acidity and because of possible hydrogen bonding. Since estrone has an electron absorption coefficient of 0.03 (very low) (10), it is unlikely that the keto group affects its solubility except in a minor way.

Solubility studies of estrone in the thirteen organic solvents listed in Table 4 are complete, and studies of estradiol (1,3,5(10)-estratrien-3,17β-diol) and of estriol(1,3,5(10)-estratrien-3,16α,17β-triol) have begun.

SUMMARY

Solubilities of estrone in thirteen organic solvents were investigated at various temperatures, including 0°, 15°, 25°, 30°, and 40°C, depending upon boiling and freezing points. Gas chromatography, the method used, is compared to the method of evaporation of solvent and weighing of residue. Results and theory are discussed.

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Ascending Paper Chromatography of Sulfated Acid Mucopolysaccharides

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Because of the failure of paper electrophoresis to show clear separations of small amounts of acidic mucopolysaccharides (AMPS) (1), the use of paper chromatography was investigated. At the time this work was initiated there were no methods available for reproducible separation of the chondroitin sulfates A (Ch-S-A), B, or C, heparin (Hep), heparitin sulfate (H.S.) or keratosulfate (K.S.) by paper chromatography (2, 3). Since then, a descending method has been developed (4), and a more complex method using glass paper has been reported (5).

The method reported below allows simple reproducible separation of Ch-S-B, H.S., Ch-S-A or C, and K.S. in ascending systems. Additionally, changes in temperature and ethanol concentration have allowed discrete separation of single AMPS spots from others for differentiation and identification when desirable, and fragments of peptide-AMPS may be manipulated.

MATERIALS AND METHODS

The glucuronic acid potassium salt from Matheson, Coleman & Bell when compared with anhydrous glucuronolactone from Eastman Chemicals gave color intensities 16% lower, accounting stoichiometrically for two moles of water per mole of potassium glucuronic acid. Hexosamines were obtained, standardized and quantitated as previously described (6).

The carbazole (C) reaction of Dische (7) was used to measure uronic acid content of AMPS and the naphtholresorcinol (N) reaction of Pelzer and Staib (8) was used to indicate the presence of Ch-S-B by comparison with the carbazole reaction. Low C/N ratios were indicative of Ch-S-B (9).

Sodium salts of Ch-S-A and Ch-S-B were obtained from Dr. Albert Dorfman, keratosulfate preparations from Dr. Karl Meyer and Dr.

Alfred Linker (10), sodium heparin and crude H.S. from the Upjohn Company, a purified H.S. from Dr. Karl Meyer, and hyaluronic acid from Sigma Chemical Company. Additional standards of Ch-S-A were prepared from bovine nasal septum by the method of Malawista and Schubert (11); human Ch-S-B and heparitin sulfate were prepared from urine of children with gargoylism (12).

One child (C.D.) was the genetic type of this disorder (Hurler's syndrome) in which Ch-S-B and H.S. were excreted in excess. The second child (D.B.) was the genetic type, in which large amounts of H.S. were excreted without Ch-S-B being present (12–16). The urine of children with gargoylism is a source of readily available human AMPS. Normal children excrete only Ch-S-A or -C in small amounts with occasional traces of Ch-S-B and H.S. (18, 19). These AMPS are polydisperse in molecular size, but relatively free of covalently linked peptides (17).

The AMPS were precipitated from urine similar to the methods of others (15, 20) using cetyltrimethylammonium bromide (CTMAB) at pH 4–5. The cationic detergent-AMPS complex was broken by triturating 3 times with 2% sodium acetate in 95% alcohol and the crude sodium AMPS salts were washed with ethanol and ether and dried. Further purification and fractionation was carried out by reprecipitating the AMPS from 0.2% aqueous barium acetate solution by stepwise ethanol fractionation at 20% and 80% concentration in the cold (4°C). Conversion of barium salts to sodium AMPS was possible by addition of excess sodium carbonate. Following removal of barium carbonate by centrifugation, the AMPS solution was acidified with liberation of excess CO_2 and the resulting AMPS sodium salts were precipitated by addition of 5 vol of 95% ethanol. These salts were washed free of excess sodium acetate by several washings with ethanol, dried with ether, and desiccated over silica gel and paraffin by storage at —20°C.

Paper Chromatography Method

Conditions: The following method was based initially on the differential solubility of potassium salts of AMPS in aqueous ethanol solution in PH range of 4–6.

The pH of the solvent systems was critical. Higher pH resulted in considerable tailing of AMPS spots and lower pH caused faster migration so that separation was not clearcut. Temperature increase resulted in higher R_f values. Increase in ethanol concentration slowed migration and made the spots more discrete. Optimal temperature for solvent systems #1 and #2 was 25°C and for #3 and #4 was 37°.

The addition of disodium ethylenediaminetetraacetic acid (DEDTA)

to the solvent systems caused the spots to be more uniform and increased migration of Ch-S-B specifically.

Prespotting of the paper with 10% glucose solution eliminated some of the fixation of the AMPS to the site of application.

Because of the weight of solvent on paper, Whatman 3MM withstood manipulation best. Separations of spots were most discrete when run against machine direction. Different batches of paper sometimes behaved differently and required adjustment of buffer-to-ethanol ratios to compensate for the paper effect.

Solutions: Potassium acetate (KAc)/acetic acid (HAc) buffer of pH 4.0 ± 0.05 was prepared from $0.2\,M$ solutions (90 ml KAc and 410 ml HAc diluted with water to 1 liter); 95% ethanol was used as obtained commercially. A 10% glucose solution was kept frozen until use. Standard AMPS solutions were made to a concentration of 2.5 mg/ml.

The metachromatic staining solution was 0.05% (w/v) azure A in aqueous 2% HAc (v/v). Aqueous 2% HAc was used for a clearing solution.

Solvent Systems: (#1) Acetate buffer/ethanol ratio 4/3. DEDTA was added to concentration of $0.0011\,M$. The final pH 4.80 ± 0.05. (#2) Acetate buffer/ethanol ratio 1/1. DEDTA $0.0011\,M$. Final pH was 5.00 ± 0.05. (#3) Acetate buffer/ethanol ratio 4.3/3. DEDTA $0.0011\,M$. pH 4.70 ± 0.05. (#4) Acetate buffer/ethanol ratio 1.2/1. DEDTA $0.0011\,M$. pH 6.01 ± 0.05.

AMPS Application and Development of Chromatograms: With the aid of a hair dryer 20 μl of 10% glucose solution was spotted at the site of origin and dried thoroughly. AMPS solution (5 μl containing 10–30 μg AMPS) was spotted over each glucose spot and dried. Systems #1 and #2 were used for 40 cm chromatograms, which were developed in tall battery jars by ascent of the solvent front for approximately 16 hr at 25°C. With systems #3 and #4, 5–15 μg of AMPS was applied to 15 cm papers that were developed for 4–6 hr at 37°.

The developed chromatograms were dried in an oven (90°C), stained by immersion in acidic azure A solution, dripped of excessive stain, cleared in 2% aqueous acetic acid, and dried at room temperature.

Bands of AMPS were developed for elution by streaking the AMPS along the bottom of the paper and developing the chromatograms. Sufficient AMPS for monosaccharide analysis could be eluted if the developed chromatograms were dried at room temperature instead of by oven drying, which tended to fix the AMPS to the paper. Application of glucose was omitted because it interfered with monosaccharide determination in the eluates.

TABLE 1
Colorimetric Analysis of AMPS Preparations

Standard preparation	Uronic acid				Hexosamines			
	% by wt.	Carbazole, μmole	Naphthol-resorcinol, μmole	C/N	% by wt.	Glucosamine, μmole	Galactos-amine, μmole	U/H
Ch-S-A (Good)	33%	4.2	0.32	14	26%	0.0	3.6	1.16
Ch-S-A (Dorfman)	34%	4.6	0.35	13	26%	0.0	3.6	1.28
Ch-S-B (Dorfman)	15%	2.0	4.9	0.4	27%	0.0	3.8	0.52
Heparin Na (Upjohn)	37%	4.8	0.29	17	19%	2.6	.3	1.66
Heparitin sulfate (Meyer)	38%	3.4	0.27	12	27%	2.6	0.0	1.31
C.D. 20% EtOH		2.6	3.9	0.7		0.0	6.0	.43
D.B. 20% EtOH		2.6	0.2	13		1.8	0.1	1.36
C.D. 80% EtOH		2.8	1.5	1.9		.72	1.3	1.35
D.B. 80% EtOH		4.9	0.33	15		2.52	1.3	1.28

RESULTS

The colorimetric analyses of the AMPS standards are given in Table 1. The per cent composition of uronic acid by carbazole (7) and total hexosamine are in accord with reports of others (21) using similar methods. Differential hexosamine determination (6) showed, as expected, only galactosamine in the chondroitin sulfates, and glucosamine for Hep. and H.S. The presence of iduronic acid characteristic of Ch-S-B is indicated by a low C/N ratio (9). The AMPS containing glucuronic acid have high ratios.

Ch-S-B was further characterized by a low uronic acid/total hexosamine ratio (U/H), because iduronic acid develops only about half as much color intensity with the carbazole method as does glucuronic acid (21). Heparin has been reported to give increased colors with carbazole (7). The expected increased U/H ratio was seen with the standard sodium heparin preparation.

From the urine of children with gargoylism the AMPS resulting from barium acetate-20% ethanol precipitation (20% fraction) had a monosaccharide composition similar to the standard Ch-S-B in the preparation from C.D. and to H.S. in the preparation from D.B. The preparations from 80% ethanol fractions appeared to be mixtures. This preparation from C.D. contained monosaccharide composition suggestive of Ch-S-B and H.S. and that from D.B. appeared to be H.S. and Ch-S-A.

Paper Chromatography

System #1 at 25°C separated Ch-S-B, H.S. spots from Ch-S-A, and K.S. solvent front material (Fig. 1). Commercial heparin and purified heparin have always shown separation of two spots, one of which was a long slow migration streak and the other with an R_f value identical with the H.S. preparation of Meyer. Similar results were obtained by the method of Spolter and Marx (4).

System #3 at 37°C showed similar separations of these AMPS but the H.S. spot was less distinctively separated from trailing solvent front material and Ch-S-B had a relatively higher R_f (Fig. 2).

System #2 at 25°C separated Ch-S-A as a spot discrete from solvent front migrating K.S. The other AMPS remained close to the origin (Fig. 3).

System #4 at 37°C showed K.S. to migrate as a distinct spot with all other sulfated AMPS staying close to the origin (Fig. 4). K.S. did not migrate if prespotting with glucose was omitted.

Chromatography with system #1 of the AMPS preparations from children with gargoylism showed the 20% fraction of C.D. to cochromatograph only with Ch-S-B. The 80% fraction from this patient showed

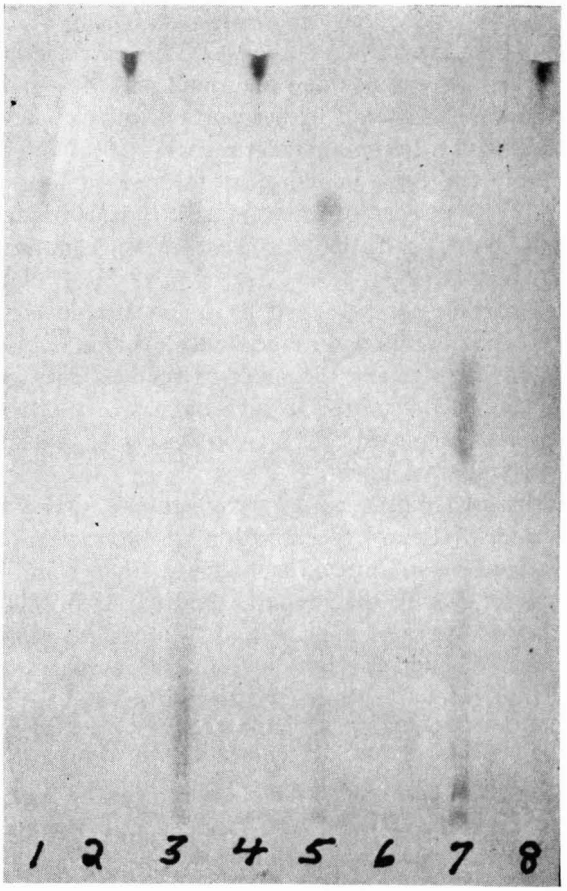

FIG. 1. Photograph of 40 cm chromatogram run at 25°C for 18 hr: (1) H.S. (Meyer), (2) Ch-S-A (Dorfman), (3) Na heparin (Upjohn), (4) K.S. (Linker), (5) H.S. (Upjohn), (6) hyaluronic acid (Sigma), (7) Ch-S-B (Dorfman), (8) Ch-S-A (Good). Buffer/ethanol, 4/3. Spots are dark purple on light blue background.

spots of Ch-S-B and H.S. with a small amount of solvent front material.

The 20% fraction of D.B. separated into a heparin streak and an H.S. spot; the 80% fraction cochromatographed with H.S. and showed solvent front material, presumably Ch-S-A. In solvent system #2 a Ch-S-A spot was seen.

Further proof of the identity of the chromatographed spots of different AMPS was obtained by applying streaks of AMPS at the origin and developing the chromatogram. The edges of the paper were cut, dried, and stained to localize the bands of AMPS. The areas corresponding to the bands were eluted with water and differential hexosamine

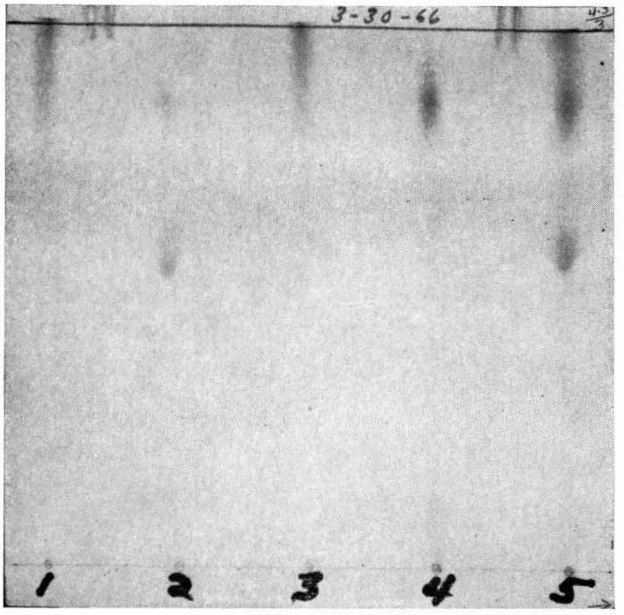

FIG. 2. 15 cm chromatogram run at 37°C for 5 hr: (1) Ch-S-A, (2) Ch-S-B, (3) K.S., (4) H.S., (5) mixture 1–4. Buffer/ethanol 4.3/3.

determinations carried out on the eluates. The eluates from the Ch-S-B band showed galactosamine and no glucosamine, from the H.S. band only glucosamine. From the AMPS preparations from urine of the children with gargoylism, the band migrating as Ch-S-B showed only galactosamine, the band migrating as H.S. showed only glucosamine, and the solvent front material showed a mixture of galactosamine and glucosamine.

DISCUSSION

The method described in this paper has been developed over the past five years with various adaptations and has been useful for identification of sulfated AMPS from different biological sources. Because the method is sensitive to variations in the conditions mentioned earlier, standard AMPS preparations are always run, even though characteristic R_f values are possible with freshly made solutions.

Ch-S-B under some circumstances has shown two spots, a very slow migrating spot and a spot that had the characteristic R_f for this preparation from any source. The exact reason for these separations was not apparent, but seemed probably related to the size of the AMPS polymer in relationship to the remaining adhering peptide. AMPS preparations from various sources contained some residual peptide, which influenced

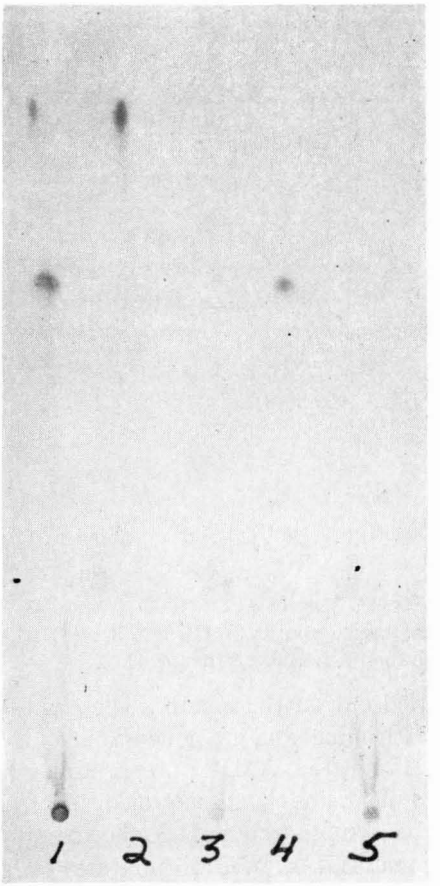

FIG. 3. 40 cm chromatogram run at 25°C for 18 hr: (1) Mixture of Ch-S-A, K.S., Ch-S-B, H.S., (2) K.S.; (3) H.S., (4) Ch-S-A, (5) Ch-S-B. Buffer/ethanol 1/1.

the migration of AMPS in these systems. Tissues from a number of biological sources (tissues from children with mucopolysaccharidosis, human umbilical artery, bovine and porcine nasal septum) have been subjected to hydrolysis with papain followed by precipitation with acetone or CTMAB and conversion to sodium salts. Spots typical of AMPS known to be present in these tissues were seen. In addition, other streaks and spots were seen which were not typical of any standard AMPS. Further extractions of these preparations with cold dilute alkali demonstrated that these streaks disappeared and spots characteristic of the AMPS appeared. These studies indicated that it was necessary for the AMPS preparations to be relatively free of covalently bonded protein for characteristic migration. The presence of other acidic muco-

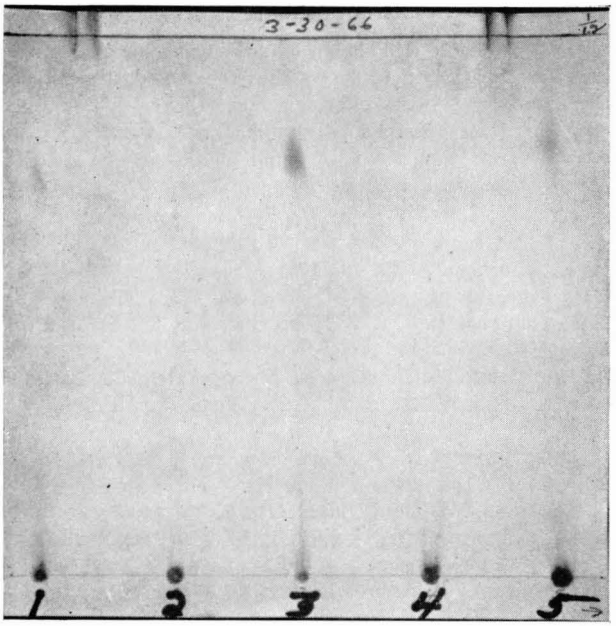

FIG. 4. 15 cm chromatogram run at 37°C for 5 hr: (1) Ch-S-A, (2) Ch-S-B, (3) K.S., (4) H.S., (5) mixture 1–4. K.S. migrates discretely. Buffer/ethanol 1.2/1.

proteins such as those containing sialic acid contaminating AMPS preparations from the urine of children (19) has not appreciably affected the AMPS migration in systems #1, 2, and 3. Divalent salts such as barium profoundly slowed migration, and presumably proteins with positive charges would affect the AMPS resolution. Versenate was introduced to reduce this intermolecular binding, but did not eliminate it, and the AMPS salts had to be monoionic for characteristic migration.

As with the method of Spolter and Marx (4), the paper has influenced migration, but this has been seen only with Ch-S-A, which on batches of newer paper gave two spots migrating closely together near the solvent front in system #1.

SUMMARY

Separation of acidic mucopolysaccharides by ascending paper chromatography has been possible in systems of acetate buffer and ethanol. The method will separate 5 to 30 μg mixtures of AMPS. Purified preparations of AMPS will give characteristic migration allowing identification of 2 μg quantities. AMPS from biological sources may be separated after removal of covalently bonded protein and divalent ions.

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The Reaction of Carbazole with Carbohydrates[1]

I. Effect of Borate and Sulfamate on the Carbazole Color of Sugars

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The carbazole reaction in H_2SO_4 is a convenient and commonly used method for the quantitative determination of hexuronic acids. The original description of this reaction by Dische (1, 2) has been modified by several authors (3, 4). All these methods require "hydrolysis" of the carbohydrates in H_2SO_4 before the development of the carbazole reaction. The temperature at which the "hydrolysis" takes place has been said to differentiate the hexuronic acids in glycosaminoglycans[3] (GAG) (5, 6). After "hydrolysis" at high temperatures, the carbazole color developed in either the presence or the absence of borate (3, 4) at room temperature (2) or in boiling water (3, 4). Green (7) working in my laboratories noted that sulfamate depressed the carbazole color with glucuronic acid to a lesser degree than that with glucose.

The following experiments were performed to study the kinetics of the carbazole reaction with hexoses and with compounds containing hexuronic acid at various temperatures and to evaluate the effect of borate and sulfamate on the sensitivity and specificity of the carbazole reaction with hexuronic acids and GAG.

MATERIALS AND METHOD

Freshly prepared, purified, commercial samples of hyaluronic acid (from vitreous humor, Worthington); chondroitin sulfate (from bovine nasal septum, Sigma); heparin, glucuronic acid, galacturonic acid, glu-

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[3] The nomenclature of Balazs and Jeanloz.

cose, galactose, and mannose (from Nutritional Biochemical Corporation); and the barium salt of mannuronic acid[4] (from Bios Laboratories) were used. β-Heparin was a gift from Dr. Roger W. Jeanloz.

The concentrated H_2SO_4 (Baker Analyzed) used in this study was 96.4%. The 1 M borate buffer, pH 8.4, was prepared by addition of potassium hydroxide to boric acid. The sulfamate solution was prepared either by adding 100 ml distilled water to 60 or 100 gm of sulfamic acid, followed by the addition of potassium hydroxide pellets until all sulfamic acid was dissolved, or by adding 100 ml of water to 60 gm of ammonium sulfamate. The latter solution was more convenient to prepare. When the effect of borate or sulfamate was evaluated, each 1 ml of carbohydrate solution contained 0.1 ml of the 1 M borate and/or 0.1 ml of the sulfamate solutions. Therefore, these carbohydrate solutions were 0.1 M borate and 0.4 M sulfamate (B & S). Five or 6 ml of the H_2SO_4 was added to 1 ml of aqueous or B & S sample. The reaction took place in 19 mm test tubes and the absorbancies were measured in a B & L Spectronic-20 or in 10 mm cuvets in a Beckman DU or Beckman DB spectrophotometer against appropriately prepared blanks. When serial dilutions of carbohydrates were studied, the slopes (b) of absorbancies (y) versus concentration (x), y on x, were calculated on the basis of five concentrations by the least-squares method:

$$b = \frac{\Sigma xy}{\Sigma x^2}$$

From b one can calculate the absorbance of the desired amount of carbohydrate or vice versa from $y = bx$ or $x = y/b$, where y = absorbance, b = slope, and x = concentration in μmoles of hexuronic acids, hexoses, and pentoses and in μg of GAG per tube.

Depending upon the type of vessel used, the temperature in the "boiling" water bath varied from 93° to 98°C. There was high humidity in the immediate vicinity of the test tubes that contained hygroscopic H_2SO_4; consequently, the final concentration of water in the H_2SO_4 could not be ascertained when these test tubes were kept in the "boiling" water bath or were cooled in a laboratory where a water bath was boiling. Therefore, all reactions took place in heated, insulated copper blocks[5] built to fit the test tubes. The temperature in these copper blocks varied ± 1°C during the reactions.

Allowing for volume changes on mixing, the final concentrations of H_2SO_4 were 84.5% in the 5:1 and 87% in the 6:1 dilutions. The samples

[4] Molecular weight of 523 was assumed.

[5] The copper blocks were built in the instrument shop of Emory University School of Medicine.

were either kept in ice water, during, and cooled by constant shaking, after, the addition of H_2SO_4 or were mixed and placed promptly in copper blocks which were set at 60° or at 100°C. The "hydrolysis" was allowed to proceed for various periods of time. The tubes were cooled and 0.2 ml of 0.2% carbazole in ethanol was added. The tubes were thoroughly mixed and the color reaction developed for different periods of time: (*1*) at 100°, 60°, or 45°C in copper blocks; (*2*) at 31°C in the thermostatically controlled cuvet housing of a Beckman DU and DB spectrophotometer; or (*3*) at 37° or 31°C in an incubator in 19 mm diameter Coleman tubes.

RESULTS

(*1*) *Effect of H_2SO_4 Concentration*

The absorbancies at 525 mμ were consistently higher when 5 ml, rather than 6 ml, of H_2SO_4 was added to 1 ml of aqueous sample. For glucuronic or hyaluronic acid the increase in absorbancies was greater; but for chondroitin sulfate and heparin this increase was less than could be

TABLE 1

Effect of H_2SO_4 Concentration on Absorbancies at 525 mμ of the Carbazole Reaction with Carbohydrates in Water and in 0.1 *M* Borate

	In water			In 0.1 *M* borate		
	6:1[a]		5:1[a]	6:1[a]		5:1[a]
Compound	37°	100°	100°	37°	100°	100°
Glucuronic acid (20 μg)	0.225	0.245	0.340	0.315	0.340	0.460
Hyaluronic acid (60 μg)	0.250	0.280	0.360	0.325	0.365	0.480
Chondroitin sulfate (60 μg)	0.240	0.255	0.280	0.310	0.350	0.350
Heparin (60 μg)	0.255	0.325	0.350	0.250	0.325	0.365

[a] H_2SO_4: aqueous sample ratios.

This was a typical experiment. Color developed at 37°C for 2 hr or at 100° for 10 min after a 15 min "hydrolysis" in H_2SO_4 at 100°. Absorbancies were measured in a Spectronic-20 spectrophotometer.

accounted for only by increased concentration of the "chromogen." The absorbancies which developed at 37°C in 2 hr were less than those that developed at 100°C in 10 min (Table 1). However, interference by neutral sugars was considerably greater when the color developed at 100° in 10 min, instead of 37° in 2 hr.

(*2*) *Stability of the Carbazole Color*

The absorbancies of the carbazole color at 525 mμ remained stable at room temperature for several hours. The absorbancy gradually de-

clined during the next 24 hr, but 95% of the original absorbancies could be recovered by heating the samples again at 100°C for 10 to 15 min.

(3) *Effect of "Hydrolysis"*

When H_2SO_4 was added to aqueous carbohydrate solutions, some heating occurred even when the tubes were immersed in ice. Nevertheless, for the purposes of this study, these tubes will be considered as "unhydrolyzed" and listed under zero-minute "hydrolysis."

The time of "hydrolysis" had a significant effect on the development of maximum absorbancies at 525 mμ after the subsequent reaction with carbazole. "Hydrolysis" increased the subsequent carbazole color of

TABLE 2
Effect of "Hydrolysis" for 0, 4, 6.5, or 12 Minutes in 84.5% H_2SO_4 at 100°C on Absorbancies[a] of Carbazole Color[b] at 525 mμ with Aqueous Carbohydrates

Compound	Duration of "hydrolysis," min			
	0	4	6.5	12
Glucuronic acid (0.1 μmole)	0.145	0.170	0.180	0.190
Galacturonic acid (0.1 μmole)	0.265	0.240	0.240	0.235
Mannuronic acid (0.1 μmole)	0.190	0.165	0.165	0.160
Heparin (100 μg)	0.415	0.390	0.390	0.380
Chondroitin sulfate (100 μg)	0.235	0.270	0.295	0.305
Hyaluronic acid (100 μg)	0.215	0.240	0.260	0.270
β-Heparin (100 μg)	0.200	0.190	0.190	0.185

[a] Absorbancies were corrected according to the least-squares estimates on the basis of 5 serial dilutions.

[b] The carbazole color was developed at 100°C in 10 minutes.

glucuronic acid, hyaluronic acid, and chondroitin sulfate. However, "hydrolysis" depressed the absorbancies of the subsequent carbazole color at 525 mμ of heparin, β-heparin, galacturonic acid, and mannuronic acid (Table 2). In the case of mannuronic acid and β-heparin no further increase in final absorbancy was obtained when the carbazole was suspended in the aqueous carbohydrate solutions, which were frozen in a mixture of dry ice and acetone, and the H_2SO_4 was added in the cold.

Twelve-minute "hydrolysis" gave maximum absorbancies for glucuronic acid, hyaluronic acid, and chondroitin sulfate when the carbazole reaction took place at 100°C in 10 min. When carbazole was added to samples that were previously "hydrolyzed" and then cooled in ice, a typical carbazole color developed at the interface. This reaction was not observed without prior "hydrolysis."

(*4*) *Effect of Temperature on the Carbazole Reaction in 84.5% H_2SO_4*

After 12 min of "hydrolysis" at 100°C and cooling, the carbazole reaction developed rapidly at 31°. In the absence of "hydrolysis": (*1*) The typical carbazole color did not develop during the first 4 hr with glucuronic acid and chondroitin sulfate. (*2*) The absorbancies of the carbazole color at 525 mμ rapidly developed with mannuronic acid, galacturonic acid, and heparin. At 4 hr their absorbancies at 525 mμ

TABLE 3

Development of Absorbancies of "Hydrolyzed" and "Unhydrolyzed" Hexuronic Acids and Glycosaminoglycans (GAG) at 525 mμ at 31°C at 1, 4, and 24 Hours after Addition of Carbazole[a]

Compound	1 hour		4 hours		24 hours	
	H	UH	H	UH	H	UH
Glucuronic acid (20 μg)	0.260	0.020	0.190	0.040	0.300	0.080
Galacturonic acid (20 μg)	0.250	0.100	0.185	0.250	0.280	0.350
Mannuronic acid (62 μg)	0.220	0.130	0.195	0.240	0.230	0.265
Heparin (50 μg)	0.180	0.070	0.140	0.150	0.220	0.240
Chondroitin sulfate (50 μg)	0.170	0.010	0.140	0.030	0.175	0.060
Hyaluronic acid (50 μg)	0.235	0.045	0.180	0.065	0.340	0.160
β-Heparin (100 μg)	0.170	0.060	0.120	0.070	0.240	0.315

[a] H = "hydrolyzed," UH = "unhydrolyzed."

To 1 ml aqueous carbohydrates 5 ml H_2SO_4 was added with cooling (UH) or was heated at 100°C for 12 min (H) and cooled; 0.2 ml of 0.2% carbazole in ethanol was added to all tubes and the color developed at 31°.

were the same or greater than their absorbancies when they were "hydrolyzed" for 12 min at 100°C before the carbazole reaction. (*3*) "Hydrolysis" of heparin did not affect the absorbancies at 525 mμ at 4 and 24 hr after the addition of carbazole. (*4*) Absorbancies of the carbazole color at 525 mμ developed slowly with "unhydrolyzed" hyaluronic acid and were one-third of the absorbancies of the "hydrolyzed" tubes (Table 3).

At 31° and 37°C, the biphasic nature of the carbazole reaction with glucuronic acid and GAG became readily apparent. The carbohydrates were "hydrolyzed" for 12 min at 100°C in 84.5% H_2SO_4, were cooled and carbazole was added.

At 31°C, the absorbancies increased rapidly and reached an early peak within 20 min. Then the absorbancies at 525 mμ began to fade and reached a low point after 5 hr. Again the absorbancies increased and reached a second peak after 72 hr. Indeed, little change occurred between 48 and 80 hr (Fig. 1). The absorption spectra of the early and late peak of absorbancies were the same as those which developed at 100°C.

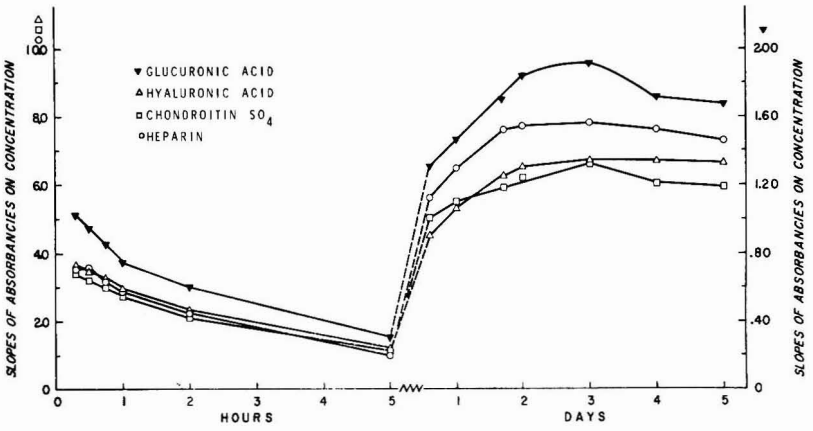


FIG. 1. Effect of time on development of absorbancies at 525 mμ at 31°C. Aqueous carbohydrates were "hydrolyzed" in 84.5% H_2SO_4 for 12 min at 100°C and cooled, and 0.2 ml of 0.2% carbazole in ethanol was added. The absorbancies for glucuronic acid were determined in the thermostatically controlled cuvet housing at 31°C of a Beckman DU spectrophotometer in glass-stoppered cuvets. The absorbancies of GAG developed at 31°C in an incubator and were determined in a B & L Spectronic-20 spectrophotometer in 19 mm Coleman cuvets.

When 6 ml, instead of 5 ml, of concentrated H_2SO_4 was added to 1 ml of aqueous hexuronic acid, a similar pattern of changes in absorbancies was observed. At 37°C, higher absorbancies developed at 2 hr in 87% H_2SO_4 (6:1 dilution) than in the 84.5% H_2SO_4 (5:1 dilution), despite the greater concentration of hexuronic acid in the latter dilution.

The transient decrease of absorbancies after the development of the first peak of the carbazole color was more marked and was more prolonged at 5°C.

The absorbancies of the second peak at 31°C were similar to those that developed at 100°. However, the development of the former took 2 to 3 days and that of the latter took 10 min. Under the conditions of the experiments, borate did not increase the rate of development of peak absorbancies at 31°. The absorption spectra of the carbazole color were similar whether these developed at 31° or at 100°C. Addition of 2

ml of water (8) after development of the carbazole reaction with hexuronic acid or GAG either at 31° or at 100°C did not abolish the color but shifted the primary absorption peak from 525 to 535 mμ.

When the carbazole reaction proceeded at 60°C, maximum absorbancies were reached in 8 hr with glucuronic acid and in 5 hr with GAG. These were higher than the maximum absorbancies that developed in 10 min at 100° and in 73 hr at 37°C.

The carbazole reaction with glucose, galactose, and mannose gave characteristic absorption spectra. All three sugars had peaks in their absorption spectra at 535, 430, 385 mμ and between 320 and 340 mμ. The relationships of these peak absorbancies with glucose, galactose, and mannose were different. The 535 mμ peak was higher for glucose, similar for galactose, and lower for mannose than their respective 430 mμ peaks. Similar observations have been made under different conditions by Seibert and Atno (9) and by Gurin and Hood (10).

(5) *Effect of Borate and/or Sulfamate*

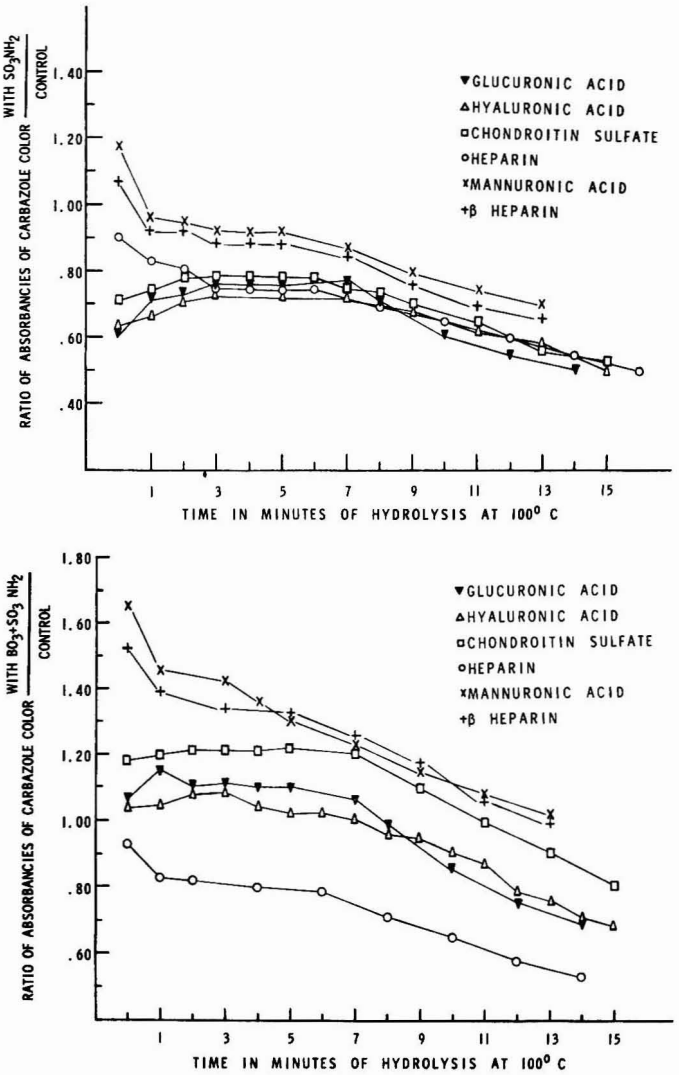
Borate depressed and sulfamate prevented the development of the carbazole color with glucose, galactose, and mannose. The inhibitory effect of sulfamate was more pronounced when these hexoses were "hydrolyzed" at 100°C before the addition of carbazole. "Hydrolysis" before the addition of carbazole had less effect on borate than on sulfamate depression of the absorbancies at 535 mμ. A typical experiment is recorded in Table 4. Sulfamate, with or without borate, had little effect

TABLE 4
Effect of Borate and/or Sulfamate on Carbazole Reaction with Hexoses

Hexose	Duration of "hydrolysis," min[a]	Per cent of control[b]		
		Borate	Sulfamate	Borate and sulfamate
Glucose (0.580)[b]	0	70	26	30
	4	—	0	0
	10	70	0	0
Galactose (0.300)[b]	0	85	30	30
	4	—	3	2
	10	75	0	0
Mannose (0.250)[b]	0	76	45	43
	4	55	16	0
	10	55	5	0

[a] Tubes were cooled on ice (i.e., zero-minute "hydrolysis") or were heated at 100°C after addition of 5 ml concentrated H_2SO_4 to 1 ml hexose in 0.1 *M* borate and/or 0.4 *M* sulfamate.

[b] Absorbancies of 100 μg of hexose at 525 mμ after a 10 min "hydrolysis" and 10 min carbazole reaction at 100°C in 19 mm cuvets in a Spectronic-20 spectrophotometer.



FIGS. 2 and 3. Aliquots (1 ml) of aqueous carbohydrates containing sulfamate (Fig. 2, *top*) and sulfamate plus borate (Fig. 3, *bottom*) were mixed with 5 ml concentrated H_2SO_4 with cooling (unhydrolyzed) or with 1 to 15 min "hydrolysis" at 100°C. The control contained no sulfamate or borate; it was "hydrolyzed" at 100° for 12 min. All tubes were cooled in ice, 0.2 ml of 0.2% carbazole in ethanol was added to each tube, and the tubes were heated for 10 min at 100°. The absorbancies at 525 mμ of sulfamate-containing tubes were divided with that of the controls. The absorbancies of the triplicates in this experiment were within 5% of each other.

on the absorbancies at 430 mμ of the carbazole-hexose reaction and increased their absorbancies at 320 mμ.

Sulfamate inhibited the development of absorbancies at 525 mμ of the carbazole reaction with glucose if it was added to the *aqueous sugar* before H_2SO_4. When sulfamate was added to glucose after heating in H_2SO_4, it depressed, but did not effectively inhibit, the development of the carbazole color.

The combination of borate and sulfamate effectively prevented the development of significant carbazole color at 525 and 535 mμ of up to 1.0 μmole of glucose, mannose, galactose, fructose, xylose, ribose, deoxyribose, fucose, sialic acid, and amino acids. (L-arginine, L-cysteine, DL-leucine, DL-serine, DL-tyrosine, β-alanine).

Borate increased the carbazole color both at 37° and at 100°C in both the 5:1 and 6:1 H_2SO_4 dilutions. The absorbancies were higher in the 5:1 than in the 6:1 diluted samples, as was the case in the absence of borate (Table 1). Borate did not increase the absorbancies of heparin in 87% H_2SO_4; but it had a significant, although small, effect in 84.5% H_2SO_4.

NaCl (0.4 to 2.1 *M*) depressed the absorbancies of the carbazole color at 525 mμ *only* with heparin. This depression occurred both in 87% and 84.5% H_2SO_4 with or without borate and/or sulfamate.

The effect of "hydrolysis" on the carbazole reaction with GAG and hexuronic acids in the presence of sulfamate with or without borate is illustrated in Figures 2 and 3.

The duration of "hydrolysis" prior to the addition of carbazole played a significant role in the development of the carbazole color at 525 mμ in the presence of sulfamate. More than 7 min of hydrolysis" progressively depressed the subsequent carbazole color. The absorbancies of the subsequent carbazole reaction did not vary significantly when the samples were "hydrolyzed" for 4 to 6.5 min (Table 5).

When the carbazole reaction was performed in the presence of borate and sulfamate and the tubes were heated for 4 or 6.5 min at 100°C before the addition of carbazole, the presence of hexoses up to 1.0 μmole per tube had no significant affect on the absorbancy of either hexuronic acids or GAG at 525 mμ. In a typical experiment the slope (b) of absorbancy on concentration from 0.03 to 0.3 μmole per tube of glucuronic acid was $b = 269$. The slope of equal mixtures of glucuronic acid and galactose in the same range was $b = 276$. The slope of equal mixtures of glucuronic acid and mannose in the same range was $b = 275$. The absorbancy at 525 mμ of the carbazole reaction of 500 μg of glycoprotein (bovine) fraction VI (Nutritional Biochemical Corporation) was reduced from 0.140 to 0.02 when the reaction occurred in the presence of borate and sulfamate after 4 or 6.5 min of "hydrolysis."

TABLE 5

Effect of "Hydrolysis" for 0, 4, 6.5, or 12 Minutes in 84.5% H_2SO_4 at 100°C on Absorbancies[a] of Carbazole Color[b] in the Presence of Borate and/or Sulfamate

Compound	Anions[c]	Duration of "hydrolysis," min			
		0	4	6.5	12
Glucuronic acid	S	0.130	0.160	0.160	0.125
(0.1 μmole)	B/S	0.230	0.260	0.260	0.180
Galacturonic acid	S	0.270	0.225	0.215	0.185
(0.1 μmole)	B/S	0.265	0.220	0.215	0.185
Mannuronic acid	S	0.185	0.150	0.145	0.125
(0.1 μmole)	B/S	0.280	0.230	0.220	0.180
Heparin	S	0.360	0.300	0.300	0.225
(100 μg)	B/S	0.375	0.310	0.310	0.230
Chondroitin sulfate	S	0.200	0.225	0.220	0.165
(100 μg)	B/S	0.340	0.355	0.350	0.275
Hyaluronic acid	S	0.205	0.230	0.230	0.165
(100 μg)	B/S	0.380	0.380	0.380	0.290
β-Heparin	S	0.175	0.155	0.150	0.125
(100 μg)	B/S	0.295	0.245	0.235	0.180

[a] Absorbancies were corrected according to the least-squares estimates on the basis of 5 serial dilutions.

[b] The carbazole color was developed at 100°C in 10 min.

[c] S = 0.4 *M* sulfamate. B/S = 0.1 *M* borate and 0.4 *M* sulfamate.

RECOMMENDED PROCEDURES

The following is the recommended procedure for routine analysis for hexuronic acids or polymers containing hexuronic acid:

(1) Prepare the carbohydrates in 0.4 *M* sulfamate and 0.1 *M* borate and place 1 ml in matched cuvets or test tubes, or pipet 0.8 ml of aqueous carbohydrate into matched cuvets or test tubes and add 0.1 ml each of 4 *M* sulfamate and 1 *M* borate and mix.

(2) Add with mixing 5 ml of concentrated H_2SO_4 (test each batch of H_2SO_4 to ensure that it does not contain oxidants; these oxidants give a green color with carbazole under the conditions of this procedure).

(3) Heat the tubes at 100°C for 6.5 min.

(4) Cool in ice water.

(5) Add 0.2 ml of 0.2% carbazole in ethanol and mix thoroughly.

(6) Heat at 100°C for 10 min.

(7) Cool in air and determine the absorbancies at 525 mμ.

The color is stable for several hours. After 16 to 24 hr, the color fades; but 95% of the original absorbancies at 525 mμ can be recovered if the tubes are heated again for 10 to 15 min at 100°C.

Occasionally, sulfamate precipitated in cold tubes. At first this resulted in a thin layer of turbidity just below the surface. The precipitate gradually involved the entire sample. After several hours, the tubes became clear again as the precipitate settled to the bottom. If the precipitate developed in curvets which could be read without transferring and disturbing the precipitate at the surface, the absorbancies of these tubes at 525 mμ were valid. However, the tubes could be heated again at 100°C for several minutes to redissolve the precipitate. The absorbancies at 525 mμ were not affected by the additional heating required to dissolve the precipitate, as these absorbancies were not significantly different from their replicates, which did not contain precipitate. Precipitation of sulfamate was promoted by agitation of cold tubes that were exposed to the atmosphere long enough to accumulate water at the surface of the 84.5% H_2SO_4. However, precipitates did not form when 5 ml of H_2SO_4 was mixed with 1 ml B & S solutions with vigorous agitation at ice-water temperatures.

Heparin reacted with carbazole in H_2SO_4 at low temperatures in the presence of borate and sulfamate under conditions in which glucuronic acid and other GAG, which were available for study, did not react. The color development was slow at 31°C. However, the carbazole color was visible with heparin in 15 min at 45° when no color had yet developed with chondroitin sulfate. Consequently, heparin and chondroitin sulfate can be independently estimated in the presence of each other by the following method:

(1) Place 1 ml of the mixture of heparin and chondroitin sulfate in 0.4 M sulfamate and 0.1 M borate in matched Coleman cuvets.

(2) To each 1 ml add 5 ml of H_2SO_4 during constant cooling in ice.

(3) Add 0.2 ml of 0.2% carbazole in ethanol and mix thoroughly.

(4) Heat the tubes for 15 min at 45°C and read the absorbancies promptly at 525 mμ.

(5) Heat the tubes for 10 min at 100°C.

(6) Cool the tubes in air and read the absorbancies at 525 mμ.

The calculations were based on the observation that, with heparin, 23% and, with chondroitin sulfate, 5% of the absorbancy that developed after 10 min at 100°C was detected after 15 min at 45°. The concentrations are calculated on the basis of two unknowns and two equations:

$$0.23H + 0.05C = A_{45} \quad (1)$$

$$H + C = A_{100} \quad (2)$$

where A_{45} = combined absorbance at 525 mμ after 15 min at 45°C,
A_{100} = combined absorbance at 525 mμ after 10 min at 100°C,

H = absorbance at 525 mμ due to heparin after 10 min at 100°C, and

C = absorbance at 525 mμ due to chondroitin sulfate after 10 min at 100°C.

The solution is:

$$H = \frac{A_{45} - 0.05A_{100}}{0.18} \quad \text{or} \quad C = \frac{0.23A_{100} - A_{45}}{0.18}$$

Under the same conditions β-heparin after 15 min at 45°C developed 13% of the absorbancies that developed after 10 min of additional heating at 100° (see also Table 6).

TABLE 6
Estimation of Heparin and of Chondroitin Sulfate in the Presence of Each Other

Chondroitin sulfate, μg	Heparin, μg	Absorbancy (A)[a] Observed A_{45}	Observed A_{100}	Calculated $H = \frac{A_{45} - .05A_{100}}{0.18}$
0	100	0.120	0.525	0.522
20	100	0.125	0.630	0.519
40	100	0.130	0.735	0.518
60	100	0.135	0.840	0.516
80	100	0.140	0.950	0.514
100	100	0.150	1.050	0.541
100	0	0.027	0.520	0.005

[a] A_{45} = absorbancy after 15 min of heating at 45°C. A_{100} = absorbancy after 10 min of heating at 100°. See text for method.

The absorbance at 525 mμ after full color development following 10 min at 100°C that is due either to heparin or to chondroitin sulfate in the presence of each other can be separately calculated:

$$0.23H + 0.05C = A_{45} \quad (1)$$

$$H + C = A_{100} \quad (2)$$

where H = absorbance at 525 mμ of heparin after 10 min at 100°C, and C = absorbance at 525 mμ of chondroitin sulfate after 10 min at 100°. Solving the two equations simultaneously by determinants:

$$H = \frac{A_{45} - 0.05A_{100}}{0.18} \quad \text{or} \quad C = \frac{0.23A_{100} - A_{45}}{0.18}$$

DISCUSSION

The recommended procedures for the carbazole reaction require prior heating of GAG or hexuronic acids in a "boiling" water bath for 20 min (2), 15 min (3) or 10 min (4). The optimum time for "glucurone, chondroitin sulfate, or hyaluronic acid" was said to be 15–20 min,

whereas, with borate the time was reduced to 10 min (3). This heating was thought to "hydrolyze" the polymers (2–4). The observations in this paper are not compatible with the suggestion that the GAG, but not the hexuronic acids, need to be "hydrolyzed" before the carbazole reaction can take place. For the sake of brevity the word "hydrolysis" is used in this paper to mean heating the carbohydrates in H_2SO_4 before the carbazole reaction; it does not mean hydrolysis of GAG to liberate hexuronic acids.

The carbohydrates that gave higher absorbancies with carbazole in H_2SO_4 at 100°C without "hydrolysis" than with "hydrolysis" also reacted readily with carbazole at 31° without prior heating. This pattern of reaction with carbazole is similar to that described by Dische (6), where the carbohydrates were "hydrolyzed" in about 81% H_2SO_4 for 90 sec at 60°C. In that procedure, as described in this report, glucuronic acid and chondroitin sulfate A and C at lower temperatures developed little absorbancy with carbazole without prior vigorous heating.

The behavior of heparin and galacturonic acid was similar in these experiments, as has been noted in other studies (8). The exception was that 0.4 to 2.1 *M* NaCl depressed the carbazole color only with heparin. Mannuronic acid and β-heparin also reacted in an analogous manner under the conditions of these experiments, although iduronic, rather than mannuronic, acid has been isolated from β-heparin (chondroitin sulfate B) (11).

The carbazole reaction with hexuronic acids and GAG is a biphasic one. When the carbazole color is measured after a 2 hr incubation at 31°C or "room temperature," the absorbancy of the first reaction is determined. When the color is developed for 10 min at 100°, the second reaction is measured. When "unhydrolyzed" galacturonic, mannuronic acid, heparin, or β-heparin reacted with carbazole at 31°, the first component of the biphasic carbazole reaction was missing and the absorbancies of the second reaction were higher than that of the "hydrolyzed" controls.

The reported abolition of the carbazole color with hexuronic acids or GAG after the addition of 2 ml of water (8) was not observed.

SUMMARY

The reaction of carbazole with hexuronic acids or with polymers containing hexuronic acid in H_2SO_4 develops more rapidly and has greater color when it proceeds at higher temperatures. However, nonspecific interference due to sugars is greater at higher temperatures. Borate increases the carbazole color with hexuronic acids and with glycosaminoglycans (GAG), but not with galactose and mannose.

Sulfamate supresses the carbazole reaction with hexoses under conditions in which it may or may not depress the color yield with hexuronic acids or GAG. The combination of borate and sulfamate increased the sensitivity and assured the specificity of the carbazole reaction with hexuronic acids. The duration of heating prior to the carbazole reaction is critical.

The difference in the kinetics of the carbazole reaction permits the separate estimation of heparin and chondroitin sulfate A and C in the presence of each other.

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The Reaction of Carbazole with Carbohydrates[1]

II. Effect of Borate and Sulfamate on the Ultraviolet Absorption of Sugars

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When hexoses or hexuronic acids were heated in H_2SO_4, absorption peaks developed in the ultraviolet (1). When glucuronic acid was heated in H_2SO_4, an absorption peak developed at 300 mμ (2, 3). It has been suggested that this peak was caused by formation of 5-formylfuroic acid (2). This compound was thought to be the "chromogen" that reacted subsequently with carbazole to give an absorption peak at 525 mμ. Hexoses in hot H_2SO_4 gave a product with a spectrum like that of furfural (1, 2) and developed absorbancies at 525 mμ when carbazole was added (4, 5), although peak absorbancies were 10 to 15 mμ higher (4, 6). In our studies the development of the absorbance at 525 mμ after addition of carbazole to hexoses and pentoses in hot H_2SO_4 was prevented by borate and sulfamate (4).

The following experiments were performed to study the relationship between the ultraviolet absorbancies of carbohydrates in H_2SO_4 and the development of the carbazole reaction. This information may allow us to develop methods for the independent estimation of both hexose and hexuronic acid, or their polymers, in the presence of each other by a single, simple procedure.

MATERIALS AND METHOD

The material and methods used were the same as described in the accompanying paper (4). The exceptions were that: (*1*) β-heparin (Ro 1-2232/715) was a gift of W. E. Scott of Hoffmann-La Roche, Inc., and (*2*) chondroitin sulfate was prepared from bovine nasal cartilage

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after papain digestion according to Meyer *et al.* (7) and Seno *et al.* (8) and 20% ethanol-precipitable calcium chondroitin sulfate was used.

Unless otherwise stated, all carbohydrate solutions were freshly prepared from samples kept several days in a vacuum desiccator. The solutions were made by dissolving the carbohydrates in water (aqueous) or in 0.1 *M* borate and 0.4 *M* sulfamate (B & S) (4). Both solutions were stable for at least 14 days at 4°C. H_2SO_4 (5 ml) was added to each 1 ml of carbohydrate in water or in B & S; thus the reactions took place in 84.5% H_2SO_4. Unless otherwise stated, for the development of ultraviolet absorbancies aqeous carbohydrates were heated at 100°C for 12 min in 84.5% H_2SO_4 and carbohydrates in B & S were heated for 6.5 min at 100° in 84.5% H_2SO_4.

To make the data compact, yet adequately descriptive, slopes were calculated by the least-squares method rather than presenting absorbancies for specific concentrations or series of graphic illustrations. Each experiment consisted of serial dilutions from 0.025 to 0.3 μmole of hexuronic acid or hexose or 20 to 200 μg of glycosaminoglycans (GAG) per tube. The slopes were based on five points not varying more than 5% from the slope. Since the lines crossed zero both on the abscissa and the ordinate, the following formula was used:

$$b = \frac{\Sigma xy}{\Sigma x^2}$$

where b = slope, x = concentration (i.e., μmoles of hexose or hexuronic acid or μg of GAG per tube), and y = absorbancy. The reproducibility of the data on samples prepared days or weeks apart is described by the standard deviations of the means of the slopes. From the slope one can readily recover the original data by the equation $y = bx$ or $x = y/b$, assuming a value for either x or y, respectively.

Some experiments were based on triplicate analysis. Such experiments were repeated four or more times and were included in this report only if their variance was not larger than that reported for the slopes.

The absorbancies were determined in a Beckman DB spectrophotometer and the absorption spectra were recorded on a Photovolt Varicord Model 43 linear/log recorder.

RESULTS

When aqueous hexuronic acids and GAG were heated in H_2SO_4, a single ultraviolet absorption peak at 298 mμ developed with aqueous hexuronic acids; but several additional absorption peaks developed with aqueous GAG (Fig. 1A). However, a single absorption peak at 318 mμ developed in hot H_2SO_4 with hexuronic acids and GAG in B & S. The

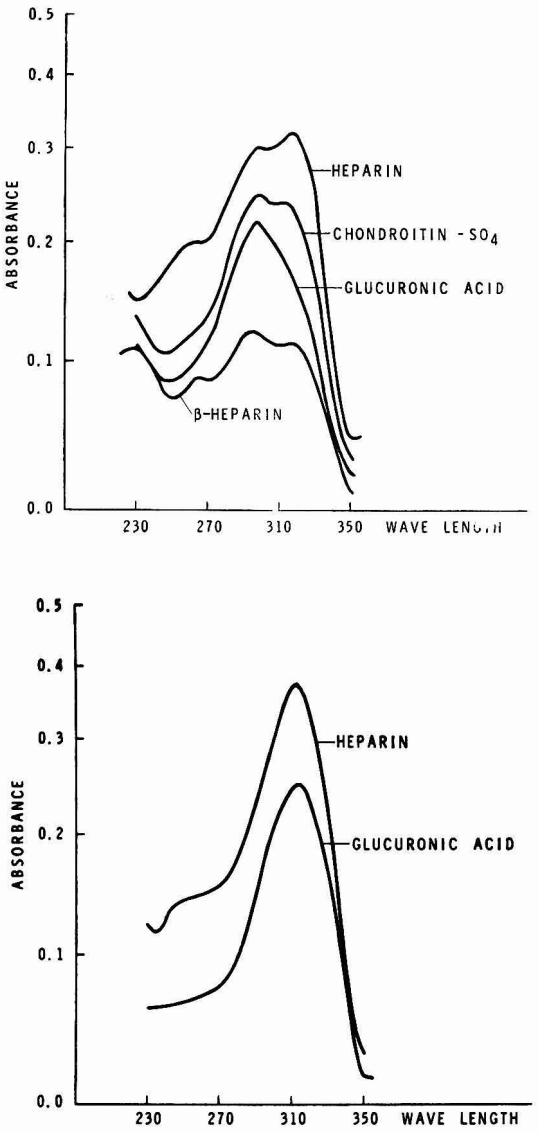

FIG. 1. Spectra of glucuronic acid and heparin (A, *top*) after 12 min "hydrolysis" in 84.5% H_2SO_4 at 100°C, and (B, *bottom*) after 4 min "hydrolysis" in 84.5% H_2SO_4 at 100° in the presence of borate and sulfamate. In the presence of borate and sulfamate the GAG had a single major absorption peak which was similar to that of hexuronic acids. The absorption spectra of the other GAG were the same as that of heparin only in the presence of borate and sulfamate (condition B). The absorption spectra of galacturonic and mannuronic acids were the same as that of glucuronic acid both under conditions A and B.

spectra of glucuronic acid and heparin shown in Figure 1B are typical examples.

The absorbancies were linear with concentration in aqueous carbohydrates at 298 mμ and in B & S at 318 mμ (both at 100°C in 84.5% H_2SO_4) and at 525 mμ after the carbazole reaction (Fig. 2).

The duration of heating had a significant effect on the development of these absorbancies. Absorbancies at 318 mμ of hexuronic acids and GAG in B & S increased during the first 7 min of heating in H_2SO_4. Heating for 10 additional minutes did not significantly increase the

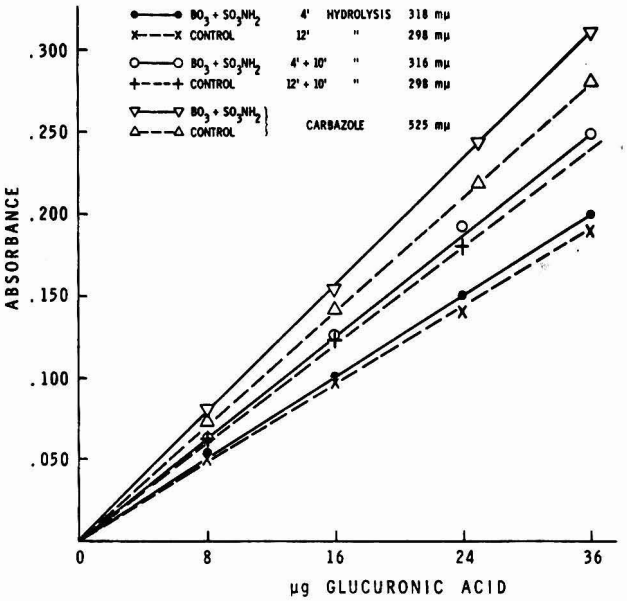

FIG. 2. Glucuronic acid (Na, monohydrate, 8 to 36 μg) was "hydrolyzed" in 84.5% H_2SO_4 at 100°C for 12 min in aqueous carbohydrates and for 4 min in B & S. The absorbancies were determined at 298 and 318 mμ, respectively; 0.2 ml of 0.2% carbazole in ethanol was added to one set of tubes and 0.2 ml of ethanol to another set. All tubes were heated at 100°C for 10 min, cooled, and the absorbancies determined at their respective absorption peaks.

absorbancies at 318 mμ. However, heating more than 7 min depressed the absorbancies at 525 mμ of the subsequent carbazole reaction. Under the same conditions, the absorbancies at 318 mμ began to decline when galactose or mannose in B & S was heated over 7 min. Typical examples are given in Table 1.

When heat production was prevented during the addition of H_2SO_4, two types of reactions were observed: (*1*) the absorbancies with glu-

curonic acid, hyaluronic acid, and chondroitin sulfate in the ultraviolet were less than 5% of that which developed in hot H_2SO_4; (*2*) the absorbancies with galacturonic acid, mannuronic acid, heparin, β-heparin, galactose, mannose, and fucose were more than 20% of that which developed in hot H_2SO_4.

When carbohydrates in B & S were heated in H_2SO_4 from 1 to 20 min, the absorption peak was at 318 mμ for glucuronic acid, galacturonic acid, mannuronic acid, hyaluronic acid, heparin, chondroitin sulfate, β-heparin, glucose, and galactose. The absorption peaks were at 306 mμ for mannose and at 328 for fucose. No ultraviolet absorbancy developed with

TABLE 1

Effect of Duration of Heating Hexuronic Acids, Hexoses, or GAG in 84.5% H_2SO_4 on Absorbancies at 318 mμ and on the Subsequent Carbazole Color at 525 mμ

Minutes at 100°C	Glucuronic acid, 0.15 μmole		Galactose, 0.3 μmole		Hyaluronic acid, 130 μg	
	318 mμ	525 mμ	318 mμ	525 mμ	318 mμ	525 mμ
2	0.170	0.360	0.860	0.020	0.280	0.530
4	0.260	0.390	0.890	0.010	0.385	0.510
5	0.275	0.390	0.880	0.005	0.405	0.510
6	0.300	0.395	0.890	0.005	0.440	0.510
7	0.320	0.400	0.885	—	0.440	0.510
8	0.330	0.370	0.850	—	0.440	0.500
9	0.320	0.340	0.830	—	0.440	0.485
10	0.320	0.310	0.820	—	0.430	0.475

The carbohydrates were in 0.1 *M* borate and 0.4 *M* sulfamate. H_2SO_4 (5 ml) was added to each 1 ml carbohydrate, heated at 100°C for 2 to 10 min, cooled in ice, and read at 318 mμ; 0.2 ml of 0.2% carbazole was mixed with each and after 10 min at 100° the absorbancies were read at 525 mμ.

sialic acid, 2-deoxyribose, serine, or *N*-acetylhexosamines. However, when aqueous glucosamine·HCl was heated in 84.5% H_2SO_4, five absorption peaks developed in the ultraviolet. The absorbancy of glucosamine·HCl at 318 mμ was not affected by borate and sulfamate.

The maximum absorbancies in the ultraviolet were significantly greater when the H_2SO_4 was added to carbohydrate in B & S rather than to aqueous carbohydrates under the same conditions.

The means and the standard deviations were computed for replicate determinations of the slopes of absorbancies versus concentrations for hexuronic acids and GAG (Table 2) and for hexoses, pentoses, hexosamines, amino acids, etc. (Table 3). The maximum absorbancies at 318 mμ (A_{318}) in 84.5% H_2SO_4 were: (*1*) less than the absorbancies of the carbazole color at 525 mμ (A_{525}) with glucuronic acid, galacturonic acid, hyaluronic acid, and chondroitin sulfate; (*2*) the reverse with man-

TABLE 2
Absorbancies at 318 mμ and the Carbazole Color at 525 mμ of Hexuronic Acids and GAG in B & S[a]

Compound	N	A_{318}/A_{525}	Slopes (mean ± S.D.)[b] 318 mμ	525 mμ	ε
Glucuronic acid	14	0.76	2.05 ± 0.05	2.68 ± 0.11	1.53×10^4
Galacturonic acid	7	0.94	2.00 ± 0.07	2.13 ± 0.14	1.21×10^4
Mannuronic acid[c]	7	1.08	2.31 ± 0.09	2.21 ± 0.11	1.27×10^4
Hyaluronic acid	7	0.89	3.42 ± 0.16	3.84 ± 0.18	—
Chondroitin sulfate	7	0.90	3.28 ± 0.08	3.62 ± 0.17	—
Heparin	7	1.39	4.39 ± 0.12	3.15 ± 0.20	—
β heparin	7	1.09	2.64 ± 0.06	2.41 ± 0.08	—

[a] ε = molar absorbance at 525 mμ, N = number of sets of determinations. The carbohydrates were in 0.1 M borate and 0.4 M sulfamate. H_2SO_4 (5 ml) was added to 1 ml of carbohydrate, heated at 100°C for 6.5 min, cooled, and read in a Beckman DB spectrophotometer in a 10 mm cuvet at 318 mμ (A_{318}); 0.2 ml of 0.2% carbazole in ethanol was added with mixing and the tubes were heated at 100° for 10 min, cooled, and read at 525 mμ (A_{525}) as above.

[b] The slopes (b) were calculated by the least-squares method on the basis of five points. Each tube contained from 0.025 to 0.3 μmole of hexuronic acid or 20 to 200 μg of GAG. From the slope the original data can be calculated as $y = bx$ (absorbancy = slope × concentration).

[c] The molecular weight of barium mannuronate was assumed to be 523.

TABLE 3
Absorbancy at 318 mμ of Hexoses, Pentoses, Amino Acids, and Some Other Compounds in B & S after 6.5 Minutes at 100°C in 84.5% H_2SO_4

Compound	N[a]	Slopes[b] at 318 mμ ± S.D.
D-Glucose	7	4.00 ± 0.11
D(+)-Galactose	7	2.98 ± 0.09
D(+)-Mannose[c]	7	1.47 ± 0.07
D-Levulose	5	2.96 ± 0.09
D(−)-Ribose	4	1.50 ± 0.06
D(+)-Xylose	5	2.64 ± 0.07
D-2-Deoxyribose	—	0
L-Fucose[d]	5	2.21 ± 0.09
Sialic acid	—	0
DL-Serine	—	0
D-Glucosamine · HCl	4	0.13
N-Acetylglucosamine	—	0

[a] N = number of sets of determinations.

[b] The slope was calculated by the least-squares estimate on the basis of four dilutions up to 0.3 μmole per tube (see text).

[c] Maximum absorbancy for mannose, 305 mμ, $b = 160 \pm 8$.

[d] Maximum absorbancy for fucose, 328 mμ, $b = 258 \pm 9$.

nuronic acid, β-heparin, and heparin. The ratio of these two absorbancies (A_{318}/A_{525}) for mannuronic acid was the same as that for β-heparin. The absorbancy at 318 mμ of heparin was about 40% higher than that of the subsequent carbazole reaction at 525 mμ ($A_{318} = 1.39A_{525}$). The absorbancy of hyaluronic acid and chondroitin sulfate at 318 mμ was about 10% lower than the corresponding absorbancies of the subsequent carbazole color at 525 mμ ($A_{318} = 0.9A_{525}$).

When hexoses in B & S were heated in 84.5% H_2SO_4, the absorbancy of galactose at 318 mμ was twice that of mannose, was the same as that of fructose, and was 75% that of glucose. The maximum ultraviolet absorbance of mannose, however, was at 305 mμ, where the absorbance

TABLE 4
Effect of NaCl on Absorbancies of Heparin and Glucosamine · HCl

Compound	Amount	318 mμ	525 mμ
Heparin, B & S[a]	150 μg	0.675	0.475
Heparin, B & S, in 1.2 *M* NaCl	150 μg	0.595	0.355
Glucosamine · HCl, B & S	1 μmole	0.130	0
Glucosamine · HCl, B & S, in 1.2 *M* NaCl	1 μmole	0.090	0

[a] B & S = in 0.1 *M* borate and 0.4 *M* sulfamate.

To 1 ml of carbohydrate 5 ml H_2SO_4 was added and heated at 100°C for 6.5 min, cooled, and read at 318 mμ; 0.2 ml of 0.2% carbazole in ethanol was added, mixed, and heated at 100° for 10 min, cooled, and read at 525 mμ.

was 10% higher than at 318 mμ. No ultraviolet absorbancies were found with D-2-deoxyribose, sialic acid, *N*-acetylglucosamine, or serine under the same conditions (Table 3). None of the compounds in Table 3 developed a carbazole color up to 1 μmole under the same conditions as the compounds in Table 2.

With the exception of heparin, NaCl had no effect on the absorbancies either in the ultraviolet or following the carbazole reaction at 525 mμ of other hexoses, hexuronic acids, or GAG either aqueous or in B & S when placed in 0.4, 1.2, or 2.1 *M* NaCl and heated in 84.5% H_2SO_4. In all three concentrations, NaCl depressed the carbazole color 33% with aqueous heparin and 25% with heparin in B & S (Table 4). NaCl depressed the 298 mμ peak but did not affect the 316 or 265 mμ peaks of aqueous heparin; however, it depressed 12% of the 318 mμ peak of heparin in B & S. The absorbancies of glucosamine·HCl at 318 mμ were depressed by 0.4 to 2.1 *M* NaCl. This was the case whether glucosamine-HCl was aqueous or in B & S.

When equal amounts of glucuronic acid and galactose or glucuronic acid and mannose in B & S were mixed and heated at 100°C for 6.5

TABLE 5
Absorbancies at 318 mμ of Glucuronic Acid (Gl-UA), Galactose (Gal), and Mannose (Man) in B & S alone and in Combination after 6.5 Minutes at 100°C in 84.5% H_2SO_4

μmole of each per tube	Gl-UA (1) obs.	Gal (2) obs.	Man (3) obs.	Gl-UA + Gal (4) obs.	Gl-UA + Gal (1 + 2) theor.	Gl-UA + Man (5) obs.	Gl-UA + Man (1 + 3) theor.
0.06	0.120	0.175	0.090	0.295	0.295	0.215	0.210
0.12	0.245	0.350	0.185	0.600	0.595	0.425	0.430
0.15	0.310	0.440	0.230	0.735	0.750	0.550	0.540
0.18	0.380	0.525	0.270	0.890	0.905	0.640	0.650
0.24	0.500	0.710	0.350	—	—	—	—
0.30	0.630	0.890	0.450	—	—	—	—
b[a]	2.09	2.95	1.49	4.94	5.04	3.59	3.58

[a] b = slope (see text).

min, the absorbancies at 318 mμ of the combinations were not different from those of the sum of the individual sugars (Table 5). When increasing amounts of glucuronic acid in B & S were mixed with decreasing amounts of hexose in B & S, the absorbancies at 318 mμ were within 5% of theoretical after 6.5 min at 100°C in 84.5% H_2SO_4. The absorbancies due to glucuronic acid at 525 mμ after the subsequent carbazole reaction were not affected significantly by the presence of hexose. An example with glucuronic acid and galactose is illustrated in Table 6.

The absorbancy at 318 mμ which developed when glucuronic acid in B & S was heated in 84.5% H_2SO_4 was 76% of that which developed at

TABLE 6
Absorbancies[a] of Galactose and Glucuronic Acid in B & S at 318 mμ and at 525 mμ in Inverse Concentrations

μmoles of galactose + glucuronic acid	318 mμ obs.	318 mμ theor.[b]	525 mμ obs.	525 mμ theor.[c]
0.24 + 0.06	0.805	0.830	0.165	0.160
0.18 + 0.12	0.735	0.770	0.315	0.321
0.12 + 0.18	0.740	0.730	0.485	0.482
0.06 + 0.24	0.645	0.675	0.655	0.643
b[d]	—	—	2.71	2.68

[a] Absorbancies were measured at 318 mμ after 6.5 min at 100°C and at 525 mμ after addition of 0.2 ml of 0.2% carbazole in ethanol to each tube and color development at 100° for 10 min.

[b] Calculated from Table 5.

[c] Calculated from Table 2.

[d] b = slope (see text).

525 mμ with the subsequent carbazole reaction ($A_{318} = 0.76A_{525}$). Under these conditions the absorbancies at 318 mμ of the combinations of glucuronic acid and hexose were the sum of their respective absorbancies. Hexoses do not give a carbazole color under these conditions. When 76% of the absorbancy of the carbazole color at 525 mμ is subtracted from the absorbancy at 318 mμ before the carbazole reaction, the remainder of the absorbancy at 318 mμ is not due to the glucuronic acid. To test this hypothesis equal amounts of glucuronic acid and galactose (Table 7) or glucuronic acid and mannose (Table 8) in B & S, separately and

TABLE 7
Estimation of Galactose in Equal Mixtures of Galactose (Gal) and Glucuronic Acid (Gl-UA) in B & S[a]

	Absorbancies								
	318 mμ						525 mμ	
μmole of each per tube	(1) Gl-UA + Gal obs.		(2) Gl-UA calc. from (6)		(3) Gal theor.	(4) Gal obs.	(5) Gl-UA obs.	(6) Gl-UA + Gal obs.
0.06	0.300	−	0.130	=	0.170	0.175	0.165	0.170
0.12	0.605	−	0.250	=	0.355	0.350	0.325	0.330
0.15	0.735	−	0.320	=	0.415	0.440	0.405	0.420
0.18	0.880	−	0.370	=	0.510	0.525	0.480	0.490
b	4.92	−	2.09		2.84	2.92	2.69	2.76

[a] The absorbance of galactose in mixture of galactose and glucuronic acid was calculated by subtracting from the absorbancy observed at 318 mμ after 6.5 min at 100°C in 84.5% H_2SO_4 76% of the absorbancy observed at 525 mμ after the carbazole reaction (i.e., Gal $A_{318} - 0.76A_{525}$). The absorbancies were measured at 318 mμ after 6.5 min at 100°C in 84.5% H_2SO_4 and at 525 mμ after the carbazole reaction. *b* = slope (see text).

in combination, were heated in 84.5% H_2SO_4 at 100°C for 6.5 min. The absorbancies were measured at 318 mμ (A_{318}); 0.2 ml of 0.2% carbazole in ethanol was mixed with each tube and heated at 100° for 10 min. After cooling, the absorbancies at 525 mμ (A_{525}) were determined. The absorbancies at 525 mμ of the combinations were not different from that of glucuronic acid alone (columns 5 and 6, Tables 7 and 8). Seventy-six per cent of absorbancy at 525 mμ (A_{525}) was the absorbancy of glucuronic acid at 318 mμ (Gl-UA, A_{318}) under these conditions (column 2, Tables 7 and 8). When 76% of A_{525} is subtracted from the observed absorbancy at 318 mμ of the combination of glucuronic acid and hexose, it gives the calculated absorbancy at 318 mμ due to galactose (Gal, A_{318}), (A_{318}–$0.76A_{525}$ = Gal A_{318}), or mannose (Man, A_{318}), (A_{318}–$0.76A_{525}$ = Man A_{318}), under these conditions (column 3, Tables 7 and 8). These theoretical absorbancies were within 5% of the observed absorbancies

TABLE 8
Estimation of Mannose in Equal Mixtures of Mannose (Man) and Glucuronic Acid (Gl-UA) in B & S[a]

	Absorbancies					
	318 mμ				525 mμ	
μmole of each per tube	(1) Gl-UA + Man obs.	(2) Gl-UA calc. from (6)	(3) Man theor.	(4) Man obs.	(5) Gl-UA obs.	(6) Gl-UA + Man obs.
0.06	0.215	0.133	0.082	0.09	0.165	0.175
0.12	0.425	0.250	0.175	0.185	0.325	0.330
0.15	0.530	0.310	0.220	0.230	0.405	0.405
0.18	0.640	0.380	0.260	0.270	0.480	0.500
b	3.55	2.10	1.45	1.52	2.69	2.75

[a] The absorbance of mannose in mixture of mannose and glucuronic acid was calculated by subtracting from the absorbancy observed at 318 mμ after 6.5 min at 100°C in 84.5% H_2SO_4 76% of the absorbancy observed at 525 mμ after the carbazole reaction (i.e., Man $A_{318} - 0.76A_{525}$). b = slope (see text).

at 318 mμ of galactose (column 4, Table 7) or mannose (column 4, Table 8).

DISCUSSION

Too little is known about the effect of borate (3) or sulfamate on carbohydrates in hot H_2SO_4 to warrant speculation on their mechanism of action. The shift of the ultraviolet spectra of hexuronic acids, hexose, and GAG in H_2SO_4 by the presence of borate and sulfamate offers a valuable analytical tool since the carbazole reaction has been made specific for hexuronic acids by borate and sulfamate. The relationship between the maximum ultraviolet absorbancies in H_2SO_4 and the carbazole color at 525 mμ was not the same for glucuronic acid and for polymers containing glucuronic acid. This ratio was of a similar order of magnitude for glucuronic acid (0.76), for hyaluronic acid (0.89), and for chondroitin sulfate (0.9); but the ratio was considerably higher (1.39)) for heparin (Table 2). Some of the differences between the reactivity of the sugars in the free form versus the reactivity in the polysaccharides may be due to the glycosidic linkages. The great difference between the ratios with glucuronic acid and heparin again emphasizes the atypical reactivity of heparin which has been noted by others (3, 9). However, the presence of *N*-sulfated glucosamine in heparin may contribute to the anomalous behavior when compared to other GAG-containing *N*-acetylhexosamines. The effect of NaCl on heparin and glucosamine·HCl, but not on *N*-acetylglucosamine, lends support to this suggestion. The higher A_{318}/A_{525} ratios of chondroitin sulfate and hyal-

uronic acid were probably due to the presence of galactose and/or xylose in the region of linkage of the polysaccharide chain to peptide (10, 11).

SUMMARY

Hexuronic acid and hexose can be independently estimated in mixture with each other by the carbazole reaction in the presence of borate and sulfamate.

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Enzymatic Determination of Oxalic Acid-C^{14} in Plant Material

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Older methods of determination of oxalic acid in plant material consist of many analytical steps and are thus laborious. In 1957 Shimazono and Hayaishi (1) described an enzyme from wood-rot fungus *Collybia velutipes,* which catalyzes the reaction:

$$\begin{matrix}\text{COOH}\\|\\\text{COOH}\end{matrix} \rightarrow \text{HCOOH} + \text{CO}_2$$

The enzyme, specific for oxalic acid, has since been used for determining oxalic acid in plasma (2), in beer (3), and in urine (4, 5). In the present paper it is shown that this enzyme can be used for rapid and accurate measurement of the amount and specific activity of total and water-soluble oxalic acid in plant material.

EXPERIMENTAL

Enzyme. Collybia velutipes (strain S) was grown and harvested, and the enzyme was prepared through "the first acetone fraction" according to the procedure described by Shimazono and Hayaishi (1). "The first acetone fraction" was dissolved in 0.4 *M* potassium citrate buffer, pH 5.0. This enzyme solution was stable for several weeks at 5°C.

Plant Material. Spinach plants (*Spinacia oleracea* L.) grown in sand culture (three plants in one pot) were placed in a growing chamber. A partial vacuum was created in the chamber by means of an aspirator and 10 μC of CO_2 was drawn into the chamber to an initial concentration of CO_2 of about 0.05%. Illumination was maintained for 2 hr at a level of 10,000 lux. Air was then drawn through the chamber and exhausted in a ventilated hood for 1 hr, in order to reduce any remaining $C^{14}O_2$. The plants were removed for analysis; the leaves were washed in water, gently blotted, and dried at 100–105°C for 20 hr; the dried spinach material was ground to a fine powder.

Extraction of Oxalic Acid. For determination of total oxalic acid, 50–100 mg of spinach powder was extracted with 2 ml 3.0 *N* HCl for 1 hr at 60°C. The mixture was then passed through a glass filter. The clear solution was cooled in an ice-water bath for 10 min and a stream of air was bubbled through the clear solution for 10 min to wash out any dissolved $C^{14}O_2$. For determination of water-soluble oxalic acid, 50–100 mg was extracted with 2.0 ml water. It was treated in the way mentioned above and 0.4 ml was used for the determination.

Manometric Method. The carbon dioxide evolved through decarboxylation of oxalic acid was determined by the conventional Warburg technique at 37°C. The enzyme was placed in the main chamber of a Warburg flask along with 600 μmoles of potassium citrate buffer, pH 5.0.

TABLE 1
Experimental Protocol Showing the Calculations Carried Out

Determination of	Total oxalic acid		Water-soluble oxalic acid	
Spinach extracted, mg	83.6		60.2	
Hyamine added	—	+	—	+
CO_2 volume evolved				
Total, μl	224	15	215	13
Difference, μl	209		202	
Difference, μmole	9.33		9.02	
Oxalic acid in spinach dry matter, gm/ 100 gm	10.25[a]		6.87	
Activity of CO_2 evolved, cpm		1196		1302
Specific activity of oxalic acid, cpm/10 μmoles		2564[b]		2887

[a] $9.33 \times \frac{90.04}{8.36 \times 10^3} \times \frac{100}{98} \times 100 = 10.25.$

[b] $1196 \times \frac{10}{9.33} \times 2 = 2564.$

The substrate was placed in the side arm with 600 μmoles of hydrochloric acid. After temperature equilibration for 15 min, enzyme and substrate were mixed, giving a final pH value of 3.2 in a total volume of 2.2 ml. Readings were made every 10 min until the reaction was complete, usually within 1 hr. Two Warburg flasks were prepared for each determination; in one of them the gas exchange was measured; in the other the evolved $C^{14}O_2$ was trapped in 50 μl 1 *M* Hyamine hydroxide in methanol entirely absorbed on a 2×5 cm piece of Whatman No. 1 paper. This flask was shaken another 15 min after the reaction was ended to make sure that $C^{14}O_2$ was quantitatively absorbed. This second flask was also used as a control vessel (Table 1).

Liquid Scintillation Counting. The paper from the Warburg flask was carefully removed from the center well and dropped directly into a counting vial containing 1 ml 1 *M* Hyamine hydroxide in methanol and 15 ml toluene containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-2′-(5′-phenyloxazolyl) benzene. The counting vial was shaken for 10 min to extract the $C^{14}O_2$ absorbed on the paper. Then the vial was counted in a Packard Tri-Carb liquid scintillation spectrometer model 3101 with an efficiency of 70% (determined by internal hexadecane-C^{14} standard).

RESULTS AND DISCUSSION

Stoichiometry of the Reaction. In Figure 1 the rate of oxalic acid decarboxylation of 10 μmoles of standard calcium oxalate is shown. Table 2 shows the relationship between the amount of sodium oxalate

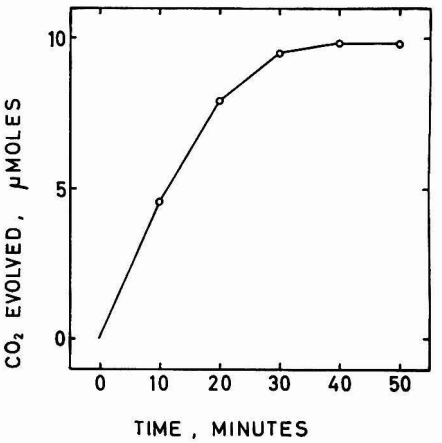

FIG. 1. Rate of oxalic acid decarboxylation of 10 μmoles of calcium oxalate.

added to the Warburg flasks and the amount of carbon dioxide evolved. It is evident that the observed carbon dioxide yields from pure oxalates are less than the expected value, or on the average 98%. Thus all values

TABLE 2

Relationship between Amount of Sodium Oxalate and Evolved Carbon Dioxide (each figure represents the mean value from Two Analyses)

Oxalic acid added, μmoles	CO_2 evolved, μmoles	Recovery, %
2.50	2.42	96.8
5.00	4.87	97.4
7.50	7.43	99.1
10.00	9.86	98.6
		Mean: 98.0

reported below have been corrected by multiplication by 100/98. Such a deviation is usually obtained with most enzymic methods using the Warburg technique (6).

Liquid Scintillation Counting. To test the reliability of the trapping and counting technique, aliquots of standard oxalic acid-C^{14} were pipetted into the side arm of Warburg flasks and carrier oxalic acid was added to a

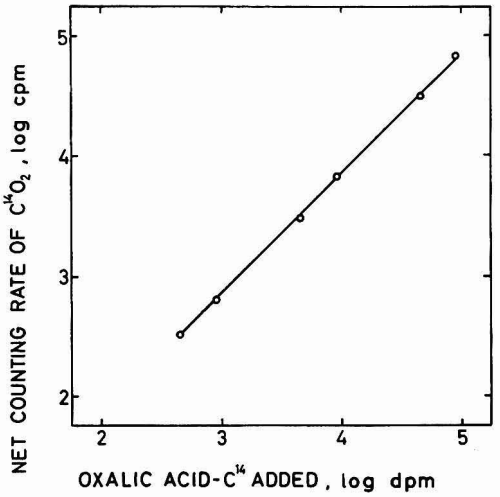

FIG. 2. Relationship between amount of activity in oxalic acid-C^{14} and recovered $C^{14}O_2$ after enzymic decarboxylation in Warburg flasks with a total amount of oxalic acid of 10 μmoles per flask.

total content of 10 μmoles. From Figure 2 it is evident that the recovery of $C^{14}O_2$ was constant over a wide range of activities.

In another experiment a constant amount of oxalic acid-C^{14} but with a varying amount of carrier oxalic acid was added to all the Warburg flasks. Table 3 shows that the recovery of $C^{14}O_2$ was independent of the amount

TABLE 3

Effect of Total Oxalic Acid Concentration on Recovery of Radioactivity from Warburg Flasks Containing Oxalic Acid-C^{14}

(initial radioactivity 10,000 dpm)

Total oxalic acid-C^{14} per flask, μmoles	Net counting rate, cpm	Counting efficiency, %
1.5	7027	70.3
3	7037	70.4
6	6977	69.8
12	6966	69.7
15	6991	69.9
		Mean: 70.0

of oxalic acid in the Warburg flasks, and that the average counting efficiency was 70.0% with backgrounds of about 30 cpm.

Reproducibility. To test the reproducibility of the results, 6 determinations were carried out on total and water-soluble oxalic acid in a finely ground sample of dried spinach. Table 4 gives the mean values and the

TABLE 4
Mean Values and Coefficients of Variation of Six Determinations of Amount and Specific Activity of Total and Water-Soluble Oxalic Acid in Dried and Ground Spinach

Oxalic acid fraction	Mean oxalic acid, gm/100 gm	Coefficient of variation, %	Specific activity, cpm/10 μmoles	Coefficient of variation, %
Total	10.29	2.9	2600	2.4
Water-soluble	6.74	2.0	2976	2.4
Water-insoluble[a]	3.55	—	1886	—

[a] Calculated from total and water-soluble oxalic acid.

coefficients of variation of the amount and specific activity of total and water-soluble oxalic acid.

Time of Extraction. The dried and ground spinach sample was extracted for 1 hr for determining both the total and the water-soluble oxalic acid. To check whether this time was optimal, some determinations were made

TABLE 5
Recovery of Oxalic Acid Added to Dried and Ground Spinach

Oxalic acid fraction	Oxalic acid in spinach, gm/100 gm	Oxalic acid added, gm/100 gm	Oxalic acid recovered, gm/100 gm	Recovery, %
Total	10.29	10.00[a]	10.21	102.1
	10.29	20.00[a]	19.22	96.1
Water-soluble	6.74	7.00[b]	6.77	96.7
	6.74	14.00[b]	14.28	102.0
			Mean:	99.2

[a] Oxalic acid added as calcium oxalate.
[b] Oxalic acid added as sodium oxalate.

after a 4 hr extraction. The values obtained were in good agreement and thereby indicated that a 1 hr extraction time was sufficient.

Recovery of Added Oxalic Acid. The mean recovery (4 analyses) of sodium oxalate and calcium oxalate added to a dried and ground sample was 99.2% (Table 5).

Calculation of Specific Activity. Table 1 shows an experimental protocol with the calculations carried out. It should be stressed that the evolved

activity of CO_2 was only half of the activity of oxalic acid, since half of the activity remained in the Warburg vessel as formic acid.

Specificity of the Enzyme. Some investigators have found the enzyme to be specific for oxalic acid (1, 2), while others have found that it is necessary to use inhibitors in the Warburg vessels to obtain reliable results. Hass and Fleischmann (3) used sodium fluoride and potassium cyanide to avoid the uptake of O_2 and evolution of CO_2 from compounds other than oxalic acid in beer. Mayer *et al.* (4) used ethylenediaminetetra-acetic acid disodium salt (EDTA) to secure high recoveries of oxalic acid added to urine.

The use of inhibitors was found unnecessary in this work, since the uptake of oxygen was of no significance, and since the recovery of oxalic acid added to spinach was in the expected range.

SUMMARY

A rapid and accurate manometric method for determining the amount and specific activity of total and water-soluble oxalic acid utilizing oxalic decarboxylase from *Collybia velutipes* (wood-rot fungus) is described. Two Warburg flasks were used for each determination; in one of them the gas exchange was measured, and in the other the liberated $C^{14}O_2$ was trapped on a paper moistened with 1 *M* Hyamine hydroxide in methanol. The paper was studied in a liquid scintillation counter with high counting efficiency.

ACKNOWLEDGMENT

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Separation of Native and Denatured DNA on Columns of Silk Fibroin

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Separations of native and denatured DNA can be performed on columns of methyl-esterified albumin supported on kieselguhr (1, 2), on columns of IRC-50, a polycarboxylic acid resin in the Mg^{++} form (3, 4) and on columns of hydroxyapatite (5, 6). On a micro scale this separation can also be done by means of a density gradient of CsCl in the ultracentrifuge (7), and by filtration on membranes of nitrocellulose (8). Some of these methods do not lend themselves readily to scaling up, and some at least are tedious to perform.

In a search for a method of separating native and denatured DNA which would be simple, rapid, and capable of dealing with large quantities of material, we investigated the binding of DNA to silk fibroin. Columns of suitably pretreated fibroin allow the unretarded passage of native DNA, while denatured DNA is bound. The latter can be eluted quantitatively. Up to 20 mg of DNA (half of it denatured) has been fractionated in a single run.

MATERIALS AND METHODS

Pretreatment of Silk. Silk "noils" (a low-grade form of raw silk) were obtained from the American Silk Spinning Company, Woonsocket, Rhode Island. The silk fibers were cut into 1–5 mm lengths with scissors. To remove sericin and other impurities, the cut silk was boiled 1 hr in water and drained (9), then resuspended in water and autoclaved at 15 psi for 1 hr. After further rinsing with water, the silk was suspended in a 0.5% solution of "neutral" laundry soap, autoclaved at the same pressure for 3 hr, and again rinsed several times with water (10). The resulting crude fibroin was dried in air, extracted in a Soxhlet apparatus for 48 hr with methanol/benzene (2/3 by volume), and again dried in air (11). The fibroin was then oxidized by shaking it overnight at 37° in 6% H_2O_2, adjusted to pH 1.9 with H_3PO_4 (11). The oxidized fibroin was washed with water until the pH of the washings was 6, dried in air, and

stored ready for use. (Hydrolysis with 1 N HCl for 1 hr at 60° could be substituted for the oxidation step to provide an approximately equally active product.)

Preparation of Columns. To prepare a column for the separation of native and denatured DNA, the fibers were washed successively with 0.05 M potassium phosphate buffer, pH 7.4, 0.1 N HCl, water until the pH of the washings reached about 3.8, and finally 0.1 M sodium acetate buffer of pH 3.8, and poured into a chromatographic tube closed at the bottom with a sintered glass disc. The fibers were packed by weighting them with a glass piston of about 100 gm weight until the volume of the bed decreased no further. After packing, the column was washed with 0.2 M $MgCl_2$.

Samples of DNA. Three kinds of DNA were used: Calf thymus DNA obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, *Escherichia coli* DNA prepared by the method of Marmur (12), and ϕX 174 DNA prepared by the method of Sinsheimer (13) from bacteriophage ϕX 174 purified by the method of Rueckert, Zillig, and Huber (14). Radioactive *E. coli* DNA was prepared from bacteria grown for 8 hr in the medium of Lehman *et al.* (15) containing 50 μc ^{32}P as orthophosphate per liter at a final specific activity of 10 μc/mg P. Denatured DNA was prepared by heating a solution of native DNA (0.15 mg/ml) in 0.015 M NaCl, 0.0015 M sodium citrate for 15 min in a water bath at 100°, and rapidly cooling it. Concentrations of DNA were measured by measuring the absorbance at 260 mμ and assuming that a 0.01% solution of native DNA had an absorbance of 2.

Deoxyadenylate-Thymidylate Copolymer. This DNA-like polymer was synthesized from thymidine 5′-triphosphate-α-^{32}P (International Chemical and Nuclear Corp., City of Industry, California) and deoxyadenosine 5′-triphosphate by the method of Schachman *et al.* (16), using a preparation of DNA polymerase from *E. coli* purified to stage 5 by the method of Lehman *et al.* (15).

RESULTS

Preliminary Experiments to Establish Optimum Conditions for Adsorption of Denatured DNA. To investigate the adsorptive capacity of silk fibroin for denatured DNA under various conditions, columns of various diameters and lengths were prepared, and solutions of denatured DNA (approximately 2 mg/ml) were allowed to flow through the columns. The difference between the amount of denatured DNA put onto the column and that recovered after passage through the column was the capacity of the column.

The type of pretreatment given the fibroin markedly affected its

ability to adsorb denatured DNA. As stated under "Methods," either oxidation or slight hydrolysis after treatment with soap gave an adsorptive product. Treatment with HCl and methanol (as used in esterifying albumin (1)) was also effective. Products of lower capacity were obtained by hydrolysis with H_2SO_4, oxidation with $KMnO_4$, irradiation with UV, or treatment with NH_4OH. Inactive products were obtained if the treatment with soap was omitted before hydrolysis with HCl, or if acetylation with acetic anhydride, or treatment with sodium lauryl sulfate, or prolonged autoclaving was substituted for the oxidation.

The capacity of H_2O_2-oxidized fibroin for denatured DNA was also affected by the pH of the final wash before the column was packed. The lower the pH, the higher was the capacity, but since exposure to pH values lower than 3.7 is known to denature DNA (17) a pH value of 3.8 was selected for the final wash.

The effect of the concentrations of $MgCl_2$ used to wash the columns and as solvent for denatured and native DNA was studied. At concentrations of $MgCl_2$ below 0.01 *M*, denatured and native DNA were both retained by the column, and the binding was only partially reversible. At concentrations between 0.01 and 0.1 *M* progressively less native DNA was retained, while denatured DNA was still bound efficiently, though reversibly. At $MgCl_2$ concentrations of 1 *M* or higher, the retention of denatured DNA appeared to be lower. (The fact that denatured DNA was not completely retained may have been due to partial renaturation of the denatured DNA. When *E. coli* DNA was heat-denatured and cooled over the course of 18 hr to promote renaturation, only 15% was retained by the column.) On this basis, the concentration selected for routine use was 0.2 *M* $MgCl_2$.

When all the conditions investigated were adjusted to give optimum adsorption, the capacity of a fibroin column was found to be approximately 1 mg of denatured DNA per gram of fibroin, and was independent of the shape of the column. The capacity for denatured DNA did not appear to be influenced by the presence of native DNA.

Desorption of denatured DNA from fibroin columns was most efficient when 0.05 *M* potassium phosphate buffer, pH 7.4, was passed through the column.

Separation of Native and Denatured DNA. In order to determine the efficiency of separation of native from denatured DNA, two mixtures of native and denatured *E. coli* DNA were prepared, one containing ^{32}P-labeled native DNA and unlabeled denatured DNA, the other containing unlabeled native DNA and ^{32}P-labeled denatured DNA. All operations were carried out at room temperature, about 25°. In each case about 0.5 mg of DNA, 60% of it native, 40% denatured, was dissolved in 20 ml

of 0.2 *M* $MgCl_2$ and applied to a column of H_2O_2-treated fibroin 9 cm long and 0.8 cm in diameter. When the solution had entered the column, a reservoir containing 0.2 *M* $MgCl_2$ was connected to each column to give a hydrostatic head of 40 cm H_2O to the bottom of the column. This

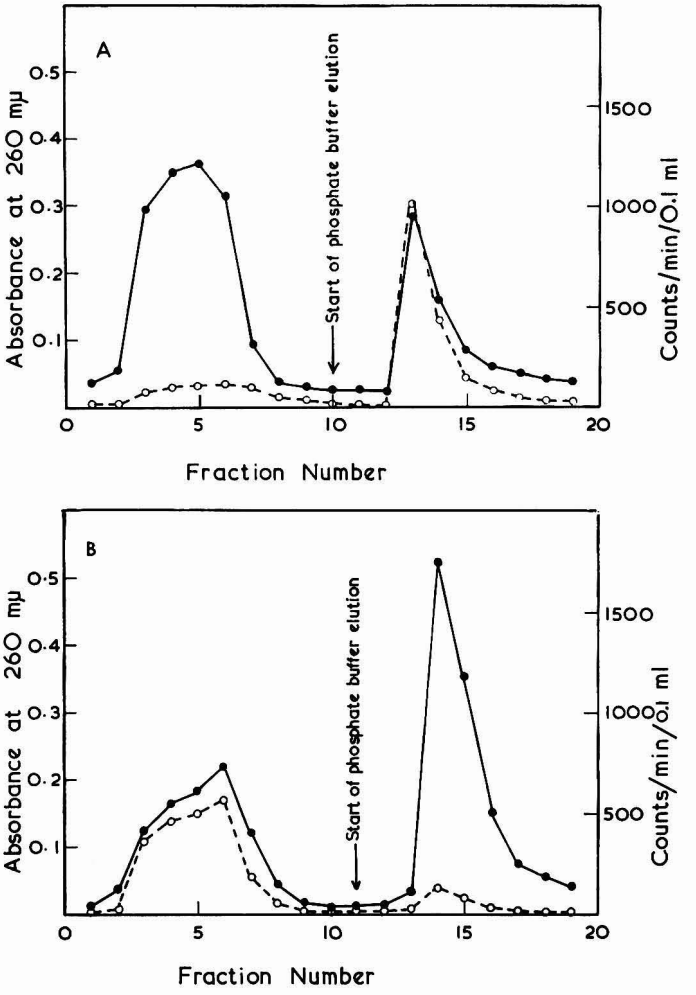

FIG. 1. Separation of native from denatured *E. coli* DNA. Mixtures of native and denatured DNA (0.5 mg in total) were applied to columns of H_2O_2-treated fibroin, 9 cm long × 0.8 cm diameter. Elution was begun with 0.2 *M* $MgCl_2$ and followed with 0.05 *M* potassium phosphate buffer, pH 7.4, at the point shown. In A, ^{32}P-labeled denatured DNA and unlabeled native DNA were put on the column; in B, ^{32}P-labeled native DNA and unlabeled denatured DNA were used. (●) Absorbance values. (○) Counts per minute per 0.1 ml of effluent.

resulted in a flow rate of approximately 0.5 ml/min. Fractions (5 ml) were collected and their absorbance at 260 mμ and radioactivity content were measured. The results are shown in Figure 1. From Figure 1A it can be seeen that 22% of the labeled denatured DNA applied was found in the native DNA peak. This was probably renatured DNA. The converse experiment (Figure 1B) shows that 12% of the labeled native DNA was found in the denatured peak. This may be due to a small amount of denatured DNA in the preparation of native DNA, or perhaps to "tailing" of the first peak. The recoveries of UV-absorbing material in each peak are shown in Table 1.

In order to check the hypothesis that the small amount of supposedly denatured DNA which appeared in the first peak was renatured, a sample of φX 174 DNA was applied to a column. None of the UV-absorbing material emerged from the column until the elution with phos-

TABLE 1
Recoveries of DNA from Columns
(conditions as for Figure 1)

Expt. No.	DNA applied to column, μg		DNA recovered from column, μg	
	Native	Denatured	Peak 1	Peak 2
A	366	177	443	187
B	170	320	194	314

phate buffer was begun. The fact that all of this genuinely single-stranded DNA was eluted as denatured DNA suggests that the material in the heat-denatured preparation of DNA which was eluted with native DNA was not single-stranded.

In a similar experiment a mixture of native and heat-denatured calf thymus DNA (neither labeled) was separated into two peaks. The fractions from each peak were pooled and dialyzed against 0.15 *M* NaCl, 0.015 *M* sodium citrate. Upon heating, the material in the first peak increased in absorbance at 260 mμ by a factor of 1.35, while that in the second did not increase. This indicates that the first peak contained native, and the second denatured DNA.

Separation of Renatured Deoxyadenylate-Thymidylate Copolymer and Denatured DNA. The regularity of structure of synthetic deoxyadenylate-thymidylate copolymer (poly-dAT) allows it to renature even when rapidly cooled after heat denaturation. It was therefore expected that a mixture of ordinary DNA and poly-dAT could be separated on a column of H_2O_2-treated fibroin after heating and quick cooling. A mixture of about 0.1 mg of *E. coli* DNA and 0.1 mg poly-dAT (the latter labeled with ^{32}P), dissolved in 0.50 ml of 0.015 *M* NaCl, 0.0015 *M* sodium

citrate, was heated at 100° for 10 min, rapidly cooled, and diluted to 5 ml with 0.2 *M* $MgCl_2$. The mixture was then applied to a column of H_2O_2-treated fibroin, and elution carried out as above. As anticipated, all the radioactivity appeared in the first peak, indicating that the eluted *E. coli* DNA was entirely free of poly-dAT. One might expect, however, that a little of the *E. coli* DNA would be renatured and appear in the first peak, on the basis of the experiments reported above.

DISCUSSION

The method of separating denatured and native DNA described here has advantages of speed and simplicity over previously reported methods, and also offers the possibility of being scaled up to any desired degree. There seems to be no inherent reason why columns of silk fibroin of any size could not be used, and even the small columns used in our work have a very considerable capacity.

The separation achieved on the fibroin columns seems to depend only on the secondary structure of the DNA. Attempts to separate either native or denatured DNA's of different base compositions, or intact from sheared DNA, were not successful. Separations of different kinds of RNA are still being investigated.

The mechanism by which silk fibroin binds denatured but not native DNA in the presence of 0.2 *M* $MgCl_2$ is not understood. Silk fibroin is isoelectric over the pH range 4–8 (9). It therefore seems unlikely that simple electrostatic binding between DNA and fibroin should occur under our conditions. The pH of the $MgCl_2$ solutions used was 5.7–5.8. Buffers of the same pH containing no magnesium did not allow denatured DNA to be bound. Frankel and Crampton (3) suggest that the binding of native DNA to polycarboxylic resin is due to the formation of a chelation complex of the phosphate of DNA, the carboxylic acid groups of the resin, and magnesium ion. Under their conditions, however, denatured DNA was not bound, whereas native DNA was retained by the column. The fact that modified fibroin under our conditions binds denatured DNA more firmly than native suggests that this kind of explanation cannot be applied to our results.

SUMMARY

Columns of suitably pretreated silk fibroin adsorb denatured DNA from 0.2 *M* $MgCl_2$ solution, but allow unretarded passage of native DNA. Denatured DNA can be eluted from such columns with dilute phosphate buffer. These columns can be used to separate native and denatured DNA. The capacity of the columns is about 1 mg of denatured DNA per gram of fibroin.

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ANALYTICAL BIOCHEMISTRY 19, 157–165 (1967)

The Implication of Acid Concentration in Lipid Phosphorus Determinations[1]

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Solvent-free lipid fractions, cut out spots from paper chromatography sheets, and scrapings from thin-layer chromatography (TLC) plates are frequently the lipid-bearing sources used for determination of phosphorus in lipids. These are usually digested with either perchloric acid (70–72%) or concentrated sulfuric acid followed by addition of a few drops of hydrogen peroxide.

On occasion, variations in the estimation of phospholipid phosphorus may be attributed to the amount of acid left after digestion or during color development. The influence of sulfuric acid concentration in phosphorus assay using an ascorbic acid reducing method (1) and an aminonaphthosulfonic acid reducing method (2) has been examined briefly. Chen *et al.* (1) noted that, when the sulfuric acid concentration was within the range 0.5–1.0 *N*, the color development was proportional to the phosphorus concentration. Dawson (3), using aminonaphthosulfonic acid, simply gave a volume range of perchloric acid which was adequate for the estimation of phosphorus in paper chromatographic spots. Having difficulties with a varying loss of perchloric acid during digestion of commercial feeds, Reiser (4) neutralized the residual perchloric acid, after digestion, with sodium hydroxide and added a fixed amount of sulfuric acid to the neutralized solution.

Aside from these scattered elements of information, a thorough review or study of the implication of acid concentration in phosphorus assay has not been reported. This paper demonstrates the influence of acid concentration in phosphorus determination by using two different assay methods and different acids. The acid effect was examined in the presence as well as in the absence of silica gel G since the phosphorus content of lipid components separated on a TLC plate may be determined without first eluting the lipid from the adsorbent. The conditions

[1] Publication No. 3874 from the Michigan Agricultural Experiment Station.

for digesting lipid-bearing materials with perchloric acid are further discussed.

MATERIALS AND METHODS

Silica Gel G Plates

Silica gel G (Merck) was washed with methanol/chloroform/formic acid, 2/1/1 (v/v/v), according to Parker's procedure (5). For preparation of chromatoplates the washed silica gel (25 gm) was placed in a Virtis 45 homogenizer with 75 ml of distilled water and homogenized for 5 min at a rheostat setting of 25. The slurry prepared in this fashion was coated on the plates with a Desaga applicator. The plates made with this washed silica gel were used in most experiments unless otherwise indicated.

In one experiment with unwashed silica gel G, the plates were coated with a slurry of 1:2 silica gel:water. In all cases only those plates were used which were apparently smoothly and evenly coated.

Fiske-SubbaRow Reagent

1-Amino-2-naphthol-4-sulfonic acid (Eastman Organic Chemicals) was purified as described by Fiske and SubbaRow (6). The reducing reagent was prepared according to the method described by Bartlett (2).

Aspiration of the Silica Gel Layer and Digestion

In experiments including silica gel G, the silica gel was first digested with perchloric acid (70%) and the acid was driven off completely before adding a known amount of phosphorus and developing the color with subsequently added acid.

A silica gel layer from a TLC plate, 6 cm^2 for each sample unless otherwise indicated, was directly aspirated into a 30 ml Kjeldahl digestion flask containing 0.5 ml of perchloric acid by the procedure of Rouser *et al.* (7). A small piece of cotton, washed with distilled water, methanol, and chloroform and air-dried, was used as a tube plug in place of Kimwipe to prevent the adsorbent from passing out of the flask during aspiration.

The flask contents were digested at a mild heat setting (2–4 position) on an electrically heated digestion rack (Laboratory Construction Company, model A). When the digestion was completed and the digestion fluid became colorless, the excess perchloric acid was evaporated rapidly by increasing the heating rate ("high" position on an ordinary Kjeldahl digestion rack) and introducing a piece of glass tubing into the

neck of the Kjeldahl flask, the other end of the tubing being extended to the water pump fume head.

Phosphorus Determination

With or without the digested silica gel a known quantity of phosphorus as estimated from Na_2HPO_4 was determined with different amounts of acid by the procedure employed by Parker (5) and the method of Rouser *et al.* (7). The major difference in the two methods is in the reducing reagent used: Parker used 1-amino-2-naphthol-4-sulfonic acid (see Fiske-SubbaRow reducing reagent) whereas Rouser *et al.* used ascorbic acid. The concentration of ammonium molybdate in the total volume of each procedure is comparable. The total volume in color development is 10 ml for Parker's procedure and 9.9 ml for the method of Rouser *et al.* The absorbance was measured at 820 mμ after zero adjustment with water using a Beckman DU spectrophotometer.

RESULTS AND DISCUSSION

Effect of Acid Concentration in the Absence of Silica Gel

Concentrated sulfuric acid, 70% perchloric acid, and a 1:1 mixture of the two acids were used for determining the concentration effect since these are most commonly used for digestion of organic matter. As shown in Figures 1 and 2, phosphorus color development is acid-dependent regardless of the kind of reducing reagent employed. Each acid shows a similar pattern in acid dependence although at a different acid level, but the ultimate absorbance at the plateau of each curve is nearly the same. Therefore, the amounts of acid which give readings corresponding to the horizontal straight line part of each acid curve are considered to be appropriate for that acid for the phosphorus assay method used: a constant color reading can be obtained with those acid concentrations. At acid concentrations below those appropriate for development of phosphorus color reaction, molybdate is directly reduced to a blue color and at concentrations which are too great phosphorus color development is inhibited. Chen *et al.* (1) and Bartlett (2) observed a similar phenomenon with different concentrations of sulfuric acid.

The differences in the pattern among the three acid curves could be due to the differences in acid strength because the plateaus lie between pH 4.0 and 3.8 of the reaction mixture with the three acids tried and by both methods of phosphorus determination.

Although the reducing reagent is different in the two methods, there are no significant differences in pattern of acid-dependent curves and

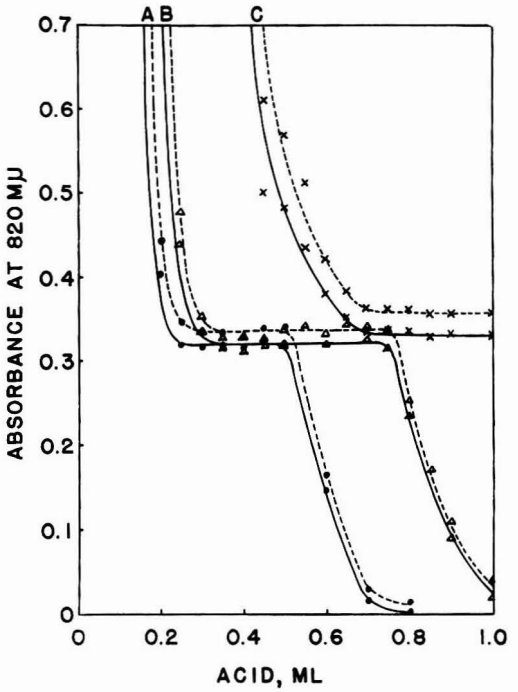

FIG. 1. Effect of acid concentration in phosphorus color development with 1-amino-2-naphthol-4-sulfonic acid—Parker's procedure: (A) concentrated H_2SO_4 (- - -) without correction for reagent control, (—) after correction for control; (B) 1:1 mixture of concentrated H_2SO_4 and 70% $HClO_4$ (△- - -) without correction, (△—) after correction; (C) 70% $HClO_4$ (- - -) without correction, (—) after correction. The amount of phosphorus was 4 μg for each.

the absorbance of the ultimate phosphorus color value at the proper acid concentrations.

Effect of Acid Concentration in the Presence of Silica Gel

Figure 3 shows that the curves with silica gel are somewhat different from the ones obtained without silica gel. If the readings are not corrected for controls, there are no straight-line segments indicating fixed absorbance for a given phosphorus concentration at the acid levels tested except for the mixture of sulfuric acid and perchloric acid, whereas a plateau was observed with each acid in the absence of silica gel even without correction for controls. However, the control-corrected readings give curves of nearly the same pattern as those without silica gel except that the phosphorus color development begins to be inhibited with a lesser amount of acid. The absorbance at plateaus of the corrected curves

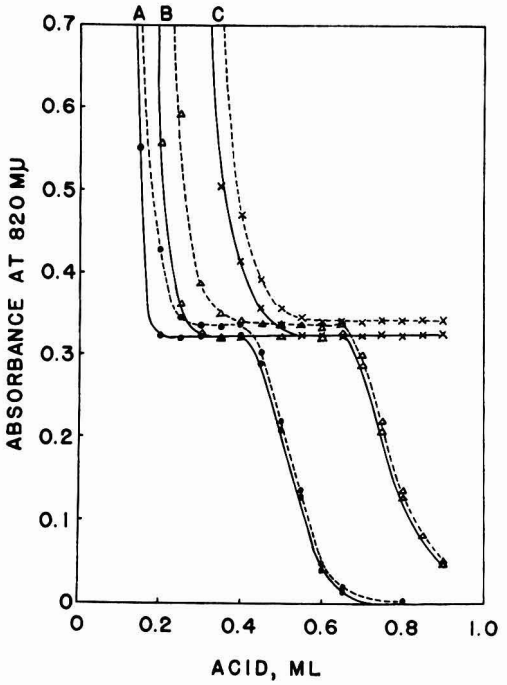

FIG. 2. Effect of acid concentration on phosphorus color development with ascorbic acid—method of Rouser *et al.* Explanations for the curves are as in Figure 1.

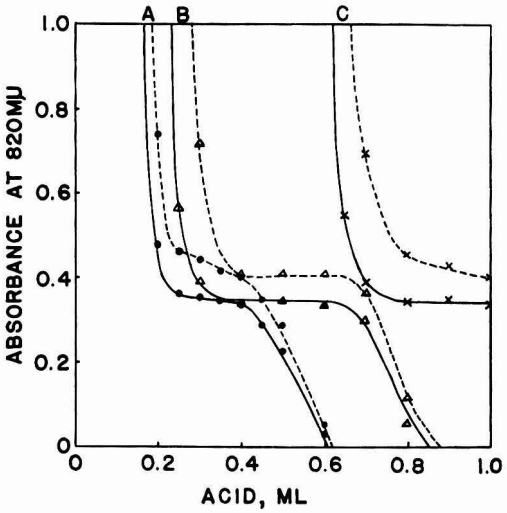

FIG. 3. Effect of acid concentration on phosphorus color development in the presence of silica gel G—Parker's procedure: (A) concentrated H_2SO_4 (- - -) without correction for control, (—) after correction; (B) 1:1 mixture of concentrated H_2SO_4 and 70% $HClO_4$ (△- - -) without correction, (△—) after correction; (C) 70% $HClO_4$ (- - -) without correction, (—) after correction. The amount of phosphorus was 4 μg for each.

TABLE 1
Proper Acid Concentration Ranges for Phosphorus Determinations[a]

P assay method	Acid	Range of acid volume, ml	pH of reaction mixture
Parker	H_2SO_4 (conc.)	0.25–0.50	4.05–3.80
	$HClO_4$ (70%)	0.70–	4.00–
	1:1 H_2SO_4:$HClO_4$	0.35–0.75	3.98–3.80
Rouser *et al.*	H_2SO_4 (conc.)	0.20–0.40	4.00–3.83
	$HClO_4$ (70%)	0.55–	4.00–
	1:1 H_2SO_4:$HClO_4$	0.30–0.65	3.99–3.82

[a] With 4 μg of P as estimated from Na_2HPO_4 used.

is about the same as that without silica gel. The same results were obtained with the method using ascorbic acid as reducing reagent.

Even with an increased quantity of silica gel and a greater concentration of phosphorus, the phosphorus color values, corrected for controls,

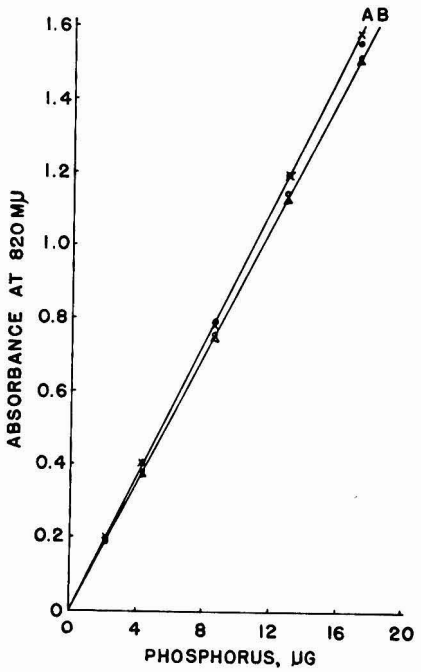

FIG. 4. Phosphorus color standard curve with and without silica gel G: (A) Parker's procedure using 1 ml of 70% perchloric acid and Fiske-SubbaRow reducing reagent (●—●) without silica gel, (X—X) with silica gel; (B) method of Rouser *et al.* using 0.9 ml of 70% perchloric acid and ascorbic acid as reducing reagent (○—○) without silica gel, (△—△) with silica gel.

at the proper acid concentrations were comparable with those without silica gel.

Standard Curve with and without Silica Gel

Additional experiments confirmed that the presence of silica gel does not affect the results when a proper level of acid is used and when an adequate control for the amount of silica gel used is included. A phosphorus standard curve was determined with and without silica gel using both Parker's procedure and the method of Rouser *et al.* Perchloric acid

TABLE 2
Phosphorus Color Readings for Standard Curve with and without Silica Gel G[a]

P, μg	Absorbance at 820 mμ[b]		
	Without silica gel	With washed silica gel[c]	With unwashed silica gel[c]
2.18	0.193	0.202	0.198
4.36	0.403	0.401	0.391
8.72	0.792	0.783	0.778
13.08	1.194	1.193	1.202
17.44	1.554	1.583	1.592
Control	0.026	0.067	0.068

[a] With 1.0 ml of perchloric acid (70%) using Parker's procedure.
[b] Absorbance corrected for each control.
[c] 6 cm^2 layer.

was used for color development, 1.0 ml for the former method and 0.9 ml for the latter. Control values were subtracted from sample readings. As shown in Figure 4, the presence of silica gel does not change the standard curve in either method.

Even in the presence of unwashed silica gel G, the corrected readings gave the same standard curve (Table 2). Therefore, silica gel G need not be washed with methanol/chloroform/formic acid for direct phosphorus determination of TLC spots, which is advantageous because unwashed silica gel makes more uniform plates.

Remarks on Digestion Procedure

The three acids used here are almost equally satisfactory for phosphorus assay of inorganic materials. However, perchloric acid and the mixture of perchloric acid plus sulfuric acid would be advantageous over sulfuric acid for determining phosphorus in organic materials such as phospholipids since digestion is rapid with the two acids and the proper volume range of these acids for phosphorus estimation is wider.

Digestion with sulfuric acid could be speeded by addition of a few drops of hydrogen peroxide, but even boiling for 15 min after digesting is complete may be insufficient to destroy all the residual hydrogen peroxide, and the residual hydrogen peroxide results in variations in the estimation of phosphorus (8).

Washed cotton was used in these experiments instead of Kimwipe or similar light paper in aspirating silica gel G layer because cotton disintegrates readily in perchloric acid due to its loose texture and thus avoids local heating at an early stage of digestion, which could bring about an explosion. When 2–3 cm^2 of Kimwipe, closely folded, was used and the heating rate was relatively high (on an ordinary Kjeldahl digestion rack) from the beginning of digestion, a minor explosion occasionally occurred owing to local heating in the presence of the closely folded Kimwipe. When Kimwipe was completely disintegrated by holding it a longer time in perchloric acid before starting digestion, it was then possible to proceed with a moderately high heating rate.

As already demonstrated, the amount of an acid left after digestion of evaporated lipid fractions, aspirated TLC spots, or paper chromatographic spots should be adequate to obtain dependable and reproducible results. If the acid, particularly perchloric acid and an acid mixture containing perchloric acid, evaporates in appreciable amount during digestion, the resulting phosphorus concentration in the lipid sample will be estimated to be much higher than it should be since the phosphorus standard curve is usually made using inorganic phosphate without necessity of digestion.

When digestion was carried out with perchloric acid at a very low heat setting of an electrically heated digestion rack, there was no significant decrease in the acid volume. As Rouser *et al.* (7) digested TLC spots with 0.9 ml of perchloric acid (72%) under similarly mild conditions, they may have encountered little difficulty due to acid evaporation during digestion.

SUMMARY

The effect of acid concentration in lipid phosphorus determinations by the ascorbic acid reducing method and aminonaphthosulfonic acid reducing method, was reinvestigated in the presence and absence of silica gel G using sulfuric acid, perchloric acid, and a 1:1 mixture of the two acids. The concentration range of each acid or acid mixture within which a constant phosphorus heteropoly blue reading can be obtained with a given concentration of phosphorus is presented for both methods of phosphorus assay. Although the pattern of the three acid effect curves was different, the plateaus (the adequate concentration or volume

ranges) of the curves lay in the same pH range of the reaction mixture with the three acids and by the two methods of phosphorus determination. The acid effect was the same with or without silica gel when carefully controlled corrections were made for the amounts of silica gel used.

The importance of the proper amount of acid in phosphorus color development is particularly emphasized when perchloric acid or an acid mixture containing perchloric acid is used for digestion of lipids or scrapings from TLC plates because appreciable quantities of perchloric acid may be lost unless the digestion conditions are controlled. Necessary precautions for digestion procedures are discussed.

ACKNOWLEDGMENT

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Ultramicro Method for Nonconjugated 17-Hydroxycorticosteroids in One-Half Milliliter of Monkey or Human Plasma

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Previous methods based on the Porter-Silber reaction for the determination of plasma 17-hydroxycorticosteroids (17-OHCS) have had the disadvantage of requiring relatively large blood samples and have not been extensively evaluated for specificity and accuracy toward cortisol. The present method represents improvements in these areas. The method is an ultramicro modification of an existing procedure (1), with some simplification, and one purpose of this paper is to report the solution of apparatus and other problems in scaling this procedure for routine ultramicro measures. In addition, the validation of the method against a double-isotope derivative assay for cortisol, both for an extensive series of monkey plasmas (*Macaca mulatta*) and for a lyophilized normal human plasma, has provided a quantitative statement of the specificity and accuracy with which the method measures cortisol. Since the present method has been found to compare closely in results with certain other Porter-Silber based procedures, this quantitative validation would seem to be at least generally indicative of the accuracy and specificity of these other, much used procedures.

MATERIALS AND METHODS

Materials

Dichloromethane (Matheson, Coleman & Bell, b.p. 39.5–40.5°C) was redistilled and stored in all-glass brown bottles; 5 ml evaporated to dryness should leave no visible residue.

Absolute ethanol (Commercial Solvents Corp., Gold Shield brand, USP, NF) was used as obtained in one-pint bottles.

Phenylhydrazine hydrochloride was decolorized with charcoal, recrystallized three times from absolute ethanol, and stored in a brown bottle in a desiccator.

Benzene, thiophene-free, was washed with 0.1 volumes of concentrated sulfuric acid until the acid no longer discolored, then neutralized with 1 *N* sodium hydroxide and water, dried over sodium sulfate and sodium metal, redistilled at 79°C and stored in all-glass brown bottles.

Glass wool (Pyrex) and granular anhydrous sodium sulfate (ACS) were both washed in three changes of redistilled dichloromethane and stored dry.

Cortisol, chromatographically pure, 1 μg/ml, was stored frozen in 1.0 ml aliquots of distilled water for use as standards.

Porter-Silber reagent: 2 vol of a mixture of concentrated sulfuric acid and water (310:190) was mixed with 1 vol of absolute ethanol and stored. The reagent consisted of approximately 1 mg of phenylhydrazine hydrochloride per milliliter of this solvent mixed immediately before use.

Apparatus

Repipets (Labindustries, Berkeley, Calif.) were used for speed and accuracy in dispensing solvents.

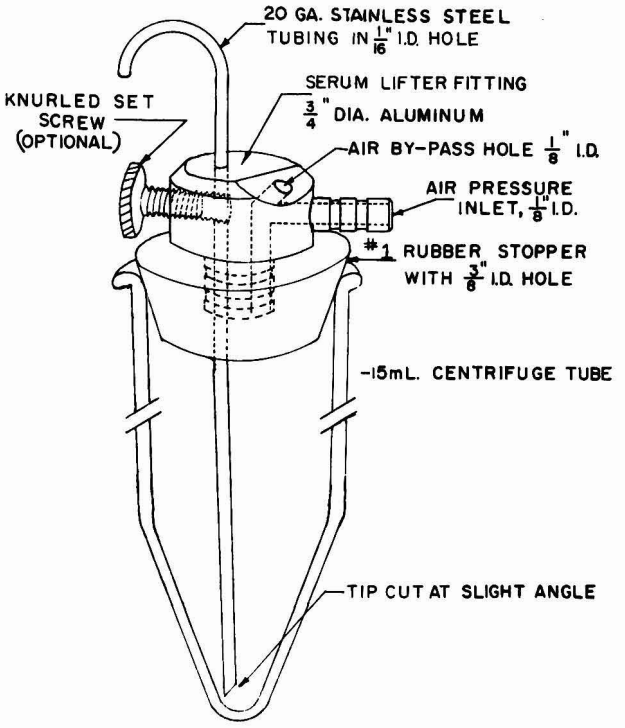

FIG. 1. Line drawing of the serum lifter as positioned in a test tube for removal of the lower solvent layer.

Fritted-disc funnels had a 28 mm top diameter and 1.5 cm long stems cut square and tipped with coarse fritted-glass discs.

Serum lifter, designed in final form by Dr. Max Sweat, University of Utah, and described in Figure 1 with his permission, was used for transferring solvent layers via air pressure.

Polyethylene pipets were pulled from 5/16 in. tubing. Tips were pulled to about 0.5 mm i.d. and the upper end was molded for coupling to a glass syringe by quickly squeezing the flame softened end together and pushing it onto the glass tip of the syringe.

Aspirator, used for removing dichloromethane completely from 0.04 ml of reaction mixture, consisted of a 20-gage stainless steel tube held vertically in a clamp and tipped at the lower end with 3 cm of P.E. 100 polyethylene tubing drawn at the free end to about 1/4 its normal diameter. The upper end of the tubing was bent in an inverted U and connected to a vacuum pump.

A Beckman DU spectrophotometer was modified with an automatic positioning carriage and cell compartment, supplied with a custom 0.5 mm diameter pinhole mask (Gilford Instruments, Inc., Oberlin, Ohio). Additional equipment (Pyrocell Manufacturing Co., New York City) included a Bessey-Lowry cell holder and custom-made Y-cuvets holding a minimum of about 0.030 ml in the 1 mm wide by 5 mm high by 10 mm long light-path stem of the Y.

Washing Procedures

All glass and plastic ware were submerged overnight in 10% 7× (Linbro Chemical Co., New Haven, Conn.), rinsed 5–10 times with distilled deionized water, and air dried. The fritted-glass funnels and the 3 ml test tubes involved in the color reaction were additionally soaked in sulfuric acid before final rinsing and drying. Finally, the Y-cuvets were soaked in sulfuric acid the hour before use and were subsequently rinsed between samples with distilled, deionized water and then absolute ethanol (forcefully delivered from squeeze bottles), the outside dried with lens paper, and the inside blown dry with air filtered through glass wool. *Proper and careful cleaning of the cuvets was critical.*

Procedure

A series of solvent partitions was used to extract and purify the 17-OHCS before subjecting them to the color reaction. We found it convenient to process the samples in squads of 6 through to the combined water stage (see below), hold them frozen there until 30–36 tubes were so collected, and then to carry these through the remaining steps

together; 2 blanks, 6 standards of cortisol in water, and 28 plasma samples of 0.5 ml each were generally processed together and required 3–4 days. Sample sizes other than 0.5 ml have been run (*a*) by diluting smaller samples to 0.5 ml with water or (*b*) by dividing larger samples into 0.5 ml aliquots and subsequently combining the initial 5 ml dichloromethane extracts and evaporating the total volume down to 5 ml. The steps of the procedure were as follows:

(*1*) Each sample in a 15 ml conical centrifuge tube was extracted by adding 5 ml of dichloromethane, vortexing for 20 sec, centrifuging for 1 min, and transferring the lower, dichloromethane layer to a clean 15 ml tube using the serum lifter. The serum lifter was inserted while holding the upper end of the 20-gage tubing shut with the thumb, and the dichloromethane was completely transferred by then placing the thumb on the air by-pass hole. Any drop of plasma accidentally transferred was removed by the next step of the procedure. Between each sample, the tubing of the serum lifter was flushed inside and out with dichloromethane delivered through a 22 gage needle from a syringe. Very occasional emulsion layers in the samples necessitated a second dichloromethane extraction and evaporation to bring the combined extract volume to about 5 ml.

(*2*) Each dichloromethane extract was partitioned for no longer than 15 min against 0.5 ml of 0.2 *N* sodium hydroxide. The dichloromethane was then serum lifted onto 1 gm of anhydrous sodium sulfate held over a glass-wool plug in a funnel (39 mm top diameter) and collected with a 0.1 ml wash of the funnel in a clean 15 ml tube. The dichloromethane was evaporated at 45°C under a stream of nitrogen and the residue concentrated in the tube tip by washing down and evaporating 3 times with 0.5 ml of dichloromethane.

(*3*) The residue was dissolved in 0.4 ml of water and partitioned against 0.2 ml of benzene. The water layer was transferred to a clean 15 ml tube using a polyethylene pipet controlled by a greased 1 ml syringe. Individual pipets were used for each tube and were retained for all subsequent transfers. The benzene layer was reextracted with 0.2 ml of water and the water extracts were combined and frozen until the time when the run was to be completed.

(*4*) Each water extract was extracted once with 2.4 ml of dichloromethane. The dichloromethane phase was pipetted through 0.5 gm of anhydrous sodium sulfate in a fritted-disc funnel and collected with a 0.1 ml funnel wash in a 3 ml conical centrifuge tube.

(*5*) The dichloromethane was evaporated to dryness at 45°C under a stream of nitrogen and the residue was immediately washed down with exactly 0.5 ml of dichloromethane dried with sodium sulfate. From this

point on, all operations were performed at 20° in an atmosphere of 50% or less relative humidity to avoid erratic color reactions.

(*6*) The dichloromethane was extracted with 0.040 ml of Porter-Silber reagent and every drop of the dichloromethane was immediately discarded by aspiration. The reaction was either incubated at 20°C for 12–20 hr or at 60° for 30 min.

(*7*) The absorbances of each sample and of the second blank were measured against the first blank at 370, 390, 410, 430, and 450 mμ.

Calculations

All absorbances were corrected for the mean of the blanks by algebraically substracting from them half the reading of the second blank. Then, to avoid interference from nonspecific linear background absorbance, two Allen-corrected (2) absorbances, Q_{20} and Q_{40}, were calculated for each standard and blank, according to the equations:

$$Q_{20} = 2(A_{410}) - A_{390} - A_{430}$$
$$Q_{40} = 2(A_{410}) - A_{370} - A_{450}$$

where A_{410} equaled the blank-corrected absorbance observed at 410 mμ, etc. The apparent amount of cortisol in μg/100 ml plasma in each sample was then calculated for each of these Q values by the following formula and averaged:

$$\mu g/100 \text{ ml} = \text{sample } Q \times \frac{\mu g \text{ cortisol in standard}}{\text{ml of sample} \times \text{standard } Q}$$

The reason for computing 17-OHCS concentrations based on two corrected absorbances per sample was to utilize the discrepancy between these two concentrations to detect cases in which the nonlinear curvature itself was not the same as that observed for cortisol standards. It was found for a series of cortisol standards of concentrations 0, 20, 40, 60, 80, and 100 μg per 100 ml that three times the standard deviation of the absolute discrepancy between the Q_{20} and Q_{40} based 17-OHCS values amounted, respectively, to 4.9, 5.2 5.6, 6.0, 6.3 and 6.7 μg per 100 ml. Therefore, if the Q_{20} and Q_{40} based 17-OHCS values for any sample disagreed by more than these standard discrepancies, the sample value was discarded. In our hands, 10–15% of sample values was lost by this test. Only one of the duplicate samples generally available for each plasma was assayed initially, and discarded values could therefore generally be replaced by processing the duplicate sample later.

RESULTS AND DISCUSSIONS

Reports of assay methods vary surprisingly in the type and form of data submitted in evaluation of the assay. As an attempt at a system-

atic and standardized account, this report is organized around the set of assay criteria listed by Loraine (3): specificity, accuracy, precision, sensitivity, and practicability (see below for definitions). In addition, technical evaluations justifying certain aspects of the procedure are reported, as well as comparisons of the results of this method with those of other published procedures.

Technical evaluations. The Beer-Lambert law was found to hold for cortisol standards taken through the procedure, up to concentrations equivalent to 120 μg per 100 ml, thus supporting the assumption of linearity embodied in the formula for calculating 17-OHCS concentration.

Recovery of cortisol in water taken through the procedure in parallel with the plasma samples for six runs averaged $62 \pm 3\%$ (S.D); although not systematically evaluated, the recovery of cortisol added to plasma was of similar magnitude. Recovery has previously sometimes been overvalued in assay criteria, perhaps through its implicit use as an index of accuracy. In the present case, this recovery factor is low enough to require compensation by the use in the 17-OHCS calculation formula of cortisol standards processed in parallel with plasma samples in each run. This compensation avoids effects of recovery on specificity or accuracy. However, any *variation* in recovery would affect precision, and *low* recovery would affect sensitivity; nevertheless, it will be seen below that both precision and sensitivity are adequate in the present method.

Finally, an assessment has been made on the over-all adequacy of solvent purity and apparatus cleanliness, by measuring background color in the Porter-Silber reaction. The water blanks for 19 successive runs had an average absorbance ranging from 0.170 at 370 mμ to 0.60 at 450 mμ, about four times that noted for the Porter-Silber reagent alone. This was felt to be not high enough to justify further efforts in cleanliness, particularly in view of the over-all adequacy of the method in measuring 17-OHCS detailed below.

Specificity. Specificity and accuracy are in part aspects of the same problem. Specificity refers to the extent to which other compounds interfere with the measurement of the desired compound, and the Porter-Silber reaction has been extensively evaluated for compounds other than cortisol which will produce interference. The use of Allen-corrected absorbances provide a partial guard against nonspecific interference, i.e., interference from substances which have Porter-Silber absorbance curves different from cortisol. The possible presence of such nonspecific interference has been evaluated for the present method by comparing absorbance curves of plasma extracts and cortisol for 19 successive runs each containing 8 plasma samples and 2 cortisol standards. Since the

interest was in whether the sample and standard absorbance curves had the same shape, each individual curve was converted to a "proportional" curve by dividing each absorbance of the curve by the absorbance at 410 mμ. Given the Beer-Lambert relationship at all five wavelengths, as did in fact hold, all concentrations of cortisol yield the same proportional curve. The average proportional curve for the samples (for which A_{410} averaged 0.230) and that for the 0.50 μg standards (for which A_{410} averaged 0.515) were nearly identical, as seen in Figure 2, indicating

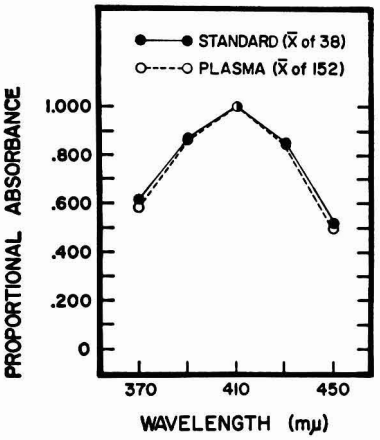

FIG. 2. Mean curves of the Porter-Silber absorbance at each wavelength divided by the absorbance at 410 mμ both for cortisol standards of 0.50 μg and for extracts of 0.50 ml of monkey plasma.

negligible systematic nonspecific chromogens. Nevertheless, substances giving the same specific absorbance curve as cortisol might still be present and interfering.

To evaluate this latter possibility, 17-OHCS values obtained by the present method were compared to cortisol values obtained by a double isotope derivative method for a set of 25 monkey plasmas taken in duplicate (5 normal plasmas, 4 following ACTH stimulation, 12 from sick or dying monkeys, 2 from pregnant monkeys, and 2 from monkeys stressed by restraint); all 50 samples were number coded and assayed blind.

Cortisol values were obtained by a double isotope derivative assay done by New England Nuclear Biomedical Assay Laboratories, Inc. The procedure of this assay was to add to each sample a known trace amount of cortisol-4-^{14}C and then to form the monoacetate derivative using tritiated acetic anhydride of known specific activity. Three paper chromatographic separations were performed, following which the cortisol

monoacetate was oxidized to cortisone monoacetate and chromatographed again; the sample cortisol concentration was then calculated from the ^{14}C and ^{3}H contents measured by scintillation counting.

As a test of the specificity of the color test data, a comparison of the standard and sample proportional absorbance curves found them to be nearly identical (similar to those of Fig. 2). Nevertheless, the correlation of the color test data with the isotope data (Fig. 3) indicated that the

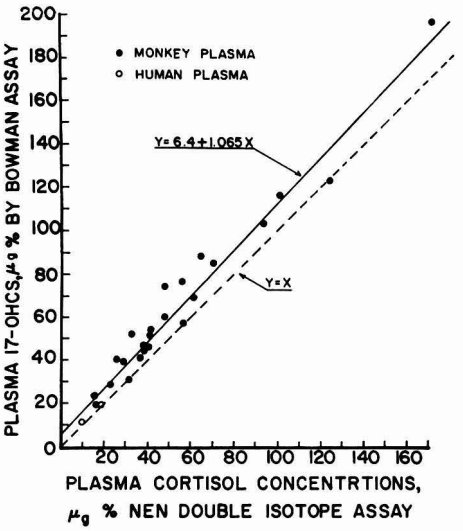

FIG. 3. Comparison of the 17-OHCS values with the cortisol values obtained for 25 monkey plasmas and 2 human plasmas.

17-OHCS values of monkey blood plasma obtained by the present method tended to be higher than the corresponding cortisol values determined by the double-isotope derivative method. The least-squares linear regression (Fig. 3) yielded the following equation for estimating cortisol values from the 17-OHCS data:

$$\text{cortisol in } \mu g/100 \text{ ml} = 0.939 \text{ (17-OHCS in } \mu g/100 \text{ ml} - 6.38)$$

The error of 6.38 μg per 100 ml was independent of cortisol concentration, which suggests that it was not of adrenocortical origin. The source of this error is not presently known. After subtracting this constant error, 93.9% of the remaining 17-OHCS value was then accounted for as cortisol, and the remaining 6.1%, since it was proportional to the cortisol concentration, seems likely to have been of adrenocortical origin (e.g., perhaps 11-deoxycortisol, etc.).

Accuracy. Accuracy is best measured as the statistical deviation of

the assay measurement around the true concentration of the desired compound. Linnig and Mandel (4) have pointed out that the accuracy with which a biochemical assay estimates true concentrations of a specific chemical cannot generally be assumed to be given by the replication error (precision) of the estimate. To determine in the present case the closeness with which the 17-OHCS method could estimate cortisol, cortisol concentrations were estimated, using the regression equation of Figure 3, from the 17-OHCS values of each of the 25 plasmas for which isotope-determined cortisol values were known. The difference between each cortisol estimate and its corresponding isotope-determined cortisol value was converted to its percentage of the cortisol value. The S.D. of these per cent differences was ±16.3%. Subtracting the variance attributable to replication error of the isotope method left a S.D. of ±14.0% as the accuracy of the 17-OHCS assay for cortisol estimation. Subtracting further the variance attributable to the 17-OHCS replication error left a S.D. of ±12.6%, which probably reflected the variation in amounts of Porter-Silber chromogens due to noncortisol components in the various plasma extracts.

Precision. Precision of the 17-OHCS method, as the pooled S.D. of replicate determinations about their respective means, was ±9.8 μg per 100 ml for the plasmas graphed in Figure 3. The S.D. of the isotope assay of the same plasmas was ±9.3% μg per 100 ml. Since, however, a scatter plot indicated that the S.D. increased with increasing 17-OHCS concentration, and the bulk of the replication error was contributed by a few samples of high concentration, it seemed desirable to calculate a coefficient of variation, (S.D./mean)100, for each sample and to pool these coefficients for an estimate of replication error. These coefficients were essentially without trend from plasma 17-OHCS concentrations of 11 to 197 μg per 100 ml and the pooled estimate of these coefficients yielded a value of ±10.0% as the replication error for the present method, and a value of ±11.8% for the isotope derivative method. Similar estimates of 17-OHCS replication error for other plasma sets have been found as follows: ±7.5% for 29 aliquots of a single monkey plasma of mean 17-OHCS value of 65 μg per 100 ml, stored frozen and assayed from time to time over a 3 year period, ±9.0% for the plasmas on which Figure 2 was based (mean concentration of 33.6 μg per 100 ml and range of 15–45 μg per 100 ml), and ±5.3% for the 3 lyophilized human plasmas of the Eik-Nes cooperative comparison of 17-OHCS assays (see below).

Sensitivity and Range. The present method has been found capable of measuring as little as 10 μg per 100 ml of 17-OHCS in a 0.5 ml sample of plasma, and of detecting as little as 5 μg per 100 ml. These values

correspond to limits of 0.05 and 0.025 μg of cortisol per sample. Although the upper limit of concentration adhering to Beer's law varied somewhat with humidity, 120 μg per 100 ml could generally be measured accurately in 0.5 ml of plasma.

Practicability. Since the procedure described employs only solvent extractions for purification, and uses easily fabricated or commercially available equipment, it would appear feasible for many laboratories. It is at least as economical as most current manual steroid procedures. The microcuvets and spectrophotometer equipment are particularly simple and reliable in use, provided care is exercised in cleaning the cuvets and the pinhole mask and the Bessey-Lowry cell carriage have been adjusted to direct the spectrophotometer light beam through the center bottom of each cuvet. The 40 μl of Porter-Silber reagent has been pipetted both with commercial micropipets and with more expensive microburets without change in the precision of the method. The only unusual requirement is the need for low humidity during the last steps of the method, and this can be attained if necessary by the use of one of the inexpensive glove-equipped lab bench dry boxes now available.

Comparison with Other 17-OHCS Methods. By the present method, the normal 9:00 a.m. 17-OHCS blood plasma concentration in rhesus monkeys was found to be 31.9 ± 1.07 (S.E.) μg per 100 ml for an N of 50 monkeys. Other methods employing the Porter-Silber reaction have reported 9:00 a.m. values of 37.7 ± 1.3 μg per 100 ml for 36 monkeys (5), 34 ± 2.1 μg per 100 ml for 10 monkeys (6), and 36.8 ± 1.88 μg per 100 ml for 29 monkeys (7). The present method thus yielded values for the monkey substantially in agreement with or slightly lower than, those of other Porter-Silber procedures.

The present procedure has also been applied to human plasma as part of a cooperative project conducted by Dr. Kristen Eik-Nes at the University of Utah to compare various 17-OHCS assay methods. On three lyophilized plasma samples, "B," "1," and "2" (of which "B" was a normal human plasma, "1" was "B" plus cortisol in an amount of 5 μg per 100 ml, and "2" was "B" plus cortisol in an amount of 10 μg per 100 ml), the present method obtained values of 12.8 ± 0.27 (S.D.), 17.2 ± 0.65 and 21.7 ± 1.20 μg per 100 ml (each value is the mean of 6 replicates). This was in good agreement with the 13 sets of lowest values obtained by various procedures reported from 20 other laboratories. These 13 sets had over-all means and S.D. between procedures of 12.6 ± 0.22, 18.0 ± 0.44 and 23.3 ± 0.60. Two of these plasmas ("B" and "2") were included, after storage at 4°C in the lyophilized state for 1 year, as part of the double-isotope comparison described

above for the monkey plasma. The cortisol (isotope) values were 10.0 and 18.4 μg per 100 ml while the 17-OHCS values now were 11.0 and 19.0 μg per 100 ml. In this case approximately 94% of the total 17-OHCS values could be accounted for as cortisol, suggesting (although with this limited sample, not proving) that the constant error of 6.38 μg per 100 ml observed with the monkey plasma did not occur with this normal human plasma.

SUMMARY

A procedure based on the Porter-Silber reaction and capable of measuring 17-hydroxycorticosteroids (17-OHCS) in 0.50 ml or less of monkey or human plasma is described. This procedure has been in routine use for 5 years in studies on the monkey, and has been found to yield values similar to those given by other, frequently used, Porter-Silber based methods. The method also agreed with other 17-OHCS methods in values obtained on a lyophilized normal human plasma. In addition, a comparison with values obtained by a double-isotope derivative procedure indicated for monkey plasma an average 82% of the 17-OHCS value to be accounted for as cortisol (specificity). Furthermore, the standard deviation around true cortisol values of cortisol estimates calculated from the 17-OHCS values by a least-squares linear regression equation was $\pm$14% (accuracy). These data provide one of the most complete quantitative statements to date establishing the specificity and accuracy for cortisol of a Porter-Silber based method.

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ANALYTICAL BIOCHEMISTRY 19 (1967)

SHORT COMMUNICATIONS

Separation of Human Plasma Lipoprotein by Electrophoresis on Cellulose Acetate

On the basis of the paper electrophoretic patterns of the plasma lipoproteins, familial hyperlipoproteinemias in man have been classified into five different types (1). However, a need exists for a simpler, rapid, high-resolution technique for the fractionation of the plasma lipoproteins as an aid to their further study.

Although the ultracentrifuge provides the highest degree of sophistication for the fractionation of lipoproteins, the cost in time and equipment is impractical for the screening of large numbers of samples. Several methods of zone electrophoresis utilizing paper (2), starch gel (3), and polyacrylamide gel (4, 5) also have been employed for plasma lipoprotein separation. However, none of these techniques adequately fulfils all the existing needs. Cellulose acetate electrophoresis (6) has been previously successful for general α- and β-lipoprotein separations (7, 8) but a method will be described now that can resolve unfractionated human plasma into its additional lipoprotein fractions commonly found in clinical studies.

A barbital buffer solution at pH 8.6 and ionic strength 0.05, 1% in albumin and 0.001 M in EDTA, was employed. The cellulose acetate strips (Gelman Sepraphore III, 6 $\times$ 1 in.) were soaked in buffer at 4°C for 10 min, blotted dry, and placed in a Gelman Deluxe electrophoresis chamber. Samples of 5 μl of whole plasma or individual lipoprotein fractions were applied by means of a Gelman sample applicator. The optimum conditions for electrophoresis of a general sample of unknown complexity were 240 V (20 V/cm) for 90 min at room temperature. The current was about 2.5 mA/strip. Voltages from 180 to 360 V (15 to 30 V/cm) for different periods of time (60–150 min) were also used in order to achieve better resolution of unusual lipoprotein patterns.

A staining solution of oil red O in methanol was prepared by addition of 0.2 gm of the dye to 350 ml of methanol, which was then brought to a boil under a condenser so that loss of solvent was minimized. Distilled water

(150 ml) was added with stirring and the mixture was stirred continuously while cooling to near room temperature, then filtered and kept at about 39°C. The strips were removed from the electrophoresis chamber, placed immediately in the staining solution, and kept 6–7 hr at 37–39°C, even though the bands could be detected in most cases within a few hours.

The stained strips were bleached for 3 min in 200 ml of a 5% aqueous acetic acid solution to which 10 ml of 5% sodium hypochlorite had been added. After rinsing 3 times in tap water they were dried between paper toweling and glass plates at room temperature for 24 hr. They were

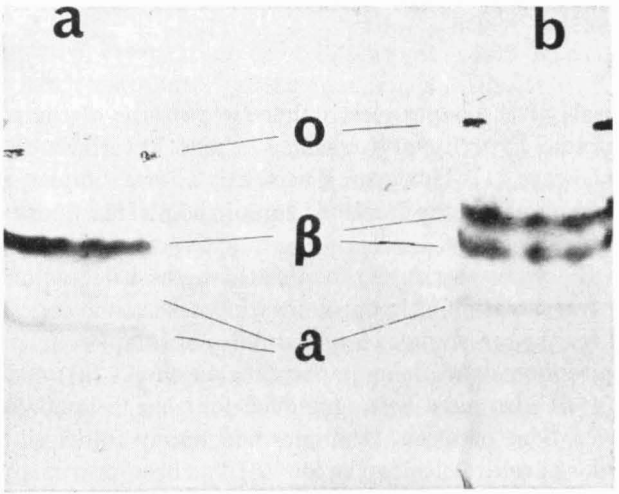

FIG. 1. Cellulose acetate electrophoresis patterns of unfractionated human plasma: (a) normal subject showing two α-bands, (b) subject with endogenous hypertriglyceridemia showing a pre-β-band ahead of the normal β-band. Important α- and β-bands are labeled ("O" indicates origin). Conditions: 240 V (20 V/cm), 90 min.

prepared for permanent mounting by being rinsed in a 15% solution of acetic acid in methanol, which cleared the opaque cellulose acetate and left the red lipoprotein bands on a transparent cellulose strip. A photograph of the wet, bleached strips possibly might make an equally useful permanent record. The use of protein stains or prestaining (9) was not sensitive enough for the small sample sizes used.

Figure 1a illustrates the electrophoretic pattern on cellulose acetate of the plasma of a normal individual collected after an overnight fast. In addition to the expected β- and α-bands, a slower moving second α-band is present. Ultracentrifugation of similar normal plasma showed that this band was in the fraction of density 1.10–1.21. Figure 1b shows a patient with the additional pre-β-band (10), but only the faster moving α-band.

If nonfasting plasma was collected, in either case, it was found that the chylomicrons did not migrate and were detected at the origin. All bands were identified by satisfactory comparison of their migration rates against individual plasma fractions prepared by ultracentrifugation (11). Chylomicron fractions were also prepared by starch gel electrophoresis (12).

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Detection and Determination of 4-Hydroxyskatole Sulfate Ester[1]

The hydroxyindoles are of considerable clinical interest (for review see reference 1) and have been related to various pathological and psychiatric conditions. Methods have been described previously for the determination of 5-hydroxyindoles, notably 5-hydroxytryptamine and 5-hydroxyindoleacetic acid (cf. reference 2, p. 204) and the 6-hydroxyindoles (3, 4).

A method for the determination of the four hydroxyskatoles has been described (5). However, these compounds are excreted as sulfate conjugates and must, therefore, be hydrolyzed to the corresponding hydroxyskatoles before they can be assayed by the method of Heacock and Mahon (5). The sulfate esters of the 5-, 6-, and 7-hydroxyskatoles are readily converted to the hydroxyskatoles by enzymic hydrolysis. However, the sulfate ester of 4-hydroxyskatole is not hydrolyzed by this procedure (6) and must be assayed directly. Therefore, although the sulfate conjugates of 5-, 6-, and 7-hydroxyskatoles have been detected in urine, it has not been possible to establish the presence of the 4-hydroxyskatole conjugate (7, 8). The present communication describes a procedure for the detection and determination of 4-hydroxyskatole sulfate ester in urine. This method may be used in conjunction with the method of Heacock and Mahon (5) for the assay of the other three isomers.

Materials. 4-Hydroxyskatole (9) and its sulfate ester (6) were prepared by methods described in the literature. The alumina G (maximum grain size 40 μ) and silica gel G (grain size 10–40 μ) were obtained from E. Merck A. G., Darmstadt, Germany. The alumina was prepared as a slurry in phosphate buffer (pH 7.0). Glusulase[2] was obtained from Endo Laboratories, Richmond Hill, New York.

Procedure. Urine (10 ml) was adjusted with concentrated sulfuric acid to pH 2.5 and extracted with water-saturated *n*-butanol (5 $\times$ 10 ml). The combined extracts were evaporated to dryness on a rotary flash evaporator at 40–45°C. The residue was dissolved in water (2 ml), aqueous sodium acetate (0.2 *M*, 2 ml) was added, and the solution was incubated for 1 hr at 38° with Glusulase.

[1] The acid sulfate esters of the hydroxyskatoles are generally described in the medical literature as "sulfatoxyskatoles," although they are more correctly named hydroxyskatole sulfate esters according to chemical nomenclature.

[2] Glusulase is an enzyme preparation having both glucuronidase and sulfatase activity.

After incubation, protein was precipitated by the addition of ethanol (10 ml) to the hydrolyzate, followed by a further incubation for 1–2 min. The solution was then centrifuged and the precipitate was separated and washed with ethanol (2 × 1 ml). The combined washings and supernatant were evaporated to dryness at 30–40°. The residue was dissolved in water, HCl (2 *N*, 5 drops) was added, and the solution was extracted with ethyl acetate (5 × 4 ml). The aqueous mother liquors were then extracted with water-saturated *n*-butanol (5 × 4 ml).

The combined butanol extracts were evaporated to dryness at 40–45° and the residue dissolved in methanol (0.5 ml). A sample (50 μl) of this methanolic solution was spotted on to a silica gel G thin-layer plate and chromatographed (development time 3 hr) using *n*-butanol/ethanol/cyclohexylamine (76/3/6) as the developing solvent. The plate was then dried in a cold-air current for about 10 min and sprayed with a modified Ehrlich reagent (*p*-*N*,*N*-bis-(2-chloroethyl)aminobenzaldehyde, 1% in methanol/HCl (3/1)) (10). Any sulfate ester of 4-hydroxyskatole which is present then appears as a blue spot (R_f 0.60).

Estimation of 4-Hydroxyskatole Sulfate Ester. After drying briefly in a cold-air current, the zone containing the 4-hydroxyskatole sulfate ester was excised and eluted with methanol (1.3 ml) and a further amount (0.2 ml) of the color reagent was added. Maximum color development was obtained after the solution had been allowed to stand for 1 hr; the intensity of the color was measured from the absorbance at 620 mμ of the solution. The concentration of 4-hydroxyskatole sulfate ester was estimated by comparing this absorbance with a calibration curve. The reference solution for this measurement was prepared in the way described above for the sample solution, but using a clear zone of the silica gel G on the plate. Absorbance measurements were obtained with a Unicam SP800 spectrophotometer equipped with a scale expander.

The above extraction and chromatographic procedures were applied to a series of solutions of authentic 4-hydroxyskatole sulfate ester (up to 3 μg/ml) in urine and the same urines alone. A calibration curve was obtained by plotting the absorbances at 620 mμ (due to added 4-hydroxyskatole sulfate ester) against the concentrations of added 4-sulfate ester.

Results and Discussion. Most of the commonly used indole color reagents were examined for their usefulness in the colorimetric assay of the sulfate ester of 4-hydroxyskatole. The modified Ehrlich reagent (*p*-*N*,*N*-bis-(2-chloroethyl)aminobenzaldehyde) (10) was found to be the most suitable on the basis of the stability and intensity of the absorbance in the visible region of the colored product.

No satisfactory paper chromatographic system has yet been reported for the separation of the four isomeric sulfate esters of the hydroxy-

skatoles. An attempt was made, therefore, to develop a thin-layer chromatographic method for the resolution of mixtures of these compounds. Approximately 200 solvents in combination with silica gel G or alumina as adsorbent were examined for their usefulness in the thin-layer chromatographic separation of these compounds. The best separation was obtained when methyl acetate/methanol/cyclohexylamine (3/4/2) was the developing solvent and alumina was the adsorbent. This system permitted a partial resolution of mixtures of the four isomers into three fractions: 4- (R_f 0.80), 6- (R_f 0.52), and the 5- (R_f 0.64) and 7- (R_f 0.68) isomers which were partially overlapped. This system was not considered to resolve the four isomers sufficiently well to provide a basis for an assay procedure. For the purposes of the assay, a solvent system was therefore sought that gave the best separation of the 4-sulfate ester from the hydroxyskatoles and other related substances; e.g., indican, found in urine, this solvent was found to be *n*-butanol/ethanol/cyclohexylamine (76/3/6) with silica gel G as adsorbent.

The isomeric sulfate esters of the hydroxyskatoles are hydrolyzed by dilute mineral acid to yield the corresponding hydroxyskatoles (6) but the application of this method to urine extracts results in the destruction of these compounds. The sulfate conjugates of 5-, 6-, and 7-hydroxyskatoles may be hydrolyzed by Glusulase, a reaction that occurs without complications when these compounds are present in urine extracts. This reaction has been used as the basis of an examination of urines for the sulfate esters of 5-, 6-, and 7-hydroxyskatoles (7, 8). The 4-sulfate ester may, therefore, be separated from its isomers by hydrolysis of its isomers by Glusulase, followed by extraction of the resulting hydroxyskatoles. The mother liquors then contain any 4-sulfate ester present. This isomer is detected after thin-layer chromatography of a sample of the *n*-butanol extract from the mother liquors followed by spraying with the modified Ehrlich reagent, and is assayed colorimetrically from measurements of the absorbance at 620 mμ of the solution obtained after eluting the appropriate zone from the chromatogram. The calibration curve obtained with urine specimens to which known amounts of authentic 4-hydroxyskatole sulfate ester had been added and then carried through the assay procedure is shown in Figure 1. In this way, concentrations of the 4-sulfate ester corresponding to 0.5 μg/ml of urine may be estimated ($\pm$5%). Considerably lower levels (ca. 0.05 μg/ml) may be detected visually on the chromatograms. The over-all recovery of 4-hydroxyskatole sulfate ester added to urine was 75%.

Until recently *in vivo* hydroxylations of positions 4 or 7 of the indole nucleus were not recognized in animals. Hydroxylation at C-7 in skatole has now been shown to occur in man (7). Physiologically active plant

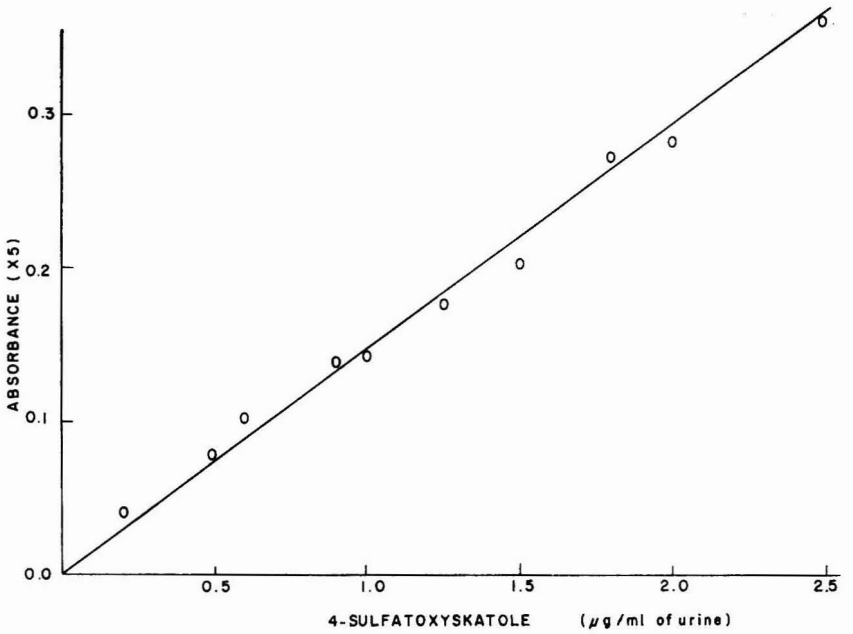

FIG. 1. Calibration curve at 620 mμ for 4-hydroxyskatole sulfate ester added to urine.

alkaloids which contain the 4-hydroxyindole structure have been isolated, e.g., mitragynine, psilocin (4-hydroxy-ω-*N*,*N*-dimethyltryptamine), and its 4-*O*-phosphate ester, psilocybin. The method described above will make it possible to determine whether, in man, skatole is also hydroxylated at position 4. When used in conjunction with the method of Heacock and Mahon (5), the present method will permit a comprehensive study of the metabolic hydroxylation of skatole and should lead to confirmation, or otherwise, of the suggested relationships between hydroxyskatole excretion and various clinical disorders.

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Improved Method for the Fluorometric Estimation of Spermidine[1]

This laboratory's interest in the occurrence of the aliphatic polyamine, spermidine, in tissue, arose in part from the finding (1, 2) that spermidine is a major contaminant in the (*n*)-butanol extraction of tissue for the *o*-phthalaldehyde (OPT) fluorometric method (3) used in the determination of histamine. The fluorophore resulting from condensation of spermidine and OPT provides a sensitive method for the estimation of this compound in extracts of tissue and biological fluids (1, 2). Histamine can be separated from spermidine by ion-exchange chromatographic purification of tissue extracts (1, 2) on cellulose-phosphate (Cellex-P), a weak cation-exchange resin. In previous studies, recovery of spermidine was accomplished by elution of the column with 0.1 *N* HCl (1) or 0.2 *N* HCl (2, 4). Kremzner has shown that Cl^- quenches the fluorescence of the spermidine-OPT product, and for optimal results he therefore removed the hydrochloric acid by flash evaporation (4).

The following communication reports a simplified method, used for the estimation of spermidine in *Lactobacillus casei*, which circumvents the need for evaporation of hydrochloric acid. This is accomplished by direct elution with nitric acid.

[1] This research was supported by United States Public Health Service, National Institute of Neurological Diseases and Blindness, Grant Number, 5 ROI-NB-06053.

Methods. Lactobacillus casei was grown by the method of Birnbaum and Lichstein (5) with the following modification: Biotin (10^{-2} μg/ml medium) was added. Suitable samples of *L. casei* containing 2.5 to 20 nanomoles of spermidine were taken from the growth medium and centrifuged at 3020 *g* for 5 min in a refrigerated centrifuge. The resulting

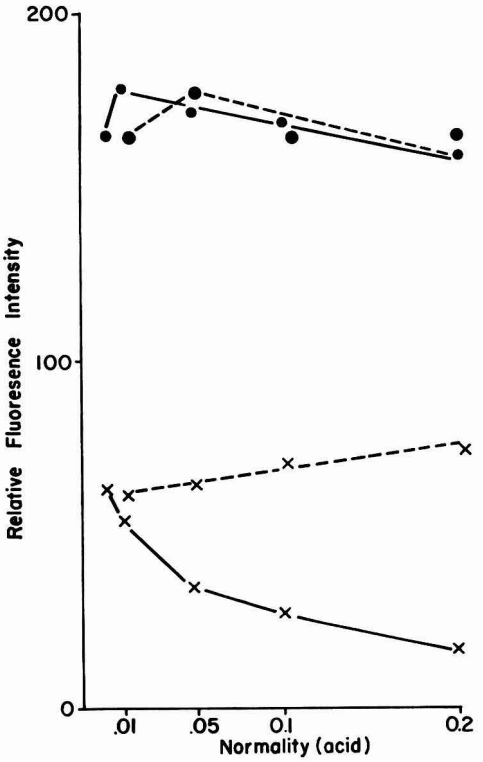

FIG. 1. Relative fluorescence of 1 ml each of histamine (●) (1 nanomole) and spermidine (×) (10 nanomoles) in differing concentrations of hydrochloric acid (———) and nitric acid (- - - -) after reactions with OPT as described under "Methods." Histamine: activation 350 mμ, emission 450 mμ. Spermidine: activation 350 mμ, emission 400 mμ.

pellet was washed 3 times with cold saline, and the final pellet homogenized in 5 ml of 0.4 *N* $HClO_4$. The acid extract was recentrifuged, and the clear supernatant prepared for ion-exchange chromatography by neutralization with solid K_2CO_3. The precipitated $KClO_4$ was removed by centrifugation, and the final supernatant adjusted to pH 6.1 by means of a glass electrode.

The chromatographic purification of spermidine was achieved by a con-

tinuous gradient eluant generated as follows: 100 ml of 0.5 *N* HNO_3 contained in an aspirator bottle (i.d. 8 cm) was allowed to flow into a second bottle (i.d. 5 cm) containing 100 ml of water and a magnetic stirring bar. The output from this second bottle was connected to the top of the ion-exchange chromatographic column. Fractions of effluent (0.8 ml) were collected with a fraction collector. The contents of each tube were directly assayed for spermidine by adding 0.4 ml of 1.0 *N* NaOH,

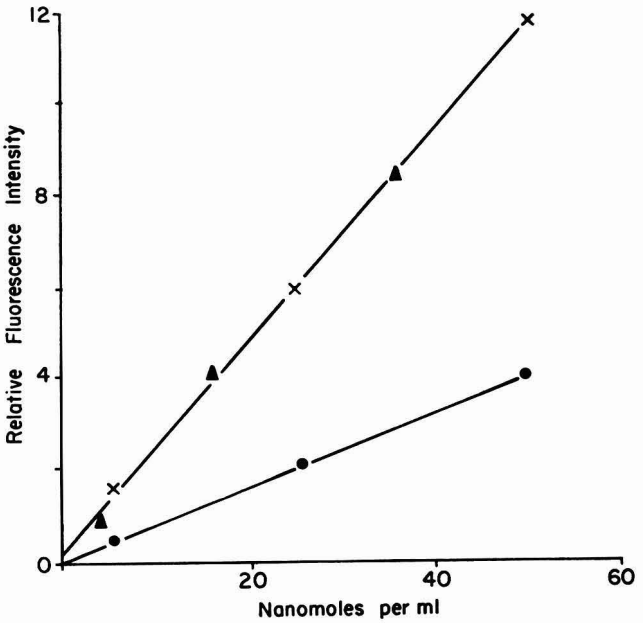

FIG. 2. Standard curve of spermidine fluorescence in the presence of 0.1 *N* HCl (●), 0.1 *N* HNO_3 (×), and 0.1 *N* H_3PO_4 (▲). Fluorophore developed as described under "Methods." Activation 350 mμ, emission 400 mμ.

followed by 0.05 ml of 1% OPT in methanol. After 3 min, 0.2 ml of 3.5 *M* H_3PO_4 was added and the fluorescent emission read at 400 mμ when activated at 350 mμ (Aminco Bowman spectrophotofluorometer) (4). Spermidine was recovered in fractions 32 through 42. The peak recovery corresponded to a point on the gradient elution curve equivalent to 0.1 *N* HNO_3. Known amounts of spermidine carried through this procedure showed 95% to 96% recovery.

Results and Conclusions. Figure 1 shows that HNO_3 from 0.01 to 0.20 *N* does not quench the histamine or spermidine fluorophore; similar concentrations of hydrochloric acid significantly decrease the fluorescence of the spermidine fluorophore (4), but not the histamine fluorophore. The spec-

tral characteristics of either the histamine-OPT or the spermidine-OPT fluorophore do not change when nitric acid is substituted for hydrochloric acid.

Figure 2 demonstrates the linearity of fluorescence intensity with increasing spermidine concentrations in 0.1 *N* HCl, 0.1 *N* HNO_3, and 0.1 *N* H_3PO_4. This graph shows a 3-fold increase in sensitivity in the estimation of spermidine by use of nonhalogen acids. Kremzner (4) has likewise observed no quenching with phosphate ion.

Using the nitric acid rather than hydrochloric acid for the chromatographic purification of spermidine results in a significant increase in sensitivity of this fluorometric method. It further eliminates the need for evaporation of hydrochloric acid.

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Preparation of Starch Gels Containing 8 Molar Urea

Starch gels suitable for electrophoresis of proteins according to Smithies (1) can readily be prepared using buffer solutions containing up to 8 *M* urea (e.g., reference 2). The presence of urea in the starch gels has been shown to improve resolution of proteins in some cases (e.g., reference 3) and can also be used to eliminate multiple bands when these are due to different degrees of aggregation of a single subunit. However, difficulties arise if gels of accurately controlled pH and ionic

strength are required in the presence of high concentrations of urea. Ammonium cyanate formed from the urea, particularly on heating, ammonium carbonate produced by hydrolysis of the cyanate, as well as ammonia resulting from the biuret reaction, all affect the pH and ionic strength of the solution (4). The effect on pH is, of course, particularly marked for acid gels.

These reactions can be inhibited by Zeocarb 225 resin in the H^+ form, as can be seen from the following. A solution of urea (British Drug Houses, commercial grade) was prepared (9.5 *M*) without heating. The pH was found to be 10 and the resistance in a standard conductivity

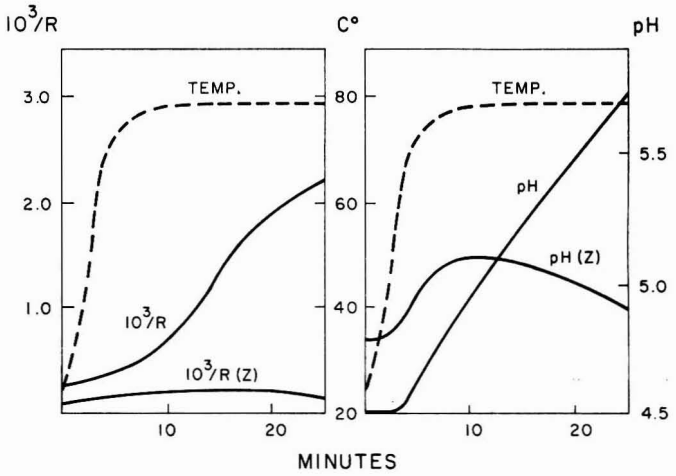

FIG. 1. Effect of heating 9.5 *M* urea solution at 80°. The pH and resistance (*R* measured in a standard conductivity cell) of a 9.5 *M* solution of urea was measured as the solution was heated in an 80° bath. The curves marked with (Z) were obtained in solutions containing 2% (w/v) Zeocarb 225 H^+ resin (16-50 mesh).

cell, 8000 ohms (the cell was not calibrated in terms of absolute conductivity, but all measurements were taken in the same cell at the same temperature and used only for comparative purposes). The pH was adjusted to 4.5 with acetic acid and the solution immersed in a water bath at 80°. Changes in temperature, pH, and resistance with and without the presence of Zeocarb 225 H^+ resin (2% w/v) were followed with time (Fig. 1). Without the resin the final resistance was equivalent to a 50 m*M* sodium acetate buffer, but in its presence the rise in pH and drop in resistance were reduced to negligible proportions. A modified procedure was thus adopted for preparation of gels containing 8 *M* urea whereby resin is present in the mixture as long as any heating is required, but is removed before addition of the buffer.

To prepare 500 ml of gel containing 8 *M* urea and 12% starch, urea (288 gm) was stirred with 150 ml water containing 2 gm Zeocarb 225 H^+ resin (16–50 mesh) and heated until completely in solution (60°). Starch (72 gm) was suspended in 146 ml water in a 2 liter round-bottom flask and the urea solution, containing the suspended resin, was added with vigorous swirling. The mixture was then heated in an 80° water bath with constant swirling for about 10 min, and cooled in a water bath at room temperature for 20 to 30 min. In the presence of urea the gel is slow to set and remained fluid at this point even though the temperature had dropped to 40°. The resin was now filtered off through a muslin cloth and, as a check, the resistance was always measured at this stage (4000 to 6000 ohms in the standard cell). To 450 ml (five-sixths of the total volume) 50 ml sodium acetate buffer (pH 4.0, *I* 0.10) was added, the solution well mixed, degassed rapidly, poured into a tray and allowed to set overnight.

In this way the pH of the gels could be controlled to within ±0.1 of a pH unit and the maximum variation in conductivity was equivalent to about a ±0.002 change in ionic strength. The final pH of the gel was approximately 0.8 of a unit higher than that of the buffer solution used (for a discussion of pH in the presence of high concentrations of urea see reference 5).

This technique was used to study the effect of urea on the electrophoretic behavior of ovomucoid which shows multiple bands on starch gel electrophoresis only over a very narrow range of pH and ionic strength. It was found that the electrophoretic pattern and the isoelectric point of ovomucoid were very little altered in the presence of 8 *M* urea. However, the ovomucoid-trypsin complex is dissociated in 6 *M* urea (6).

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An Inexpensive Luer-Fitting Chromatographic Column

This communication describes a chromatographic column of simple design, which has been used widely in our laboratory and in others at the National Institutes of Health. Its advantages over those columns generally available commercially are: (*1*) a small dead space between the sintered glass disc and the column outlet, thus minimizing mixing space and loss of resolution; (*2*) an outlet which conveniently allows connection to commercially available, inexpensive plastic three-way stopcocks, and connector tubing with Luer fittings; (*3*) a simple in-

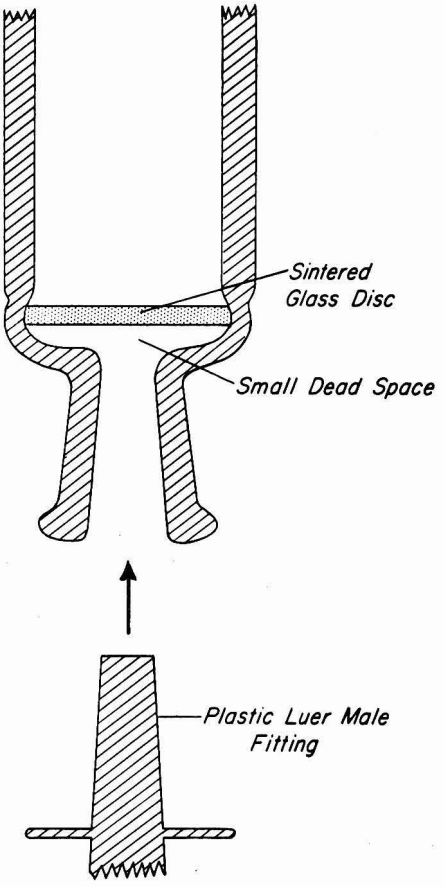

FIG. 1.

expensive design. The design of the column is illustrated in Figure 1.

These columns have been manufactured with reproducible precision in the NIH glass shop[1] with a forming tool, machined to the shape of a male Luer fitting. With this tool (Fig. 2) the column fittings are easily shaped from Pyrex glass tubing and are then annealed to glass columns.

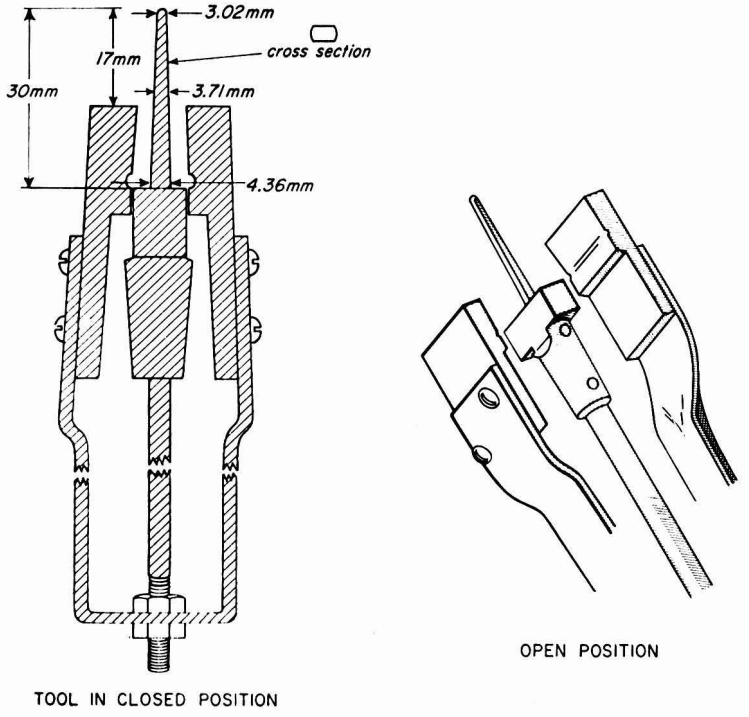

FIG. 2.

The male end of a plastic three-way stopcock or Luer connector tubing is inserted into the column outlet (Fig. 3), and the column eluate can then be diverted to a colorimeter or fraction collector. We have found it convenient to anneal these Pyrex fittings to glass reagent bottles, thus enabling us to use the tubing and stopcocks for our eluant inflow systems.

We use nylon three-way stopcocks and polyvinyl intravenous tubing with nylon Luer-fitting connections[2] for work with relatively neutral aqueous solvent systems. By removing the polyvinyl tubing and thread-

[1] We wish to acknowledge the excellent workmanship and cooperation of Mr. William Kump and the NIH glass shop in the development and production of these columns. These columns will be manufactured commercially by Ace Glass Incorporated, Vineland, N. J.

[2] Available from Pharmaseal Labs, Glendale, Calif.

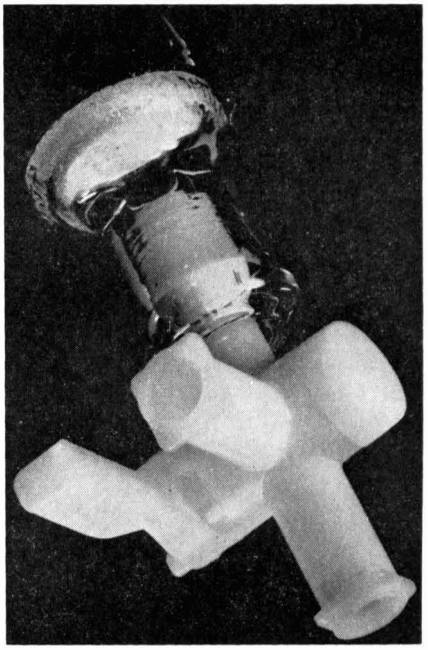

FIG. 3.

ing Teflon spaghetti tubing[3] through the nylon fittings, one can readily obtain a system resistant to most aqueous and nonaqueous solvents with the exception of phenol, strong acids, and alkali, to which the nylon is not resistant. For use with these latter solvents and for high-pressure systems, Kel-F and Teflon valves, connectors, and tubing are commercially available,[4] but they are considerably more expensive than the polyvinyl-nylon system.

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[3] The tubing used has an i.d. of 0.068 in. and an o.d. of 0.092 in.; available from Federal Scientific Co., Inc., Kensington, Md.

[4] Available from Hamilton Co., Whittier, Calif.

Determination of Fructose and Fructose-Yielding Carbohydrates with Cold Anthrone

Fructose reacts at room temperature with anthrone (1). This reaction can be used for quantitative determination in carbohydrates that yield fructose upon hydrolysis, and for the determination of fructose in the presence of glucose and other carbohydrates that do not react at room temperature.

Procedure. On the day of use, mix 150 mg anthrone with 100 ml diluted sulfuric acid (760 ml sulfuric acid in 300 ml water). Evaporate the carbohydrate solution to approximately 0.1 ml, but not to dryness; mix with 3 ml reagent; and, after 1–1.5 hr, read at 620 mμ against an identically treated fructose standard (20–50 μg). At least 90% of the maximum optical density is reached in 10 min at 40°, in 1 hr at 30°, in 2 hr at 25°, and in 4–5 hr at 20°C.

TABLE 1

Optical Density Produced by Reaction of Carbohydrates with Anthrone after 1.5 Hours at 27°C (Cold) or 15 minutes at 90° (Hot), Measured in a Coleman Junior with 12 mm Round Cuvets

	Cold			Hot	
Carbohydrate	μg	Fructose equivalent	Optical density	μg	Optical density
Fructose	50	1	0.69	50	0.73
Inulin	50	1	0.67	50	0.76
Sucrose	95	1	0.70	47.5	0.72
Melizitose dihydrate	150	1	0.75	50	0.75
Raffinose pentahydrate	165	1	0.73	55	0.66
Sorbose	50		0.51	50	0.54
Glucose	50	0	0.00	50	0.72
Maltose monohydrate	100	0	0.00	50	0.74
Melibiose dihydrate	105	0	0.00	52.5	0.61
Glycogen	100	0	0.00	50	0.70
Fucose	50	0	0.08	50	0.70
Rhamnose	50	0	0.08	50	0.70
Galactose	50	0	0.00	50	0.43
Mannose	50	0	0.00	50	0.37

The total carbohydrate content can be determined after heating with anthrone for 15 min at 90°C (1). In this case it is recommended to use as a standard the carbohydrate that is most prevalent in the mixture. Table 1 demonstrates that the difference in reaction velocity at room

temperature and 90° allows quantitative differentiation between keto and aldohexoses. Hydrolysis proceeds so rapidly with the acid reagent at room temperature that the fructose moiety of inulin, sucrose, melizitose, and raffinose reacts with the same speed as does free fructose. When the fructose moiety of a carbohydrate has been determined with cold anthrone, the reaction can be completed by heating the same tube at 90°. This procedure can be used for the identification of sugars eluted from chromatograms. Fructose can be determined in a 10-fold excess of glucose (Table 2). When fructose constitutes no more than a few per

TABLE 2
Optical Density of Glucose-Fructose Mixtures Reacting with Anthrone

Fructose, μg	Glucose, μg	Optical density at 27°C, 1.5 hr
50	—	0.71
50	50	0.70
50	500	0.73
50	2000	0.90
—	500	0.04
—	2000	0.23
		at 40°C, 10 min
50	—	0.75
50	500	0.75

cent of the total carbohydrate mixture, it is recommended to measure first the total amount with hot anthrone and then to add this amount to the fructose standard in order to correct for overassessment due to a slow reaction of aldohexoses with cold anthrone.

ACKNOWLEDGMENTS

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A Multitemperature Bath[1]

A bath is described which produces a range of temperatures simultaneously. It was developed for the purpose of studying the temperature dependence of chemical reactions but has application in any investigation relating response to a range of temperatures.

Two types of multitemperature baths are presently available. The bath described by Oppenheimer and Drost-Hansen (1) is limited to samples of about 10 ml, while that of Miller and Davey (2) may lack sufficient precision for some applications. The system described has a well volume up to 1500 ml and control of ± 0.05°C or better.

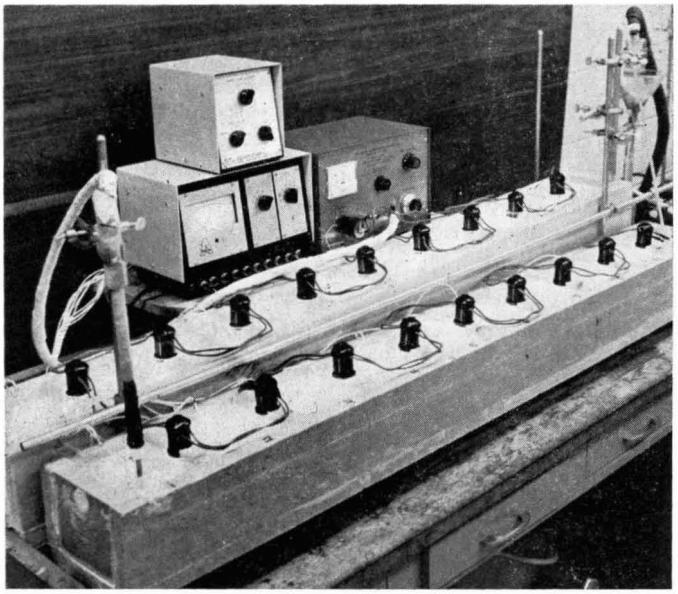

FIG. 1. Multitemperature bath containing 20 temperature wells. Each well is 12.5 × 12.5 × 10 cm deep. Right-hand corner shows gravity feed from external bath source and fiberglass filter. Left-hand corner shows controller probe position.

The bath is shown in Figure 1. The hull and the well partitions were constructed from 2.5 cm polystyrene foam board (Dow Chemical Co., Styrofoam SB) and joined together with General Electric RTV-102 silicone rubber. Transparent acrylic tubes of 3.7 cm i.d. and 12 cm depth were mounted in the partitions. These formed water jackets for 40 W in-

[1] Supported in part by the Research Board, University of Illinois.

candescent showcase bulbs, wired in parellel, which served as heaters. Epoxy resin was applied to the bulb-socket joints to prevent corrosion. A section 1.6 cm deep was removed from each cylinder to form an overflow on the upstream side. A slot 2.0 $\times$ 0.3 cm was sawed out on the downstream side. These details are shown in Figure 2.

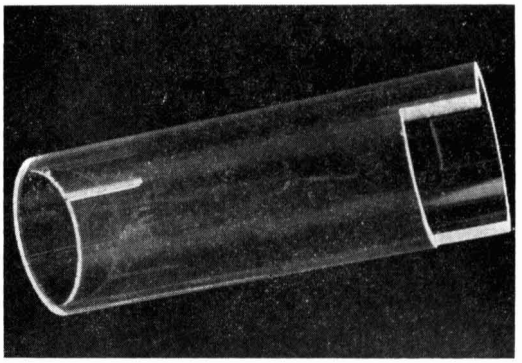

FIG. 2. Lucite cylinders used as temperature jackets. The cylinder is 12 cm long with an i.d. of 3.7 cm.

Operation. Water is fed at a constant rate (gravity feed) from an external constant-temperature circulation bath ($\pm$0.05°C) into the first well, where it mixes and flows by gravity into the first jacket to gain heat. It then flows through the slot into the second well, where it mixes to produce a slightly higher temperature than in the preceding one. The process continues through the remaining wells, the water finally returning to the external bath for cooling and recirculation. A flow rate of 250 ml/min was found adequate to prevent thermal stratification in the wells.

Because of head loss across the slots, it was necessary to mount the bath on a slight incline. An incline just under the total head loss across the bath is best. To prevent plugging of the slots, the water was passed through a fiberglass filter.

A constant water feed is attained by pumping water from the circulation bath to an overflow container. Water then siphons from the overflow container to the first well, the siphon rate is regulated by the height of the overflow container and the diameter of the siphon tube. The overflow is caught in a funnel and filtered through fiberglass. In selecting a circulation bath it is useful to note that for a flow rate of 250 ml/min the cooling requirement is 60 BTU/hr/°C. For a 20°C rise through the multitemperature bath, a 1200 BTU/hr cooling would be required.

The temperature gradient was controlled with a Bayley proportional controller. This controller varies the voltage across the bulbs to correct

for fluctuations of room temperature and flow rate. The controller probe is mounted in the middle well, in the water moving most rapidly. This assures the most rapid response of the probe. To minimize heat loss due to evaporation, covers (Parafilm, American Can Co., with holes to accommodate reaction vessels) were placed on the water surface of each well.

With baths with either 10 or 20 wells, temperature control in all wells of ±0.05°C was obtained. This degree of temperature control was found in all parts of the wells except for an area immediately in front of the outflow slot. The temperature variation was measured with thermistors (time constant 5 sec). Due to heat exchange with the room, the temperature gradient is slightly nonlinear. Baths of this type have been used with well sizes ranging from 12.5 × 12.5 × 10 cm deep to 4.5 cm wide × 11 cm long × 12 cm deep. With a bath with 20 wells the largest temperature range used was 25° and the smallest was 10°. The lowest temperature used was 10° and the highest 45°.

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Separation of Choline from Some Bases on Dowex Columns

Since choline is important in metabolic processes, it is desirable to be able to isolate and quantitate this substance. Some quantitative assay methods are relatively insensitive and others may lack specificity. A commonly used assay method is the periodide test (1), which is very sensitive for detection of relatively small amounts of choline but which also gives positive reactions with many bases. We have utilized a column chromatographic method for the separation of some of the interfering compounds. This method is fast and reproducible and gives almost complete recovery of the substances. The general approach was to chromato-

graph different bases on the Dowex 50-X8 column of a Beckman Spinco amino acid analyzer and to collect the fractions with a fraction collector. The bases were then quantitated by the periodide method.

Experimental Procedure. The solid compounds were taken to constant weight in a vacuum desiccator. A stock solution of each compound, with concentrations ranging from 0.165 to 7.14 μmole/ml, was prepared in the same buffer that was used as eluant in the Dowex columns. This buffer was the pH 5.28 citrate buffer that Spackman, Moore, and Stein (2) used to elute basic amino acids from Dowex columns, but with three important exceptions. Caprylic acid and Brij-35 were deleted because these substances interfered with the periodide test. Phenol is not used in our amino acid analyses, and was also omitted from the buffer. The Dowex 50-X8 column, bead form, 6.5 × 0.9 cm, was the same column that is usually used to separate the basic amino acids in the Beckman Spinco amino acid analyzer. The column was equilibrated with the buffer, some of which was taken from the column outflow for use as reagent blanks in the periodide test.

In order to determine the position of elution, a 1 ml aliquot of a stock solution was placed on the column. The flow rate was 68 to 70 ml/hr and the eluant was collected in 1 ml aliquots in a fraction collector (Gilson Medical Electronics, Middleton, Wisconsin). These aliquots were analyzed by the periodide method of Hayashi *et al.* (1), but with a slight modification. The addition of 0.2 ml of acetate buffer was omitted from the procedure. Potassium iodide (0.1 ml of 0.1 *M*) was added to each 1 ml eluant fraction and to 1 ml of eluant buffer for a reagent blank. Then extraction into 4 ml of ethylene dichloride reagent (0.5% I_2 in ethylene dichloride) was done. Absorption was measured at 385 mμ with a Gilford spectrophotometer 300 (Gilford Instrument Co., Oberlin, Ohio).

Recovery of each of the bases from the column was determined in separate experiments. The absorption of 1 ml of stock base was measured by the periodide method. One milliliter of stock solution was applied to the column and the absorption of each fraction was measured. Since preliminary studies indicated linear standard curves, the ratio of the sum of the absorbancies of the fractions to the absorbance of the stock solution was taken as per cent recovery. Molar extinction coefficients were calculated in order to compare the relative absorbancies of the periodide reaction products.

Experimental Results and Discussion. The compounds tested, the concentrations of stock solutions, the calculated molar extinction coefficients, and the average per cent recoveries are shown in Table 1. Figure 1 shows the positions of elution of ten bases tested; eight are completely separable from choline. The trailing edge of betaine overlapped the leading edge of choline. More complete separation of these two compounds might be

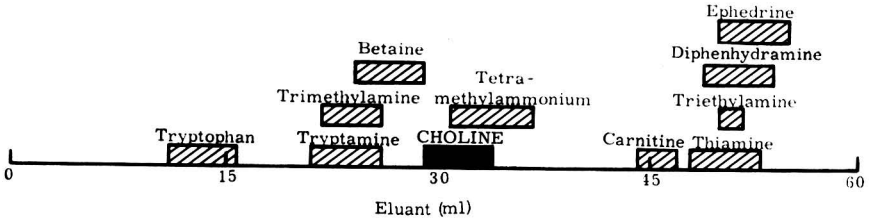

FIG. 1. Eluant pattern of different bases from Dowex 50-X8 column. The quantities of material applied to the column are given in Table 1, to demonstrate the number of milliliters of eluant in which the compounds were detectable with the periodide test.

effected by collecting appropriate aliquots in the fraction collector. The only base which significantly overlapped choline was tetramethylammonium. However, a longer column than 6.5 cm, and alteration of the pH of the buffer, may effect complete separation. The data for the amino acids lysine, histidine, and arginine are not included because these amino

TABLE 1
Concentrations, Calculated Molar Extinction Coefficients and Per Cent Recoveries

Compound	Stock concentration, μmole/ml	Molar extinction coefficient[a]	Average % recovery
Choline chloride	0.358	4,050	99.9
Thiamine hydrochloride	0.165	14,545	92.0
D,L-Carnitine chloride	0.252	6,865	95.4
Tetramethylammonium bromide	0.324	4,877	99.0
Betaine hydrochloride	0.348	4,023	95.2
Diphenhydramine hydrochloride	0.342	3,392	80.1
Trimethylamine hydrochloride	0.523	3,231	93.8
D,L-Tryptophan	0.489	2,822	103.0
Ephedrine sulfate	0.466	2,210	87.3
Tryptamine hydrochloride	1.509	1,643	73.3
Triethylamine hydrochloride	7.143	377	98.0

[a] 385 mμ, Gilford spectrophotometer 300.

acids are not periodide positive. The usual elution sequence on these columns is lysine, histidine, ammonia, and arginine. Choline elutes between histidine and ammonia.

The usefulness of this system is that it takes less than one hour for elution of all the bases tested, and in most instances the separation from choline is complete.

ACKNOWLEDGMENTS

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A Method for the Mass Production of Density Gradients

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New developments in gradient centrifugation such as the zonal centrifuge (1) offer highly sophisticated systems with nearly ideal density gradient centrifugation for the rapid resolution and isolation of complex mixtures of particulates. Swinging-bucket rotors, however, because of their high capacity and general wide applicability to currently available centrifuges, have continued to be a powerful tool for the separation of complex mixtures of particulates. In contrast to the zonal-type rotors, swinging-bucket rotors are generally simple to operate. As an aid to the utility of swinging-bucket rotors, many devices have been suggested for the preparation of continuous density gradients of varying complexity (2–7). Except for the system of Albright and Anderson (2) none of these meets the following criteria for nonzonal gradient applications: (*1*) rapid production of large numbers of density gradients; (*2*) versatility, that is, the preparation of both simple and complex gradients; (*3*) ease of operation; and (*4*) commercial availability. This paper reports on a system having these features, based on the adaption of a model 131 gradient pump[1] coupled to a centrifugal loading device adapted from Albright and Anderson (2). With this system, twelve density gradient tubes (30–60 ml) can be formed in 10 min. Gradient shapes are altered merely by inserting a new template.

METHODS AND MATERIALS

Gradient Machine. A commercial instrument was employed as the basic gradient pump system (Fig. 1). The program cylinder is connected to the minimum density solution. The output of the program cylinder is controlled by the program cam. The program cams are cut from brass shim stock (0.010″). Figure 2 shows the various types of cams that have been employed to produce density gradients of varying shapes. The mixing cylinder pumps pure solution of maximum density

[1] Model 131, special high-capacity gradient pump, Beckman/Spinco.

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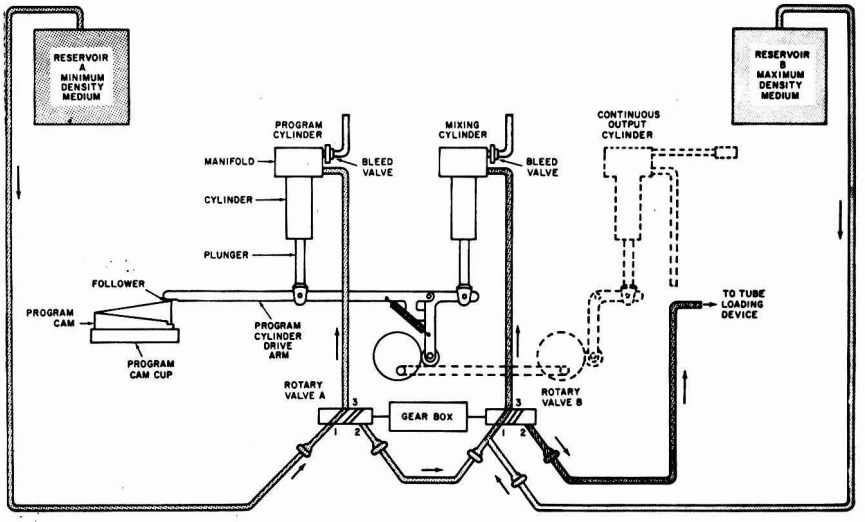

FIG. 1. Flow diagram of modified model 131 gradient machine. Continuous output cylinder has been removed from the flow scheme.

when the program cylinder is arrested. This is accomplished by the arrest mechanism or the program cam (Fig. 1). With no restrictions on the cylinder stroke of the program cylinder (that is, the follower movement is not restricted by the program cam) the gradient machine outflow is pure minimum density solution. Variations in the program cam height (Fig. 1) between the maximum (program follower and plunger arrested) and the minimum (program follower and plunger traverse at maximum) are reflected in an increase in density with a decrease in movement of transverse program cylinder drive arm and follower. Two changes were made in the basic 131 gradient instrument: (*1*) the continuous output cylinder was disconnected from the solution line (Fig. 1) and the outflow from rotary valve B was run directly into the loading head (Fig. 3); (*2*) the index pin on the inner side of the program cam cup (Fig. 1) was moved 75° to permit the addition

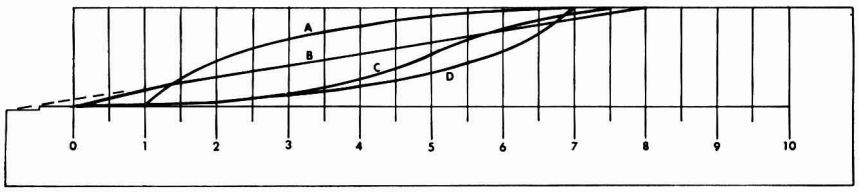

FIG. 2. Program cam shapes required to produce exponential gradients (A and D), linear gradient (B), and logarithmic gradient (C). Stages 0 to 10 are shown on the horizontal axis.

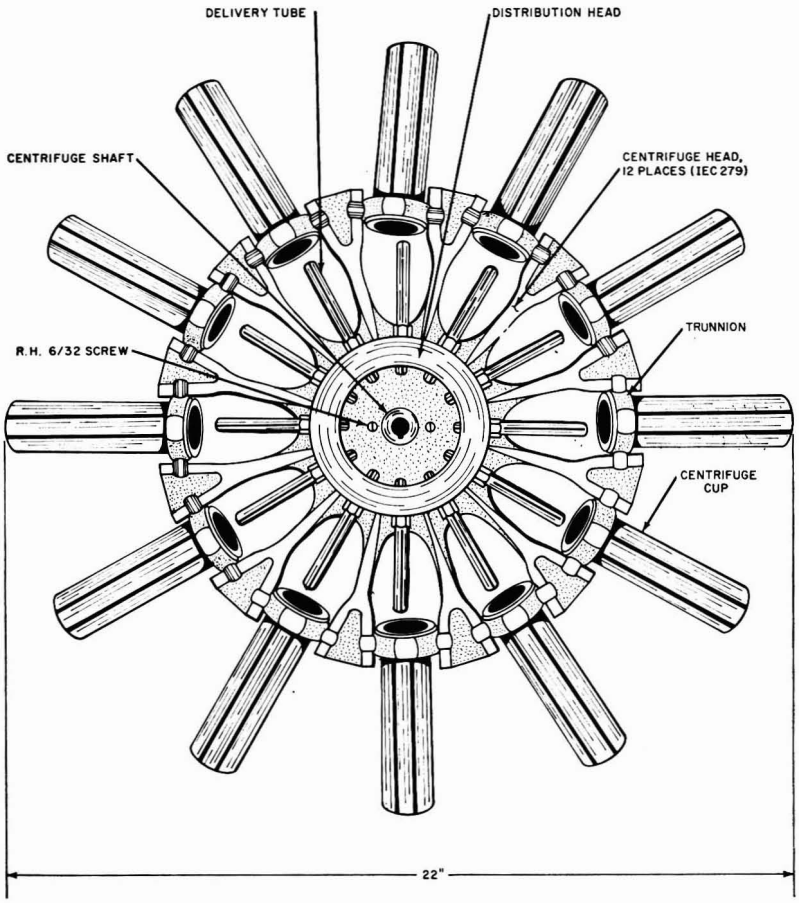

FIG. 3. Loading assembly. Loading head fastened to IEC 279 centrifuge head. Delivery tubes are welded to polyethylene funnels leading to the centrifuge tubes. Centrifuge cups and trunnions are radially arranged as during actual loading operations while the entire assembly is rotating at 1200 rpm.

of a 20 A micro switch to shut down the centrifuge and gradient machine after pumping the prescribed volume of the program.

Tube Loading Device. The mixed gradient outflow from the gradient machine was run directly into the loading head shown in Figure 3. The circular loading head (Fig. 4) was machined from brass and plated with chromium. The main loading head was fastened with brass screws to a 12-place IEC 279 centrifuge head[2] (Fig. 3). The trunnions and cups (100 ml capacity) were standard IEC 350 and 340, respectively. The rubber cup cushions were removed and a polyethylene delivery tube

[2] International Equipment Co., Boston, Mass.

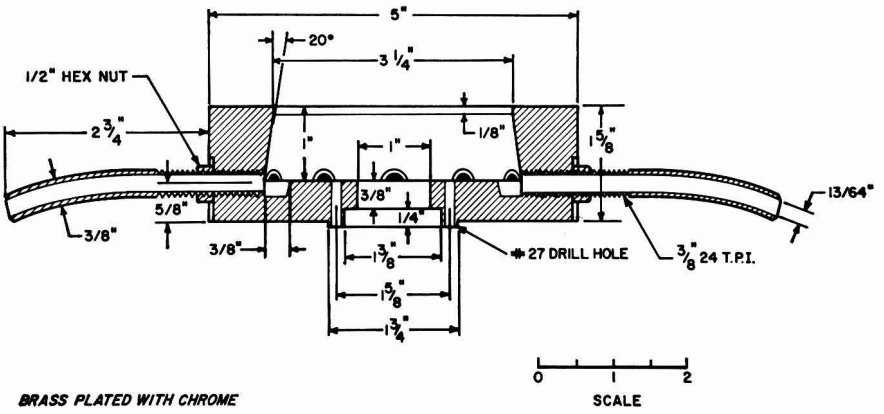

FIG. 4. Cross section of loading head. All parts to scale (in inches).

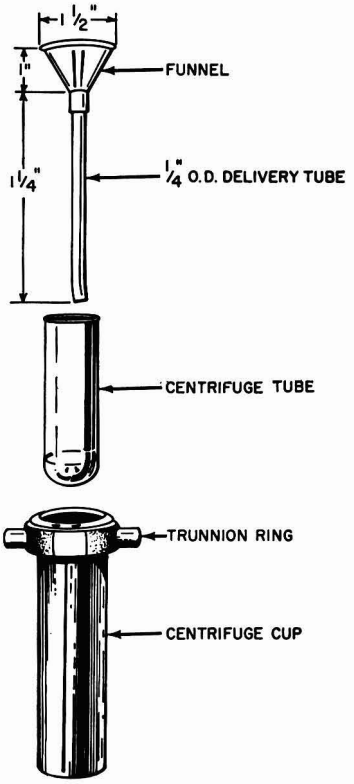

FIG. 5. Tube loading assembly: polyethylene funnel, delivery tube, centrifuge tube, and centrifuge cup with trunnions.

¼″ o.d. was welded with a soldering iron to a polyethylene funnel ½″ in diameter (Fig. 5). The polyethylene tubes were cut so that the delivery orifice was ¼″ from the bottom of the cellulose centrifuge tubes. The complete loading assembly (loading assembly and swinging bucket head, Fig. 3) was mounted in a size 2, model V International centrifuge. The formed gradients were pumped into the rotating loading assembly through a hole drilled in the centrifuge cover.

MODE OF OPERATION

The program cam drive gears were disengaged and the cam cup rotated by turning the driven shaft by hand until the starting index of the program cam was directly under the program cylinder follower pin. Next the pump was turned on for approximately 30 sec to clear the lines leading from rotary valve A to rotary valve B and the outflow tube. Since the displacement of the program follower arm was unrestricted at this stage, pure minimum density medium cleared the lines and valves. The clearing output solution was discarded. Once the

TABLE 1
Programmed Volumes for Centrifuge Bottles

Rotor	Tube volume, ml	Tube dimension	Programmed delivery volume, ml
SW25.1	30	1 × 3″	250
SW25.2	60	1¼ × 3½″	500
#30	38	1 × 3½″	250
TR-250[a]	200	2¼ × 5½″	1000
RC-3[b]	650	3¼ × 6⅛″	500

[a] Bottles loaded in Albright-Anderson (2) 4-place centrifuge assembly.
[b] Bottles loaded individually.

lines had been cleared of maximum density solution (except for the line leading from the maximum density solution reservoir to rotary valve B), the gradient pump was stopped. The centrifuge with its loading head and swinging buckets loaded with empty centrifuge tubes was accelerated to a minimum speed of 1200 rpm, the program cup drive gears were engaged, and the pump started with the gradient outflow leading to the loading head. When the programmed gradient was completed, a micro switch connected in series with the gradient machine and centrifuge shut down both instruments. The centrifuge was permitted to stop without braking, the polyethylene funnel and delivery tubes were carefully withdrawn, and the completed gradients and tubes removed and stored at 4°C until used. Delivery volumes and centrifuge tubes were selected according to need (Table 1). The Albright-Anderson

loading head (4-place) (2) was used for 250 ml centrifuge tubes. The programmed volume was 1 liter. Larger volume tubes (650 ml each) were loaded directly while the bottle rested on a laboratory bench with the delivery tip extending to ¼″ of the bottom.

Changes in delivery volume were made by altering the gear train driving the program cam according to the table given in the model 131 manual (Ref. 8).

CALIBRATION OF GRADIENTS

A 2 *M* sucrose solution containing Evans blue solution equivalent to approximately 640 Klett Units was used as the maximum density medium. The minimum density medium was distilled water. The various templates were mounted on the program cam cup. The prepared density gradient tubes were mounted in a rack and 5 ml increments were taken from the bottom of the completed gradient tube. The sampling device was a 10 ml syringe connected to a blunt No. 13 needle through a three-way stopcock. Eight 5 ml samples were drawn into the syringe from the bottom of the gradient tube and discharged into separate Klett tubes. This process was repeated with additional colorimeter tubes until the gradient tube was emptied. The optical densities of the samples were measured in a Klett colorimeter using a No. 56 filter.

RESULTS

A one factor analysis of variance was carried out on the individual optical density values for each 5 ml layer in the gradient tube. The results of this comparison of gradient distribution from tubes within runs and between runs is shown in Table 2. The individual least significant difference (LSD) for the between-runs data is 19 Klett Units out of 640. The LSD for within-runs data is 10 Klett Units. The LSD for a comparison between both sets of curves is 13 Klett Units, indicating that the individual datum of each (each point is an average of five replicates) is not statistically different. These LSD values indicate that the density averages of the 5 ml layers within the gradient tubes do not differ statistically from each other when compared within runs and between runs.

Variations in the top 5 ml and bottom 5 ml of the prepared gradient tubes were compared in Table 3. The top and bottom 5 ml of tubes prepared during one run varied from the minimum density solution by plus $3.22 \pm 0.80\%$ at the top and $95.4 \pm 0.99\%$ of the maximum density solution at the tube bottom. Density variations in gradient tubes between runs in the top and bottom 5 ml were plus $4.66 \pm 1.65\%$ for the minimum density layer and 96.2% of the maximum density layer. These

TABLE 2
Variability[a] in Klett Units between Formed Gradients[b]

Reproducibility within runs	Reproducibility between runs
17	25
36	42
82	82
174	174
316	321
451	453
516	520
516	520
LSD 19	LSD 10

For comparisons between both curves
LSD 13

[a] Means of five replicate curves (case averages).
[b] Logarithmic gradients.

variations are relatively minor and for most preparative isolations procedures no corrections are necessary.

Attempts to reduce the dilution of the top and bottom gradient layers by increasing the length of the delivery tube (Fig. 5) to less than ¼" from the centrifuge tube bottom resulted in the delivery tube bearing against the bottom of the centrifuge tube. When the delivery tube–centrifuge tube bottom distance is maintained at ¼", the outer edge of the delivery funnel is the bearing surface during the loading operation.

The program cams (Fig. 2) produced the gradients shown in Figure 6. Cam A produced the exponential type curve A shown in Figure 6. Cam B consisted of a straight line run from stage 7.5 (Fig. 2) to the tip of the cam (dashed portion). At stage 1, the cam edge was cut to stage 0

TABLE 3
Variability[a] in per Cent of Total Klett Units between Upper and Lower Limits of Centrifuge Tubes[b]

	Variations within runs		Variations between runs	
	Top	Bottom	Top	Bottom
	2.59	94.4	2.96	96.2
	2.78	96.2	4.63	96.2
	2.59	96.2	4.26	96.2
	4.07	96.2	4.07	96.2
	4.07	94.4	7.40	96.2
Means	3.22 ± 0.80	95.4 ± 0.99	4.66 ± 1.65	96.2 ± 0

[a] Top or lower 5 ml aliquots of individual tubes.
[b] Logarithmic gradients (template C).

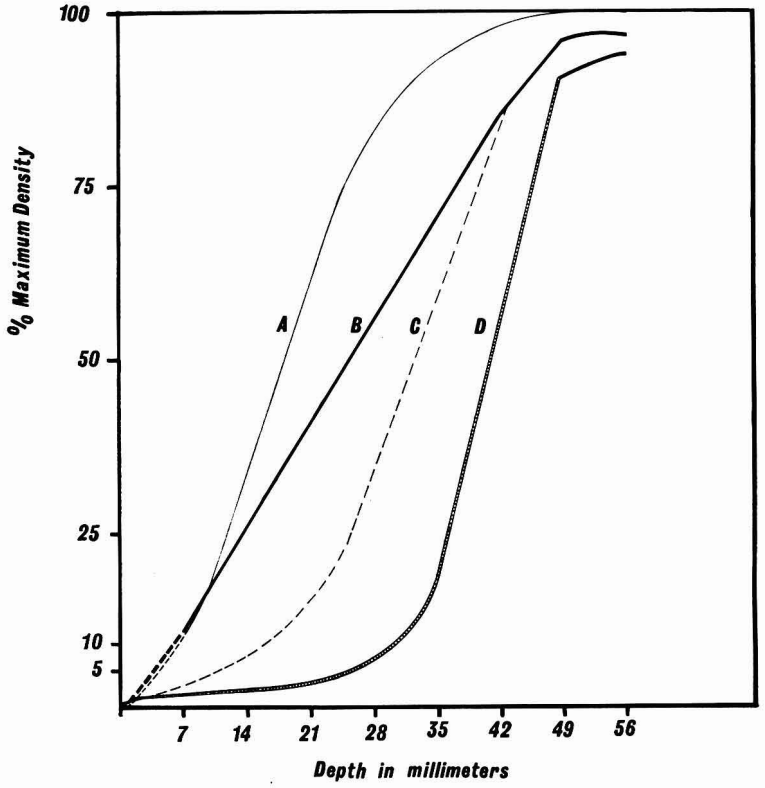

FIG. 6. Curves describing gradients produced by cams A and D (exponential), cam B (linear) and cam C (logarithmic).

to produce a linear response at the top layer of the gradient rather than the initial log type response shown by curve C in Figure 6. Logarithmic gradients (Fig 6, template C) were produced with cam C (Fig. 2). Exponential curves of type D in Figure 6 were produced with cam D shown in Figure 2.

DISCUSSION

The results show the excellent reproducibility of the density gradient production system. With the removal of the continuous output cylinder from the flow train, significant improvement was noted in reducing the gradient machine holdup volume, since this cylinder discharges only three-fourths of the accumulated solution and retains one-fourth of the mixture. Further reductions in mixing were made by adjusting the length of the polyethylene delivery tube (Fig. 5) to within ¼″ of the bottom of the centrifuge tube in the centrifuge cup. Table 2 shows that very little

mixing occurs. The density variations of approximately 5% from the two starting solutions is acceptable for all particulate separations employed in this laboratory. Of greater significance is the gradient reproducibility between runs which was not distinguishable statistically from normal variation within runs (Table 2). Variations in the volumes delivered to the individual centrifuge tubes within the same run were minimized by standardizing the loading assembly speed at 1200 rpm.

The density gradient tubes are stable for at least five days at 4°C. The gradients lose their shape and density characteristics if they are held at room temperature.

Changes in gradient shape can be made rapidly by inserting the appropriate cam. Alterations in maximum gradient density can be made by substituting the appropriate solution reservoirs. No involved computations are necessary to calculate the new densities since the top and bottom densities will be within 5% of the starting solutions. The relative densities along any specific depth can be taken directly from Figure 6. The above features permit rapid alteration in gradient shape during a fractionation run to make critical separations of specific components. The centrifuge (IEC size 2, model V) employed in this study is commonly available in most biochemical laboratories and offers the additional feature of being easily washed free of spills.

The gradient machine when employing the Albright-Anderson (2) loading head also has been employed for loading 250 ml centrifuge bottles which have been used in 4- and 6-place swinging-bucket heads. No variations were observed in either gradient shape or densities with changes in the delivery volumes from the 250 to 1000 ml ranges.

SUMMARY

Liquid density gradients can be mass produced by converting a commercially available gradient pump to a tube loading device. Centrifuge tubes are loaded while under a field of gravity to stabilize the gradient being formed. The top layer of the prepared gradient tubes did not exceed the starting solution density by 5%. The bottom of maximum density solution was never less than 5% of the maximum density solution. Density gradient curves from tube samples between and within runs are highly reproducible. This gradient production and tube loading system is highly flexible and permits rapid alterations in gradients during a fractionation run for optimum separation of cellular particulates.

ACKNOWLEDGMENTS

I am indebted to N. G. Anderson (Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.) for information on the use of the gradient machine

for zonal centrifuge applications. Figure 1 is published with permission of Beckman/Spinco, Palo Alto, California.

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ANALYTICAL BIOCHEMISTRY 19, 211–225 (1967)

Trypsin, Chymotrypsin, and Total Proteolytic Activity of Pancreas, Pancreatic Juice, and Intestinal Contents from the Bovine[1]

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Proteolytic enzyme assays involving the pancreas are dependent on complete extraction and solubilization of the zymogen granules prior to activation. The activation kinetics of purified trypsinogen (Tg) and chymotrypsinogen (ChTg) have been thoroughly studied, and reviewed by Neurath (1) and Northrop *et al.* (2). However, the optimum activation conditions for a mixture of zymogens in a crude extract of bovine pancreatic homogenate or juice have not been adequately characterized. Activation times ranging from 15 min at 37°C to 24 hr or more at 0 to 5° have been used (3, 4). Intestinal contents contain a wide variety of substances that could interfere with enzyme assays. Spectrophotometric esterase assays for trypsin (T) and chymotrypsins A and B (ChT) in rat intestinal contents have been performed (4), but difficulties arose in the present study with one-week-old calves irrespective of diet and older calves fed rations containing soybean protein.

This report compares several procedures for extraction of Tg and ChTg from pancreatic tissue, activation and assay for Tg, ChTg, and total proteolytic activity. Methods employed to remove interfering substances in small intestinal contents, which produced an apparent esterase activity, are presented.

MATERIALS AND METHODS

(*i*) *Homogenization of Pancreas*

Pancreatic tissues from calves or cows were cooled on ice and about 0.1 gm samples accurately weighed; 10 ml of ice-cold 0.15 *M* NaCl or dis-

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tilled water, containing 0 or 0.1% Triton X-100,[3] was added and the mixture homogenized in a Potter-Elvehjem ground-glass tissue grinder. A portion of the homogenate was centrifuged at 1300*g* for 30 min at 4°.

(*ii*) *Activation of Tg and ChTg*

Activation of Tg and ChTg with a crude enteropeptidase (enterokinase) for 15 min at 37°C according to the method of Howard and Yudkin (3) produced low T and ChT activity and several other methods were investigated. Three dilutions of pancreatic homogenate were prepared (0, 1:1, 1:2) and pancreatic juice from another experiment (5) was diluted in 0.15 *M* NaCl to contain from 0.1 to 0.8 mg protein/ml—the protein determined by absorption at 280/260 (6). Activation was carried out at 37 or 5°C with crude enteropeptidase or cystalline T. Enteropeptidase was prepared by mixing 2% Viodenum[4] (w/v) in 0.1 *M* Tris buffer (tris (hydroxymethyl)aminomethane), pH 7.2, preincubated at 37° for 30 min and centrifuged to remove solid material. A stock solution of 0.3 mg T[5]/ml in 0.001 *N* HCl was diluted in the above Tris buffer to contain 0.02 or 0.04 mg T/ml. The Tris buffer contained 50% glycerol or 0.05 to 0.1 *M* $CaCl_2$ (7) in some experiments. Equal volumes of diluted zymogens and activating solution were mixed and T and ChT activities determined at specified intervals. Toluene was added during prolonged activation periods.

(*iii*) *Enzyme Assays*

Total proteolytic activity was determined by the release of trichloroacetic acid (TCA) soluble products from a casein substrate and measuring absorbance (*A*) at 280 mμ (8). The method of Howard and Yudkin (3) was modified by incubating the active enzyme solutions or crystalline T in 5 ml of 0.3 or 1% casein solution at 37°C for 10 or 20 min. Enzyme activity was expressed as μmoles tyrosine[6] equivalent (280 mμ) released/min/gm pancreatic tissue. The method of Miller and Johnson (9) was modified to convert the normal curvilinear enzyme response to a linear relationship with different enzyme concentrations.

Esterase activities of T and ChT were assayed using a recording spectrophotometer, and toluene arginine methyl ester (TAME[7]) and benzoyl

[3] Alkylphenoxypolyethoxyethanol, Rohm and Haas, Philadelphia, Pa.

[4] Produced by Viobin Corporation, Monticello, Ill.

[5] Trypsin, 2× crystallized, salt free, 1-250 R, Nutritional Biochemicals Corp., Cleveland, Ohio.

[6] L-Tyrosine, A grade, lot 36337, Calbiochem, Los Angeles, Calif.

[7] TAME (*p*-tosyl-L-arginine methyl ester-HCl, lot F1155) and BTEE (*N*-benzoyl-L-tyrosine ethyl ester, lot R 4386-R), purchased from Cyclo Chemical Corp., Los Angeles, Calif.

tyrosine ethyl ester (BTEE[7]), respectively, as substrates (10). The procedures used were essentially those outlined by Worthington Biochemical Corp. (11), except for comparisons of dissolving BTEE in 2 to 56% methanol (v/v). Dissolution in 20% or less methanol was accomplished by first added methanol to the BTEE followed by slow addition of water with mixing. Crystalline α-ChT[8] dissolved in 0.001 N HCl was also used to compare BTEE hydrolysis at different methanol concentrations.

(*iv*) *Apparent Esterase Activity of Intestinal Contents*

The supernatant fluid of intestinal contents from many calves produced a marked curvilinear change in A with TAME or BTEE and persisted when the substrates were replaced by distilled water or 56% methanol, respectively. This apparent esterase activity was compared to a similar response with steer bile, certain purified bile salts, and water extracts of calf milk replacer diets containing dry skim milk or soybean protein. Removal of interfering substances from intestinal contents was attempted by the following procedures: (*1*) dialysis against 0.15 M NaCl for 12 hr, (*2*) acid precipitation (pH 4) by dropwise addition of 2 N HCl or 10% acetic acid followed by dialysis of the supernatant against 0.001 N HCl for 12 hr, (*3*) precipitation by solid $(NH_4)_2\,SO_4$ to 0.1 M concentration and addition of 2 N NaOH and excess solid $CaCl_2$ until a constant pH of 8 was attained, and (*4*) use of a slurry of Sephadex G-25 (nonbead).[9] Recovery of T and ChT activities in the supernatant fluids was determined for each procedure. Fat was extracted from oven-dried samples of diets with petroleum ether (65–110 b.p.) for 20 hr. The fat-free extract was mixed with water and the supernatant tested for apparent esterase activity.

RESULTS

(*i*) *Homogenization of Pancreas*

The supernatant of pancreatic tissue homogenized in 0.15 M saline contained only 71% of the T and 65% of the ChT activity, compared with the homogenate or supernatant from saline plus 0.1% Triton X-100 (Table 1). Chymotrypsin activity in saline homogenates was less than the activity in homogenates with saline plus Triton X-100. Triton X-100 induced a marked clearing of the homogenates, presumably due to lysis of zymogen granules (12).

[8] α-ChT crystallized, salt free (bovine pancreas), A grade, lot 52245, Calbiochem, Los Angeles, Calif.

[9] A. B. Pharmacia, Uppsala, Sweden.

TABLE 1
Comparative Release of Trypsinogen and Chymotrypsinogen from Pancreatic Tissue by Saline and Saline–Triton X-100

Treatment	Trypsin		Chymotrypsin		
				Ratio	
	Trypsin units[a]	Ratio saline to Triton X-100	Chymotrypsin units[b]	Saline to Triton X-100	Chymotrypsin to trypsin
Saline					
homogenate	4.91[c]	1.00	1.09	0.88	0.25
supernatant	3.49	0.71	0.81	0.65	0.26
Saline–Triton X-100					
homogenate or supernatant	4.91	1.00	1.24	1.00	0.28

[a,b] One unit equals hydrolysis of 1 μmole TAME or BTEE (in reaction mixture of 26% methanol, v/v) min/mg pancreatic tissue dry matter.
[c] Average of 5 samples/treatment.

Trypsin and ChT activities were lower in both the homogenates or supernatants of tissues homogenized in distilled water, compared with saline plus Triton X-100 (Table 2). Addition of Triton X-100 to the water apparently did not effect rapid rupture of the zymogen granules

TABLE 2
Comparative Release of Trypsinogen and Chymotrypsinogen from Pancreatic Tissue by Water and Saline–Triton X-100

Treatment	Trypsin		Chymotrypsin		
				Ratio	
	Trypsin units[a]	Ratio of water to saline–Triton X-100	Chymotrypsin units[b]	Water to saline–Triton X-100	Chymotrypsin to trypsin
Saline-Triton X-100 vs. Water					
Saline–Triton X-100 homogenate or supernatant	3.14[c]	1.00	0.94	1.00	0.30
Water					
homogenate	2.48	0.79	0.75	0.80	0.30
supernatant	1.62	0.52	0.69	0.73	0.43
Saline-Triton X-100 vs. Water-Triton X-100					
Saline–Triton X-100 homogenate or supernatant	2.83[d]	1.00	0.89	1.00	0.31
Water-Triton X-100					
homogenate	2.88	1.02	0.76	0.85	0.26
supernatant	1.93	0.68	0.76	0.85	0.39

[a,b] One unit equals hydrolysis of 1 μmole TAME or BTEE (in reaction mixture of 26% methanol, v/v)/min/mg pancreatic tissue dry matter.
[c,d] Average of 2 samples, but pancreatic tissue from different calves are represented in *c* and *d*.

since there was reduced enzyme activities in the supernatant. More ChT than T activity was retained in the supernatant of tissues homogenized in distilled water as shown by the higher ratios of ChT-to-T (Table 2).

(ii) Activation of Tg and ChTg

Activation rates of Tg and ChTg in pancreatic homogenates or juice, with constant amounts of enteropeptidase, were inversely proportional to the concentration of inactive zymogens present (Figs. 1 and 2, Table 3). Pancreatic juice samples containing 0.54 mg protein/ml at 37°C in the absence of $CaCl_2$ were completely activated in less than 30 min followed

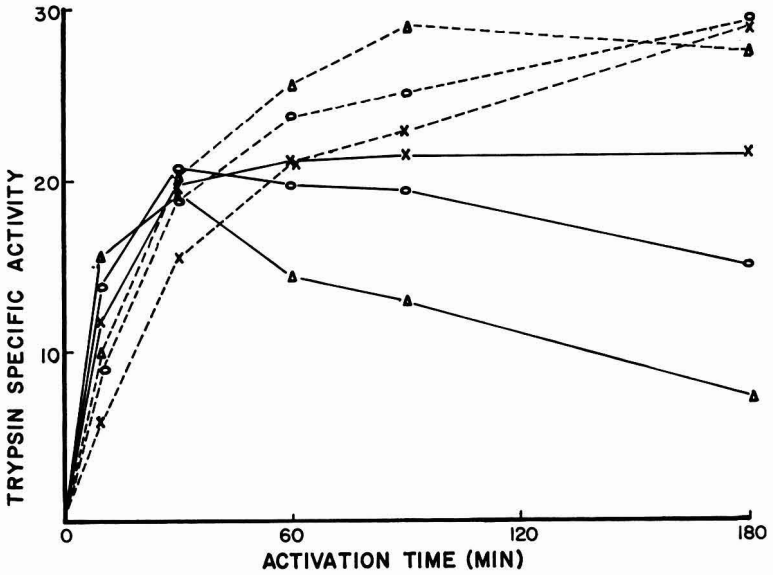

FIG. 1. Effect of pancreatic juice protein concentration and $CaCl_2$ on activation of trypsinogen by enteropeptidase at 37°C: (—) no $CaCl_2$, (---) 0.05 *M* $CaCl_2$ final concentration, (×) 0.13 mg protein/ml, (○) 0.27 mg protein/ml, (△) 0.54 mg protein/ml. One trypsin unit on ordinate equals hydrolysis of 1 μmole TAME/min/mg pancreatic juice protein.

by rapid loss of enzyme activity. Activation of Tg and ChTg in samples containing 0.13 mg protein/ml were not complete until 60 and 120 min, respectively, and T activity was maintained for 180 min or more (Figs. 1, 2). The initial rate of conversion of Tg to T was reduced by 0.05 *M* $CaCl_2$, compared with no $CaCl_2$ at 37°, and 0.13 or 0.27 mg pancreatic juice protein/ml was not fully activated by 3 hr incubation (Fig. 1). However, maximum activity and stability of both T and ChT were enhanced by 0.05 *M* $CaCl_2$. An intermediate effect was observed with

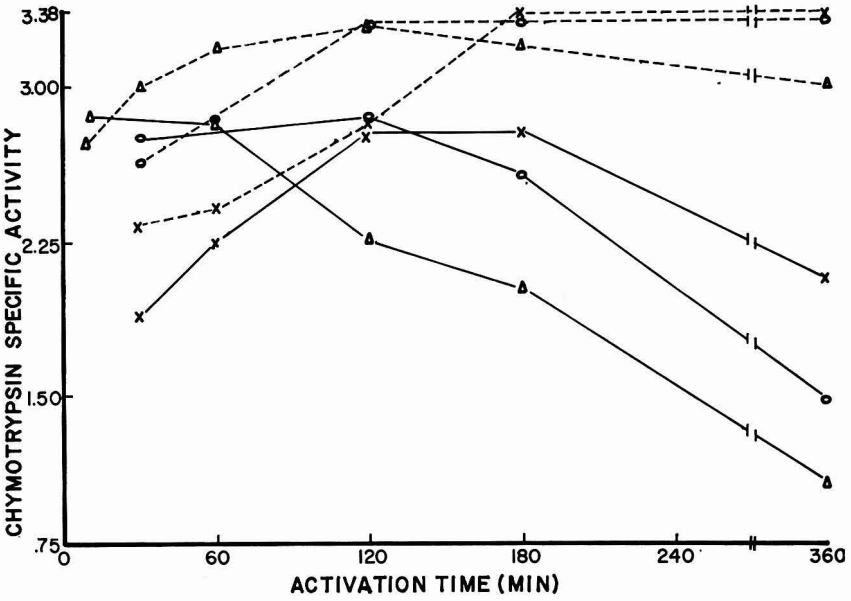

FIG. 2. Effect of pancreatic juice protein concentration and $CaCl_2$ on activation of chymotrypsinogen by enteropeptidase at 37°C: (—) no $CaCl_2$, (- - -) 0.05 *M* $CaCl_2$ final concentration, (×) 0.13 mg protein/ml, (○) 0.27 mg protein/ml, (△) 0.54 mg protein/ml. One chymotrypsin unit on ordinate equals hydrolysis of 1 μmole BTEE in reaction mixture of 26% methanol (v/v)/min/mg pancreatic juice protein.

TABLE 3

Activation of Pancreatic Juice Trypsinogen and Chymotrypsinogen with Enteropeptidase and $CaCl_2$ at 5°C

Activation time, hr	Pancreatic juice dilutions, mg protein/ml			
	0.25	0.50	0.75	Average
		Chymotrypsin units/mg protein[a]		
1–2	5.10[b]	5.28	5.09	5.16
7–8	5.11	5.10	5.09	5.10
24	4.58	4.62	4.62	4.61
48	4.81	4.91	4.79	4.84
		Trypsin units/mg protein[c]		
24	9.40	11.34	12.24	10.99
48	10.69	12.90	13.50	12.36
120	13.84	14.79	14.77	14.47
170	14.70	14.50	14.40	14.53
235	14.94	14.32	13.61	14.29

[a,c] One unit equals hydrolysis of 1 μmole of BTEE in reaction mixture of 4.7% methanol (v/v) and TAME/min, respectively.

[b] All values average of 4 pancreatic juice samples.

0.025 M $CaCl_2$ (not shown in Figs. 1 and 2). Glycerol markedly reduced the rate of activation of Tg, with no effect on the final T activity.

Activation of ChTg in pancreatic juice containing 12.4 to 23.4 mg protein/ml and diluted to 0.25, 0.50, and 0.75 mg protein/ml was complete after 2 hr at 5°C, in the presence of 0.05 M $CaCl_2$ (Table 3). Activity was reduced somewhat by 24 or 48 hr. Activation with enteropeptidase or crystalline T resulted in comparable ChT activity. Complete activation of Tg did not occur until 120 hr for samples containing 0.50 and 0.75 mg protein/ml and 170 to 235 hr in the case of 0.25 mg protein/ml (Table 3). A gradual loss in T activity of the more concentrated samples was evident after 120 hr. Comparable T activities have been achieved by activating pancreatic juice diluted to 0.1 mg protein/ml at 37°C for 6 hr, or 0.25 to 0.50 mg protein/ml at 5° for 170 hr, with enteropeptidase plus 0.05 M $CaCl_2$. Autoactivation with crystalline T always resulted in lower and more variable T activities with each dilution of pancreatic juice. Although the enteropeptidase solution contained a trace of ChT activity, no T activity was present.

Spontaneous activation of pancreatic juice or tissue homogenates did not occur. Repeated freezing and thawing of nonactivated, undiluted pancreatic juice exerted no detrimental effect on T and ChT activity, and in some cases, increased enzyme activity.

(*iii*) *Enzyme Assays*

The normal curvilinear relationship between purified T concentration and casein digestion was made linear by plotting $A + A^{3/2}$ at 280 mμ in terms of micromoles tyrosine equivalent released per minute (Fig. 3). Deviations from linearity with higher levels of enzyme have been attributed to limiting substrate (9). Similar plots were obtained at several different dilutions of pancreatic homogenate but were linear over a much wider range of casein digestion (Fig. 4). A differential response to substrate concentration was apparent between the purified T and the mixture of proteolytic enzymes. The measured enzyme activities of purified T above 15 μg/ml were elevated when using 1.0% casein as compared with 0.3% casein (Fig. 3). Release of TCA soluble products was always less than, or equal to, the higher substrate concentration at each level of activated pancreatic homogenate (Fig. 4). An illustration of the effectiveness of the $A + A^{3/2}$ transformation is presented in Table 4. A fourfold increase in enzyme concentration reduced relative activity by 20% using A values directly, whereas no change was evident when using the term $A + A^{3/2}$. Total proteolytic activity was also 120 to 180 units greater after the linear transformation. Rate of casein digestion/min during 10 or 20 min incubation was equalized using this calculation.

The linear relationship between T and ChT concentration and TAME or BTEE hydrolysis, respectively, reported previously (10) was confirmed. A marked curvilinear absorption change was evident on mixing the buffer, substrate, and enzyme, when the BTEE was dissolved in 56% methanol (v/v), making a final concentration of 26% methanol in the reaction mixture. After 2 to 3 min a reasonably linear response to

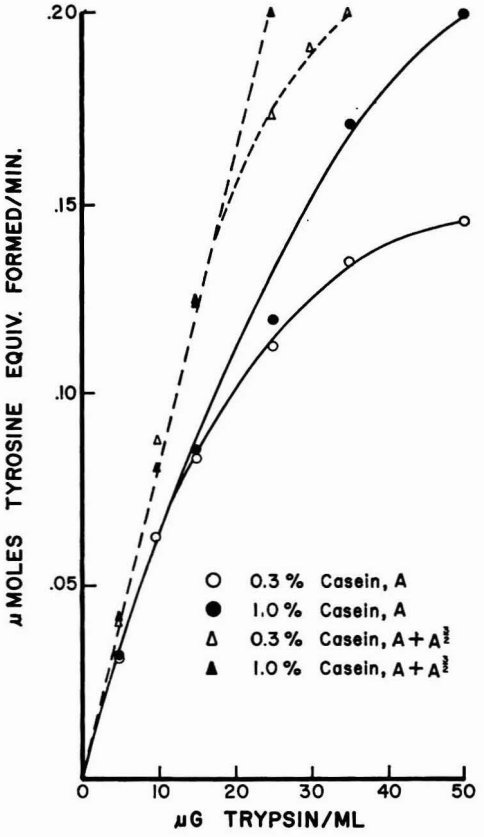

FIG. 3. Digestion of casein by crystalline trypsin and transformation to a linear response by substituting absorbancy (A) plus $A^{3/2}$ in place of A.

enzyme concentration was obtained. This apparent spontaneous hydrolysis of BTEE which was reported previously (10) was negligible when BTEE was prepared in 10% methanol (v/v), or a final concentration of 4.7%, and also a more linear reaction rate was recorded. Rate of BTEE hydrolysis in 31 assays of pancreatic juice was 2.16 ± 0.13 (standard deviation) times greater in 4.7% methanol than in 26%. The ratios of ChT-to-T activities (maximum values from Table 3) were

TABLE 4
Total Proteolytic Activity Based on Absorbancy (A) at 280 mμ vs. $A + A^{3/2}$

Samples,[a] ml	Enzyme units[b]		Relative activity[c]	
	A	$A + A^{3/2}$	A	$A + A^{3/2}$
0.10	290	416	100	100
0.20	263	419	91	101
0.30	242	414	83	100
0.40	228	409	79	98

[a] Bovine pancreatic homogenate, 5.45 mg tissue/ml.
[b] One enzyme unit equals 1 μmole tyrosine equivalent released/min/gm tissue.
[c] Activity for lowest enzyme concentration set at 100.

raised from 0.16 to 0.35 by using the lower level of methanol. The BTEE did not remain in solution when dissolved in 2 or 5% methanol. In contrast to activated pancreatic juice, methanol concentration had a variable effect on the hydrolysis of BTEE by crystalline α-ChT. Dissolution in 20% methanol increased α-ChT activity by 1.6 to 2.0 times that with 56% methanol, whereas the 10% level decreased enzyme activity below that of the 56% concentration.

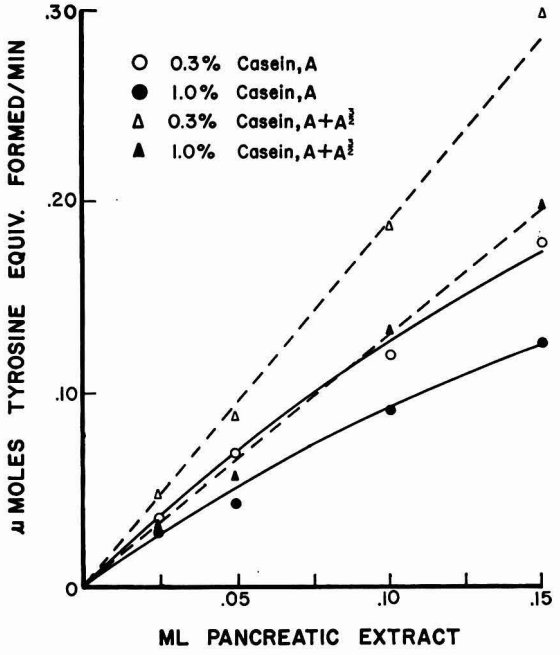

FIG. 4. Digestion of casein by activated pancreatic homogenate and transformation to a linear response by substituting absorbancy (A) plus $A^{3/2}$ in place of A.

(*iv*) *Apparent Esterase Activity of Intestinal Contents*

Apparent hydrolysis of TAME or BTEE by enzymes in intestinal contents from one-week-old calves or from those fed soybean protein was not linearly related to reaction time. A rapid initial nonlinear increase in *A* was obtained and was similar to that shown in Figure 5. A similar type of reaction was also found with a water extract of diets containing either milk or soybean protein, abomasal contents, steer bile, Na glycocholate, Na deoxycholate, or intestinal contents in the absence of TAME

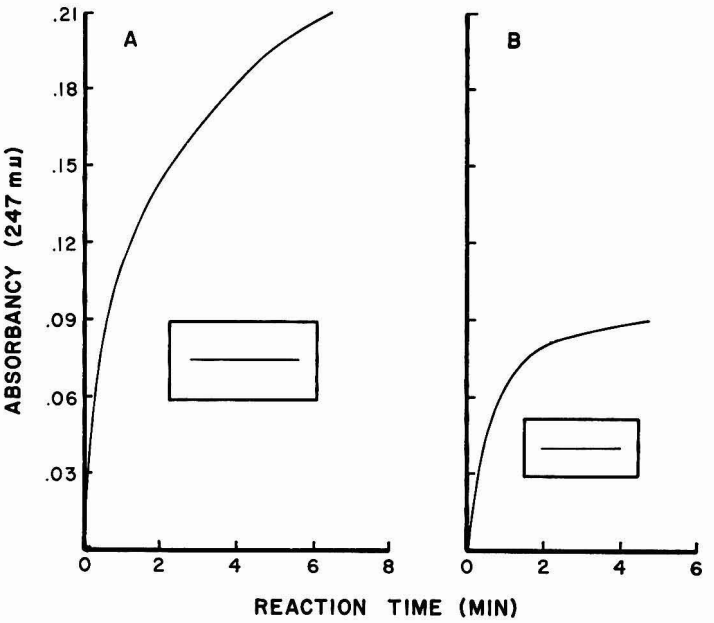

FIG. 5. Apparent esterase activity of intestinal contents and purified bile salts: (A) Trypsin assay of intestinal contents without TAME; insert, no $CaCl_2$ in Tris buffer. (B) Na glycocholate in pH 8.1 Tris buffer, 0.01 *M* $CaCl_2$; insert, no $CaCl_2$ in Tris buffer.

or BTEE (Fig. 5). This apparent esterase activity was dependent on the presence of $CaCl_2$ in the Tris buffers. Sodium cholate or Na taurocholate resulted in no *A* change at 247 mμ but Na taurocholate caused a slight *A* change at 256 mμ, in the presence of methanol. Solutions of Na glycocholate and Na deoxycholate, but not Na cholate or Na taurocholate, were precipitated by $CaCl_2$ or acidification to pH 4. Appearance of cloudiness in the reaction mixture and the above results strongly implied that the *A* change or apparent esterase activity was due to a precipitate formed involving $CaCl_2$ and certain bile salts or other substances in

intestinal contents. This reaction was not due to hydrolysis of the synthetic substrate esters, TAME and BTEE. Contents from the lower part of the small intestine contained less substances which interfered with esterase assays for T and ChT than those from the upper small intestine. *In vitro* incubation of intestinal contents also decreased the nonenzymic apparent esterase activity. The interfering substances were apparently degraded by intestinal enzymes or bacteria, or both (13).

Some correction factor or method of removing interfering substances from intestinal contents was necessary to determine the true T and ChT esterase activities. This was accomplished by performing blank reactions in which water replaced TAME, and 10 or 56% methanol (v/v) replaced the BTEE solution. However, the nonlinear reaction rate for the duration of the assay introduced considerable error in this procedure. Precipitation of calcium ions from the Tris buffer could also reduce the activity of T and ChT (14). Omission of $CaCl_2$ from the Tris buffers was subject to the same error. Adjustment of intestinal contents to pH 4 with 2 *N* HCl caused extensive precipitation, including nearly all of the protein. Interfering substances which remained in the supernatant were completely eliminated by dialysis. A major disadvantage of acid precipitation was the loss of 30% or more of the T and ChT activities from the supernatant, which were recoverable by dissolving the precipitate in 0.15 *M* NaCl. Dialysis of whole intestinal contents or addition of Sephadex G-25 were relatively ineffective. Ether extraction of dry diets did not remove the factor(s) reacting with $CaCl_2$.

Adjustment of intestinal contents first to pH 8 by 2 *N* NaOH, followed by adding excess solid $CaCl_2$, resulted in precipitate formation as well as a marked drop in pH. Additional NaOH and $CaCl_2$ were added until a constant pH, near 8.0, was achieved. The presence of 0.1 *M* $(NH_4)_2SO_4$ resulted in still greater precipitation of substances under the above conditions. The blank reaction for BTEE was reduced still further by lowering the final concentration of methanol in the reaction mixture from 26 to 4.7% (v/v). The easterase activities of T and ChT in the supernatant of intestinal contents, corrected for *A* changes of blank reactions, were increased by 78 and 27%, respectively, by treatment of the contents with $(NH_4)_2SO_4$, $CaCl_2$, and pH 8 (13).

DISCUSSION

Proper enzyme assays of pancreatic tissues require complete rupture of the zymogen granules and release of the enzymes during homogenization. The hypotonic effect of distilled water did not produce maximum T and ChT activities in the homogenate, even after the addition of Triton X-100. Homogenization in 0.15 *M* NaCl containing Triton X-100

caused a clearing of the homogenate and there was no loss of T or ChT activity in the supernatant when the homogenate was centrifuged. Holtzer *et al.* (15) reported that 0.02% Triton X-100 released all the amylase from isolated mouse pancreatic zymogen granules. Suspension of isolated zymogen granules in distilled water liberated most of the amylase (15, 16), but most of the lipase and proteases remained bound to the insoluble material (16). The differential solubilization of these enzymes is not completely understood. Since 0.1% Triton X-100 exerted no inhibitory effect on enzyme activity in this study, as well as on several liver enzymes (17), inclusion of this compound with 0.15 *M* NaCl should be followed.

Ideally, identical T and ChT activities per unit of pancreatic tissue or juice should be attained when several dilutions are assayed. Depending on the concentration of zymogens present and length of activation, particularly at 37°C, considerable error will be inherent by activating for an arbitrarily assigned time as illustrated in Figs. 1 and 2. The presence of 0.05 *M* $CaCl_2$ depressed the activation rate of Tg by enteropeptidase, as noted previously (7), but maximum activity and stability of T and ChT were enhanced during a much longer activation time. Optimum results were obtained by activation with enteropeptidase and 0.05 *M* $CaCl_2$ at 0 to 5°C for 2 to 6 hr for ChTg and 5 to 7 days for Tg. These conditions approached the "rapid" activation of ChTg in which the formation of α-ChT would be small (1). A reasonable estimate of the enzyme activity of pancreatic juice, and consequently the dilutions required to attain optimum activation conditions, can be obtained from the protein content. A correlation coefficient of 0.89 has been reported between pancreatic juice protein and T or ChT activity (5).

A procedure developed to produce a linear response between enzyme concentration and total proteolytic activity (9) was modified by plotting $A + A^{3/2}$ in terms of micromoles of tyrosine equivalent released from a casein substrate. A linear enzyme response (μmoles tyrosine equivalent released/min/gm pancreatic tissue) was then achieved over a 4-fold enzyme concentration and two incubation times. The inadequacy of a crystalline T standard, compared with a tyrosine standard was illustrated in Figures 3 and 4, and has been attributed to a more rapid decrease of casein digestion with time by crystalline T than a mixture of proteolytic enzymes (18). Commercial preparations of casein must be treated to remove ribonuclease, which introduced error in determining total proteolytic activity of tissue extracts by this method (19). The ability of pancreatic juice ChT to hydrolyze BTEE was increased 2.2-fold by decreasing the concentration of methanol from 26 to 4.7% (v/v) in the reaction mixture (BTEE dissolved in 56 or 10% methanol). Kauf-

man *et al.* (20) noted only a small decrease in BTEE hydrolysis by ChT on raising the methanol in the reaction mixture above 25%. Almost a 4-fold increase in hydrolysis of acetyltyrosine ethyl ester (ATEE) by ChT A or B has been observed on changing the methanol from 30 to 5% (v/v) (21). Bovine carboxypeptidase A from tryptic activation of procarboxypeptidase A was reported to contain esterase activity with ATEE (22). Hydrolysis of BTEE by activated porcine procarboxypeptidase A was attributed to impurities of a ChT-like enzyme (23). The relative effects of procarboxypeptidase A and different forms of ChT (α,π,δ) in bovine pancreatic tissue or juice and methanol concentration in the reaction mixture on rate of BTEE hydrolysis have not been determined.

Ratios of ChT-to-T esterase activities have been used to express the relative amounts of these enzymes synthesized and secreted by the pancreas. These ratios have ranged from 1 to 2 in rats (4) to 0.2 in calves (5), but caution must be exercised in making such comparisons. Esterase activity of ChT was shown to differ markedly between hydrolysis of ATEE and BTEE, and concentration of BTEE was critical (20). The level of methanol in the reaction mixture and the possible contribution of procarboxypeptidase A to esterase activity must also be considered.

The nature of intestinal contents represents a crude material for enzyme assays. A change in turbidity due to the enzyme mixture per se or reaction with the substrate or buffer introduces considerable error in spectrophotometric assays. The pH-stat method of determining esterase activities of T and ChT was not investigated. However, this method would not be affected by changes in turbidity. Interfering substances were partially removed by $CaCl_2$, $(NH_4)_2SO_4$ and adjustment of intestinal contents to pH 8. Furthermore, the ability of $CaCl_2$ or $(NH_4)_2SO_4$ to increase the T and ChT activity of the supernatant may be associated with the release of these enzymes complexed with digesta or dietary protein, or by reduction of competition between native proteins and synthetic substrates.

SUMMARY

A procedure was developed for assaying total proteolytic activity, or esterase activities of trypsin and chymotrypsin in bovine pancreas, pancreatic juice, and intestinal contents. Trypsinogen and chymotrypsinogen were released from pancreatic tissue by homogenization in 0.15 *M* NaCl containing 0.1% Triton X-100. The homogenate was centrifuged and diluted to contain 0.3 to 1% tissue. Pancreatic juice was diluted in saline to 0.25 to 0.75 mg protein/ml. Maximum enzyme activity and stability were obtained by activating with a crude enteropeptidase solution con-

taining 0.05 M $CaCl_2$ at 5°C for 2 to 6 hr for chymotrypsinogen and 5 to 7 days for trypsinogen. The normal curvilinear response of enzyme concentration and casein digestion was transformed to a linear process by plotting absorbancy $(A) + A^{3/2}$, using a tyrosine standard at 280 mμ. Rate of BTEE hydrolysis by activated pancreatic juice was 2.2 times greater in 4.7% methanol than in 26%, v/v (BTEE dissolved in 10 or 56% methanol). Interfering substances in intestinal contents which produced an apparent esterase activity, in the absence of enzyme substrates, were attributed to increased turbidity as a result of calcium complexing with certain dietary constituents or bile salts, or both. Addition of $CaCl_2$, $(NH_4)_2SO_4$, and adjustment of the intestinal contents to pH 8 removed most of the interfering substances from the supernatant, and also augmented trypsin and chymotrypsin activity in most samples.

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Determination of Traces of Proline in Biological Fluids[1]

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All the previous methods of determining proline without isolating it from the material analyzed are burdened with an error caused by the interference of other amino acids (1–3). Wren and Wigall (4) recently reported a modification of the method of Chinard (5); this modification also is not completely specific for proline, as it does not abolish the influence of hydroxproline and tyrosine on the color reaction.

In the present work the estimation of proline is based on the color reaction with isatin after proline isolation by means of combined column-paper chromatography (6). Potato starch was used as absorbant in the columns as previously applied by Stein and Moore (7, 8) for separation of amino acids. This method is especially adjusted for determination of slight amounts of this compound in biological material, even in samples containing 2 μg of proline and a considerably higher amount of other amino acids.

EXPERIMENTAL

Apparatus

1. Chromatographic columns 0.9 by 32 cm.
2. Containers, 50 ml in volume, connected with particular columns directly or by means of plastic tubing.
3. An automatic device (Fig. 1) for placing the eluate from the column on separate sheets of paper, moving continuously at the rate of 0.75 cm/hr. To evaporate the eluate deposited on the paper, a stream of warm air is blown under each column (6).
4. Glass tanks for ascending chromatography.

Reagents and Adsorbents

1. Isatin reagent, consisting of 0.2 gm of isatin dissolved in 96 ml of 80% (v/v) aqueous acetone solution with the addition of 4 ml of glacial

[1] Presented at the Third Meeting of Federation of European Biochemical Societies, April 4–7, 1966, Warsaw, Poland.

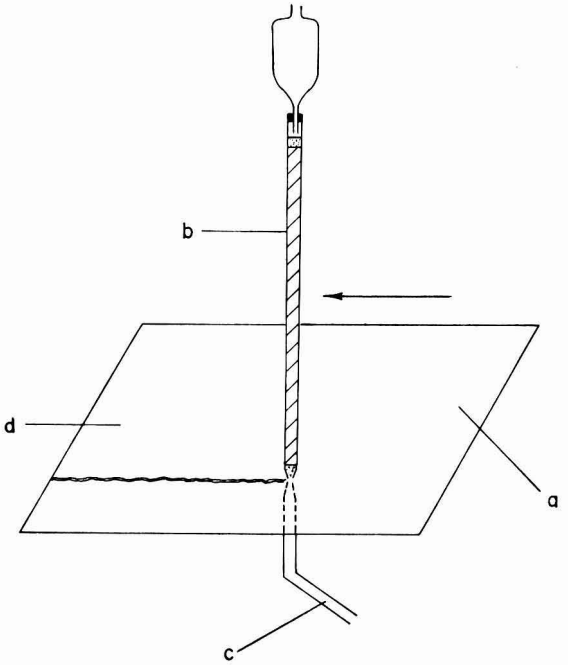

FIG. 1. Diagram of apparatus for placing an eluate from the chromatographic column on a moving paper sheet: (*a*) sheet of paper; (*b*) column; (*c*) tube blowing warm air under the column outlet; (*d*) trace formed by the eluate which dropped and dried.

acetic. The solution can be stored in a capped flask in the refrigerator over 30 days.

2. Phenol saturated with water. Freshly distilled phenol is shaken with water and left until the phenol layer clarifies. The solution stored in a dark flask is stable at room temperature over 20 days.

3. Alcohol solutions: (*a*) 10% (v/v) aqueous solution of *n*-propanol; (*b*) 80% (v/v) aqueous solution of n-propanol; (*c*) 10% acidic solution of *n*-propanol (up to 90 ml of distilled water, 10 ml of *n*-propanol, and 2.0 ml of 1 *N* HCl).

4. Potato starch, thoroughly washed water, dried in air, ground in a mortar, and sieved.

5. Whatman No. 4 paper.

6. Sulfonated polystyrene resins Amberlite IR-120.

Procedure

Preparation of Material. The sample was deproteinized by adding 9 vol of absolute ethanol. The precipitated protein was centrifuged and

a known volume of the cleared supernatant was diluted with an equal volume of water and desalted on cation-exchange resin Amberlite IR-120 (9, 10). Amino acids bound on the ion exchanger were eluted with 6 *N* ammonia solution; the eluate thus formed was evaporated to dryness.

Preparation of Columns. A thick suspension of starch in 80% *n*-propanol was poured into the column up the desired level (30 cm). The column filled with starch was washed with acidified 10% solution of *n*-propanol until an acidic eluate began to flow out; then the adsorbent was washed with 20 ml of 10% *n*-propanol. After chromatography, the column was regenerated by letting through it about 30 ml of acidified 10% propanol and washing with about 20 ml of 10% propanol. The regeneration may be repeated and the column may be used an unlimited number of times within several months.

Analytical Procedure. The deproteinized and desalted sample was dissolved in about 1 ml of 10% propanol and placed in the column prepared in the manner described above. Immediately after the solution had soaked in, about 0.5 ml of 10% propanol was added to rinse the inner walls of the column and the column was connected to a container with 40 ml of 10% propanol. The solution should flow out of the column at a rate of about 1 ml/hr. This can be achieved by placing the container at a suitable height above the column. After 36 hr, proline and even the slower traveling amino acids flow out of the column. The sheet on which the substances were automatically placed in the form of a narrow band was subjected to ascending chromatography twice for 24 hr in 80% propanol. After chromatography, the paper was inbibed with isatin solution, partially dried at room temperature for 20 min, and subsequently developed in a chamber containing air saturated with water vapor at 70° for 25 min. Under such conditions proline formed a blue spot, which was cut on into narrow strips and placed in the tube. The yellow background was removed by twice rinsing out with 5 ml of absolute ethanol. After complete removal of the second portion of ethanol, the blue spot was eluted with 5 ml of phenol-saturated water. The process was carried out in darkness. In 10 min the content of the tube was gently mixed and within the next 5 min it was mixed once more and measured directly in the photometer.

The blank was prepared with slip of the paper with the yellow background in the same way as the test proper. Maximum absorption of color solution was at wavelength 610 mμ. A Pulfrich colorimeter with Elpho attachment (photoelectric cell) and I-58 filter was used.

The adequate amount of proline for determining extinction was obtained from the standard curve prepared for various quantities of

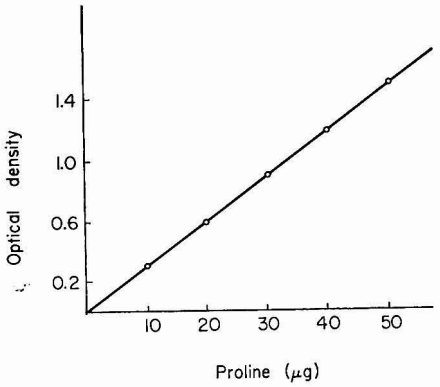

FIG. 2. Relation between amount of proline and optical density.

standard solution according to the above-mentioned procedure. The colorimetric chambers used were 10 mm thick and the correlation between extinction and proline content was linear within 2.5 to 50 μg of proline in the sample (Fig. 2).

RESULTS

Estimation of the error of the method was performed for samples containing 2.5 to 50 μg of proline. Table 1 shows the reproducibility of the method. The variation coefficient was lower than 5%; only for the samples below 5 μg was it higher. The error of the determination does not exceed 2%. In order to evaluate the losses of proline in the course of

TABLE 1
Estimation of Error of the Method in Determination of Proline Subjected to Column-Paper Chromatography and Color Reaction with Isatine

Taken, μg	Found, μg	No. of determinations	Standard deviation, μg	Relative S.D., %	Accuracy	
					μg	%
2.5	2.55	5	0.21	8.2	0.05	2.0
5.0	5.02	5	0.24	4.8	0.02	0.4
10.0	10.14	5	0.40	3.8	0.14	1.4
25.0	24.80	5	0.40	1.6	0.20	0.8
50.0	49.78	5	1.08	2.2	0.22	0.4

the column-paper chromatography, five replications of the analyses were carried out for each respective sample containing 2.5, 5.0, 10.0, and 25.0 μg of proline. Five other samples with the same amounts of proline were simultaneously placed on the paper and the determination was carried out under the same conditions without chromatography. The results

TABLE 2
Losses of Proline in the Course of Column-Paper Chromatography[a]

Sample	Without chromatography, μg	After chromatography, μg	Recovery, %
1	2.5	2.45	98
2	5.0	5.05	101
3	10.0	9.88	99
4	25.0	24.90	100

[a] The values under the heading "Without chromatography" were obtained by placing on the paper appropriate amounts of proline dissolved in 0.1 ml of solution. Measurements were performed immediately after the color reaction with isatine.

obtained from two series of determinations differed only by 2%. It means that there were no losses during chromatography (Table 2).

Determination in Biological Fluids

Urine. The application of column-paper chromatography permits complete separation of proline from other chemical compounds in the urine.

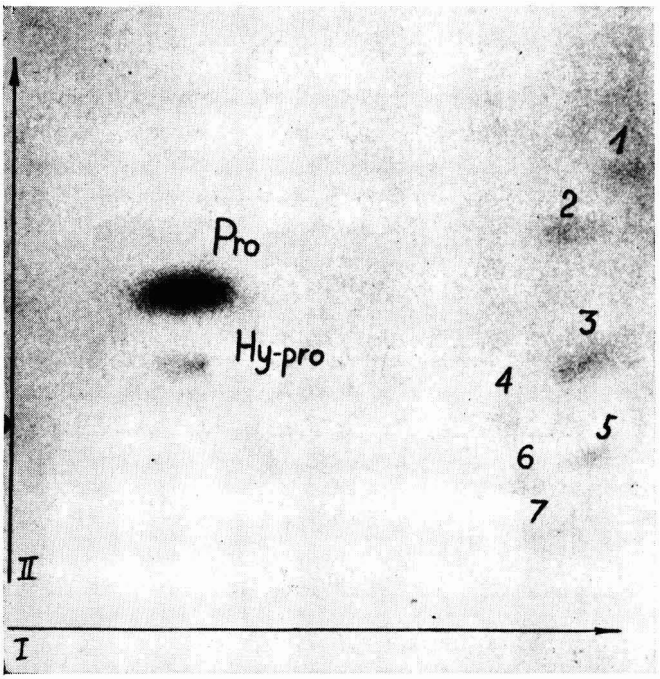

FIG. 3. Chromatogram of 10 ml of deproteinized and desalted human urine of healthy adult individual: (I) direction of placing the eluate from the column; (II) ascending chromatography. Pro = proline, Hy-pro = hydroxyproline; (1-7) amino acids and other compounds giving a color reaction with isatin.

Figure 3 represents a chromatogram developed by isatin obtained from 10 ml of urine. The first amino acids flowing out from the column were proline and hydroxyproline, which were separated by ascending chromatography. As the result of both these processes proline is located in a place distant from the spots formed by other compounds.

The results obtained for five different samples of urine from a healthy adult individual are shown in Table 3. The concentration of proline

TABLE 3
Results of Proline Determinations in Five 10 ml Samples of Urine and Recovery of Proline Added to Each Sample

Sample	Proline concn., μg/10 ml	Proline added, μg	Proline found, μg	Proline recovered	
				μg	%
1	4.2	5.0	9.4	5.2	104
2	7.4	5.0	12.5	5.1	102
3	5.6	5.0	10.5	4.9	98
4	10.5	5.0	15.7	5.2	104
5	6.0	5.0	10.9	4.9	98

for these samples ranged from 0.42 to 1.05 μg/ml. To each urine sample 5 μg of proline was added and the total amount of proline was determined. The determination was repeated twice for each sample; Table 3 gives the averages obtained from two determinations. The recovery of the proline added was on the average 101% (Table 3). It means that there were no losses of proline during deproteinization and other manipulations of the material analyzed. In order to evaluate the accuracy and the precision of the determination of proline in urine, five simultaneous analyses were performed.

Serum and Other Biological Fluids. Serum was deproteinized and desalted in the same way as the samples of urine and 0.5 ml samples of serum were analyzed. Proline on chromatograms gave a distinctly separated spot, as on a chromatogram of urine. The proline values for five different serum samples ranged from 15 to 24 μg/ml. The calculated variation coefficient for five samples of the same serum amounted to

TABLE 4
Error of the Method in Determinations of Proline in Biological Fluids

Material	Volume, ml	Found, μg	Relative standard deviation, %
Urine	10	8.4	4.9
Plasma	0.5	10.9	4.1
Cerebrospinal fluid	10	7.2	4.8

4.1%. In addition, the method was applied to determine proline in the cerebrospinal fluid and in deproteinized hydrolyzates of urine. For proline determination 10 and 0.2 ml samples of cerebrospinal fluid and of hydrolyzed urine were taken, respectively.

In both fluids analyzed the variation coefficient did not exceed 5% (Table 4).

DISCUSSION

The methods of proline determination used so far are not adapted for analysis of samples containing a few micrograms of proline in the presence of many times higher concentrations of other amino acids. For this reason most authors concluded the absence of proline in normal adult human urine or decided that its concentration was too low to be determined by accessible procedures (11, 12).

The additional separation by paper chromatography makes possible a reliable isolation of proline from the material examined; and the sensitivity of the color reaction of proline with isatin on the paper allows a determination of small quantities of proline starting from 2 μg in the investigated samples; and the results are independent of the other amino acids present. Although the ion-exchange chromatographic method as developed by Stein, Moore, and Speckmann (13) is to date the most satisfactory method for amino acid analysis, the use of the isatin reagent instead of ninhydrin in proline determination increases the sensitivity of the reaction.

This method, which uses combined column-paper chromatography, is suitable for analysis of urine, blood, and protein hydrolyzates with a low proline content:

For determining proline, 10 ml of *urine* was used. Analysis of smaller volumes increases the relative error with regard to trace proline quantities.

When determining *blood serum* proline, it is not necessary to use just 0.5 ml samples. Analysis of larger volumes, e.g., 2–3 ml of serum, also gives chromatograms with a well separated proline spot.

For determination of proline in *blood serum or in some other material with a relatively high content of this amino acid,* as for instance gelatin hydrolyzate, the previously elaborated method (14, 15) with the use of one-dimensional paper chromatography may be applied.

The time of performance of column-paper chromatography of proline is 3 days, but use of a device with several columns permits carrying out several analyses simultaneously. The high sensitivity and small errors in the determinations predestine the method for examination of small concentration changes of this amino acid in biochemical processes.

SUMMARY

A method for proline determination is described. The procedure was especially adjusted to determine small amounts of this amino acid in biological material. The first step of the analysis is the isolation of proline from other compounds by column-paper chromatography, and then the color reaction with isatin is performed. An amount of proline beginning with 2 μg in the sample may be determined by this method.

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ANALYTICAL BIOCHEMISTRY 19, 234–242 (1967)

Thin Layer and Column Chromatographic Group Separations of Steroids as Trimethylsilyl Ethers. Isolation for GLC Analysis of Pregnanediol and Estriol in Pregnancy Urine

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Gas-liquid chromatographic (GLC) separation methods are now widely used for qualitative and quantitative analyses of steroid mixtures. However, it is often desirable to carry out a preliminary separation or fractionation of crude mixtures by thin-layer or column chromatographic (TLC or CC) methods. The combination of one or more high capacity, low resolution separations and a final low capacity, high resolution separation is highly effective for analytical purposes.

TLC or CC separations of steroids are usually based upon polarity differences. Steroids of biological origin, for example human urinary steroids, include a wide range of polarities, ranging from simple mono- or bifunctional compounds (cholesterol, 16-androstene-3α-ol) to highly substituted steroids such as those of the adrenocortical group. Many procedures have been devised for the fractionation of such mixtures into classes or individual components according to the degree of polarity. Acyl derivatives have been frequently used in chromatographic separations to reduce both the degree and the range of polarity, thus facilitating group separations and incidentally improving the recovery of the more polar steroids during the elution process.

Simple group separations employing trimethylsilyl ether (TMSi) derivatives provide a new way of achieving preliminary fractionation. Although these compounds are susceptible to acid-catalyzed hydrolysis, they are sufficiently stable to permit their use in TLC and CC separations (1–3). Polyhydroxy steroids with unhindered groups are con-

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verted into ethers, while ketone groups are unchanged.[3] The over-all effect is to transform steroids of widely different polarity into derivatives of relatively low polarity, while preserving differences due to unreactive functional groups. After TLC or CC separation, the TMSi derivatives may be used in analytical studies by GLC and GLC-mass spectrometric methods (5).

METHODS

Derivatives. Trimethylsilyl ethers were prepared in the following way. The steroid (0.4 mg) or the residue comprising steroids derived from human pregnancy urine (20–50 ml) was dissolved in dry pyridine (0.2 ml). The solution was treated with hexamethyldisilazane (0.2 ml) and trimethylchlorosilane (0.04 ml) (6) and left overnight in a stoppered tube (overnight standing at room temperature is required for complete reaction of the 11β-hydroxyl group). The reaction mixture was evaporated at a temperature below 50°C with the aid of a stream of nitrogen.

For examination by TLC, the residue was extracted with dichloromethane (0.2 ml) and the supernatant solution was used for application to the chromatoplate. In some experiments the supernatant solution was evaporated to dryness and the residue was purified by vacuum sublimation in a cold-finger type of apparatus; this procedure was not essential, but it was employed to ensure the absence of nonvolatile contaminants from urinary extracts. No change in composition of steroid trimethylsilyl ether mixtures was observed by GLC analysis after sublimation.

For column chromatography, the residue was triturated with 2 ml of hexane/ethyl acetate/hexamethyldisilazane (95/5/2). The mixture was centrifuged and the supernatant solution was removed. A second similar extraction (2 ml) was carried out and the combined organic extracts were applied to the column.

Trimethylsilyl ethers were characterized by their mobilities in TLC and by gas chromatographic retention data. Where further evidence of structure was required, mass spectra were determined by combined gas chromatography-mass spectrometry, using an Atlas CH4 instrument modified by Ryhage (7).

Chromatographic Procedures. Thin-layer chromatography was carried out on Adsorbosil-3 (silica gel containing 10% calcium sulfate; Applied Science Laboratories, Inc.). No appreciable differences in R_f values were

[3] Small amounts of enol ethers may be formed from 3-keto steroids when pyridine is used as a solvent; this reaction does not occur when dimethylformamide is the solvent. Enol ether formation has not been observed for the steroids under study in this paper (4).

observed whether chromatography was effected at 4°C or at room temperature (22°C). Examples of solvent systems are noted in Table 1. The same solvents could be used for elution of TMSi ethers from the plates.

In the analyses shown in Figure 1, elution of each zone was carried out with ethyl acetate/methanol (1/1). The eluted fractions were evaporated with the aid of a nitrogen stream, and the residues were treated with pyridine and silylating reagents (hexamethyldisilazane and trimethylchlorosilane) in the proportions used for the original mixture. After reaction overnight the derivatives were isolated as described above and dissolved in dichloromethane for GLC analysis.

Column chromatography was carried out on silicic acid, washed and activated as described by Horning, Williams, and Horning (8) (Unisil, 200–325 mesh, Clarkson Chemical Co.). The sample was applied to the column (10 gm of silicic acid) in hexane/ethyl acetate/hexamethyldisilazane (95/5/2) and the first fraction (20 ml) was eluted with the same solvent mixture. A second fraction (30 ml) was eluted with the same solvents in 40/60/2 proportion, respectively. The solvents were removed with the aid of a nitrogen stream, and the residues were taken up in dichloromethane. The composition of each fraction was determined by GLC analysis.

Gas-liquid chromatographic separations were carried out with Barber-Colman models 10 and 5000. The columns were 6 ft $\times$ 4 mm (isothermal separations) and 12 ft $\times$ 4 mm (temperature-programmed separations) glass U-tubes. The packing was 1% SE-30 on 100–120 mesh Gas Chrom P, prepared according to the usual procedure of this laboratory (9). An argon detector was used for the isothermal studies and a flame ionization detector was used for the temperature-programmed urinary steroid studies.

For quantitative work with urinary steroid extracts an internal standard is required. For isothermal analyses the usefulness of the flame ionization detector is well known and the suitability of a temperature-programmed separation, with a 1% SE-30 column and a flame ionization detector, for the analysis of steroid mixtures has been established (10). Response factors for pregnanediol di-TMSi ether and estriol tri-TMSi ether should be determined locally with reference to the experimental conditions employed.

Extraction of Urinary Steroids. Urine (20–50 ml) was buffered to pH 5.2 with acetic acid-sodium acetate, treated with an enzyme preparation (0.5 ml of Glusulase, Endo Laboratories, Inc.) and incubated at 37°C for 24 hr. A further 0.5 ml of Glusulase was added and incubation continued for an additional 24 hr. The urine was then extracted with

ether, using a continuous extractor, for 3 hr. The extract was washed with 5% aqueous sodium bicarbonate solution (0.1 vol), dried with anhydrous sodium sulfate, and evaporated to dryness in a rotary evaporator below 50°C. The residue was treated directly by the standard trimethylsilylation procedure.

RESULTS AND DISCUSSION

Relationships between TLC chromatographic properties and the structure of representative steroid TMSi derivatives are evident from the data in Table 1. Steroid TMSi ethers with no additional oxygen-containing functional group are not readily separable from one another in TLC systems in which polarity differences form the basis of the chromatographic separation. This is an expected effect; the TMSi

TABLE 1
Typical R_f Values Observed for Thin-Layer Chromatography of Steroids as TMSi Ethers[a]

Steroid derivative	R_f values				
	(A)	(B)	(C)	(D)	(E)
5-Androstene-3β, 17β-diol (diTMSi)	0.88	0.90	0.73	0.60	0.40
5-Pregnene-3β, 20β-diol (diTMSi)	0.88	0.90	0.73	0.65	0.47
5β-Pregnane-3α, 20α-diol (diTMSi)	0.91	0.90	0.72	0.63	0.48
5β-Pregnane-3α, 17α, 20α-triol (3, 20-diTMSi)	0.78	0.82	0.66	0.27	0.13
5-Androstene-3β, 17β-diol-16-one (diTMSi)	0.77	0.72	0.63	0.29	0.10
5α-Androstane-3α-ol-17-one (TMSi)	0.76	0.57	0.49	0.32	0.17
5β-Androstane-3α-ol-17-one (TMSi)	0.69	0.45	0.45	0.16	0.08
5-Androstene-3β-ol-17-one (TMSi)	0.71	0.45	0.41	0.29	0.18
5β-Androstane-3α, 11β-diol-17-one (diTMSi)	0.72	0.44	0.46	0.17	0.08

[a] Solvent systems: (A) chloroform, (B) hexane/ethyl acetate/hexamethyldisilazane/pyridine (95/5/2/0.5), (C) cyclohexane/ethyl acetate (90/10), (D) benzene/chloroform (80/20), (E) cyclohexane/chloroform (70/30). Visualizing agent: 1% ceric sulfate-10% sulfuric acid spray.

derivatives are highly mobile and hydrocarbon-like in polarity. As a class these steroids may be distinguished readily from compounds containing an additional ketone or free hydroxyl functional group. However, the only type of alcohol group that does not form a TMSi ether under the conditions employed in this study is one that is highly hindered; in the example shown in Table 1, the effect of such unreactive hydroxyl group on the mobility is markedly dependent upon the solvent system. The R_f values in systems B and C, respectively, of 0.82 and 0.66 for 5β-pregnane-3α,17α,20α-triol (pregnanetriol) as the TMSi derivative, in contrast to values of 0.90 and 0.73 for TMSi ethers of hydroxyl-substituted steroids containing only ether groups, indicate that

the tertiary 17α-hydroxyl group does not react to form a derivative. This is confirmed by the more pronounced differences in R_f values observed for other solvent systems (Table 1). This is significant from the structural point of view (the reaction of cortol to form a penta-TMSi ether has been reported in one instance (1); we did not observe reaction of the tertiary 17α-hydroxyl group of pregnanetriol in the course of the present study). For the group separations described in this paper, solvent systems have been selected in order to include pregnanetriol with other hydroxylic steroids.

The presence of a ketone group leads to a decreased R_f value, as expected. Differences in R_f value exist among derivatives of a group of 17-ketosteroids of the androstane series, but the mobilities of these derivatives are all lower than those observed for steroids containing only TMSi ether groups.

An 11β-hydroxyl group is present in many steroids of adrenocortical origin, and it is well known that this group has a lower reactivity (for steric reasons) than a steroid 3-equatorial or 3-axial hydroxyl group. The mobilities (Table 1) of the TMSi derivative of 5β-androstane-3α,11β-diol-17-one (11β-hydroxyetiocholanolone) compared with those for 5β-androstane-3α-ol-17-one (etiocholanolone), indicate unequivocally that the 11β-hydroxyl group has been converted to an 11β-TMSi ether group. In contrast, Gardiner and Horning (11), in a description of a new GLC separation method for human urinary metabolites of the adrenocortical group, reported that the 11β-hydroxyl group was unreactive in a trimethylsilylation reaction. The reactivity of the 11β-hydroxyl group has been investigated separately, and both mono- and di-TMSi derivatives have been prepared and characterized. The di-TMSi ether, which is formed when trimethylchlorosilane is added to the reaction mixture, is the derivative referred to in Table 1. No hydroxyl group absorption was found in the infrared absorption spectrum, and the mass spectrum (obtained with a gas chromatograph-mass spectrometer combined instrument) showed a molecular ion at m/e 450. The mono-TMSi ether showed the expected hydroxyl absorption near 3630 cm^{-1} (infrared absorption spectrum in carbon tetrachloride) and a molecular ion at m/e 378 in the mass spectrum. For the objective of the present work, the reaction conditions of choice were designed to convert an 11β-hydroxyl group to the TMSi ether. 11β-Trimethylsilyloxy steroids of the androstane series show approximately the same mobility as the corresponding steroids without substitution at the 11-position.

Trimethylsilyl ethers undergo acid-catalyzed hydrolysis to yield the original hydroxyl-substituted compound. The ease of hydrolysis for different kinds of TMSi derivatives has not been investigated in detail,

but from laboratory experience it is believed to be as follows: TMSi esters (of carboxylic acids) > TMSi oxime ethers > TMSi phenol ethers > TMSi alcohol ethers. In this study, the stability of steroid alcohol TMSi ethers during TLC and CC separation conditions was examined by TLC and GLC analytical techniques. Figure 1 shows GLC analyses of a mixture of TMSi derivatives before and after a

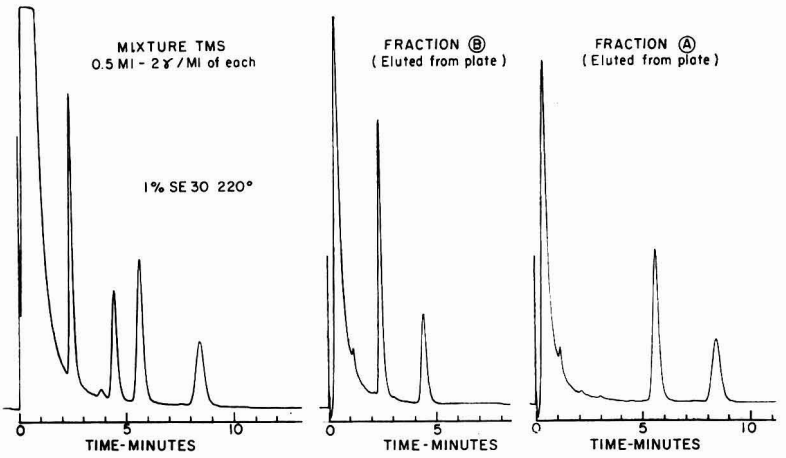

FIG. 1. GLC analyses of steroid mixtures before and after group separation by thin-layer chromatography. *Left panel:* original mixture of TMSi derivatives of 5β-pregnane-3α,20α-diol, 5β-pregnane-3α,17α,20α-triol, 5β-androstane-3α-ol-17-one, and 5β-androstane-3α,11β,diol-17-one. *Right and center panels:* bands eluted by ethyl acetate/methanol (1/1) from thin-layer chromatograms, and retreated by the standard trimethylsilylation procedure. The TLC separation was carried out with hexane/ethyl acetate (9/1). *Right panel,* fraction A (R_f 0.70–0.95): the peaks are due to the TMSi derivatives of 5β-pregnane-3α,20α-diol and 5β-pregnane-3α,17α,20α-triol. *Center panel,* fraction B (R_f 0.20–0.70): the peaks are due to the TMSi derivatives of 5β-androstane-3α-ol-17-one and 5β-androstane-3α,11β-diol-17-one. The GLC separations were carried out with a 6 ft 1% SE-30 column at 220° (nitrogen pressure, 18 psi).

TLC group separation. No hydrolysis of the TMSi ethers was observed on the plate, as determined in separate experiments in which compounds were detected by a ceric sulfate-sulfuric acid spray reagent. Partial hydrolysis occurred in the process of elution and recovery of the TMSi ethers; this was observed with a variety of solvents. There was, however, no loss of steroid, as shown by GLC analysis following retreatment by the trimethylsilylation procedure.

The same experimental approach, employing GLC analyses, was followed in a study of a group separation by CC techniques. Figure 2 shows the GLC analysis of a mixture of TMSi derivatives before and

after a group separation by silicic acid CC using elution with hexane/ethyl acetate/hexamethyldisilazane, 95/5/2, for the first fraction and the same solvents in 40/60/2 ratio for the second fraction. The addition of a small amount of hexamethyldisilazane to the eluting solvents served to protect phenolic TMSi ethers from partial hydrolysis but did not alter the nature of the separation. No evidence of hydrolysis of steroid alcohol TMSi ethers was found in CC separations effected in the absence of hexamethyldisilazane.

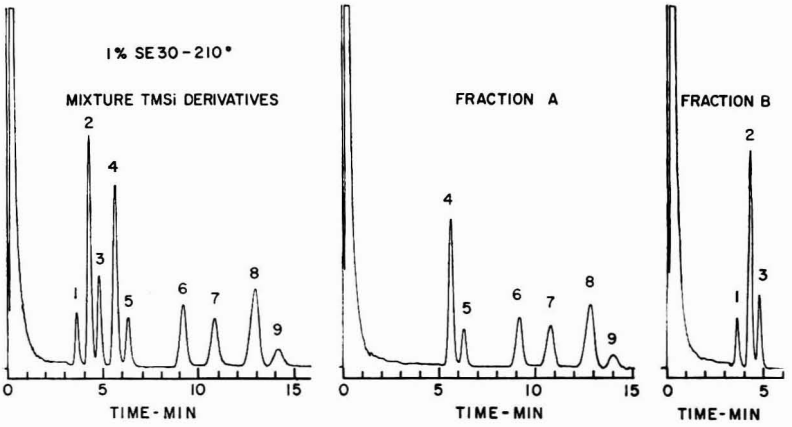

FIG. 2. GLC analyses of steroid mixtures before and after separation by CC. The TMSi derivatives are those of (1) 5β-androstane-3α-ol-17-one, (2) 5-androstene-3β-ol-17-one, (3) estrone, (4) 5-androstene-3β,17β-diol, (5) estradiol, (6) 5β-pregnane-3α,20α-diol, (7) 5-pregnene-3β,20α-diol, (8) estriol, and (9) 5β-pregnane-3α,17α,20α-triol. The derivatives were separated with a 10 gm silicic acid (Unisil, 200–325 mesh) column; the elution was carried out with hexane/ethyl acetate/hexamethyldisilazane (95/5/2) to yield fraction "A" (GLC analysis in center panel), and with hexane/ethyl acetate/hexamethyldisilazane (40/60/2) to yield fraction "B" (GLC) analyses in right panel). The GLC analyses were carried out with a 6 ft 1% SE-30 column at 210° (nitrogen pressure, 16 psi).

Many steroid mixtures may be studied by this separation method. A specific application is the determination of urinary pregnanediol and estriol during pregnancy. A number of GLC procedures have been developed for the separation and determination of pregnanediol (12) and of estriol (13), but these are usually carried out separately. A combined determination is often of interest; in instances of suspected fetal death both values are desired (14). It is possible to employ a CC separation procedure of the TMSi ethers of urinary steroids to obtain a fraction containing the derivatives of both pregnanediol and estriol. Figure 3 shows the GLC analysis of a steroid fraction obtained from pregnancy urine (second trimester) by the following method. The urinary steroids were isolated in the usual way by enzymic hydrolysis, and the entire

sample (without fractionation) was treated with hexamethyldisilazane/trimethylchlorosilane in pyridine. A CC separation was carried out to yield an initial "nonpolar" fraction (20 ml) containing only hydroxyl-substituted steroids. The GLC analysis of the "nonpolar" fraction is shown in Figure 3.

The major steroids are pregnanediol, estriol, and pregnanetriol. The small peak eluted immediately before pregnanediol corresponds in GLC retention behavior to 5β-pregnane-3α,20β-diol, but the identity of the

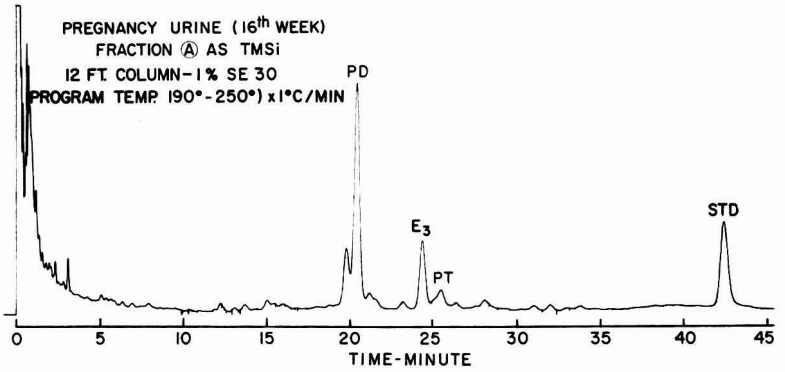

FIG. 3. GLC analysis of steroid fraction from pregnancy urine (24 hr sample, 16th week); the compounds are the TMSi derivatives of 5β-pregnane-3α,20α-diol (PD), estriol (E_3), and 5-β-pregnane-3α,17α,20α-triol (PT). The internal standard is cholesteryl butyrate. The peak eluted immediately before pregnanediol corresponds to the derivative of 5β-pregnane-3α,20β-diol, but further identification has not been made. The fraction was collected as fraction "A" by the method used for Figure 2. The urinary steroids were obtained through an enzymic hydrolysis procedure. The GLC separation was carried out with a 12 ft 1% SE-30 column with temperature programming from 190 to 250° at 1°/min. The separation may also be carried out isothermally.

compound has not been confirmed. Subject to the determination of response factors the separation may be used for quantitative purposes. Pregnanediol and estriol may thus be estimated concurrently. These applications are being further investigated.

This group separation procedure is particularly satisfactory for the isolation of steroids containing only hydroxyl groups. It is not directly useful for the study of hydroxyketosteroids of the adrenocortical type. While the methoxime-trimethylsilyl ethers of the adrenocortical hormones and their metabolites are stable under GLC separation conditions, the TMSi ethers of certain adrenocortical steroids with unprotected keto groups are not suitable for GLC analysis. The free steroids may be separated by conventional TLC methods and converted to methoxime-trimethylsilyl ethers for GLC analysis.

SUMMARY

Trimethylsilyl ethers of steroids may be separated by thin-layer and column chromatographic methods. They are suitable derivatives for the group separation of hydroxysteroids from hydroxyketosteroids prior to gas chromatographic analysis. Group separations of two model steroid mixtures are described. An application of the method has been made to the simultaneous analytical separation of estriol and pregnanediol in human pregnancy urine; the determination of these two steroids is often of interest in pregnancy studies.

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Rapid Sedimentation Equilibrium in the Ultracentrifuge Using a Modified Synthetic Boundary Cell with Step Concentrations

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Molecular weights, dispersity, and activity coefficients can be obtained by the method of sedimentation equilibrium in the analytical ultracentrifuge. This technique is generally carried out by filling the cell with a homogeneous solution. The principal disadvantage of the method is the relatively long time required to establish the equilibrium distribution of the solute. Pasternak *et al.* (1) showed that the time required to reach equilibrium can be reduced by setting up a single-step distribution in a synthetic boundary cell. Van Holde and Baldwin (2) have proposed short columns to further reduce the time to attain equilibrium, and Hexner *et al.* (3) were able to achieve equilibrium of ribonuclease in 2.2 hr by the use of their overspeeding technique. In this study, the equilibrium time was further reduced by redesigning the capillary-type synthetic boundary cell to accommodate four step-distribution concentrations. The time to reach equilibrium in the centrifuge was reduced to one-third the time required by the overspeeding technique reported by Hexner *et al.* (3) and to about one-eighth of the time when neither the step concentrations nor the overspeeding method was used.

MATERIALS AND METHODS

The Beckman/Spinco Model E Analytical Ultracentrifuge[1] was used, and the capillary-type synthetic boundary cell[2] was modified as follows: Three holes were drilled on the sample side of the cell to accommodate 0.03 ml solution each. The holes were drilled at an angle of 2.5° with the axis of the cell centerpiece so that they would empty completely. Capillary channels were made to permit flow directly from each hole into the sample sector of the cell during the initial acceleration of the

[1] Beckman Instruments, Spinco Division, Palo Alto, California.
[2] Beckman/Spinco Part No. 306076.

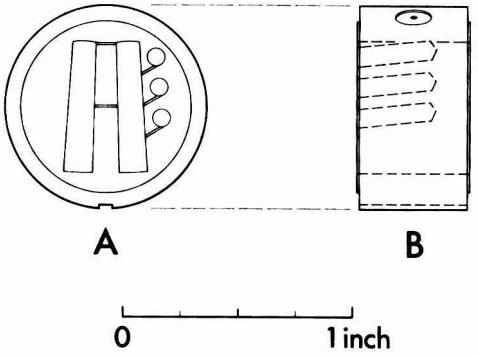

FIG. 1. (A) Front view of modified synthetic boundary cell. (B) Side view of same cell showing angle at which the filling holes were drilled.

instrument. Figure 1 illustrates this modification. The holes of the cell were filled according to the technique described by Yphantis (4).

In the experiments reported here, the sample used was lyophilized ribonuclease A.[3] The solvent was 0.025 M PO_4 buffer, pH 7.12. A 1.0% stock solution of the ribonuclease sample was diluted to make the four step-concentrations such that the average concentration in the cell was 0.5%.

Estimation of Concentration Steps for Equilibrium Distributions

Nomenclature. C = solute concentration; M = molecular weight; R = thermodynamic conversion factor, 8.314×10^7 erg $°C^{-1}$ $mole^{-1}$; r = distance from center of rotation; T = absolute temperature; $\bar{v}$ = partial specific volume; ρ = solution density; σ = effective reduced molecular weight; ω = angular velocity. *Subscripts:* app = "apparent"; b = cell bottom; m = meniscus; 0 = initial value; 1,2,3,4 = layers of solution, in order of increasing radius.

The precalculation of the equilibrium concentration distribution is based on well-known sedimentation equilibrium theory and the assumed molecular weight and partial specific volume of the solute in question. The parameter called "effective reduced molecular weight" by Yphantis (5) is computed from

$$\sigma = \frac{M(1 - \bar{v}\rho)\omega^2}{RT} \tag{1}$$

The difference in the squares of the cell bottom and meniscus radii (when the cell is filled) is computed:

$$\Delta(r^2) = r_b^2 - r_m^2 \tag{2}$$

[3] Available from Worthington Biochemical Corporation, Freehold, New Jersey.

The ratio of meniscus concentration to initial concentration is then given by:

$$\frac{C_m}{C_0} = \frac{\frac{\sigma}{2}\Delta(r^2)}{\exp\left[\frac{\sigma}{2}\Delta(r^2)\right] - 1} \tag{3}$$

The following formulae give the concentrations at the midpoints of four equal volume steps at sedimentation equilibrium, as a ratio to the meniscus concentration:

$$\frac{C_1}{C_m} = \exp\left[\frac{\sigma}{2}\frac{\Delta(r^2)}{8}\right] \tag{4a}$$

$$\frac{C_2}{C_m} = \exp\left[\frac{\sigma}{2}\frac{3\Delta(r^2)}{8}\right] \tag{4b}$$

$$\frac{C_3}{C_m} = \exp\left[\frac{\sigma}{2}\frac{5\Delta(r^2)}{8}\right] \tag{4c}$$

$$\frac{C_4}{C_m} = \exp\left[\frac{\sigma}{2}\frac{7\Delta(r^2)}{8}\right] \tag{4d}$$

The desired concentrations for the four steps, as a ratio to the initial concentration, are found by multiplying equations 4a–d by equation 3.

Adherence to these values for concentrations in the four steps should result in minimum redistribution of the solute to reach the equilibrium distribution, provided the assumed parameters were correct.

The lowest concentration was placed in the filling hole most distant from the base of the cell. The other two step-concentrations, which closely represent the equilibrium concentrations in the two center quarters of the gradient, were placed in the remaining filling holes, respectively.

After the cell was assembled, 0.02 ml of fluorocarbon FC 43 was inserted through the filling hole of the sample sector of the cell and then 0.03 ml of the fourth (highest) sample concentration. The total sample volume in the cell was 0.12 ml, which gave a column height of 3 mm. In the solvent channel of the cell, 0.16 ml of the buffer solution was inserted through the filling hole.

At the conclusion of the equilibrium experiment, C_0 (initial concentration) was obtained by removing the cell from the rotor, gently tilting it to remix the contents, adding more solvent to he solvent channel of the cell (6), and making a conventional synthetic boundary run in the same cell. This technique has proved advantageous in making the

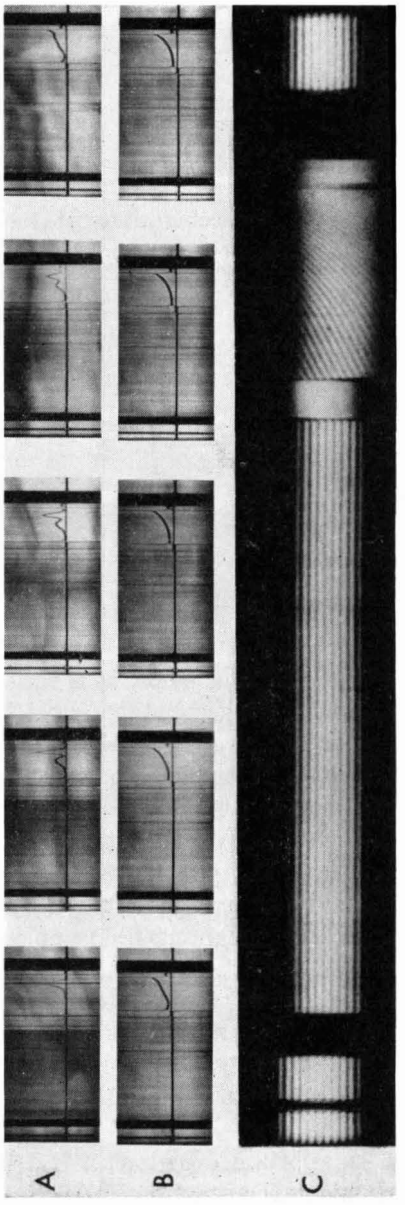

FIG. 2. (A) Representative schlieren patterns of the ribonuclease sample taken at 5,000, 10,000, 15,000–18,000, and 20,410 rpm, and 8 min after full speed of 20,410 rpm. (B) Continued sequence photography of schlieren patterns taken at 16, 32, 48, 56, and 540 min after full speed of 20,410 rpm. (C) Interference picture taken at 540 min after full speed of 20,410 rpm.

determination of C_0 under conditions most closely approximating those under which equilibrium was obtained.

RESULTS

Four replicate experiments were performed. The existence of equilibrium was detected after observing no visual change in the gradient of the last three photographs taken during each experiment. The calculated value of 13,500 ± 150 for the experiments compared favorably with the formula weight of 13,683 for ribonuclease. The equation of Lansing and Kraemer (7):

$$M_{app} = \frac{2RT}{(1 - \bar{v}\rho)\omega^2(r_b^2 - r_m^2)} \cdot \frac{C_b - C_m}{C_0}$$

was used, and the photographs taken at 48, 56, and 540 min intervals were measured. The calculated value for M_{app} reflected no change in the three intervals. Figure 2 illustrates time sequence photography as follows:

A. The first picture was taken at about 5,000 rpm, the second at 10,000 rpm, and the third at 15,000–18,000 rpm. These pictures were taken during acceleration to observe emptying of the solution from the filling holes into the sample sector of the cell. The fourth picture was taken at the speed used for equilibrium (20,410 rpm). With a continuous acceleration current of 13 amperes, the drive motor took about 3.5 min to reach the equilibrium speed. The fifth pictures was taken 8 min after reaching maximum speed.

B. Pictures 1 through 5 represent 16, 32, 48, 56, and 540 min after maximum speed.

C. The interference picture was taken at 540 min after maximum speed.

From the photographs measured, it was observed that, with the modified cell, equilibrium was attained 48 min after reaching maximum speed. A conventional cell run with ribonuclease at the same column height required 14 hr to achieve equilibrium. When the overspeeding technique was used, the reported equilibrium time was 2.2 hr (3).

Further experiments were carried out with other materials and concentrations. The observed equilibrium time gave the same results as described previously. A final experiment utilizing the meniscus depletion technique of Yphantis (5) was carried out on a solution of commercially available β-lactoglobulin.[4] Four step-concentrations were made such that the average concentration in the cell was 0.01%. The results of this experiment showed that equilibrium was attained in about 60 min.

[4] Available from Pentex, Incorporated, Kankakee, Illinois.

SUMMARY

Rapid attainment of equilibrium was achieved without overspeeding the centrifuge. A modified synthetic boundary cell was used to provide an initial multiple step-concentration distribution. By this technique equilibrium was reached in about one-third the time required for a cell having comparable column height, when compared to the reported overspeeding method. It was also shown that the equilibrium time was further reduced by about one-eighth when neither the step-concentration nor the overspeeding method was used.

ACKNOWLEDGMENTS

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ANALYTICAL BIOCHEMISTRY 19, 249–255 (1967)

Modifications of a Specific Assay for Hydroxyproline in Urine[1]

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Because essentially all the hydroxyproline in animal tissues is found in collagen, specific assays for hydroxyproline have been useful in studying the synthesis and degradation of collagen. Recently, the excretion of hydroxyproline in urine was shown to parallel changes in the metabolism of collagen, and assays for hydroxyproline in urine have been extensively used in both experimental and clinical studies (for review, see reference 1). Hydroxyproline can be conveniently oxidized to pyrrole, and the pyrrole can be converted to a relatively specific chromophore with *p*-dimethylaminobenzaldehyde (2, 3). However, because urine contains large amounts of interfering materials, the assay of hydroxyproline in urine requires at least partial isolation of the imino acid or one of the products of the oxidative assay procedure. A number of laboratories (1) have been assaying hydroxyproline in urine with the specific procedure of Prockop and Udenfriend (4) in which specificity is obtained by extracting the pyrrole into toluene. A few investigators (5–7), however, have reported difficulties with this procedure, and have suggested alternate procedures that are generally less specific. We now report several modifications of the original assay procedure (4) which make the technique more rapid without reducing the specificity or the accuracy. We also describe a new method for measuring the recoveries in individual samples with hydroxyproline-^{14}C, and review the results obtained with the present procedure in two laboratories over the past several years.

[1] This work was supported in part by a grant from Sigrid Juselius Foundation, and grants HD-00183, AM-5459, and FR-00107 from the National Institutes of Health, U. S. Public Health Service. Address reprint requests to D. J. Prockop at The Philadelphia General Hospital.

MATERIALS

Potassium Borate Buffer, pH 8.7: 61.84 gm boric acid and 225 gm potassium chloride are mixed into about 800 ml of distilled water, the pH is adjusted to 8.7 with 10 *N* and 1 *N* KOH, and the final volume is made up to 1 liter.

Alanine Solution: 10 gm alanine is dissolved in about 90 ml distilled water, the pH is adjusted to 8.7 with KOH, and the final volume is made up to 100 ml.

Chloramine T Solution (Eastman or Merck): 0.2 *M* solution in methyl Cellosolve is prepared daily.

Sodium Thiosulfate: Solution is 3.6 *M* in distilled water, and it is stored under toluene at room temperature for several weeks.

p-Dimethylaminobenzaldehyde or Ehrlich's Reagent (analytical grade; Merck or Matheson, Coleman & Bell): Concentrated sulfuric acid, 27.4 ml, is slowly added to 200 ml of absolute alcohol in a beaker and the mixture is cooled. *p*-Dimethylaminobenzaldehyde, 120 gm, is added to 200 ml of absolute alcohol in another beaker, and this solution is also cooled. The acid-ethanol mixture is then slowly stirred into the first beaker in an ice bath. The solution can be stored in a refrigerator for several weeks, and reproducible results are obtained even though the absorbance of blanks compared to water slowly increase. The crystals that precipitate when the solution is stored in a refrigerator are redissolved by warming the solution briefly. Practical-grade *p*-dimethylaminobenzaldehyde can be used if reagent is twice recrystallized from an ethanol solution to reduce the absorbance of the blank.

Culture Tubes, Screw-Capped with Teflon Liners (Kimax): The tubes are 150 × 16 mm, and have a capacity of about 20 ml. Tubes to be used for hydrolysis are marked at a level corresponding to exactly 15 ml.

ASSAY PROCEDURE

Hydrolysis: 1.0 ml of urine and 1.0 ml of concentrated HCl are placed in a screw-capped tube, and the tube is heated in a heating block or in a boiling water bath at 100° overnight. After the hydrolysis, 1 drop of 1% phenolphthalein in ethanol is added, and the sample is adjusted to a pink color with 12, 1, and 0.1 *N* KOH. The sample is then carefully diluted to the 15 ml mark on the hydrolysis tube, mixed, and centrifuged at low speed for 10 min in order to remove heavy pigments. Alternatively, the sample can be filtered through paper instead of centrifuging.

Oxidation Procedure: An appropriate aliquot of the hydrolyzed supernatant solution containing 3 to 12 μg of hydroxyproline is placed in another screw-capped tube, and distilled water is added to a final volume

of 4 ml. A water blank, and 5 and 10 μg standards of hydroxyproline are usually assayed together with each set of samples. Another drop of 1% phenolphthalein is added, and the pH is readjusted to a pale pink color with 0.1 and 0.05 *N* KOH and HCl. The solution is then saturated with KCl by adding about 3 gm of the solid with a spoon. The exact amount of KCl is not critical (see below), but the excess solid in each tube in any given assay should be approximately the same. Exactly 0.5 ml of 10% alanine solution and 1.0 ml of potassium borate buffer are added, and the solutions are well mixed. The tube is allowed to sit at room temperature for 20 to 30 min with occasional mixing in order to allow complete saturation of the solution with the KCl. The sample is then oxidized by the addition of exactly 1.0 ml of the 0.2 *M* chloramine T solution, and the sample is well mixed immediately. The tube is allowed to stand at room temperature for 25 min with occasional mixing, and then 3.0 ml of 3.6 *M* sodium thiosulfate is added. The time of oxidation should be about the same for each sample of the assay. Approximately 5 ml of toluene is added to the tube, and the tube is tightly capped with a Teflon-lined screw cap. The tube is then vigorously shaken for 5 min and centrifuged at low speed in order to separate the toluene layer. The toluene layer is removed with suction and discarded. The tube is tightly capped and placed in a briskly boiling water bath for 30 min. The tube is cooled with running tap water, and exactly 5.0 ml of toluene is added. The tube is recapped, shaken for about 5 min, and centrifuged at low speed. Exactly 2.5 ml of the toluene extract is placed in a clean test tube, and 1.0 ml of the Ehrlich's reagent is added. The sample must be rapidly mixed as the Ehrlich's reagent is added in order to prevent layering of the solvent. After 30 min of standing at room temperature, the absorbance at 560 mμ is read on a Beckman model B spectrophotometer.

RESULTS AND DISCUSSION

The present procedure involves several modifications of procedure II (4) originally recommended for the assay of hydroxyproline in urine and in other biological samples in which hydroxyproline represents less than 1% of the total amino acids or similar compounds present. The modifications consist of eliminating the humin precipitation step, of reducing all the volumes by one-half, and of changing some of the critical conditions for the oxidation and the color development. With the present modifications it is possible to assay a set of 30 to 50 samples within 5 hr. We generally have employed duplicate samples in order to reduce technical errors, and therefore we have routinely assayed 15 to 25 samples in this time. The procedure presented here can be applied directly to assay of free hydroxyproline by using unhydrolyzed samples. It can also be used

to assay hydroxyproline in tissues, but in order to hydrolyze completely proteins containing hydroxyproline, it is necessary to autoclave samples in 6 *N* HCl at 120° for at least 10 hr. It has been found more expedient to hydrolyze urine samples at 100°, since this can be carried out in the same screw-capped tubes in which they are diluted and neutralized. The humin precipitant originally suggested has been found to be unnecessary for most urine samples, and is not required unless unusual amounts of humin are present. The humin interferes somewhat in adjusting the pH with phenolphthalein, but the first adjustment is only approximate, and the pH is readjusted at a later stage on a diluted aliquot of the sample. Also, we have found that the sample need only be adjusted to within ±2 units of pH 8 unless an unusual amount of buffering substances is present. The absolute amount of solid KCl added to saturate the samples is also not critical, but duplicates agree more closely if the amounts in various tubes are comparable (±0.5 gm).

Further investigation of the oxidation time has indicated that optimal results are obtained in 20 to 30 min. The optimal time varies slightly with room temperature, but it is not critical as long as samples in one assay are oxidized for about the same time, and as long as the oxidation is for at least 20 min. Similarly, the samples must be boiled for at least 20 min, and if adding the sample tubes to the boiling water lowers the temperature significantly, the boiling time should be increased appropriately.

Although it was originally suggested that the color be allowed to develop for 15 min, the time required to obtain maximal absorbance may be slightly longer depending on room temperature. Since the chromophore is stable, a 30 min period for color development is probably preferable. The aliquot of toluene used for the color development must be transferred carefully, since small amounts of the aqueous phase will cause the chromophore to disappear rapidly. Standard curves prepared with varying amounts of hydroxyproline have occasionally been found to deviate from linearity when optical densities are greater than 0.600, and usually samples are chosen so that the measured absorbance ranges between 0.100 and 0.500. Under the conditions employed here, we usually obtain an optical density of 0.500 with 10 μg hydroxyproline when the chromophore is measured in a 1 cm light path.

As indicated previously, the assay procedure is highly specific for hydroxyproline in the presence of a 10,000-fold excess of other amino acids. The specificity is further demonstrated by the correspondence of the spectrum of hydroxyproline assayed in urine and in pure hydroxyproline (Fig. 1). It may be noted that additional absorbance peaks which are seen with less specific assays (6, 7) are not encountered.

It was recently found that the recovery of chromophore in individual

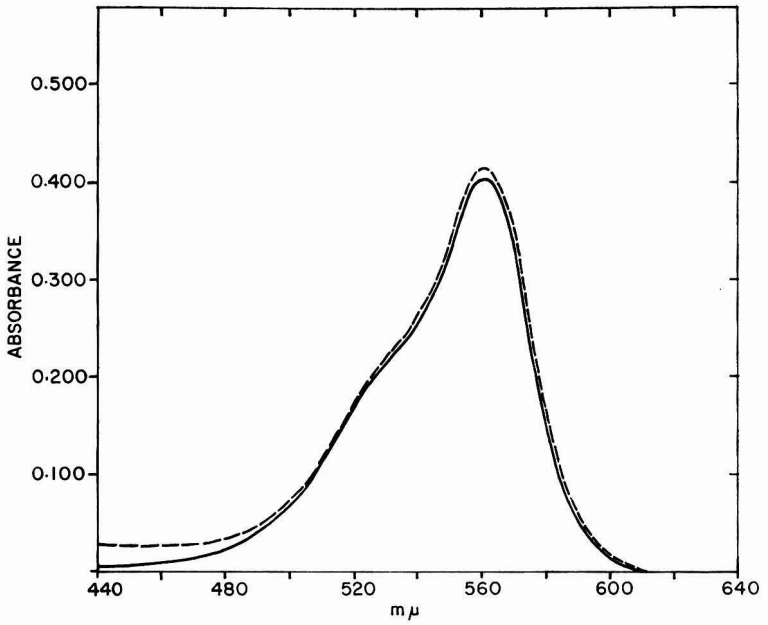

FIG. 1. Absorption spectrum of chromophore obtained with 8 μg L-hydroxyproline (solid line) and with hydrolyzed human urine (broken line). The spectra were recorded against water blank carried through the assay procedure.

samples can be measured by adding small amounts of hydroxyproline-^{14}C, and by taking a 1.0 ml aliquot of the final toluene extract for liquid scintillation counting. As shown in Table 1, the recovery of radioactive

TABLE 1
Comparison of Observed Recoveries Based on Assays of Chromophore and of Radioactivity[a]

Chloramine T, ml	Absorbance units	% [b]	Radioacivity	
			cpm	% [b]
0.10	0.095	19	227	19
0.50	0.390	78	942	78
0.75	0.479	96	1149	95
1.00	0.498	100	1210	100

[a] 10 μg hydroxyproline and 12,230 cpm hydroxyproline-2-^{14}C, 5 μc/μmole (Nuclear-Chicago), were oxidized with varying amounts of chloramine T as described in text; 1 ml of final toluene extract was mixed with 9 ml of toluene and 1 ml phosphor solution (8) for liquid scintillation counting, and 2.5 ml for development of the chromophore.

[b] Values obtained with 1.00 ml chloramine T are taken as 100%. As reported previously (4) the stoichiometric yield of pyrrole in this procedure is about 50% under optimal conditions.

pyrrole and of chromophore correspond closely when the chloramine T concentration is varied so that the over-all recovery varies from 19 to 100%. The use of hydroxyproline-^{14}C to measure the recovery in individual samples may be useful when highly impure samples are encountered, and this procedure makes it unnecessary to employ internal standards in order to estimate the recovery in individual samples.

The present procedure is more sensitive than the previously published procedure because the volumes have been reduced by one-half. However, it is possible to employ microcuvettes and to reduce the volume to one-sixth of the original procedure (4) so that 0.3 μg of hydroxyproline will give an absorbance of 0.05.

The modified procedure presented here has been used in one laboratory for a period of one year and in a second laboratory for a period of five years. Cumulative results obtained in these two laboratories are presented in Table 2. The average deviation from the mean with duplicate hydro-

TABLE 2
Cumulative Results with the Modified Procedure for the Assay of Hydroxyproline

Source	Number of samples	Average deviation from mean with duplicate assays(%)
Total hypro		
in human urine	1520	±0.8
in rat urine	1220	±1.0
Free hypro		
in human urine	330	±1.7
in rat urine	580	±1.5
in human serum	1010	±1.3
Total hypro in different animal tissues	890	±1.3

lyzed samples was ±1% in over 1000 assays of human urine and over 1000 assays of rat urine. Recovery of hydroxyproline was determined with internal standards or by additions of hydroxyproline-^{14}C in over 200 samples of hydrolyzed urine, and it was found to be 90% or greater. In duplicate assays of free hydroxyproline in urine, the average deviation from the mean was less than ±2%, and the recovery of free hydroxyproline varied from 70 to 90%. The variations of duplicate assays of free hydroxyproline in rat serum and of total hydroxyproline in various rat tissues were of about the same magnitude, and the recoveries were over 90%.

Because assays for hydroxyproline in urine, blood, and tissues are now being employed in many diverse experimental and clinical conditions in which large amounts of interfering materials may be encountered (1), the availability of a specific and convenient assay procedure is of some im-

portance. At least nine laboratories (1) that have employed the previously published procedure (4) reported values for hydroxyproline excretion in normal subjects which agree closely, and their results as well as results from a number of other laboratories suggest the procedure does provide the specificity and sensitivity required for most applications. Our own experience with the modifications described here indicates that the assay can be conveniently used for multiple measurements on a routine basis.

SUMMARY

Several modifications of a specific chemical assay for hydroxyproline in urine are presented which make the procedure more rapid without reducing its specificity or accuracy. Also, a new method for measuring the recoveries in individual samples with hydroxyproline-^{14}C is presented. Results obtained with the new procedure in two laboratories over a number of years are reviewed.

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Modification of the Nikon Microcomparator for Accurate Reading of Rayleigh Interference Patterns[1]

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In the course of studies of chemically interacting protein systems by sedimentation equilibrium in the analytical ultracentrifuge it was desired to read the Rayleigh interference patterns on the photographic plates with maximum speed and precision. In order to obtain the requisite accuracy of fringe positions for these purposes, it was necessary to read the pattern from an individual cell three to five times. To reduce the time and increase the accuracy of determining the fringe positions, the Nikon 6C microcomparator was modified so that averaging over several fringes is accomplished in a single plate reading. In addition, since the criterion for correct fringe position is displayed on an oscilloscope screen, much of the tediousness and eyestrain associated with the plate reading process is eliminated.

The plate reading process for Rayleigh interference fringes consists of recording the x-coordinates of successive fringe numbers or fractions of fringe numbers (1). The data then constitute a concentration scale of fringe number vs. position, relative to the first point recorded. In the case of sedimentation equilibrium experiments, such data can be converted by standard methods (2, 3) to determine molecular weights and assess homogeneity of the material in solution.

The apparatus is based on the principle of the moiré pattern (4), as illustrated in Figure 1. This figure shows the superposition of a Rayleigh pattern taken at sedimentation equilibrium on the corresponding baseline picture.

The positions of maximum and minimum densities in such a superimposed pattern correspond to one-half and integral fringe numbers. Since the maxima and minima in the moiré pattern result from cancellation and

[1] From the Department of Biochemistry, University of Washington, Seattle, Washington. This research was supported by a U. S. Public Health Service Research Grant from the National Institute of General Medical Sciences (GM 13401) and by funds provided by the University of Washington under Initiative 171.

coincidence of several fringes in the y-direction, the x-coordinates of such light and dark regions constitute an average over many fringes simultaneously.

From consideration of such a photograph, two possibilities for recording the x-coordinates of the fringe numbers are apparent: the baseline grid and experimental pictures can be fixed as shown in Figure 1 and a vertical slit moved along the pattern. The position of maxima and minima, which correspond to one-half and integral fringe numbers, may be detected by placing a phototube behind the pattern. Alternatively, the slit and baseline grid can remain fixed while the experimental pattern is moved. Since this second possibility also allows the y-coordinate to be varied until minimum or maximum transmission is observed, this alternative has been chosen in the apparatus described below.

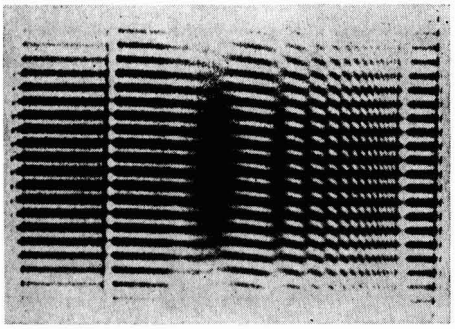

FIG. 1. Moiré bands resulting from superposition of a Rayleigh interference pattern taken at sedimentation equilibrium on the corresponding baseline pattern. The dark bands correspond to minimum light transmission, while light bands correspond to maximum transmission. Direction of sedimentation is from right to left.

MATERIALS

The macromolecule used for the sedimentation equilibrium experiments cited here was diisopropylphosphoryl-α-chymotrypsin (DIP-α-chymotrypsin) prepared by the method of Balls and Jansen (5) from 3$\times$ crystallized α-chymotrypsin purchased from Worthington Biochemicals (Lot #CD16144-5).

METHODS

Sedimentation equilibrium experiments were performed in a Spinco model E analytical ultracentrifuge equipped with an electronic speed control. The upper aperture used had a slit width of 0.178 mm and slit separation of 4.0 mm (43 fringes in the central diffraction envelop). In order to decrease exposure times, the push-pull light source slit was

opened to 0.038 mm (0.0015 in.). The optical system of the ultracentrifuge was focused at the ⅔ plane of the cell (6) to decrease the skewness in the Rayleigh fringes. Photographs were taken on IIG Kodak spectroscopic plates. Two different filters have been used to produce monochromatic light: initially the Kodak 77A filter supplied with the ultracentrifuge, while more recently a Baird-Atomic B-9 interference filter with maximum transmission at 546 mμ has been employed.

The experimental technique used for ultracentrifugation was essentially that described by Yphantis (3) except that higher initial concentrations (0.25 to 0.75 mg/ml) and lower speeds ($\sigma = 3.2$ for 3 mm solution columns) were employed.

The microcomparator used was the Nikon 6C optical comparator equipped with booster condensing lens, goniometer, zero-setting devices for the micrometers, and surface illuminator. For preliminary experiments the 50× objective lens was used, but the 100× objective has been found to increase the resolution of the apparatus and was employed for all of the experiments reported here.

The surface illuminating light source of the Nikon 6C was replaced by a light-tight box containing a RCA 1P28 photomultiplier phototube as shown in Figure 2. The photomultiplier power was supplied by a John Fluke 412B high-voltage power supply. A fixed grid of horizontal lines was mounted on the back of the slit which, in turn, is mounted approximately one-fourth inch from the wall of the microcomparator. This position was not at all critical since the diverging rays from the objective have reasonable focus at all points between the objective and wall of the microcomparator. The slit itself was constructed so as to allow independent variation of length, width, and angle of inclination. The slit width normally used for measurements was 0.038 mm.

In order to facilitate alignment of the image of the photographic plate with the slit and vertical cross-hair on the screen of the microcomparator, a unit was built which rotated the lower portion of the objective lens through small distances. This unit consisted of two short lever arms coupled by a small bolt. The two lever arms were clamped by aluminum bands on adjacent sides of the rotatable portion of the objective lens. The bolt connecting the two lever arms allowed fine rotational adjustment of the objective lens so that the image of a vertical line on the photographic plate (such as a diffraction band or a meniscus) could be precisely aligned both on the slit and on the vertical cross-hair on the microcomparator screen.

The signal from the photomultiplier was connected to one input of a Tektronix 561A oscilloscope equipped with type 2A63 differential amplifier.

To the other input of the differential amplifier 1.5 to 3 V was provided either by a battery or by a voltage reference source (Heathkit EUW 16A).

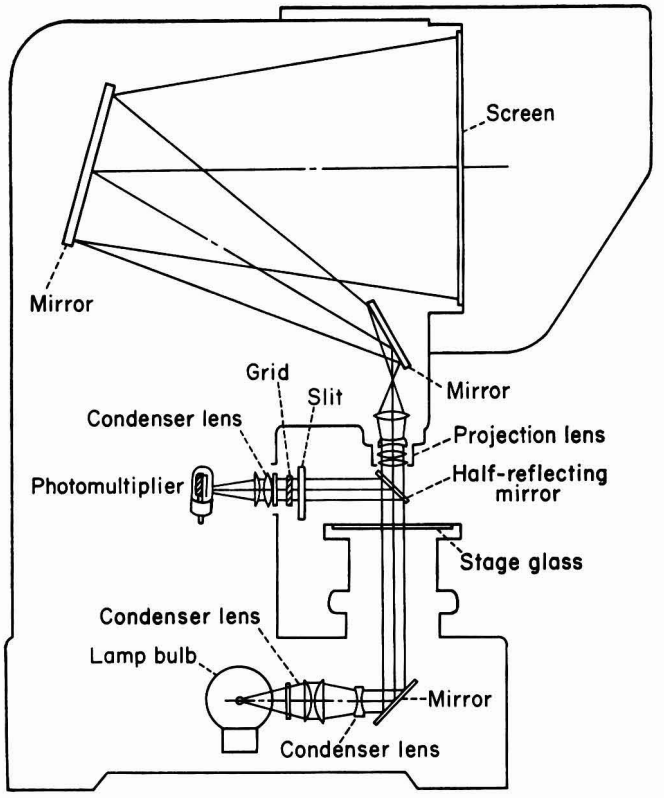

FIG. 2. Optical diagram of the modified Nikon 6C microcomparator. The surface illuminating light source has been replaced by a photomultiplier; a grid of horizontal lines and vertical slit are placed in front of the upper condensing lens as shown in the diagram.

In order to make fixed grids which have the proper fringe spacing, a camera was constructed that clamped to the slit base. This camera, made of black Plexiglas, was built to use the 2.5 × 3.5 in. film holder for the ultracentrifuge. The camera aperture was a 4.5 cm Keystone Flashmeter made by Ilex Optical Co. By means of this camera, a baseline plate from an ultracentrifuge experiment was aligned on the microcomparator stage and a picture taken on Kodak commercial film. This picture then served as the fixed grid for reading of subsequent plates until the ultracentrifuge optics were realigned. Prior to the construction of the camera, an enlargement of Ronchi rulings (Edmund Scientific Co.) served quite satisfactorily as the fixed grid.

TABLE 1
Measurement of Fringe Separation on Equilibrium and Baseline Plates[a]

Expt. No.	Plate and filter	$y_1 - y_0$		$y_2 - y_1$		$y_3 - y_2$		$y_4 - y_3$		$y_5 - y_4$	
		Av.	S.D.	Av.	S.D.	Av.	S.D.	Av.	S.D.	Av.	S.D.
I	Equilibrium 77A	277.7	4.34	278.6	4.44	275.6	3.31	—	—	274.4	4.21
II	Equilibrium 77A	—	—	279.3	4.21	278.3	2.94	276.6	2.73	275.8	3.78
III	Baseline 77A	277.3	2.32	279.0	3.07	278.6	2.96	276.9	2.74	—	—
IV	Baseline B9	276.3	1.55	275.0	2.68	276.4	3.00	276.0	3.01	—	—

[a] Column headings denote successive fringe differences as described in text. "Av." is the average fringe separation in μ, S.D. is the standard deviation of the fringe separation in μ.

In Experiment I the zero was reset on the micrometer prior to reading y_4. Experiments II, III, and IV are a set of equilibrium and baseline plates from the same ultracentrifuge run. In Experiment II, the columns have been shifted to the right to show the matching of fringe separation with experiment number III.

RESULTS

Several tests have been applied to the apparatus to test its accuracy and reproducibility. One experiment was to test the reproducibility of fringe positions in the absence of other variables. This was done simply by reading the same plate twice in an identical fashion by the same operator. This gave a standard error of 1.26 μ. The maximum deviation for this experiment was 5 μ. Reading of the same fringes by two separate operators gave 2.00 μ as the standard deviation. Realignment of the slit between two sets of readings of the same plate yielded 3.58 μ standard deviation. Here the maximum deviation was 13 μ. For these experiments, the maximum y-displacement was 1800 μ.

Another test of the apparatus consisted of the following experiment. A sedimentation equilibrium plate was read when the darkest fringe on the plate (y_0) was centered on the slit aperture. Eight light and eight dark fringes were included on the slit. This fringe was followed to the cell bottom. The plate was then reread when the next higher fringe (y_1) was centered on the slit. This process was repeated for the third, fourth, and fifth higher fringes. Data of this type are collected in Table 1 for two equilibrium and two baseline plates. It is of interest to note that, if the mean fringe separation of the first and third entries in the table are compared, for all cases using the 77A filter the fringe separation is statistically nonidentical. The same is true for the second and fourth fringes. This is not the case for the readings using the B-9 interference filter, however; this difference in fringe separation is probably due to the broader transmission band of the 77A filter compared to the B-9, giving the Rayleigh fringes some polychromatic character. This conclusion has been verified by recording the photomultiplier signal while driving the y-micrometer across the diffraction envelop for large fringe displacements.

Two experiments have been performed to test the apparatus under conditions normally used in sedimentation equilibrium experiments.

(*1*) The first of these was to test the reproducibility of the apparatus on water-water baseline plates. Normally such an experiment is performed between each sedimentation equilibrium experiment (3). Data from five such baseline experiments were combined in the following way: the average of the y-readings was made for each baseline experiment; the difference between the average of each set and the first set was subtracted from all points in that set, and the mean and standard error of the y-positions determined at each x; the cumulative standard error over the entire channel for the three channels was 3.02, 3.63, and 7.16 μ. Apparently the baseline of the third channel shifted during the course of the five experi-

ments (the probability that the third variance is identical to the other two is less than 1%).

(*2*) The second calculation of this type was performed on a single sedimentation equilibrium experiment. Three initial concentrations of DIP-α-chymotrypsin were sedimented to equilibrium and pictures taken at 512, 768, and 1024 min of centrifugation. The plates were then read using the apparatus. Normally, in routine reading of equilibrium plates, both y-readings at fixed x-positions and x-readings at fixed y-values were

TABLE 2
Variability of Fringe Positions at Sedimentation Equilibrium of DIP-α-Chymotrypsin[a]

Measurement	Channel No. 1	2	3
Initial concentration of enzyme, mg/ml	0.25	0.50	0.75
Maximum y-displacement, μ	675	1150	1210
Standard error of y-position reproducibility, μ	3.20	3.22	3.88
Number of y-coordinates	25	24	22
Standard error of x-position reproducibility, μ	—	5.30	4.40
Number of x-coordinates	—	4	12

[a] The solvent for this experiment was 0.2 M potassium phosphate, pH 6.35. Equilibrium pictures of the 3 mm solution columns were taken at 512, 768, and 1024 min of centrifugation.

made. A single fringe was followed with readings at fixed x-coordinates until the angle of the fringe was about 45°. Beyond this point, the y-micrometer was kept at its last value and the x-coordinate of the subsequent light and dark fringes recorded until the bottom of the cell was reached. For the DIP-α-chymotrypsin experiment, the calculations were made in the same fashion as was used for the baseline data reported above. Table 2 records the result of this calculation. It is clear from these data that good reproducibility was possible.

DISCUSSION

The advantages of the apparatus as illustrated by the results reported are apparent. In effect, by a single reading of the photographic plate, one performs the same operation as reading many single fringes and averaging the result.

One disadvantage of the present apparatus is that vertical streaks, diffraction bands, dust marks, etc. cause false maxima and minima in the photomultiplier output. When recording the y-coordinate of the fringes, this problem does not appear as serious as when the x-coordinates are determined. Nevertheless, in order to totally automate the plate reading process for direct computer treatment (7–9), such spurious relative

maxima and minima cannot be tolerated. Perhaps this difficulty can be overcome by use of a split-beam apparatus and two photomultipliers, one of which corrects for imperfections on the photographic plate.

At first sight it might be thought worthwhile to incorporate such a fringe detection system directly into the ultracentrifuge in a similar fashion to the scanning system used for absorption optics (10). There are two major problems which would be encountered in such a project. The first is that a very narrow slit would be required. Using the Nikon 6C with 100× objective lens, the magnification at the plane of the horizontal fringe grid is about 20-fold. Hence, for the same resolving power of such an instrument directly in the centrifuge a slit of about 1 μ would be required. Such a slit would probably be difficult to construct. The second problem for such an instrument is that the light intensities in the centrifuge are too low. At present, using the B-9 filter and the upper aperture described above, exposure times range from 8 to 16 min. Thus the photographic plate is serving as an integrator of light with time in this case. In conclusion, in order to place such an apparatus directly into the ultracentrifuge, extensive modification of the light source and optics would be required.

SUMMARY

A modification of the Nikon 6C optical comparator is described which enables the operator to determine fringe positions on Rayleigh interference patterns with greater accuracy, precision, and speed than can be obtained by other methods. Because the decision required for correct position of the fringe is a maximum or minimum in an electrical signal, much of the tediousness and eyestrain is eliminated from the plate reading process.

ACKNOWLEDGMENTS

The author would like to acknowledge the technical assistance of Mrs. Donna Georgakakos and Mr. Lawrence Tew in the construction and experimentation with this apparatus. In addition, he would like to thank Mr. R. D. Wade and Dr. D. A. Deranleau for discussion and encouragement in the course of this work.

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ANALYTICAL BIOCHEMISTRY 19, 265–271 (1967)

A Simplified Enzymic Procedure for the Preparation of Nucleoside Di- and Triphosphates[1]

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The importance of nucleoside 5′-di- and triphosphates for the synthetic processes of the cell has led to the development of numerous chemical (1–3) and enzymic (4–7) methods for their preparation. Although these methods have obvious advantages, each is subject to limitations. Thus, the chemical procedures are inefficient when mμmole or even μmole quantities of nucleotide are required, whereas the enzymic methods often require extensive purification or facilities to grow and harvest bacteria. We, therefore, would like to present another method which may in some instances supersede the previously described procedures.

These studies were the outgrowth of an investigation of the levels of deoxycytidylate deaminase in regenerating liver (8, 9), where it was found that the employment of heat denaturation to terminate reactions containing dCMP-2-C^{14} yielded higher zero time blank values than did reactions terminated with acid.[4] Further investigation revealed the elevated blanks to be due to the presence of dCDP and dCTP, products apparently resulting from the presence of heat-stable phosphotransferases in the liver extracts. Subsequent study indicated that, in addition to dCMP, all of the naturally occurring nucleotides except dTMP were converted to nucleoside di- and triphosphates in heated liver extracts.

By altering conditions, the formation of the di- or triphosphate could

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[4] Abbreviations used: UMP, UDP, UTP, uridine 5′-mono-, di-, and triphosphate; CMP, CDP, CTP, cytidine 5′-mono-, di-, and triphosphate; GMP, GDP, GTP, guanosine 5′-mono-, di-, and triphosphate; AMP, ADP, ATP, adenosine 5′-mono-, di-, and triphosphate; dTMP, thymidine 5′-monophosphate; d, deoxy-.

be favored and, as will be seen, the method is particularly useful when small quantities of highly labeled nucleotide are required.

MATERIALS

d-CMP-C^{14} was purchased from Schwarz BioResearch, Inc., Orangeburg, New York, and high specific activity UMP-C^{14} from New England Nuclear Corporation, Boston, Massachusetts.

Triethylamine bicarbonate (TEAB) solutions were prepared by bubbling CO_2 from an enclosed container with dry ice into a triethylamine-water mixture (139 ml/liter—1 *M*) until the pH was about 8.0 or less. The triethylamine was purified by distillation through a Hempel column with beads.

Creatine kinase was a generous gift of Dr. Robert Peanasky, Marquette University, Milwaukee, Wisconsin. The enzyme is now commercially available.

Creatine phosphate was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio, and dithiothreitol from Calbiochem, Los Angeles, California.

METHODS

Preparation of Enzyme Extracts. A 30% homogenate of the livers (24 gm) from 48-hr fasted rats in 1.15% KCl was centrifuged at 37,000 *g* for 30 min; 5 ml of 0.5 *M* potassium phosphate (pH 7.5) was added to the supernatant (92 ml), which was then heated to 85° and maintained at this temperature for 10 min. The suspension was cooled and centrifuged, after which the supernatant fraction was lyophilized. The residue was dissolved in a minimum volume of cold water and dialyzed against two 2-liter changes of 0.001 *M* potassium phosphate (pH 7.5). About 10 ml of active enzyme extract was obtained, which was used mainly for the preparation of nucleoside diphosphates. If nucleoside triphosphates were desired, heating the rat liver supernatant at 85° for 2 min was found to be adequate. The extracts in each case were still active at the end of 6 months, despite repeated freezing and thawing.

Chromatographic Procedures. Primarily two paper chromatographic systems were utilized: (A) Isobutyric/NH_4OH/0.01 *M* EDTA (100/60/1.6); (B) 95% ethanol/1 *M* ammonium acetate saturated with sodium tetraborate (pH 9.0) (5/2).

With (A), Whatman No. 31 paper (heavy weight) gave satisfactory separations in 4 to 6 hr; however, the cytidine nucleotides were not adequately separated from the adenine nucleotides, when CDP formation was measured. For quantitation, Whatman No. 1 was preferred, although the solvent moves considerably slower on this paper. (B) was used mainly

with reactions containing deoxyribonucleotides and the papers of choice were Whatman No. 1 or 3 MM.

Preparation of Nucleoside Diphosphates. The reaction mixtures contained the following components (μmoles): ribo- or deoxyribonucleoside 5′-phosphate, 8; potassium phosphate (pH 7.5), 50; ATP, 18.6; $MgCl_2$, 30; glutathione or dithiothreitol, 10; Versene, 0.1; heat-stable enzyme, 0.2 ml; water to bring the reaction mixture to 1.0 ml. Two drops of toluene was added to the reaction mixtures, which were incubated at room temperature for 24 to 28 hr. The course of the reaction was followed by chromatographing 20 χ of the reaction solution in solvent A for the ribonucleotides and in solvent B for the deoxyribonucleotides.

Preparation of Nucleoside Triphosphates. The components of reaction mixtures were similar to those described above except for the following changes: ATP, 0.5; $MgCl_2$, 3.0; creatine phosphate, 20; creatine kinase, 0.5 mg.

(*C*) *Ion-Exchange Separation.* A Dowex 1 carbonate (200–400 mesh, X-8), column (6 × 1 cm) was utilized with a convex TEAB gradient, at

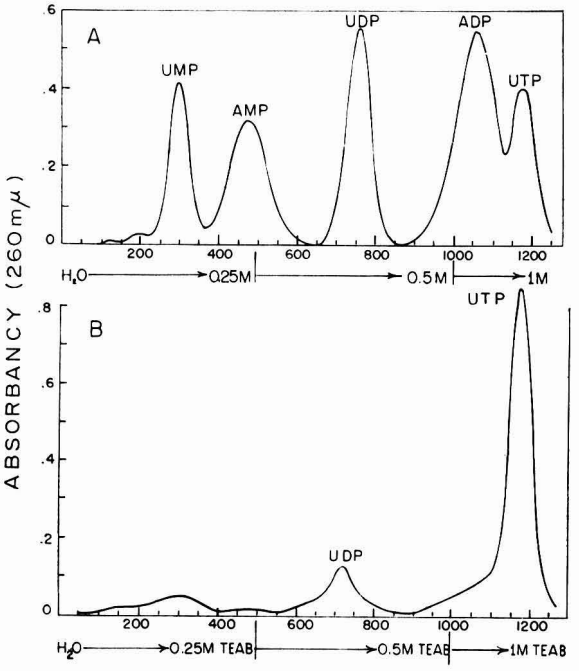

FIG. 1. Gradient elution of the products resulting from the phosphorylation of 10 μmoles of UMP (A) without the creatine phosphate/creatine kinase system and (B) with this system.

the concentrations indicated in Figures 1 and 2. The constant-volume mixer contained initially 250 ml of water.

Since guanosine nucleotides could not be isolated cleanly by this procedure, the following method was adopted from the procedure of Cohn and Bollum (10) to facilitate their recovery. For the isolation of GDP and dGDP, the reaction mixture was brought to 10 ml with water and passed through a 3×1 cm column of Dowex 1 formate. The column was then

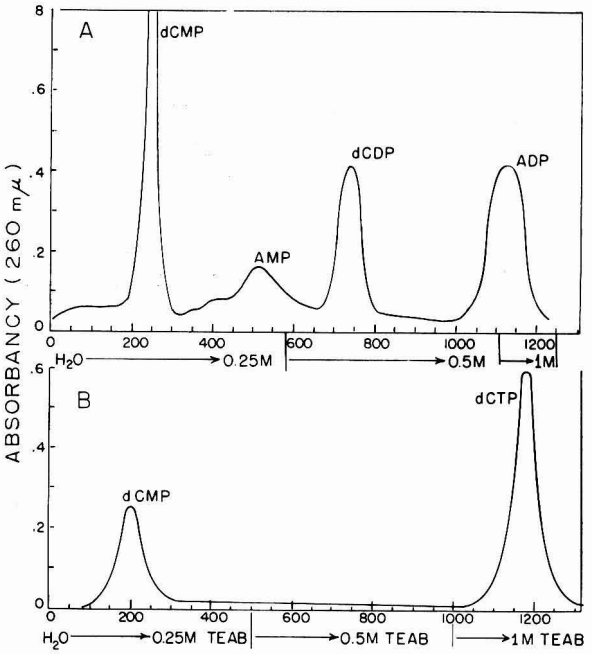

FIG. 2. Gradient elution of the products resulting from the phosphorylation of 9.4 μmoles of dCMP. (A) and (B) are the same as in Figure 1.

eluted with eight 10-ml fractions of 4 *N* formic acid to remove ADP and GMP, followed by 200 ml of 0.1 *M* Na_2CO_3 to convert the column to the carbonate form. Excess Na_2CO_3 was removed with water and the GDP was eluted with about ten 10-ml fractions of 0.8 *M* TEAB. A similar procedure was employed to isolate GTP, but the eluting agents in this case were 4 *N* formic acid +0.2 *M* ammonium formate (AmF) to remove GMP and ATP, and 2 *M* TEAB to elute GTP.

To achieve rapid separations, particularly when labeled nucleotides were desired, Dowex 1 formate columns with 0–1 *M* AmF convex gradients were employed (Fig. 3). In these cases, the radioactivity and ultraviolet light absorption patterns were monitored simultaneously with a Gilson

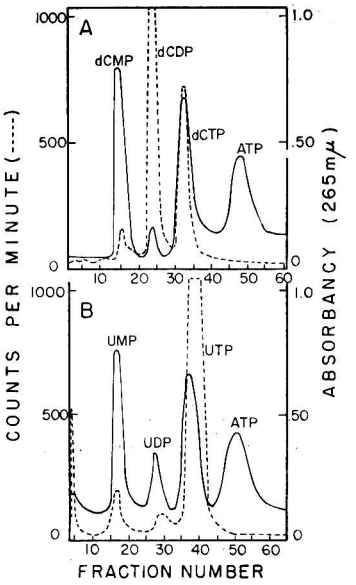

FIG. 3. Dowex 1 formate gradient elution of products following (A) dCMP-2-C^{14} phosphorylation and (B) UMP-C^{14} phosphorylation. The reaction mixture for (A) contained 2.1×10^{-3} μmole of dCMP-2-C^{14} (6.4×10^{7} dpm/μmole) and components similar to those in (B) but no creatine kinase or creatine phosphate. The incubation time was 28 hr at 25°. The reaction mixture for (B) contained (in μmoles); UMP-C^{14}, 5×10^{-4} (7.0×10^{8} dpm/μmole); potassium phosphate (pH 7.5), 50; $MgCl_2$, 3.0; mercaptoethanol, 10; Versene, 0.2; ATP, 1.0; creatine kinase, 0.2 μg; heat-stable enzyme extract 0.1 ml; total volume, 0.5 ml. Elution was effected with a convex gradient consisting of a 250-ml water-mixing chamber and a 1 *M* AmF reservoir. Markers of deoxycytidine and uridine nucleotides were included to aid in identifying the radioactive peaks.

U.V. recorder and a Nuclear-Chicago scintillation flow cell system. If recovery of the nucleotides was desired, TEAB was substituted for AmF.

RESULTS AND DISCUSSION

The broad specificity of the heat-stable phosphotransferase in rat liver suggests a similarity to the nucleoside monophosphate kinase preparation from calf liver (4). However, the latter enzymes would appear to be labile to the heating procedure described in this paper. Still, an exact comparison cannot be made because of differences in source material and the stage of purification at which stability was measured.

The presence of a nucleoside diphosphate kinase in the liver extract, which is more labile to heat than the monophosphokinase, is suggested by the greater capability of the 2-min heated preparations to form triphos-

phates than those heated for 10 min. This difference was exploited, when nucleoside diphosphates were desired.

The enhancement of triphosphate formation by the addition of ATP-generating systems, such as creatine phosphate/creatine kinase or phosphoenol pyruvate/pyruvic kinase,[5] suggests contamination of these enzymes by nucleoside diphosphokinase. Considering the length of time of the incubations, only trace amounts of the latter enzyme could contribute greatly to the formation of triphosphates. However, the reported lack of strict specificity of creatine kinase may also be responsible for the formation of some of the nucleoside triphosphates, such as CTP (11). Myokinase, which is known to be heat-stable, may be involved in purine nucleoside diphosphate formation.

TABLE 1
Isolated Nucleoside 5′-Phosphates[a]

Substrate, μmoles	A, μmoles			B, μmoles	
	Mono-	Di-	Tri-	Mono-	Tri-
UMP 8.4	1.9	4.8	1.2		8.2
CMP 9.6	2.6	6.7		3.5	6.0
GMP 11.0		5.5[b]			6.5
dUMP 9.0	7.3	2.1	0.5	6.0	3.5
dCMP 9.0	4.2	4.5		4.0	4.6
dGMP 7.6		4.1			5.9

[a] A: These nucleotides were separated from an incubation mixture containing the heated (10 min) rat liver supernatant fraction. B: These nucleotides were obtained from a reaction mixture containing the heated (10 min) rat liver supernatant fraction and the creatine phosphate-creatine kinase system. For the conditions employed and the ion-exchange separation of the nucleotides, see "Methods."

[b] 8.2 μmoles of GMP was the initial substrate concentration in this case.

It is of interest to note that dTMP-2-C^{14} phosphorylation could not be measured, which is in accord with the apparent absence of dTMP kinase in normal adult rat liver (6). Neither could the phosphorylation of 5-hydroxymethyl-dCMP and 4-*N*-hydroxy-dCMP be measured. However, cytosine arabinoside 5′-phosphate was converted to the triphosphate.

Regardless of the enzymes responsible for the formation of the triphosphates, Table 1 indicates that they are formed to varying degrees in the coupled system. AMP and dAMP have not been included, but yields comparable to those with GMP and dGMP were obtained. A problem in

[5] Both enzymes are present in the Worthington Biochemicals preparation of lactic dehydrogenase, as is myokinase and nucleoside diphosphokinase. Thus the dehydrogenase can be used in place of creatine kinase for the synthesis of the nucleoside triphosphates.

separating dADP and dATP from their respective ribonucleotides may be encountered, but periodate treatment to labilize the adenine ribonucleotides (12) may be of value in this respect.

The main drawbacks of the method pertain to the slowness of the reaction and the limited substrate specificity. However, these are more than overcome by the capacity to synthesize with a high specific activity, many of the common nucleoside di- and triphosphates, labeled in one or more positions. Since this method can be utilized at the μmole level and even much lower (Fig. 3) to provide respectable yields of nucleotides, it has an obvious advantage over many of the chemical procedures.

SUMMARY

1. A heat-stable enzyme preparation from rat liver can be used to synthesize ribo- and deoxyribonucleoside di- and triphosphates from AMP, CMP, GMP, dAMP, dCMP, dGMP, and dUMP. Cytosine arabinoside 5′-phosphate is converted to the corresponding triphosphate in quantitative yields.

2. The nucleotides are isolated by ion-exchange chromatography with the volatile buffer triethylamine bicarbonate as eluant.

3. The yields in most cases are better than 50%, making this procedure an efficient method for the synthesis of highly labeled nucleoside di- and triphosphates.

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ANALYTICAL BIOCHEMISTRY 19, 272–279 (1967)

A Direct Spectrophotometric Assay for Pyruvate Kinase[1]

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Pyruvate kinase (PK) (EC 2.7.1.40, ATP:pyruvate phosphotransferase) is one of the two enzymes in glycolysis involved in substrate phosphorylation. It catalyzes the following reaction:

$$\text{phosphoenolpyruvate} + \text{ADP} \rightarrow \text{pyruvate} + \text{ATP} \qquad \text{(A)}$$

which is essentially irreversible under conditions governing the normal economy of the cell. This enzyme has been implicated in the changes in metabolic rate of neoplastic tissue (1–3), shown to be composed of isoenzymes (4–6), and suggested to be allosteric since it consists of four protomers (7). It is therefore advantageous to have a simple, direct, continuous method of assaying this enzyme.

Previous methods of assay have been summarized by Boyer (8). The older techniques have a number of disadvantages. The measurement of phosphoenolpyruvate (PEP) as phosphate liberated by alkaline iodine solution or mercuric ion is irreproducible and discontinuous. Pyruvate measured by the color produced in reaction with 2,4-dinitrophenylhydrazine and alkalinization is also discontinuous. Pyruvic kinase coupled with the lactic dehydrogenase (LDH)–NADH system, although continuous, cannot be used for detailed kinetic analyses. A pH-stat technique has recently been developed (9, 10) but here relatively large amounts of enzyme and substrates are necessary and the stoichiometry of the hydrogen ion consumption must be rigidly determined and held constant. In the present study a spectrophotometric method is described which measures directly and continuously the disappearance of PEP at 230 mμ. The method is sensitive and simple, requires relatively small amounts of substrates and enzyme, and allows for the first time a rapid reproducible way of measuring the disappearance of substrate. A similar procedure has been used to assay enolase (11).

[1] This work was supported in part by the Cancer Research Funds of the University of California.

[2] NDEA Title IV Fellow.

MATERIALS AND METHODS

Chemicals. Pyruvate kinase (166–178 EU/mg), lactic dehydrogenase (550 EU/mg), NADH (disodium salt), and phosphoenolpyruvate (monopotassium and trisodium salts) were purchased from Calbiochem. Tris-(hydroxymethyl)aminomethane (Tris), phosphoenolpyruvate (tricyclohexylamine salt), ADP (sodium and diTris salts), and pyruvate (sodium salt) were purchased from Sigma Chemical Company. All buffer solutions were prepared from glass-distilled deionized water and reagent-grade chemicals.

The PK was found to be stable at a dilution of 1 mg/ml in 0.05 *M* Tris HCl, pH 7.5, on ice but lost activity at the rate of about 3% per hour at a dilution of 0.1 mg/ml. Since the latter dilution was used as the enzyme stock for each assay it was prepared fresh when needed from the more concentrated solution. The frozen solution of ADP (Tris salt) gave evidence of decomposition and therefore was prepared fresh each day. All other stock solutions were kept refrigerated or on ice. The potassium salt of PEP was titrated to pH 7.0 with KOH and stored on ice.

Apparatus. The pH was measured using a Radiometer (model TTT1c) pH meter equipped with a combination glass electrode and reference electrode. The reaction was followed by measuring the decrease in optical density (OD) at 230 mμ using either a Beckman DB spectrophotometer with a Sargent (model SRL) linear-log recorder or a Beckman DB-G spectrophotometer with a Beckman 10″ linear-log recorder. Both recorders were set up using the log output so that OD was recorded directly. Full scale sensitivity of 0 to 1.0 OD unit and a chart speed of 1.0 in./min were used unless stated otherwise. The cell compartment was maintained at 29.5 $\pm$ 0.25°C by means of a circulating water bath and pump. The reaction was started by the addition of enzyme to the reaction mixture and the recorder immediately activated. The change in absorbancy was observed for 4 min and initial velocities were determined by measuring the tangent to the recorded curve.

Spectroscopic analysis of PEP and pyruvate were performed on the Cary (model 15) recording spectrophotometer.

Assay. For a given assay the cuvet contained in 1 ml: $MgSO_4$, 7.2×10^{-3} *M*; KCl, 7.2×10^{-2} *M*; Tris, 0.05 *M*; PK, 1 μg; and ADP and PEP as indicated in the figures. In any one assay series the components with invariant concentrations were mixed together as a stock solution and kept on ice. The stock was prepared fresh each day. The components of the assay mixture were pipetted into a standard 1 cm^2 quartz cuvet. Following this, a quartz block 7 $\times$ 9 mm (purchased from Pyrocell) was inserted into the cuvet to give an effective light path of 1 mm. The cuvet with

insert in place was preincubated at 29.5°C for 5 min and then wiped and quickly placed in the cell chamber of the spectrophotometer. The reaction was initiated by adding 10 μl of the enzyme solution, rinsing the pipet twice in the medium, stirring, and recording the decrease in OD. All points reported are the average of duplicate determinations with an average deviation about the mean of 3%. The kinetic constants were estimated from Lineweaver and Burk (12) plots in every case by the method of least squares.

RESULTS

Considering that the UV absorption of ADP and PEP is significant at 230 mμ, the initial OD values are relatively high at high concentrations of the substrates. Furthermore, as ADP is converted to ATP there is no change in the absorbance since, at 230 mμ, both ADP and ATP have the same molar extinction coefficient (ϵ). Because the absorbance of PEP and pyruvate (Fig. 1) are different at 230 mμ, however, a decrease in OD is observed as PEP is converted to pyruvate. The molar extinction coefficients of PEP and pyruvate were determined and found to be 2.93×10^3 cm^{-1} M^{-1} and 6.26×10^2 cm^{-1} M^{-1}, respectively. These values are independent of the cationic form and of the presence of the salts at the con-

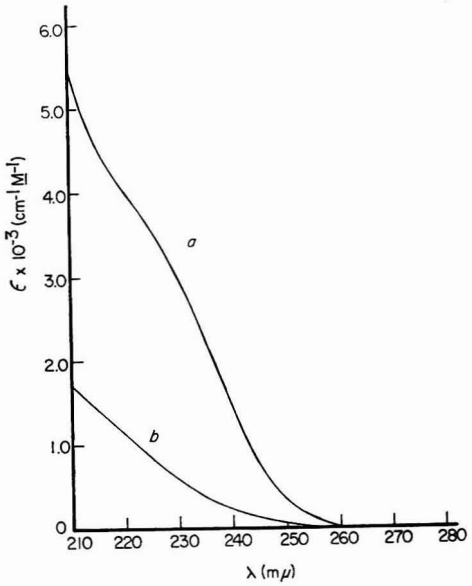

FIG. 1. UV spectra of PEP and pyruvate. The molar extinction coefficient, ϵ, was calculated from the spectrum of (*a*) sodium PEP at 2.00×10^{-4} *M* and (*b*) sodium pyruvate at 6.30×10^{-4} *M*, both in 0.05 *M* Tris HCl, pH 7.5, at room temperature.

TABLE 1
Typical Optical Densities at the Beginning and End of an Assay[a]

Substrate concentration $\times 10^4$ M		Optical density	
PEP	ADP	Initial	Final
20	8.3	0.81	0.68
0.5	8.3	0.22	0.21
6.0	28	0.88	0.84
6.0	0.7	0.20	0.16

[a] Assay performed and other components as given under "Materials and Methods."

centration used in the assay, and are in good agreement with the literature values (11 and 13, respectively). Typical initial optical densities are shown in Table 1. Even when the change in OD was only 0.01 unit the reproducibility was within the stated error.

PEP was found to follow Beer's law at 230 mμ to concentrations 25 times those used in the assay. The assay is linear with enzyme concentrations from zero to 2.5 μg/ml. Figures 2 and 3 show the Lineweaver-Burk plots for PEP and ADP, respectively, and are included to show that the assay is well adapted to substrate concentrations around the critical value for detailed kinetic studies. From these plots the Michaelis constants

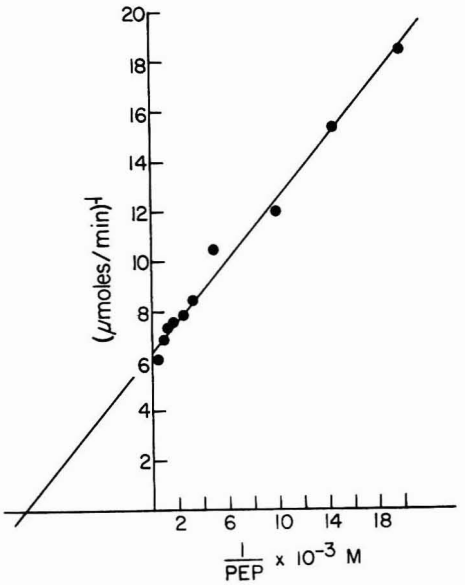

FIG. 2. Lineweaver and Burk plot of reaction rate vs. PEP concentration. The medium is as given in "Materials and Methods" plus ADP (diTris salt), 8.28×10^{-4} M. (PEP is the potassium salt.)

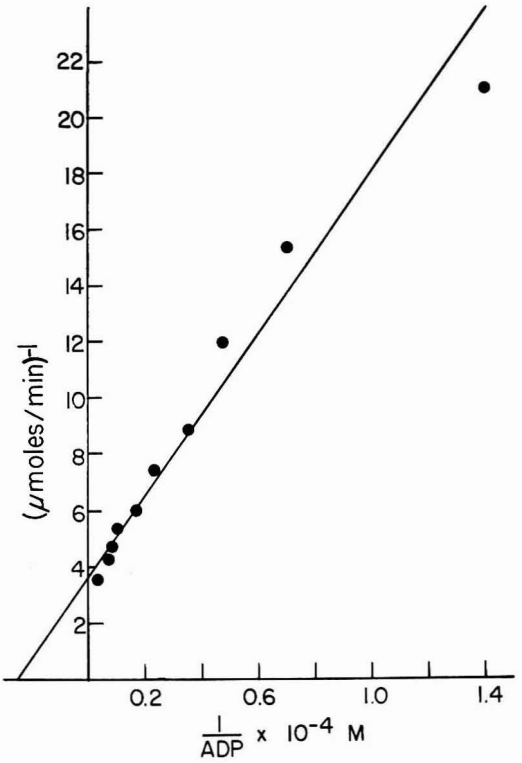

FIG. 3. Lineweaver and Burk plot of reaction rate vs. ADP concentration. The medium is as given in "Materials and Methods" plus PEP (potassium salt), 6×10^{-4} *M*. (ADP is the diTris salt.)

TABLE 2
Comparison between Direct Assay and LDH Coupled Assay

Experiment	μmoles converted/ml/min	
	Coupled[a]	Direct[b]
1	0.080	0.072
2	0.18	0.17
3	0.31	0.31

[a] Assay performed as described by Bücher and Pfleiderer (18). The activity ratio of LDH to PK was maintained at 62:1. The assay was carried out in a total volume of 3 ml and the decrease in the absorption of NADH was followed at 340 mμ. (Chart speed: 10 in./min).

[b] Assay performed as given under "Materials and Methods." In addition, each cuvet contained ADP (diTris salt), 8.28×10^{-4} *M*, PEP (potassium salt), 6×10^{-4} *M*, and NaCl, 3×10^{-4} *M* (see under "Discussion").

(K_m) were calculated for PEP and ADP, i.e., $0.961 \pm 0.072 \times 10^{-4} M$ (Fig. 2) and $3.66 \pm 1.02 \times 10^{-4} M$ (Fig. 3), respectively. They agree with the published values of $0.7 \times 10^{-4} M$ (PEP) and $3.0 \times 10^{-4} M$ (ADP) (14), and $0.915 \times 10^{-4} M$ (PEP) and $4.8 \times 10^{-4} M$ (ADP) (15). The probable error in the K_m values was calculated by the method of Livingston (16) using the standard deviations for the intercept at the ordinate and for the slope. The latter were computed by the method of Bull (17).

The spectrophotometric assay yields the same value for the rate of transphosphorylation as does the coupled reaction with LDH which was performed as described by Bücher and Pfleiderer (18) (Table 2).

DISCUSSION

At 230 mμ the substrates, products, and enzymes have an absorbance. The enzyme, even though it has an extinction coefficient of about 7×10^5 $cm^{-1} M^{-1}$ at 230 mμ assuming a molecular weight of 237,000, contributes little to the absorbance at a dilution of 1 μg/ml with a light path of 0.1 cm. The contribution of the nucleotides is significant but does not change as the ratio of ADP to ATP changes since the optical density is strictly additive throughout. Thus the change in OD as PEP goes to pyruvate is clearly observed. To obtain initial absorbancies in a range in which the spectrophotometer is most sensitive, a shorter light path was chosen as the routine procedure since increasing the blank absorption decreases the light energy. This is at the expense, however, of the observable ΔOD.

The rate in micromoles per minute was obtained from the equation:

$$\mu\text{moles/min} = \frac{\Delta\text{OD/min}}{\epsilon_{\text{PEP}} - \epsilon_{\text{pyruvate}}} \times 10^4 \qquad \text{(B)}$$

This expression was derived on the basis that the stoichiometry of PEP to pyruvate is 1:1. As the reaction proceeds from left to right in equation A, the rate of decrease of PEP is the same as the rate of increase of pyruvate. This formula was used in the calculation of the data for Figures 2 and 3 and Table 2.

With a 10 cm silica absorption cell and recording with a full scale of 0.1 OD in the Cary 15 spectrophotometer and placing the complete medium save for enzyme in both the sample and reference chambers, a measurable rate of change in the OD was observed upon addition of enzyme to the sample containing $2 \times 10^{-7} M$ PEP and $4 \times 10^{-5} M$ ADP. The other components and conditions are as given under "Materials and Methods" except that this was run at room temperature. The total change in OD was 0.004 unit whereas the noise level was set at 0.001 OD unit.

The assay was found to be directly applicable to a crude water extract of chick muscle. Proper controls must be used to correct for the presence of enzymes for which PEP and/or ADP are substrates. Nevertheless significantly different rates were observed in the presence and absence of ADP which were additive. This would correct for endogenous enolase and phosphatase. Performing the experiment in the presence and absence of CO_2 would correct for phosphoenolpyruvate carboxykinase activity.

In all cases the kinetic parameters reported were obtained using the potassium and Tris salts of the substrates since the use of the tricyclohexylamine salt of PEP suggested that it was an inhibitor and sodium ion has been shown to be an inhibitor (19). The sodium effect was verified and is the rationale for adding NaCl to the direct assay of Table 2, to compensate for the amount of sodium ions added to the coupled assay as the sodium salt of NADH.

Preliminary experiments suggest that the K_m of PEP varies with the concentration of ADP as first reported by McQuate and Utter (14). This is in support of the similar findings by Siebert *et al.* (15) and in their conclusion that the substrates are bound in the order of ADP first and PEP second. More recently, Mildvan and Cohn (20) confirmed the "rapid-equilibrium, random" kinetics proposed by Reynard *et al.* (21) using the methods of proton relaxation rate enhancement, electron paramagnetic resonance, and kinetic data. Unfortunately the results of these authors do not explain the anomalous concentration dependency of ADP on the K_m of PEP in light of random order kinetics. This conflict clearly must await further studies before it can be resolved.

SUMMARY

The enzymic transphosphorylation reaction catalyzed by pyruvate kinase is followed directly by recording the disappearance of phosphoenolpyruvate at 230 mμ. This assay has advantages over previous assays in that it is direct, simple, sensitive, and continuous and requires relatively small amounts of material. The turnover of substrate is calculated to be the same as that obtained using the lactic dehydrogenase coupled assay.

ACKNOWLEDGMENTS

The authors wish to thank Dr. R. T. Wedding for the use of his Beckman DB-G spectrophotometer, Drs. J. B. Mudd and J. N. Pitts for the use of their Cary 15 spectrophotometers, and Dr. E. A. Noltmann for helpful suggestions and criticisms.

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Acrylamide Gel-Sucrose Gradient Electrophoresis: A Useful Preparative Method

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While mixtures of proteins can easily be resolved by zone electrophoresis in acrylamide gels, recovery of the isolated proteins usually requires rather complicated apparatus, such as a flow-through preparative column (1–5) or an electrophoresis convection chamber (6). Recovery does not present any special problems when zone electrophoresis is carried out in density gradients, but the resolution obtained by this technique is poorer than when a gel is used.

We have developed a preparative method that combines the advantages of both acrylamide gel electrophoresis and sucrose gradient electrophoresis. The apparatus is simple. There is good resolution and easy recovery of separated protein with little loss or dilution.

METHODS

The electrophoresis chamber consists of a modified U-tube made from 25 mm diameter Pyrex tubing (Fig. 1). The tube is filled with a linear gradient of sucrose in 0.375 *M* Tris-HCl buffer, pH 8.8, containing 0.001 *M* disodium ethylenediaminetetraacetate (EDTA). The gradient, which goes from 60 to 20% (w/w) sucrose, is conveniently prepared with the mixing device described by Hegenauer *et al.* (7). The 20% sucrose is placed in the mixing chamber so that the least dense portion of the gradient is formed first and the heaviest last. The feeding tube from the mixing chamber reaches to the bottom of the U-tube; this ensures that identical gradients are formed in the two limbs. The buffered gradient is allowed to flow in until it enters the capillary side arm (A in Fig. 1) that is connected to each limb of the chamber. This requires about 75 ml. The

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tubing attached to each side arm is closed off; the openings at the top of the right limb and at the right end are closed off with rubber stoppers. Acrylamide gel (7.5%) in 0.375 *M* Tris-HCl, pH 8.8, containing 0.001 *M* disodium EDTA, is prepared according to Davis (8); 5.5 ml of this gel is carefully layered on top of the sucrose gradient in the left arm of the chamber. About 1.0 ml of distilled water is layered on the gel. Care should be taken to avoid entry of the gel into the capillary side arm.

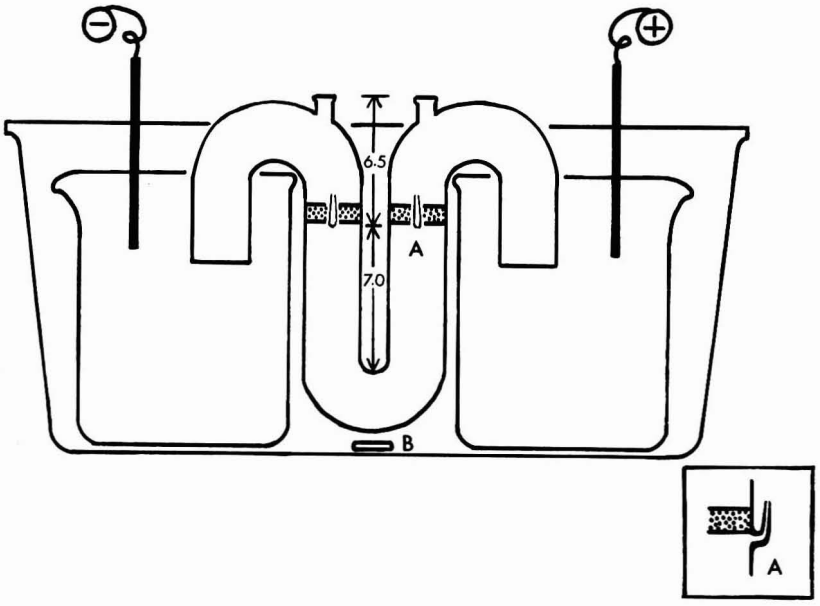

FIG. 1. Chamber for preparative acrylamide gel-sucrose gradient electrophoresis. The apparatus is accurately drawn to scale; the diameter of the glass tubing making up the chamber is 2.5 cm. The acrylamide gel is indicated by a stippled zone. The insert is a side view of side arm A. The magnetic stirring bar is indicated by B.

After the gel in the left limb has polymerized (about 15 or 20 min), the stoppers are removed and 5.5 ml of the same gel is layered on the sucrose in the right limb of the chamber. When this has hardened, the overlying water is removed from both gels, and their upper surfaces are washed with about 4 ml of water. The final gel height is about 1.2 cm. If the protein sample to be applied contains particulate matter which would distort the gel during the run, 4 ml of a spacer gel containing 2.5% acrylamide and 0.625% bisacrylamide cross-links can be polymerized on top of the running gel to prevent this distortion by preventing the particulate material from entering the running gel. The chamber is suspended between two 800 ml beakers filled with 0.025 *M* Tris-glycine buffer, pH 8.3, containing

10^{-3} *M* disodium EDTA, as shown in Figure 1. The beakers are placed in a clear plastic mouse cage (model 102, Keystone Plastics, Media, Pennsylvania) filled with ice water. The top openings of the chamber are closed with serum stoppers. The chamber above the gel is completely filled on either side with the same Tris-glycine buffer as in the beakers. This is done by withdrawing the trapped air on each side by means of polyethylene tubing attached to a 50 ml syringe. This arrangement provides for a hydrostatic equilibrium which permits the use of a short column of gel. The chamber is allowed to sit for about 1 hr in the ice bath for temperature equilibration. Cooling is facilitated by stirring the bath with a magnetic stirrer.

The sample is usually 10–40 mg of protein in 2–8 ml of water or dilute (0.02 *M* or less) buffer containing 10% sucrose or glycerol together with a little bromphenol blue dye. The maximum amount of protein that has been applied to this column is 50 mg of bovine serum albumin. The capacity for more complex protein mixtures is probably greater than this amount, since the bovine serum albumin was largely a single protein band. A modified chamber to accommodate sample volumes up to 18 ml can be made by increasing the height of the chamber above the side arms (see Fig. 1) from 6.5 to 10 cm. Suspension in the ice bath is maintained by two 1000 ml Berzelius beakers. The sample is carefully layered over the gel in the left arm of the chamber by means of a hypodermic needle inserted through the serum stopper above the gel. Platinum electrodes are placed in the buffer chamber as indicated in Figure 1, and the voltage is applied. An inexpensive pair of platinum electrodes can be obtained from Fisher Scientific Company (catalog No. 9-460). The power supply employed is a constant-voltage DC power pack developing 0–2000 V and 0–100 mA.

COLLECTION OF FRACTIONS

At the end of the run, the current is turned off and electrodes are removed. The rubber or polyethylene tubing leading to both capillary side arms is opened. A solution of 60% sucrose in water is slowly injected into the right capillary side arm from a 10 ml hypodermic syringe. Each milliliter of the heavy sucrose solution that is injected flows down to the bottom of the U-tube and forces 1 ml of the buffer in the left side of the chamber up and out through the left side arm. It is important to have the bottom of the gel just above the side arm; if it is too high, there will be a dead space that permits mixing of the proteins in different fractions.

ASSAY METHODS

Conventional methods of protein assay could not be applied directly to the fractions obtained in these experiments because of interference by the

high concentration of tris buffer in the gradient. An aliquot (usually 0.2 ml) of each fraction was pipetted into a conical glass centrifuge tube and was diluted 10-fold with water. Protein was precipitated by adding 70% perchloric acid to a final concentration of 10%, and phosphotungstic acid to a final concentration of 1%. The tubes were held in ice for 1 hr, after which the protein precipitate was removed by centrifugation, washed once with 1 ml of a solution containing 10% perchloric acid and 1% phosphotungstic acid, and dissolved in 1 ml of 0.1 *N* NaOH. The amount of protein was then determined by the phenol method of Lowry *et al.* (9). This procedure gave negligible blank values for Tris buffer alone. In preliminary experiments the recovery of bovine serum albumin was linear in the range of 19.8 to 70 μg.

A more convenient method for assaying protein in the presence of Tris buffer entails collection of the precipitated protein on a Millipore filter. The sample to be assayed is placed in a glass centrifuge tube and its volume is adjusted to 0.5 ml with distilled water; 2.5 ml of cold 5% TCA is added with mixing and the tube is kept on ice for 30 min. The suspension is then filtered through a Millipore filter (No. EHWG025) which has been soaked in distilled water. The centrifuge tube is rinsed with 3.0 ml of cold 5% TCA, which is added to the precipitate on the filter. The precipitate is then washed twice with 3.0 ml samples of 5% TCA. The filter is then transferred to a test tube containing 1.0 ml of 0.1 *N* NaOH to dissolve the protein from the filter. Aliquots of this solution are then used for the Lowry protein assay. With this membrane method, standards of bovine serum albumin have shown linearity between at least 30 and 150 μg, with recovery consistently 90% or better.

NADPH-cytochrome *c* reductase was assayed according to Horecker (10). The assay of choline acetyltransferase (choline acetylase) was modified from the radioisotopic method described by Alpert *et al.* (11). One unit of enzyme activity is the amount which will convert 10 mμmoles of acetyl-*S*-coenzyme A to acetylcholine in 1 hr. The activity of aminoacyl-sRNA synthetases was determined by a modification of the membrane method of J. F. Scott, as described by Yu and Zamecnik (12). A unit of enzyme is defined as that amount which will esterify 1 mμmole of amino acid in 10 min at 37°.

Analytical acrylamide gel electrophoresis was carried out according to Davis (8) except that no sample gel or spacer gel was used, and samples of protein containing 10% sucrose or glycerol were layered directly onto the running gel. The gels were stained with Procion blue dye (Consolidated Laboratories, Chicago Heights, Illinois), 0.5% in 7.5% acetic acid, and destained in a 1:1 mixture of 7.5% acetic acid and methanol together with a little Dowex 1 chloride resin to facilitate dye removal.

The current applied in most of these experiments ranged from 18 to 25 mA at 400–500 V. As indicated by the movement of the accompanying indicator dye, proteins entered the gel within 3 to 10 min after the current was turned on. The fastest moving protein bands took from 45 min to 1 hr to pass through the acrylamide gel and enter the top of the sucrose gradient. Because of the density gradient, the bands retained their disc shape in the sucrose solution and did not broaden to more than 2 or 3 times the thickness they had maintained in the acrylamide gel. The steepness of the sucrose gradient (about 67.4 gm/liter/cm) reduced the formation of microdroplets in the leading band (13).

The viscosity gradient associated with the change in sucrose concentration served to sharpen the rapidly moving bands. However, as pointed out by Svensson (13), closely spaced, fast-moving bands will tend to run together as the viscosity increases. This tendency can be overcome in part by imposing a reverse gradient of buffer concentration on the sucrose gradient.

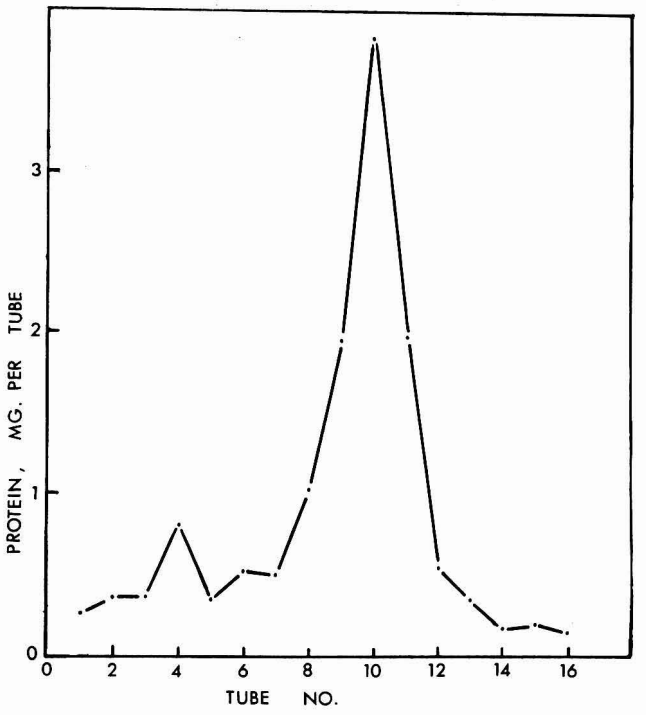

FIG. 2. Separation of bovine serum albumin monomer and dimer. The sample contained 15 mg protein in 2.4 ml 10% glycerol. A voltage of 500 V (20 mA) was applied for 2.5 hr. Sixteen samples of 1 ml were collected by displacement.

Separation of Bovine Serum Albumin Monomer and Dimer. Electrophoresis of crystalline bovine serum albumin (BSA) on acrylamide gels gives rise to several bands. These have been identified as different polymeric forms of the protein (14). The fastest moving band is the monomer. In the BSA preparation used for these experiments (Nutritional Biochemical Corporation) the monomer appeared to make up approximately 80 to 90% of the total protein. As shown in Figure 2, preparative electrophoresis gave good separation of BSA monomer and dimer, with a recovery of 88%, based on the total amount of protein applied. The isolated fractions were electrophoretically homogeneous, as indicated by analytical acrylamide gel electrophoresis.

Isolation of NADPH-Cytochrome c Reductase from Mouse Liver Microsomes. Livers from male C57 Bl/6J mice were homogenized with 0.1 *M* potassium phosphate buffer, pH 7.8, containing 10^{-3} *M* disodium EDTA (8 ml per liver). The homogenate was centrifuged at 15,000 *g* for 10 min. The resulting supernatant was centrifuged at 105,000 *g* for 75 min. The microsomal pellet was suspended in 0.03 *M* KPO_4 buffer, pH 7.8, containing 10^{-3} *M* disodium EDTA (2 ml per liver). Steapsin (Nutritional

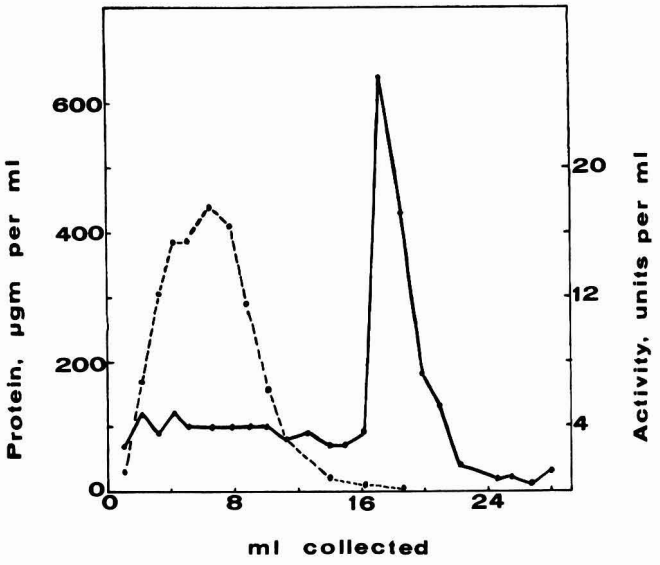

FIG. 3. Electrophoresis of microsomal NADPH-cytochrome *c* reductase. 13 mg protein (187 units of activity) was applied in 3.2 ml. The run was continued at about 500 V (18 mA) for 4.5 hr. The data are plotted so that the fraction with the highest mobility is on the right side of the figure. Protein concentrations (solid line) were determined by precipitation with perchloric and phosphotungstic acids (see "Methods"). The dashed line denotes enzyme activity.

Biochemical Corporation) was added to a final concentration of 0.7 mg/ml. The suspension was incubated at 4° for 16 hr in order to solubilize the enzyme (15). The steapsin-treated digest was centrifuged for 75 min at 105,000 *g*, and the supernatant, containing the soluble enzyme, was stored at −15°. Before electrophoresis the enzyme was desalted by passage through a column of Sephadex G-25. The eluting solution was 0.01 *M* Tris-HCl containing 10^{-3} *M* disodium EDTA.

The electrophoretic separation of the enzyme is shown in Figure 3. In several different experiments the recovery of NADPH-cytochrome *c* reductase ranged from 70 to 90%. The enzyme recovered in these experiments was homogeneous by analytical gel electrophoresis and had the same specific activity as enzyme purified by other methods by Jick and Shuster (16). Reference 16 includes an illustration of analytical acrylamide gel electrophoresis of the starting material and purified enzyme.

Purification of Choline Acetyltransferase from Lactobacillus arabinosus. An acetone powder was prepared from a frozen paste of *L. arabinosus*

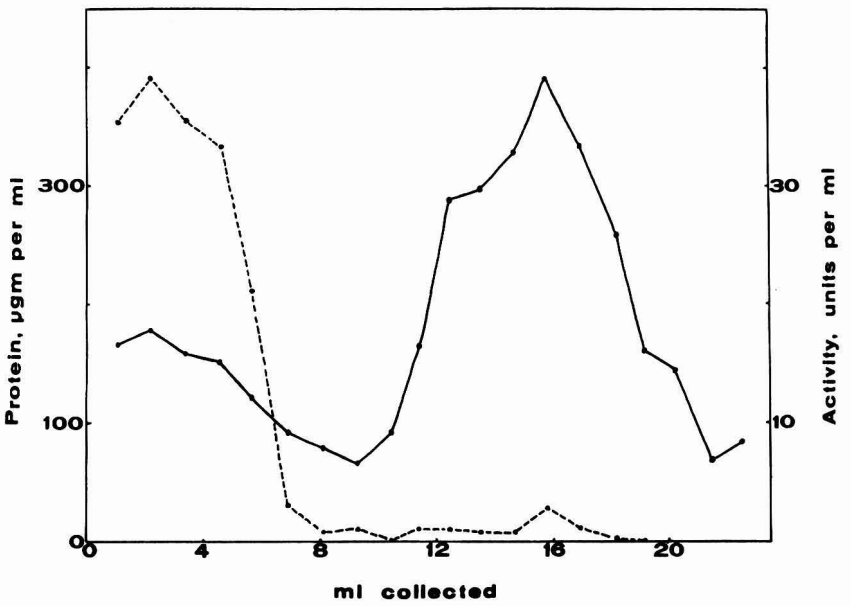

FIG. 4. Choline transacetylase purification by discontinuous collection. 18.0 ml enzyme solution containing 2430 units of activity and 12.3 mg protein was applied in 15% glycerol. After electrophoresis at 500 V (12 mA) for 5 hr, the first protein band was collected in 13.2 ml. The run was continued at 600 V (14 mA) for one additional hour, after which the enzyme was collected in 9.3 ml. The solid line represents protein determined by the membrane method (see "Methods"). The dashed line denotes enzyme activity.

cells grown and harvested by the New England Enzyme Center. The acetone powder was suspended in 0.05 *M* Tris-HCl buffer, pH 7.6, containing 0.001 *M* disodium EDTA, and was sonically disrupted in a Branson sonifier. Debris was removed by centrifugation and the extract was treated with streptomycin sulfate and recentrifuged. For the removal of small molecules the supernatant liquor was passed through a column of Sephadex G-25; the above buffer was employed for equilibration and elution. The desalted eluent protein fraction thus obtained was diluted 5-fold with 0.001 *M* disodium EDTA in water and applied to a column of microgranular DE 32 anion-exchange resin (H. Reeve Angel Inc., Clifton, N. J.) which had been equilibrated with 0.01 *M* Tris-HCl buffer, pH 7.6, containing 0.001 *M* disodium EDTA. The enzyme was eluted from the column with the same buffer containing 0.15 *M* NaCl; peak fractions were pooled and concentrated by dialysis against the same buffer containing 0.15 *M* NaCl and 30% (w/w) sucrose. The concentrated protein fraction

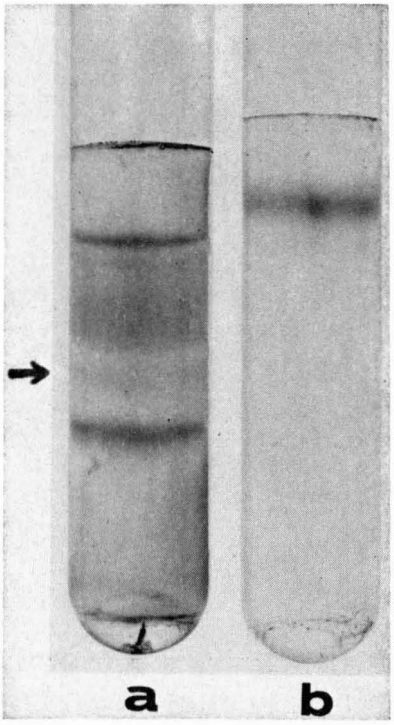

FIG. 5. Purification of choline acetyltransferase. The photographs demonstrate the appearance in analytical gel electrophoresis of 147 μg of starting material (a) and of 75 μg of the isolated enzyme (b). The arrow denotes the enzyme band in the gel of the starting material.

was desalted on a Sephadex G-25 column equilibrated with 0.001 *M* disodium EDTA in water, and the eluent was applied to the electrophoresis apparatus as described above.

In several preliminary runs, the choline acetyltransferase was not easily separated from a major protein band which preceded it. Purification was accomplished by interrupting the run just after the contaminating band had left the gel (as determined by its R_f value relative to the dye front in analytical gels), and collecting the gradient between the gel and the dye front. Following this collection, the voltage was again applied until the distance traveled by the dye front indicated that the enzyme band had left the gel. A second collection at this point resulted in nearly pure enzyme. The recovery pattern from this discontinuous collection is shown in Figure 4, where the data for the two collections are plotted contiguously in the order in which the samples left the gel. The protein with greatest mobility is on the right side of the figure. Recovery of activity was only 15%, which is consistent with total recoveries on previous runs. Purifica-

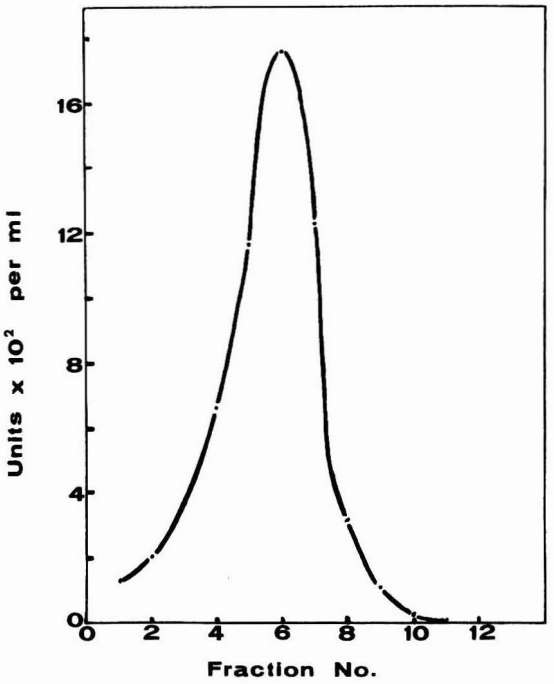

FIG. 6. Isolation of arginyl-sRNA synthetase. 1.9 ml of enzyme solution containing 3.5 mg protein (19,000 units) in 10% sucrose together with bromphenol blue dye was applied to the top of the gel. After electrophoresis for 3 hr at 500 V (15 mA), the dye front had moved 3.0 cm into the sucrose gradient.

tion of the enzyme was 1.73-fold even though about 5% of the enzyme had been inactivated.

The appearance of the starting material and purified enzyme after analytical acrylamide gel electrophoresis is shown in Figure 5. The location of the enzyme protein band in the gel of the starting material is shown by the arrow. The enzyme recovered from preparative electrophoresis appeared to be at least 90% pure; there was a trace of a faster moving component. The apparent low mobility of the purified enzyme is due to short duration of analytical electrophoresis.

Purification of Arginyl and Lysyl-sRNA Synthetases. The isolation of some amino-acyl-sRNA synthetases from *Escherichia coli* by preparative acrylamide gel electrophoresis has been reported by Calendar and Berg (17) and by Yu and Rappaport (18). We have found that the arginyl and

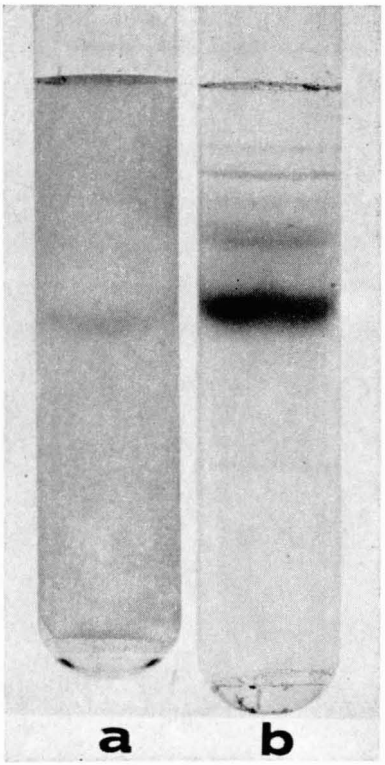

FIG. 7. Purification of arginyl-sRNA synthetase. The photographs demonstrate the appearance in analytical gel electrophoresis of the starting material (b) and the enzyme purified by gel-gradient electrophoresis (a). 145 μg of protein was applied in (b) and 33 μg in (a).

lysyl-sRNA synthetases from *E. coli* could be purified in good yield with the apparatus described in this paper.

Figure 6 shows the separation of the arginyl enzyme from a partially purified preparation that was kindly provided by M. Stephensen and J. A. Haines. The starting material had been treated with calcium phosphate gel and chromatographed on DEAE-Sephadex A-50. It had a specific activity of 5400 units/mg. Disc electrophoresis on acrylamide gel showed one major fast-moving band together with three slower bands. The enzyme isolated by preparative electrophoresis had a specific activity of 11,600 units/mg and moved as a single band when examined by disc electrophoresis. Recovery was 69%. Peak fractions were combined and dialyzed against 0.02 *M* sodium phosphate buffer, pH 7.0, containing 10^{-3} *M* EDTA. The final preparation had the same specific activity and electrophoretic

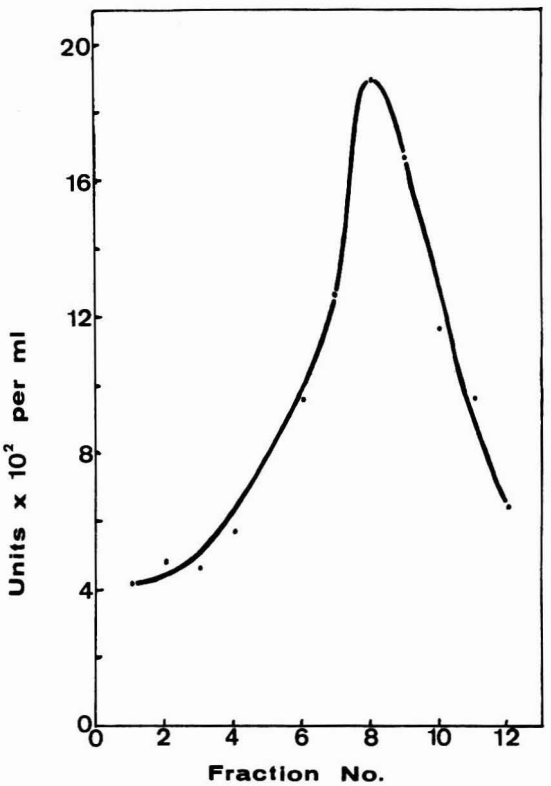

FIG. 8. Isolation of lysyl-sRNA synthetase. 2.0 ml of enzyme solution containing 17.7 mg protein (35,400 units) in 10% sucrose together with bromphenol blue dye was applied to the top of the gel. After electrophoresis for 3.5 hr at 500 V (15 mA), the dye front had moved 2.8 cm into the sucrose gradient.

mobility as enzyme that had been purified from the same starting material by gel filtration through Sephadex G-100. Analytical acrylamide gel fractionations of the starting material and product are shown in Figure 7. A detailed description of the purification of this enzyme will be published elsewhere.

The separation of lysyl-sRNA synthetase is shown in Figure 8. The starting material, provided by C. Janeway, had been treated with calcium phosphate gel and chromatographed on DEAE-Sephadex A-50 according to Stern and Mehler (19). It had a specific activity of 905 units/mg protein. Recovery by preparative gel-sucrose gradient electrophoresis was 72%. The isolated enzyme showed one major band and two very faint minor bands when subjected to analytical disc electrophoresis (Fig. 9). Both the purified arginyl- and lysyl-sRNA synthetase bound bromphenol

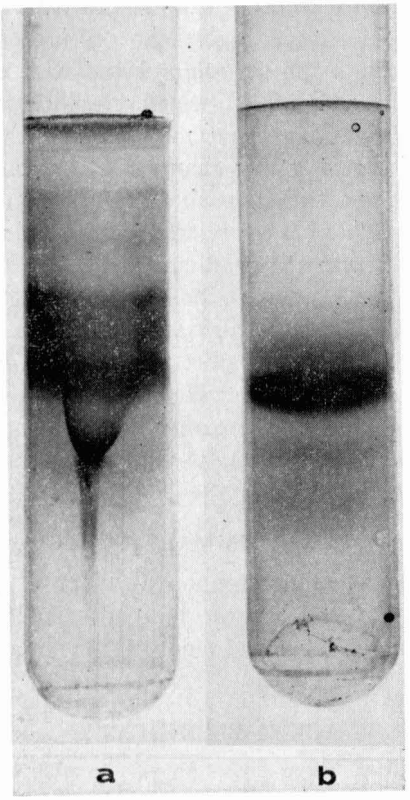

FIG. 9. Purification of lysyl-sRNA synthetase. The photographs demonstrate the appearance in analytical gel electrophoresis of 482 μg of starting material (a) and of 189 μg of the isolated enzyme (b). The coning in the gel of the starting material is an artifact that resulted from the electrophoretic destaining.

blue dye, so that the enzyme initially appeared as a blue band upon electrophoresis in acrylamide gel. As the electrophoresis continued the bound dye tended to migrate from the protein to the dye front.

DISCUSSION

Preparative electrophoresis by the method described above makes use of the major advantages of electrophoresis in both polyacrylamide gels and density gradients. Use of the gel allows the proteins to enter the gradient in discs of concentrated protein. Use of the sucrose gradient results in considerably less dilution during collection than can be maintained with a flow-through of buffer, as in the usual preparative gel methods.

The molecular sieving which occurs within the gel is not an important factor in protein mobility in the sucrose gradient and the relative mobilities of two proteins may be quite different in the two media. Hence, in the gradient, a band of a large, highly charged protein which had moved somewhat slowly through the gel might blend with or pass a band of a small, less highly charged protein which preceded it into the gradient. Should either band prove to be contaminated with the other after collection, some or all of the following changes in the conduct of the electrophoresis should serve to eliminate such contamination in subsequent runs: (*1*) *Changes in gel height:* increasing the height would retard the entrance of the second protein into the gradient, while decreasing the gel height might allow the larger protein to pass beyond the smaller one before collection. (*2*) *Changes in gel porosity.* (*3*) *Changes in the pH of the gel and/or buffers:* other practical applications of such changes are given by Raymond (14), and Williams and Reisfeld (20) give several pertinent formulations. (*4*) *Increase or decrease in the running time before collection.* (*5*) *Discontinuous collection,* as employed for choline acetyltransferase.

SUMMARY

A method of preparative electrophoresis which utilizes the advantages of polyacrylamide gels and sucrose gradients is described. Data from enzyme purifications and suggested modifications for specific problems are presented.

ACKNOWLEDGMENTS

We are indebted to Mrs. Karen Liazos for very capable technical assistance. The work on the amino-acyl-sRNA synthetases was carried out in the laboratories of Dr. P. C. Zamecnik. We are grateful for his hospitality and the help extended by his colleagues at the Massachusetts General Hospital. The assistance of Dr. S. Charm and the New England Enzyme Center in the growth of *Lactobacillus arabinosus* is gratefully acknowledged.

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ANALYTICAL BIOCHEMISTRY 19, 294–299 (1967)

Column Chromatographic Purification of Octanoyl-CoA

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Chemical (1–4) and enzymic (5, 6) syntheses of acyl-CoA's have been described; the purification of the acyl-CoA's so synthesized has usually been accomplished through the use of paper chromatographic systems (7, 8) or, in the case of long-chain acyl-CoA's, through their insolubility in acid (5, 9). In our studies on mycobacterial fatty acid synthetase systems (10) we required larger quantities of substrate than were easily available. This report describes a column chromatographic system for the purification of acyl-CoA's. The chromatographic system is based on the DEAE-cellulose column procedure of Moffatt and Khorana (11) but the lack of resolution of acyl-CoA's from the reduced and oxidized forms of CoA on DEAE-cellulose has been overcome through the use of thiol binding reagents. We report here a method for the purification of enzymically prepared octanoyl-CoA, as a model system, and the preparation and purification of ^{3}H-CoA and 1-^{14}C-octanoyl-^{3}H-CoA.

METHODS

Dithiothreitol (DTT) (6), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), CoA, sodium-1-^{14}C-octanoate (14 mc/mmole), and methyl octanoate (99.9%) were purchased from Calbiochem, Aldrich Chemical Co., P-L Laboratories, Inc., New England Nuclear Corp. and Analabs, respectively. Sodium octanoate was prepared by saponification of methyl octanoate.

The preparation of partially purified acyl-CoA synthetase (acid:CoA ligase (AMP), EC 6.2.1.3) from beef liver mitochondria has been described (6).

The purity of fatty acids was determined by gas-liquid chromatography of their methyl esters using a Barber-Colman Model 10 gas-liquid chro-

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matograph. Radioactivity was measured with a Packard Tri-Carb scintillation counter.

Ion-exchange chromatography was carried out with DEAE-cellulose (Cl^-). The elution systems are described below.

RESULTS

The chromatographic analysis of octanoate, CoA and octanoyl-CoA is shown in Figure 1. CoA and octanoyl-CoA cannot be separated by this system. CoA was retained in the reduced form by the incorporation of 0.5 m*M* DTT in the eluting solvent.

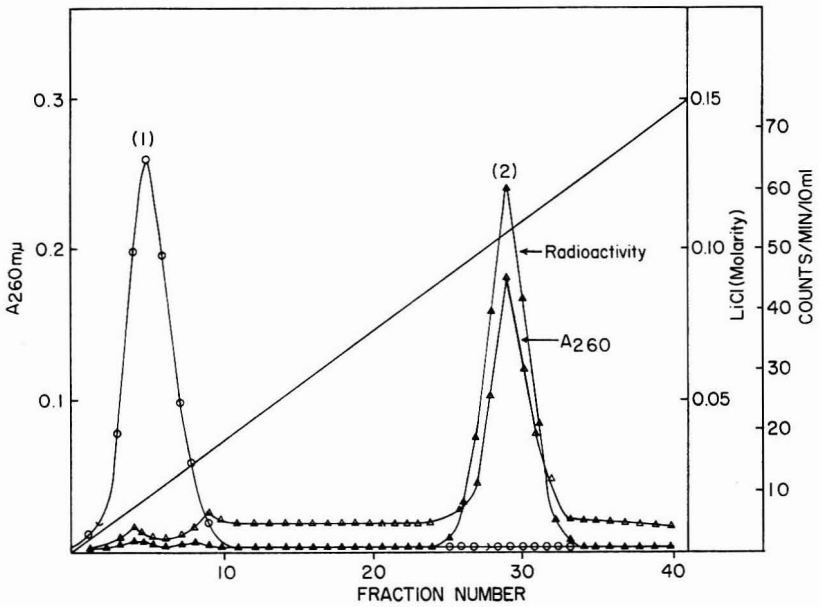
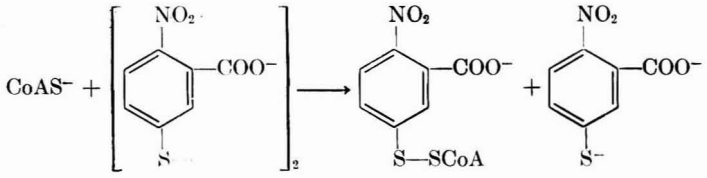


FIG. 1. Elution of octanoic-1-^{14}C acid, CoA, and 1-^{14}C-octanoyl-CoA from a column (1.2 × 15 cm) of DEAE-cellulose. An aqueous solution (1 ml) of each compound was washed into the column. Elution was initially with 100 ml of 0.5 m*M* DTT followed by 400 ml of 0.2 m*M* HCl/0.5 m*M* DTT with a linear gradient of LiCl from 0 to 0.15 *M*. Neither radioactivity nor ultraviolet absorption was found in the first 100 ml of eluting solution. The identification of peaks is as follows: (1) octanoic-1-^{14}C acid, (2) 1-^{14}C-octanoyl-CoA and CoA. Each fraction was 10 ml. The ordinate (counts/min/ml) should be multiplied by 10^3 for octanoic-1-^{14}C acid and by 10^4 for 1-^{14}C-octanoyl-CoA to attain experimental readings.

If CoA is converted to the TNB derivative it is retained on the DEAE-cellulose (Fig. 2) and can be separated from octanoyl-CoA. A mixture of 1.25 μmoles of CoA, 10 μmoles of DTNB (in *n*-propanol) and 100 μmoles of diol buffer in a final volume of 1.1 ml was permitted to react at 25°C



for 20 min following which the mixture was placed on the ion-exchange column. The deep yellow color of the Lee thionitrobenzoate (Reaction 1) is measured at 410 mμ at pH 8; the reaction is complete in less than 20 min.

Typical Procedure for Preparation and Purification of Octanoyl-CoA. The reaction mixture contained 100 μmoles of ATP, 200 μmoles of $MgCl_2$, 34 μmoles of sodium-1-^{14}C-octanoate (ca. 500 μc), 2 mmoles of diol buffer (pH 8), 40 μmoles of CoA, 20 μmoles of DTT, and 200 mg of partially purified acyl-CoA synthetase. The reaction mixture volume was 20 ml.

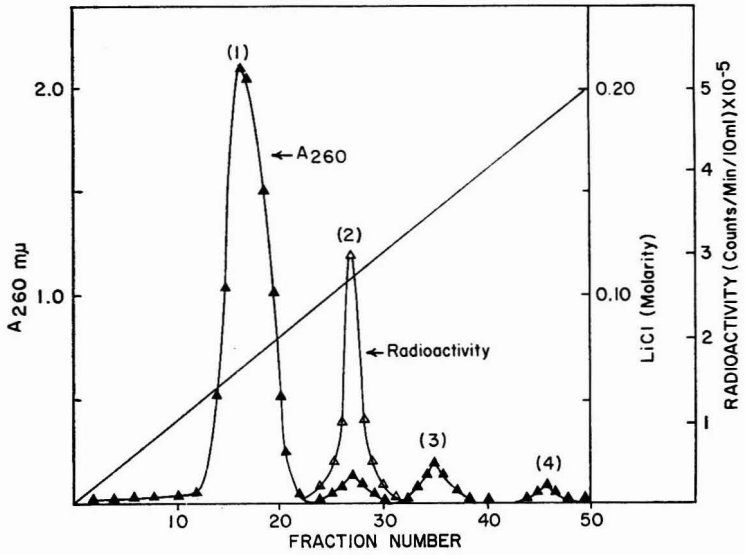


FIG. 2. Chromatography of octanoyl-CoA and the mixed disulfide of thionitrobenzoic acid and CoA on DEAE-cellulose. The reaction mixture contained 10 μmoles of DTNB (in *n*-propanol), 1.2 μmoles of CoA, 0.4 μmole of 1-^{14}C-octanoyl-CoA and 100 μmoles of diol buffer in a final volume of 1.1 ml. The reaction was carried out at 25°C for 20 min, at which time the reaction mixture was washed into the ion-exchange column. Elution was with 500 ml of 0.2 m*M* HCl with a linear gradient (0 to 0.2 *M*) of LiCl. The identification of the peaks is as follows: (1) DTNB and TNB, (2) 1-^{14}C-octanoyl-CoA, (3) mixed disulfide of TNB and CoA, (4) CoA-S-S-CoA (?). Each fraction was 10 ml.

The reagents were all mixed at 0°C, a sample was removed for CoA determination (12), and the reaction mixture was then incubated for 20 min at 38°C. In general about 30 μmoles of CoA reacts under these conditions.

After incubation the reaction was stopped by the addition of 0.5 ml of 6% $HClO_4$. The acidified mixture was lyophilized. The dried residue was extracted three times with 5 to 10 ml of pyridine/isopropanol/water (1/1/1); the extraction system was clarified through low-speed centrifugation. The combined extract was taken to dryness in a rotary evaporator at 25°C. The residue was washed three times with 10 ml of ethyl ether/ethanol (1/1) and the washed residue was dried under nitrogen. The final residue was extracted first with 2 ml of water and then with 0.5 ml of water yielding 2.5 ml of a partially purified preparation of octanoyl-CoA.

To the solution of partially purified octanoyl-CoA was added 0.5 ml of 1 *M* diol buffer (pH 8) and 2 ml (40 μmoles) of DTNB in *n*-propanol. The reaction was carried out at 25°C for 20 min, after which time the reaction mixture was placed on a column (2.0 × 20 cm) of DEAE-cellulose. Prior to elution with HCl/LiCl, the column was washed with 400 ml of water; neither radioactive nor ultraviolet-absorbing materials were eluted. The fractions containing 1-^{14}C-octanoyl-CoA were pooled and concentrated to about 10 ml. LiCl was removed by passing the concentrated solution through a column (3 × 60 cm) of Bio-Gel P-4 using water as the eluting agent.[2] The 1-^{14}C-octanoyl-CoA was recovered by lyophilization. The yield was 25 μmoles (70% based on 1-^{14}C-octanoate) of 1-^{14}C-octanoyl-CoA having a specific radioactivity of 13.8 μc/μmole. Paper chromatography of the product (8) revealed but one spot at R_f 0.73 (the R_f of CoA was 0.40); this spot contained both ultraviolet-absorbing and radioactive material.

Experiments with ^{3}H-CoA. CoA was tritiated by the Wilzbach gas exposure technique (13) at the laboratories of New England Nuclear Corporation. Pure CoA was exposed to 3 curies of T_2 at a pressure of 250 mm at 0°C for 24 hr. Labile tritium was removed with acetic acid and the product was recovered after vacuum distillation of the solvent. As received in Madison the typical specific radioactivity was about 25 μc/μmole.

In a typical experiment 25 μmoles of ^{3}H-CoA (3.4 mc) was chromatographed as described above. The elution volume was 1200 ml of 0.2 m*M* HCl/0.5 m*M* DTT with a linear gradient (0–0.15 *M*) of LiCl. Both A_{260} and radioactivity were monitored in the eluate. ^{3}H-CoA appeared at 600

[2] These exclusion characteristics suggest some degree of micellar formation for octanoyl-CoA.

ml and was completely eluted at 960 ml. This ^{3}H-CoA peak contained 52% of the A_{260} and 70% of the ^{3}H. The fraction containing ^{3}H-CoA was lyophilized to a small volume, evaporated to dryness, and then dried exhaustively over P_2O_5. LiCl and DTT were removed by extracting the dried residue with methanol/acetone (10/1). The residue was dried and dissolved in water. The yield of ^{3}H-CoA was 50% based on radioactivity and 23% based on ultraviolet absorption. The product showed but one ultraviolet-absorbing spot after paper chromatography in two solvent systems; this spot coincided with the majority of the radioactivity. ^{3}H-CoA, prior to purification, showed multiple ultraviolet-absorbing spots and the radioactivity was distributed all along the paper.

The biological activity of the purified ^{3}H-CoA was tested in the acyl-CoA synthetase system using limiting amounts of CoA with octanoate as substrate; the biological activity (43% of the ^{3}H-CoA reacted under the assay conditions) of the ^{3}H-CoA was greater than either unlabeled CoA or ^{3}H-CoA before purification. The biological activity of the ^{3}H-CoA was also tested by Dr. W. W. Cleland (Department of Biochemistry, University of Wisconsin) in a citratase (ATP:citrate oxaloacetate-lyase (CoA-acetylating and ATP-dephosphorylating), EC 4.1.3.8) assay system; 48% of the ^{3}H-CoA reacted under the assay conditions.

The purified ^{3}H-CoA showed an A_{250}/A_{260} ratio of 0.82 and an A_{280}/A_{260} of 0.21; authentic CoA showed ratios of 0.84 and 0.22, respectively (14).

The product recovered had both high biological activity and sufficient ^{3}H to allow its use in experiments.

DISCUSSION

The method described above should find application in the enzymic preparation of acyl-CoA's of intermediate chain length which are neither easily synthesized and purified by direct chemical means nor amenable to acid precipitation techniques as is palmityl-CoA. While we have not yet systematically investigated the purification of acyl-CoA's other than octanoyl-CoA we have shown that acetyl-CoA can be purified by this method.

SUMMARY

1. A column chromatographic procedure for the purification of intermediate chain length acyl-CoA's is described. Chromatography is on DEAE-cellulose and is dependent on the conversion of free excess CoA to the mixed disulfide of CoA and thionitrobenzoate. The procedure is rapid and large amounts of acyl-CoA can be handled with good recovery.

2. ^{3}H-CoA has been prepared and purified by this method. The product showed high biological activity.

ACKNOWLEDGMENTS

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ANALYTICAL BIOCHEMISTRY 19, 300–314 (1967)

An Enzymic Method for the Measurement of Inorganic Phosphate[1]

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Inorganic phosphate is involved in many metabolic reactions. Not only is it the substrate or product of numerous enzymes, but it is also capable of modifying the activity of other enzymes such as hexokinase, phosphofructokinase, and glucose-6-phosphate dehydrogenase (1–3). Consequently many authors have suggested that inorganic phosphate may be a regulator of metabolic activities. Thus, a knowledge of the true level of inorganic phosphate in tissues has assumed considerable significance.

Although many excellent methods for measuring inorganic phosphate have been developed, each has its disadvantage. In the older molybdate methods, such as that of Fiske and SubbaRow (4), some labile phosphate esters are rapidly split, due to a combination of molybdate catalysis and strong acid pH. The pH and molybdate concentration can be modified so that labile compounds are hydrolyzed more slowly (5), the phosphomolybdate complex can be rapidly separated by extraction with an organic solvent (6), or, prior to exposure to molybdate and acid, phosphate can be separated from labile esters by precipitation with calcium salts from ethanol (7, 8) or magnesia mixture. Nevertheless, the separation methods are time consuming and lack sensitivity, and there is always a possibility in the case of the molybdate methods that an unknown phosphate ester is hydrolyzed by the molybdate or by the acid used in making the extract.

The proposed method is based on enzymic reactions at neutral pH. Although in ordinary circumstances the tissue is prepared for analysis by extracting with acid or by inactivating enzymes with alkali, the method can be applied directly to whole tissue with no preliminary harsh treatment. One of the major advantages of the method is the high sensitivity attainable. On a *concentration* basis, sensitivity is limited at present only by the ability to remove inorganic phosphate from the reagents. The

[1] Supported in part by grants from the American Cancer Society (P-78) and from the National Institutes of Health (NB-00434 and NB-01352).

sensitivity on an absolute basis is practically unlimited because the indicator enzyme reaction yields TPNH,[2] which can be measured by enzymic cycling (9). A method for inorganic phosphate based on the same principle has recently appeared (10), but it is believed that there are sufficient ramifications to explore, and precautions to recommend, to justify a second presentation.

METHOD

Principle

The enzymic sequence is:

$$\text{glycogen} + P_i \rightarrow \text{glucose-1-P} \quad (1)$$

$$\text{glucose-1-P} \rightarrow \text{glucose-6-P} \quad (2)$$

$$\text{glucose-6-P} + TPN^+ \rightarrow \text{6-P-gluconolactone} + TPNH \quad (3)$$

The three reactions are carried out in a single analytical step. In ordinary applications TPNH formed is measured directly by its fluorescence or by its absorption at 340 mμ. This readily permits accurate measurement of 10^{-9} mole of P_i. Amounts in the range of 0.02 to 0.5×10^{-9} mole can be measured in a two-step reaction. For amounts smaller than this, sensitivity can be increased as needed by enzymic cycling.

REAGENTS

If the analytical blank is to be kept low, great care must be taken to avoid P_i contamination of reagents. Components of the reagent that have given trouble on this score are discussed.

Muscle phosphorylase a. Commercial preparations may be contaminated with P_i and ATPase. Although these can be removed by recrystallization, this is unnecessary.[3] The reagent composition is such as to inhibit ATPase, and P_i can be removed by dialysis: 1 ml of a 2.5% suspension of enzyme

[2] Abbreviations used: TPNH, TPN^+ = reduced and oxidized forms of triphosphopyridine nucleotide; P_i = inorganic phosphate; P = phospho or phosphate; EDTA = ethylenediamine tetraacetate; Tris = trishydroxymethylaminomethane; ATP, ADP, and AMP = adenosine-5′ triphosphate, diphosphate and monophosphate (adenylic acid).

[3] If recrystallization is preferred, the following suggestions may be helpful: a 1% suspension of crystalline rabbit muscle phosphorylase is dissolved at 38° with the addition of 0.05 vol each of 100 m*M* dithiothreitol (or mercaptoethanol) and of 1 *M* imidazole-HCl buffer, pH 6.6. Any insoluble material is removed by centrifuging, and the enzyme is allowed to crystallize at 0°. The crystals are collected by centrifuging and redissolved at 38° in the original volume of 0.02 *M* imidazole, pH 6.6, containing 1.5 m*M* EDTA and 5 m*M* dithiothreitol or mercaptoethanol. Four recrystallizations should be sufficient. Crystallization will be slow and incomplete if the pH is more alkaline than 6.7.

(preparations of Sigma Chemical Co. and of Worthington have both been used satisfactorily) is dialyzed overnight at 4° against 1 liter of 0.02 *M* imidazole-HCl buffer, pH 7.0, containing 1 m*M* EDTA, 0.5 m*M* dithiothreitol, and 0.01 m*M* 5′-AMP.

Glucose-6-P dehydrogenase. The enzyme used has been a 0.5% suspension (of about 30% purity) in 3.3 *M* $(NH_4)_2SO_4$ (Boehringer). P_i which is usually present to a disturbing degree is removed by centrifuging, resuspending in 2 vol of 4 *M* $(NH_4)_2SO_4$, centrifuging again, and then repeating the washing cycle twice more (all at 0 to 4°). The enzyme is finally suspended in the original volume of 3.3 *M* $(NH_4)_2SO_4$.

Glycogen. Rabbit liver glycogen was prepared as an 8% solution and dialyzed overnight to remove P_i against 200 vol or more of 0.025 *M* acetate buffer, pH 4.6. Some lots of *TPN*$^+$ may contain measurable P_i, as purchased, but it has been possible to select lots sufficiently free of P_i for the analytical purpose.

Imidazole. The imidazole used for making the buffer was Sigma "Grade III-Low Fluorescence Blank." Other grades are unsatisfactory unless treated with charcoal to remove a high fluorescence blank. Various lots of P-glucomutase (Boehringer) have not been badly contaminated with P_i; however it is usually worth while to centrifuge the enzyme suspension and resuspend in fresh P_i-free $(NH_4)_2SO_4$ solution.

PROCEDURES

Specific directions will be given for three different analytical procedures, each of which may serve a different purpose.

I. *Direct fluorometry.* The procedure is described for the Farrand photofluorometer equipped with Corning filter No. 5840 as the primary and Corning filter Nos. 4303 and 3387 as the secondary combination. If the stability of reading is not satisfactory (±0.3%) the fluorometer can be modified as previously described (11).

The reagent consists of 0.05 *M* imidazole-HCl buffer, pH 7.0, containing 0.08% glycogen, 0.03 m*M* TPN$^+$, 0.01 m*M* 5′-AMP, 0.02% bovine plasma albumin, 1 m*M* EDTA, 0.5 m*M* magnesium acetate, 0.5 m*M* dithiothreitol, 2.5 μg/ml of yeast glucose-6-P dehydrogenase, and 3.0 μg/ml of muscle P-glucomutase. The reagent is ordinarily prepared within a few hours of use from stock solutions and kept at room temperature. However, the reagent has been found to be still satisfactory after a day or two at 4°. To keep the fluorescence blank as low as possible the imidazole buffer is prepared and stored as a 1 *M* stock solution, and the reagent is diluted to volume with distilled H_2O of low fluorescence.

Aliquots of reagent (1 ml) are transferred to 10 × 75 mm (3 ml) Pyrex test tubes that have been selected for uniformity (close fit to holder) and

freedom from scratches. The sample is added and a reading is made at suitable sensitivity. If there is any possibility that the sample may contain significant amounts of glucose-6-P or glucose-1-P, a few minutes is allowed for these to be converted to 6-P-gluconate before making this first reading. To the sample, 2 μl of 2.5% phosphorylase is now added.[4] A second reading is made after a predetermined interval sufficient to complete the reaction (usually 20 or 30 min). Suitable blanks and standards are analyzed simultaneously, and for each the same interval is maintained between phosphorylase addition and final reading. Each day the competence of the auxiliary enzymes is tested by measuring the rate at which a glucose-1-P standard (0.002 or 0.005 mM final concentration) is converted to 6-P-gluconate. With the recommended amounts of enzyme the reaction should be half over in 30 sec at 25°, but a rate one-half or one-third of this would not prolong the assay.

The P_i content of the reagents can be easily kept as low as 0.001 mM, and TPNH fluorescence is proportional with concentration up to about 0.01 mM. Consequently the most useful range is between these limits (1 to 10×10^{-9} mole). A single level of standard (0.002 mM for example) is ordinarily sufficient for samples in the 0.001 to 0.003 mM range since TPNH formation will be proportional to P_i concentration. With higher P_i concentrations, several standard levels are recommended unless the TPN^+ concentration is increased (see below).

IA. *Direct spectrophotometry.* For even higher concentrations of P_i, the spectrophotometer may be substituted for the fluorometer with readings made at 340 mμ. The reagent is the same as that described above except that the concentration of TPN^+ is increased to 1 mM, P-glucomutase is tripled, and phosphorylase is doubled. A satisfactory range of P_i concentration is 0.02 to 0.1 mM (0.02 to 0.1 μmole if the reagent volume is 1 ml). It is recommended that calculations be based on P_i standards rather than the TPNH extinction coefficient for reasons given below.

II. *Indirect analysis.* Because of the almost inevitable contamination of reagents and even fluorometer tubes with P_i, the full sensitivity of the fluorometer cannot be utilized in the direct procedure. Therefore, with P_i samples in the range of 0.02 to 0.5×10^{-9} mole there is advantage in reducing the volume for the enzyme reaction and measuring the TPNH in a second step. The following is an example of such a procedure:

The reagent is the same as that described above except that TPN^+ and glucose-6-P dehydrogenase are omitted. Just before use, 2 μl of 2.5%

[4] If the sample to be analyzed is negligibly fluorescent and contains no significant amounts of glucose-6-P or glucose-1-P there is some advantage in adding phosphorylase to the original reagent. This permits any P_i in the reagent to react ahead of time. After making an initial reading the reaction is started by addition of the sample.

phosphorylase is added. In test tubes 50 mm long, with an inner diameter of 3 mm, is placed 10 μl of this complete reagent. The rack containing the tubes is placed in an ice bath and P_i samples, standards, and blank solutions are added, preferably all in the same volume (1 or 2 μl). The standards range from 0.05 to 0.5 $\times 10^{-9}$ mole (e.g., 1 μl of 0.05 to 0.5 m*M* P_i). The rack is transferred to a 25° water bath for 45 min, then heated for 2 min in a boiling water bath. Meanwhile 3 ml fluorometer tubes are loaded with 1 ml of 0.02 *M* Tris-HCl buffer, pH 8.0, containing 0.03 m*M* TPN^+. To achieve the best sensitivity this reagent should be prepared so that the total fluorescence blank does not exceed the equivalent of 0.2 μM TPNH. As large an aliquot as possible is now transferred to a fluorometer tube and readings are made at an appropriate high sensitivity (full scale equivalent to not more than 1 μM TPNH). To each tube is then added 2 μl of 0.025% glucose-6-P dehydrogenase. (A 0.5% suspension of stock enzyme is diluted 20-fold with 0.05 *M* Tris-HCl buffer containing 0.05% bovine plasma albumin. The diluted dehydrogenase is sufficiently stable to be usable for a week if preserved at 4°.) The second reading is made when the reaction is complete (about 10 min).

III. *Analyses with enzymic cycling.* For samples much smaller than 5×10^{-11} mole it is desirable to increase the sensitivity by enzymic cycling (9). In this case the ultimate sensitivity is limited by the degree to which the volume of the original reaction can be reduced. An illustration is given in Table 2, in which the initial volume is 1 μl.

Comment on Procedures

The composition of the basic reagent was determined by the following considerations:

Phosphorylase a kinetics. Under the conditions of assay, with P_i levels below 0.1 m*M*, the apparent Michaelis constant for glycogen is about 0.004% (12) or 0.25 m*M* calculated as glucosyl units. The concentration recommended is 20 times this level and therefore not critical. Conversely, with 0.08% glycogen, the apparent Michaelis constant for P_i is about 1 m*M*, so that the analytical reaction is first order at all P_i levels likely to be encountered. *5′-AMP* is added because it lowers the Michaelis constant of muscle phosphorylase *a* 7-fold (12). However, the concentration of AMP is kept very low, since only a small concentration is required for maximal effect and a high level can cause a progressive increase in blank. This is the result of release of P_i by at least certain phosphorylase preparations, and is presumably attributable to a contaminating enzyme.

Dithiothreitol was added (at the suggestion of Dr. Philip Needleman) to prevent reoxidation of TPNH during the analysis of certain tissue extracts. A common contaminant of glucose-6-P dehydrogenase preparations

is glutathione reductase. Tissue glutathione is almost unavoidably oxidized to a certain extent in tissue extracts and can cause erroneous results. Dithiothreitol completely overcomes this problem by keeping glutathione in the reduced state (Fig. 1).

EDTA is added as an excess above the Mg^{2+} concentration in order to prevent ATPase action. Commercial twice-crystallized phosphorylase has been found to be seriously contaminated with ATPase. Fortunately the Mg^{2+} requirement of ATPase is greater than that of P-glucomutase. Consequently it is possible practically to eliminate ATPase activity and yet provide ample mutase activity. The mutase rate is decreased 80 to 90% under analytical conditions by the EDTA. Nevertheless, the activity is such that 3 to 10 μg/ml of enzyme is sufficient, depending (as indicated above) on the P_i level. The EDTA is so effective in inhibiting ATPase and phosphatases that it is possible to obtain valid analyses of P_i in whole tissues without removal of any of the enzymes (see below).

TPN$^+$ is added in fairly generous excess because of the fact that at pH 7.0 the equilibrium of the glucose-6-P dehydrogenase reaction is only slightly more favorable to oxidation of glucose-6-P than to reduction of 6-P-gluconolactone. Moreover, the half-time for hydrolysis of the lactone at pH 7.0 and 25° is approximately 30 min. Finally TPNH is an exceedingly potent inhibitor of glucose-6-P dehydrogenase, competitive with TPN^+ (13). For all these reasons the reaction will be incomplete, and will drag slowly in the last stages, unless a TPN^+ excess of at least 4- or 5-fold is provided.

Auxiliary enzymes. Under prescribed conditions the half-time for oxidation of a low level (0.002 m*M*) of glucose-6-P is less than 10 sec and for glucose-1-P is less than 30 sec. It should be pointed out, however, that both auxiliary enzymes are inhibited by sulfate. This is particularly true of the dehydrogenase, which is 50 and 80% inhibited by 5 and 16 m*M* sulfate, respectively. Therefore an increase in the amounts of auxiliary enzymes much beyond those recommended might actually decrease the rate of reaction if that enzyme is provided as a suspension in strong $(NH_4)_2SO_4$. P-glucomutase has a very low Michaelis constant for glucose-1-P, approximately 5 μM under assay conditions. Therefore a concentration that is adequate for the low levels in the fluorometric assay is insufficient for the higher levels in the spectrophotometric assay.

TPNH oxidation and destruction. It was pointed out above that oxidized glutathione in tissue extracts can result in TPNH oxidation in the probable event of contamination of glucose-6-P dehydrogenase by glutathione reductase. There are two other possibilities for loss of TPNH after it has been formed. First, there is likelihood of contamination of auxiliary enzymes with a TPNH dehydrogenase. Second, TPNH is not completely

stable at pH 7 in the imidazole buffer. Neither presents a very serious problem. The first is minimized by using no more of the auxiliary enzymes than needed, the second has not been responsible for the loss of more than 5% of added TPNH in 30 min. Unlike the glutathione reductase problem, neither of these two potential sources of trouble is diminished by dithiothreitol. Although this is a minor matter, it is stressed since as a consequence it is desirable to base analyses carried out spectrophotometrically on carefully prepared P_i standard solutions. Otherwise a systematic error of a few per cent might result.

P_i contamination. If the method is to be used at full sensitivity all traces of P_i must be removed from glassware. The fluorometer tubes are cleaned by rinsing, boiling in 50% HNO_3 for a few minutes, and rinsing in three changes of distilled and two changes of redistilled water. Even with this treatment, if tubes have not been recently cleaned it is wise to rinse them several times in redistilled water just before use. Other glassware, especially if washed with detergent, is repeatedly rinsed before use.

Preparation of tissue samples for analysis. $HClO_4$ extracts suitably neutralized with KOH or $KHCO_3$ are readily analyzed. A satisfactory protocol is given in notes for Table 3. Trichloroacetic acid extracts are less satisfactory because salts of this acid are exceedingly inhibitory to glucose-6-P dehydrogenase (90% inhibition by a 5 m*M* concentration). Nevertheless such extracts could be used if the trichloroacetate concentration is kept as low as 2 or 3 m*M* during the assay. This is practicable with the direct fluorometric procedure. Methanolic extracts are analyzed without difficulty with most tissues, although, as will be illustrated below, neutral methanol does not extract all the P_i. Since the problem is often to prepare extracts from frozen tissues without danger of thawing, a very practical procedure is to homogenize the tissue at −20° with methanol[5] to which HCl is added at a concentration of 0.1 *N*. The HCl may be added either before homogenizing or just before centrifuging. Probably a concentration of 0.02 *N* HCl in the methanol would suffice to extract the P_i.

Methanolic extracts or suspensions of liver present a problem, since the vitamin A present is not only fluorescent but light sensitive. Consequently there is a large unstable fluorescence blank. It has been found practicable to destroy this blank in the extract or suspension by irradiation with a mercury arc (e.g., 10 min exposure at a distance of 6 cm from the arc of a General Electric Co. lamp No. AH5 is satisfactory for this purpose).

As mentioned above, since EDTA in the reagent blocks ATPase action, whole tissue dispersions can be analyzed by the direct fluorometric procedure. In this case, the problem is to get the tissue from the living state

[5] Dr. Stanley R. Nelson studied the ease of extraction of water from frozen tissues by a variety of solvents and found methanol the most satisfactory.

into the fluorometer tube without enzymic release of P_i. This can be accomplished by homogenizing in neutral methanol at —20° with a fairly tight fitting homogenizer to render tissue particles fine enough to transfer into the reagent with a constriction pipette (Table 3). At the dilution permissible, the turbidity of such a dispersion does not disturb the fluorometric analysis. No attempt has been made to carry out analyses of whole tissues spectrophotometrically, but it seems certain that the turbidity would interfere. One might, however, circumvent this problem by centrifuging before making the final reading. Major P_i tissue components appear to be quite stable in neutral methanol suspensions. Thus, no increase in P_i was observed when methanol homogenates of muscle and brain were incubated for 75 min at either 28° or 38°.

Yet another method of preparing tissues for analysis may be mentioned. This is to inactivate enzymes by heating in alkali. For example, in applying the proposed P_i method to histochemical problems (15) the frozen-dried tissue sample is pretreated by heating for 10 min at 60° in 0.02 *N* NaOH. This does not cause breakdown of P-creatine or ATP, although triose phosphates would be completely hydrolyzed.

Stability of organic phosphates during assay. Under assay conditions the following compounds were hydrolyzed at rates of not more than 1% per hour: P-creatine, ATP, ADP, AMP, dihydroxyacetone-P, 3-P-glycerate, 2-P-glycerate, P-pyruvate, and fructose diphosphate. Glyceraldehyde-P was split at a rate of 7% per hour.

ILLUSTRATIVE DATA

Reproducibility, Proportionately, Sensitivity

The fluorometric procedures permit satisfactory analyses over a wide range of concentration (Table 1) with standard deviations of less than 3%, except at the very lowest levels. It is seen that the direct procedure can be used down to levels below 10^{-10} mole but better reproducibility is attainable with less than 5×10^{-10} mole by the indirect procedure. The lowest level in Table 1 is equivalent to the P_i present in 10 μg of average mammalian tissue.

No difficulty has been found in achieving the same precision in the spectrophotometer as in the fluorometer, but much higher concentrations of P_i are of course required. The difference in sensitivity is illustrated by the fact that the optical density change at 340 mμ which corresponds to the lowest P_i concentration of Table 1 is 0.0002; this is equivalent to the absorption of only 0.05% of the light in passing through a 1 cm cell.

An advantage of the enzymic method for P_i is the fact that the enzyme sequence leads to a pyridine nucleotide step. This means that if necessary

TABLE 1
Proportionality and Reproducibility of Fluorometric Procedures for P_i

The average fluorescence reading of the first P_i level in each column was used as a basis for the "observed" entries in the rest of that column. The final volume in each case was 1 ml; therefore concentrations ranged from 4.3×10^{-6} *M* down to 0.03×10^{-6} *M*.

Direct procedure		Direct procedure		Indirect procedure	
Calc.	Obs.	Calc.	Obs.	Calc.	Obs.
10^{-11} mole		10^{-11} mole		10^{-11} mole	
435	433	54.4	53.2	79.5	79.6
	433		56.0		80.3
	437		54.2		78.3
	437		54.2		79.3
226	225	28.1	28.2	26.6	25.1
	220		28.3		25.5
	233		27.5		25.6
	233		30.0		25.9
119	121	11.6	10.7	8.8	8.7
	120		11.8		8.6
	119		12.4		8.4
	117		12.3		8.9
		4.9	5.2	2.9	3.2
			5.4		3.1
			4.9		2.4
			4.9		2.7

the sensitivity can be increased almost without limit by "enzymic cycling," i.e., by using the final pyridine nucleotide product as a catalyst in a two-enzyme system (9). The examples shown (Table 2) demonstrate that satisfactory precision can be obtained with amounts of P_i equal to that contained in 1 μg or less of average tissue. This by no means represents a limit of sensitivity. However, for satisfactory precision it is necessary to keep the volume of the original reaction small enough to maintain P_i concentrations of 10^{-6} *M* or greater. Thus, to measure P_i in the 10^{-14} mole range an initial volume of 0.01 μl would be reasonable. Analytical sequences at volumes of this magnitude can be carried out with "oil well" techniques (16).

Comparison between Enzymic and Colorimetric Methods

Mice were heavily anesthetized with phenobarbital to reduce P_i levels to a minimum, and then frozen whole in liquid N_2. The frozen tissues were powdered and extracted with $HClO_4$ by a technique which avoids thawing the tissue (11). When these extracts were analyzed both by a colorimetric

TABLE 2

Proportionately and Reproducibility of Enzymic Cycling Procedure

In procedure A, P_i was added to 5 μl of reagent in tubes of 2.5 mm bore. The reagent was similar to that described in the text for the direct fluorometric procedure except that the phosphorylase had been added to make the reagent complete. After 25 min incubation, 5 μl of 0.2 N NaOH was added to each sample and the samples were heated 10 min at 60°. Aliquots of 1 μl were added to 200 μl of TPN cycling reagent (9) in which the glucose-6-P dehydrogenase and glutamic dehydrogenase levels were reduced to 2.5 and 10 μg/ml, respectively. This was cycled for 1 hr at 38° (about 2000 cycles) and the analysis was completed as originally described (9). Procedure B was the same as A except that the first step was conducted in tubes of 2.0 mm bore with volumes of reagent and alkali reduced to 1 μl each. In addition the entire sample (2 μl) was added to the cycling reagent.

Procedure A		Procedure B	
Calc.	Obs.	Calc.	Obs.
10^{-12} mole		10^{-12} mole	
37.8	37.2	5.5	5.4
	37.3		5.5
	38.8		
26.1	27.1	1.07	1.09
	26.3		1.20
	26.7		1.04
10.5	11.3		
	10.3		
	10.9		

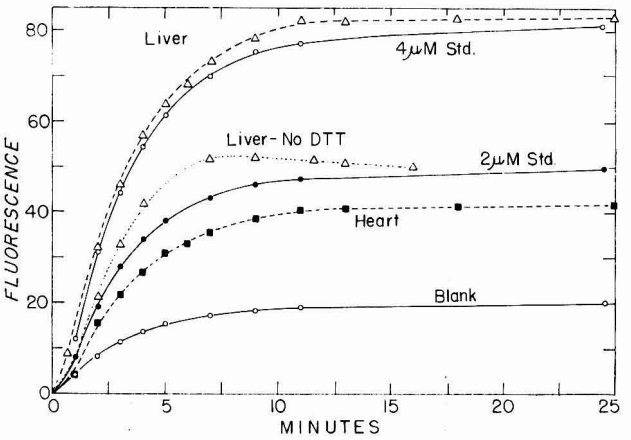

FIG. 1. Rate of fluorescence development with tissue extracts and P_i standards. The ordinate represents galvanometer readings corrected for the reading prior to addition of phosphorylase at $t = 0$. Readings are not corrected for the increase in fluorescence of the blank. Dithiothreitol was omitted from one liver sample ("no DTT"). The extracts were prepared from mouse tissues with $HClO_4$ as described in Table 3. Each sample represented approximately 1.5 mg of tissue. A volume of simulated extract equal to that of the sample (20 μl) was added to standard and blank tubes. Reagent volume in all cases was 1 ml.

TABLE 3

P_i Analyses of Four Tissues by Different Methods

Four mice (A–D) were deeply anesthetized with phenobarbital (280 mg/kg). The liver and heart were exposed and within 4 sec from the time the thoracic cavity was opened the mouse was plunged whole into liquid N_2 that had been brought to its freezing point by pumping off a portion with a vacuum pump. The tissues were dissected at −15° and powdered in a mortar chilled to liquid N_2 temperature. Samples (50 or 100 mg) were placed on top of 150 or 300 μl of 3.3 *M* $HClO_4$ that had been previously frozen in an 8 ml test tube on solid CO_2. The tubes were transferred to an alcohol bath at −10°, agitated for several minutes to extract the H_2O, then diluted and well mixed at 4° with 0.5 or 1 ml of 1 m*M* EDTA. The supernatant fluid, obtained by centrifuging, was neutralized by adding, for each milliliter, 0.32 ml of 2 *N* KOH containing 0.4 *M* imidazole base and 0.4 *M* KCl. After centrifuging off the $KClO_4$ precipitate, the supernatant extract (pH 7.0) was stored at −60° until analyzed. The methanol (MeOH) homogenates were prepared from the frozen powder in glass homogenizers at −20° (alcohol bath) with 50 vol of methanol. For the molybdate analyses, 50 μl samples of $HClO_4$ extract were added to 400 μl of 0.125 *M* acetate buffer, pH 4.3, containing 0.2 m*M* zinc acetate, and 0.1% ascorbic acid. After taking a reading at 700 mμ, 20 μl was added of a 2.5% ammonium molybdate solution containing 0.02% $CuSO_4 \cdot 5H_2O$. This is a minor modification of a published method (5). The zinc is added to compensate for EDTA in the extract (14). Readings were made at 2 and 4 min and extrapolated to zero time (see Fig. 2). For the enzymic assays in the fluorometer, 200 μl of $HClO_4$ extract or 36 μl of whole methanol homogenate was added to 1 ml of reagent. For the spectrophotometric assay 50 μl of extract was added to 400 μl of reagent.

Tissue	Molybdate assay ($HClO_4$ extr.)	Enzymic assay		
		Fluor. ($HClO_4$ extr.)	Spectr. ($HClO_4$ extr.)	Fluor. (MeOH homog.)
	mmole/kg wet weight			
Brain				
A	1.59	1.57	1.42	
B	1.98	2.04	1.94	2.03[b]
C	1.60	1.52	1.62	
D[a]	5.99	6.93	7.07	
Muscle				
A	3.53	3.78	4.05	
B	4.60	4.20	4.70	
C	3.74	3.96	3.93	3.65[b]
D[a]	8.03	7.60	8.04	
Liver				
A	3.33	3.62	3.57	
B	2.25	2.40	2.32	
C	2.99	2.76	2.87	
D	2.84	2.94	3.21	2.51
Heart				
C	3.06	3.12	3.36	4.04
D	2.67	2.81	3.22	

[a] These samples are believed to have been contaminated by a minute bone fragment.

[b] These were also analyzed after incubating the original methanol homogenates for 75 min at 28° and at 38°. No increases were observed in P_i concentration.

molybdate procedure and by the proposed enzymic procedure, agreement was satisfactory (Table 3). This is gratifying in view of the fact that in some cases the colorimetric results had to be corrected for an influence of the tissue extract on the extinction coefficient. Under the conditions of the

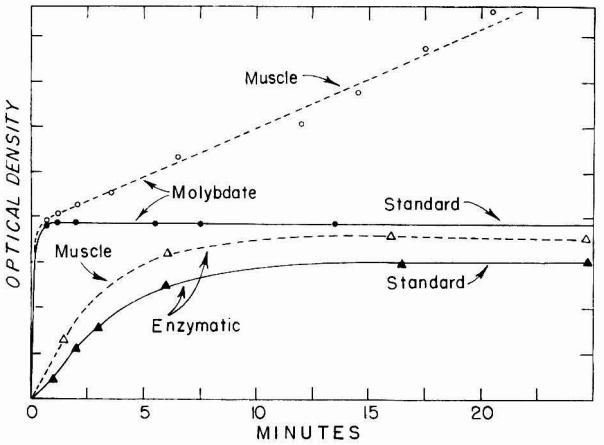

FIG. 2. Rate of increase in optical density of muscle samples and P_i standards in the molybdate and enzymic (spectrophotometric) procedures. The optical densities were measured at 700 and 340 mμ, respectively. Each curve is plotted on a somewhat different optical density scale to facilitate visualization of the rates of change.

enzymic assay there is very little hydrolysis of labile organic phosphates present in the tissue extracts examined (Fig. 1). In contrast with the molybdate method, even though the acidity is as low as possible (pH 4), there is progressive increase of color with time, particularly in the case of muscle, which is exceptionally rich in P-creatine (Fig. 2).

Measurement of P_i in Tissues without Acidification

Since the enzymic reactions are carried out at neutrality, it should be possible to measure P_i in tissues without any exposure to harsh treatment which might conceivably degrade a very unstable organic phosphate. Table 3 includes analyses of whole tissue homogenates prepared in neutral methanol. The results do not differ significantly from those obtained with $HClO_4$ extracts. If there is in fact an exceedingly acid-labile organic phosphate compound present, its concentration must be low compared to that of P_i, or it is rapidly broken down during the assay. If there is a lag in TPNH formation in whole tissue dispersions it is only a few seconds (Fig. 3).

Seraydarian *et al.* (7) reported that extracts of frog skeletal muscle made with 60 to 75% ethanol at −40° contained less P_i than $HClO_4$ extracts. We have been unable to extract all of the apparent P_i from

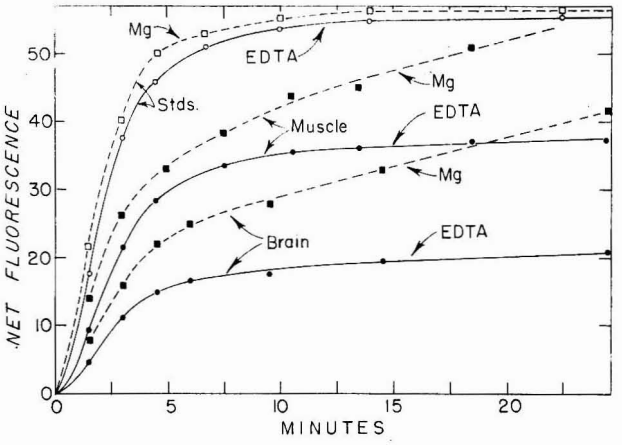

FIG. 3. Analysis of whole methanol homogenates of muscle and brain for P_i. Frozen powders of the tissue were homogenized in methanol at −20° and aliquots were added directly to enzymic assay reagent (see Table 3). "Mg" indicates Mg excess (1.5 m*M* Mg, and 1.0 m*M* EDTA). "EDTA" indicates EDTA excess (0.5 m*M* Mg, and 1.0 m*M* EDTA, or, in the case of brain, 3.0 m*M* EDTA). The total tissue dilution was about 1:1500. The fluorometric readings have been corrected for the reagent blank. Standards and blanks contained the same amount of methanol during the reaction as the tissue samples (3.5%).

tissues with neutral ethanol or methanol, although we have not duplicated the exact conditions of Seraydarian *et al.* In one experiment to test this point ethanolic extracts were made from brains either frozen quickly or

TABLE 4

Extraction of P_i from Brain with Ethanol

Mice were decapitated and the heads frozen by vigorous agitation in Freon-12 at −150°. The heads were either frozen immediately or after delays of 0.5 or 10 min. The brains were removed at −15°. Samples weighing about 75 mg were homogenized at −25° in 1 ml of 90% ethanol containing 0.025 *M* Tris base and 0.025 *M* Tris-HCl. Extract 1 is the supernatant fluid obtained by centrifuging 35,000*g* for 10 min. The precipitates were re-extracted at −25° with 1 ml of the same Tris-ethanol containing EDTA at 1 m*M* concentration (extract 2). The precipitates were finally re-extracted at −25° with 1 ml of 90% ethanol containing 0.05 *N* HCl (extract 3).

Delay in freezing, min	Extract 1, neutral EtOH	Extract 2, neutral EtOH	Extract 3, acid EtOH	Sum
		mmoles/kg		
0	0.74	0.35	1.08	2.2
	0.61	0.25	1.37	2.3
0.5	1.61	0.74	3.18	5.5
	1.34	0.71	2.88	4.9
10	3.0	0.86	9.1	13.0
	3.3	1.29	8.7	13.3

frozen after periods of ischemia to increase the total P_i (Table 4). In all cases the neutral ethanol extracted less than half the total apparent P_i. With the rapidly frozen brains this might be attributable to some very acid-labile organic phosphate, but in the case of the brains frozen after 10 min of complete ischemia the discrepancies are too great for this explanation. Other attempts have been made to solubilize, with neutral methanol or ethanol, all of the P_i that appears to be present in whole tissue dispersions. Although no way was discovered to accomplish this completely, the experiments so far suggest that this is due to a failure to extract preformed P_i rather than due to the presence of an unstable, organic phosphate insoluble in the solvents (Table 5).

TABLE 5

Inorganic Phosphate in Methanol Extracts Prepared in Various Ways

Methanol homogenates of mouse brain and muscle were prepared as described for Table 3. These were centrifuged after various additions, which were made at 0° unless noted. The imidazole buffers (imid) were all pH 7.0. In a few instances EDTA (0.2 to 1 m*M*) was also added without significant effect. The H_2O concentration at the time of centrifuging is shown.

Addition	H_2O, %	Muscle		Brain	
		SN[a]	Ppt.	SN	Ppt.
		mmole/kg			
None	2	2.82	0.51	1.40	0.61
None[b]	2			1.52	
Imid					
acetate, 0.05 *M*	7	2.94	0.61	1.43	0.61
sulfate, 0.05 *M*	7	3.05	0.45	1.48	0.51
sulfate,[c] 0.05 *M*	7			1.57	
acetate, 0.01 *M*	20			1.35	
acetate, 0.02 *M*	36			2.10	
chloride, 0.025 *M*	50	3.68	0.17	1.91	0.00
HCl, 0.025 *N*	50	4.22		2.00	
None (not centrifuged)	2	3.68		2.04	

[a] SN =

[b] Incubated 60 min at 38°.

[c] Incubated 5 min at 38°.

Seraydarian reported an increase in P_i (precipitable by $Ca(OH)_2$) in alcoholic muscle extracts after these were exposed briefly to acid treatment mild enough not to split P-creatine. However, the results given here with the enzymic assay offer little evidence for the presence of substantial amounts of organic phosphate less acid-stable than P-creatine in muscle, heart, brain or liver. This should not be taken to rule out the possible presence of an amount of very acid-labile phosphate that would be small in comparison to the P_i level.

SUMMARY

An enzymic method is described for measuring inorganic phosphate. The successive enzyme steps are those catalyzed by glycogen phosphorylase, phosphoglucomutase, and glucose-6-phosphate dehydrogenase. TPNH is finally produced from TPN^+ in proportion to the inorganic phosphate present, and is measured by its fluorescence or light absorption. The method has two advantages: (*1*) The analytical reaction takes place at neutrality thereby permitting measurement of inorganic phosphate in the presence of very unstable organic phosphates. (*2*) The method is very sensitive. In terms of concentration the sensitivity is limited mainly by the degree to which phosphate can be removed from the reagents. In terms of amount, there is no real sensitivity limit since the TPNH formed can be measured by enzymic cycling.

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An Enzymic Method for Measurement of Glycogen[1]

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Most of the current methods for determination of glycogen are based on a modification of the Pflüger method (1, 2). The glycogen is isolated by precipitating with alcohol after heating the tissue in strong KOH. After isolation the glycogen has been either measured directly with anthrone (3, 4) or indirectly by hydrolysis followed by glucose determination. Such methods have neither the sensitivity nor the specificity to be entirely adequate for analyzing tissues with relatively low glycogen content.

Predictably a method based on reactivity with glycogen phosphorylase could provide both greater specificity and sensitivity, and this has proved to be the case. Bueding and Hawkins (5) developed a method for glycogen using phosphorylase, and a similar method has been in use for several years in this laboratory (6). This report presents detailed procedures that have evolved from considerable experience with the method and from studies of possible sources of difficulty.

With the aid of appropriate, commercially available, enzymes glycogen plus P_i and TPN^+ are converted in one analytical step to 6-P-gluconolactone + TPNH (plus a small amount of glucose).[2] The TPNH is measured by its fluorescence. This provides sufficient sensitivity to measure directly the amount of glycogen present in 30 μg of brain, or 0.3 μg of liver. If less sensitivity is needed the TPNH can be measured spectrophotometrically. The specificity is such that glycogen can be determined without removal of any of the tissue constituents.

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[2] Abbreviations used: P_i = inorganic phosphate; P = phospho or phosphate; TPN^+, TPNH = oxidized and reduced forms of triphosphopyridine nucleotide; EDTA = ethylenediamine tetraacetate.

METHOD

Principle

The analytical reactions are:

$$\text{glucogen} + P_i \rightarrow \text{glucose-1-P (92\%)} + \text{glucose (8\%)} \quad (1)$$

$$\text{glucose-1-P} \rightarrow \text{glucose-6-P} \quad (2)$$

$$\text{glucose-6-P} + TPN^+ \rightarrow \text{6-P-gluconolactone} + TPNH \quad (3)$$

These reactions are carried out in a single analytical step with the aid of muscle phosphorylase *a*, amylo-1,6-glucosidase, oligo-1,4 → 1,4-glucantransferase (hereafter called transglucosylase), P-glucomutase, and glucose-6-P dehydrogenase. Commercial preparations of glycogen phosphorylase are contaminated with sufficient glucosidase and transglucosylase to degrade glycogen completely. Unfortunately commercial preparations of phosphorylase are also contaminated with amylase, which can reduce the yield of glucose-1-P. However, as Dr. Ernest Bueding kindly informed us, amylase can be inhibited with EDTA since it requires Ca^{2+} for activity (7). With this chelating agent it has proved possible to inhibit amylase effectively and yet permit P-glucomutase to function even though it requires Mg^{2+} for activity. This is made possible by the fact that the Mg^{2+} requirement of mutase is very small and can be satisfied when EDTA is in excess.

Materials

Phosphorylase a. Twice-crystallized rabbit muscle phosphorylase *a* from either Sigma Chemical Co. or Worthington has proved satisfactory in most cases. Some lots, however, may be low in glucosidase (or transglucosylase?) and the test given below is recommended. The enzymes are activated by diluting the original suspension (10 mg/ml) at least 5-fold in 0.02 *M* imidazole-HCl, pH 7.0, containing 0.02% bovine plasma albumin, 1 m*M* dithiothreitol, and 0.2 m*M* 5′-AMP and heating 60 min at 38°. The heating is required for full activation of glucosidase. Phosphorylase itself is activated very rapidly (see below). A sufficiently accurate assay of glucosidase-transglucosylase ("debrancher complex") activity is as follows: Reagent is prepared of the composition given under "Procedure" except that EDTA is omitted and TPN^+ concentration is increased to 1 m*M*. A suitable volume is placed in a spectrophotometer cell with wavelength set at 340 mμ. The phosphorylase to be tested is added to give a concentration of 10 μg/ml. After taking a reading, glycogen is added to give a concentration of 0.25 m*M* (as glucosyl units). The reaction is plotted for 15 or 20 min. The rate is rapid until the outer tiers are

gone (OD increase of about 0.6), after which the rate is slow and limited by glucosidase or transglucosylase (Fig. 1). Enzyme preparations giving rates of 0.006 optical density change per minute or more at 25° during the second phase are satisfactory. This is equivalent to about 0.1 μmole of glucose-1-P formed per milligram phosphorylase per minute.

Unfortunately the glucosidase-transglucosylase is not stable indefinitely in phosphorylase preparations. After storage for a month or longer it may be necessary to repeat the assay.

A similar test can be made in the fluorometer but it is not quite as satisfactory. With the necessarily much lower level of glycogen, the reaction kinetics for both phosphorylase and debrancher complex will

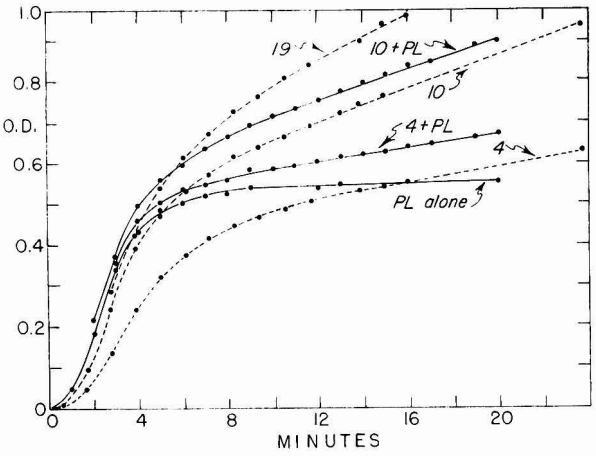

FIG. 1. Glycogen degradation by crude phosphorylase with and without added purified phosphorylase. Optical density at 340 mμ is given on the ordinate. The numerals indicate μg/ml of crude phosphorylase. "PL" indicates addition of 14 μg/ml 6-times recrystallized phosphorylase. The regular assay reagent was used with TPN^+ increased to 1 m*M* and EDTA omitted (to permit greater P-glucomutase activity). The glycogen concentration was 0.228 m*M*. The increase in optical density with purified phosphorylase alone is equivalent to 39% degradation.

approximate first order; hence the distinction between the two activities is less sharp. Furthermore, the apparent Michaelis constant for the debrancher complex is smaller than for phosphorylase; consequently there is greater overlap in activities at low substrate concentration (see below).

Crystalline rabbit muscle *P-glucomutase* and yeast *glucose-6-P dehydrogenase* of about 30% purity were purchased from Boehringer & Sons. The mutase appeared to be in the phosphorylated form so that at the recommended enzyme level it was unnecessary to add the coenzyme glucose-1,6-diphosphate. If, however, the method is to be modified for the

spectrophotometer, with higher concentrations of glycogen, it might be desirable to add coenzyme to a concentration of about 10^{-7} *M*, since the requirement for coenzyme increases at higher glucose-1-P levels (8).

Glycogen standards. These were prepared from rabbit liver glycogen (Mann Chemical Co.). As a precaution stock solutions were analyzed for glucose, although this may not be absolutely necessary. The stock solution was diluted to about 2 m*M* with 1 *N* HCl and heated 2 hr at 100° in a sealed tube. This was now assayed for glucose in a spectrophotometer by measuring the increase in absorption at 340 mμ when 5 μl was added to 500 μl of 0.05 *M* Tris-HCl, pH 8.0, containing 0.5 m*M* TPN^+, 1 m*M* ATP, 2 m*M* $MgCl_2$, 0.5 μg/ml yeast hexokinase, and 2.0 μg/ml glucose-6-P dehydrogenase. The reaction may take about 10 min for completion. (Throughout glycogen is calculated as glucosyl units, i.e., 1 mmole is equal to 162 mg.)

On long storage, particularly with repeated freezing and thawing, glycogen solutions tend to aggregate as evidenced by increasing opalescence. Such solutions assayed with phosphorylase react slowly and incompletely. If aggregated solutions are heated for 10 min at 60° in H_2O or 0.02 *N* alkali the reactivity is restored. Even diluting in 0.02 or 0.1 *N* alkali at 0° will accomplish this purpose. Heating in acid (0.03 *N* HCl) is not as satisfactory. If this precaution is not observed the use of an old glycogen standard may lead to erroneous results even though it may have been standardized by its glucose content.

Glucose-6-P. This is used as a secondary standard. Stock solutions may be standardized spectrophotometrically in the regular glycogen assay reagent provided the TPN^+ concentration is increased to 1 m*M*.

Imidazole. The imidazole used for making the buffer was Sigma "Grade III-Low Fluorescence Blank." Other grades are unsatisfactory when the highest sensitivity is required, unless treated with charcoal to remove a high fluorescence blank.

Fluorometry. The Farrand filter fluorometer model A has been used for all fluorometric determinations with Corning filter No. 5840 as the primary and Corning filter Nos. 4303 and 3387 as the secondary combination. If the stability of reading is not satisfactory (±0.3%) the fluorometer can be modified accordingly (9). Measurements are made in 3 ml (10 × 75 mm) Pyrex test tubes that have been selected for uniformity (minimal clearance in the holder) and for freedom from scratches.

Standard Fluorometric Procedure

The reagent consists of 0.05 *M* imidazole-HCl buffer, pH 7.0, containing 0.5 m*M* magnesium acetate, 1.0 m*M* EDTA, 0.04 m*M* TPN^+, 0.1 m*M* 5′-AMP, 5 m*M* K_2HPO_4, 0.02% bovine plasma albumin, 3 μg/ml P-gluco-

mutase, and 0.5 μg/ml glucose-6-P dehydrogenase. Experience has shown it is easier to keep the fluorescence blank low if the reagent is made as needed from stock solutions. For example, the imidazole-HCl buffer is kept (frozen) as a 1 *M* stock. Once prepared the complete reagent can be used for a day or two if kept at 4°.

The sample (10^{-10} to 10^{-8} mole, as glucosyl units, i.e., 0.015 to 1.5 μg) is added to 1 ml of complete reagent in a fluorometer tube. A reading is made at an instrumental setting such that the expected final readings will be nearly full scale. The first reading is made after sufficient time (2 to 5 min) for any glucose-1-P or glucose-6-P that may be present to react. (If a measure of glucose-6-P is desired, the mutase and dehydrogenase can be added after the sample rather than be incorporated in the reagent.) Phosphorylase is now added to produce a concentration of 10 to 50 μg/ml depending on the glucosidase activity of the preparation. (The higher figure is suitable for a preparation with an activity of 0.1 μmole/mg phosphorylase in the assay given above. With more active preparations the amount of enzyme is reduced in proportion, since the phosphorylase itself is not limiting.) The final reading is taken after an interval selected on the basis of the time course for a standard sample, usually 20 to 40 min. It is standard practice to measure the rate of reaction for one sample each of glucose-6-P, glucose-1-P, and glycogen, all at concentrations in the range of the samples to be analyzed. The expected half-times for the two phosphates are less than 20 sec and less than 40 sec, respectively. Having thus validated the reagent, a large number of samples (50 or more) can be carried through the analysis in a group. The results may be accurately calculated from glycogen standards, provided the glycogen stock has not been allowed to aggregate (see above). The expected yield of glucose-1-P is close to the theoretical 92%. A good check is to use standardized glucose-6-P as a secondary standard, and in the absence of a reliable glycogen standard this may be satisfactory in itself.

COMMENT ON PROCEDURES

The basic reagent is almost identical to that used for enzymic P_i determination (10) except for the substitution of P_i for glycogen. The reason for the inclusion of an excess of EDTA has already been given. The reason for including 5′-AMP is given below ("Phosphorylase Kinetics"). The necessity for a generous excess of TPN^+ and the danger of reoxidation of TPNH were discussed in the P_i paper (10). In the reagent recommended for P_i, dithiothreitol is included since there is danger that tissue extracts may contain oxidized glutathione, which in conjunction with traces of glutathione reductase in auxiliary enzymes can result in reoxidation of some of the TPNH formed. This problem has not been

encountered with tissue prepared for analysis as described below. If the problem should arise it can be solved by incorporating 0.5 m*M* dithiothreitol in the reagent.

The selection of buffer and pH is a compromise. With 0.05 *M* buffer at pH 7.0, under analytical conditions, both phosphorylase and debrancher complex were found to be more active (25 and 50%) in glycylglycine than in imidazole. On the other hand, as Najjar has shown (8), imidazole favors P-glucomutase activity (a factor of 2 or 3 compared to glycylglycine under present glycogen analytical conditions). Compared to the rate at pH 7.0, the rates at 6.6 and 7.4 were found to be 105 and 95%, respectively, for phosphorylase and 97 and 68% for debrancher complex.

The P_i concentration is not optimal for phosphorylase activity at the very low glycogen levels (11), but higher levels inhibit both P-glucomutase and glucose-6-P dehydrogenase (65% inhibition at 10 m*M*, 80% at 20 m*M* concentration).

Activation of Phosphorylase and Debrancher Complex

In the enzyme preparation, as ordinarily supplied commercially, phosphorylase is nearly inactive and debrancher activity is almost undetectable. Both activities can be restored with sulfhydril compounds. β-Mercaptoethanol and dithiothreitol (Cleland's reagent (12)) were tested for this purpose. Crude phosphorylase was diluted to a concentration of 1 mg/ml with 0.05 *M* sulfhydril compound in imidazole-HCl buffer containing 0.4 m*M* 5′-AMP. At 27°, phosphorylase was 50% activated in about 6 min with 5 m*M* mercaptoethanol and in 2 min with 10 m*M* dithiothreitol. The activation rates for debrancher complex are much slower. At 38°, activation by dithiothreitol occurred with a half-time of 9 min. With mercaptoethanol, also at 38°, debrancher activity reached 50% in 35 min, but was only 62% after 80 min. Thus dithiothreitol appears to be the preferred activating agent.

Phosphorylase Kinetics

For practical analytical purposes, the most important point about phosphorylase *a* kinetics is that the Michaelis constant for glycogen is very unfavorably affected by increase in temperature and very favorably affected by a small amount of 5′-AMP (half-maximal effect at 2×10^{-7} *M*). At 38° in the complete absence of AMP the rate with low glycogen levels is only 2 or 3% of that at 25° with AMP present (11).

At 25°, with 5 m*M* P_i and AMP present, the apparent Michaelis constant for glycogen is about 6×10^{-5} *M* in terms of glucosyl units (11). With glycogen of molecular weight 3,000,000 (for example) this would mean a Michaelis constant of 3×10^{-9} *M* in terms of actual glycogen

molecules. This not only means that low molar levels of glycogen saturate phosphorylase but that low levels of phosphorylase would "saturate" glycogen. Therefore it is to be expected that with the glycogen levels encountered in the fluorometric assay system the rate will reach a maximum with modest levels of phosphorylase. Although this has not been studied in detail it was in fact found that half-maximal activity with analytical levels of glycogen was obtained with 1.5 to 2 μg of phosphorylase per milliliter. This suggests a K_m of 6 to 8 $\times$ 10^{-9} M for the phosphorylase dimer of 250,000 molecular weight (the active form according to Wang and Graves (13)). (One might expect the molar concentration of phosphorylase required to half-saturate glycogen would be equal to the K_m for glycogen multiplied by the ratio between the glycogen and phosphorylase molecular surface areas.) From the limited studies it is calculated that under assay conditions the half-time for degradation of the outer tiers could not be reduced below about 30 sec no matter how much phosphorylase is present. The practical consequence is that there is no advantage in using much more than about 10 μg/ml phosphorylase. In

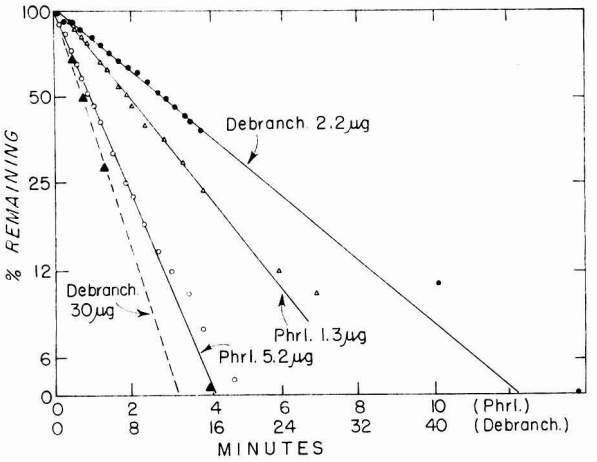

FIG. 2. Logarithmic plot of rates of degradation of limit dextrin "Debranch" and of the outer tiers of glycogen "Phrl." Measurements were made in 1 ml of regular assay medium with the amounts of enzyme shown. In the case of the "Phrl" this was 6-times crystallized muscle phosphorylase *a* with very low activity toward limit dextrin. In the case of "Debranch" this was commercial (twice-crystallized) phosphorylase containing debrancher complex activity. The amount of enzyme shown in this case refers to total protein of the preparation. For the 2.2 μg level of debrancher the reagent was supplemented with 7 μg/ml 6-times crystallized phosphorylase. The rate curves have been adjusted for lag attributable to the auxiliary enzymes. Initial glycogen concentration was 4.6 μM. Initial limit dextrin concentration was 2.5 μM expressed as glucosyl units convertible to glucose-1-P.

practice more is used in order to get enough glucosidase and transglucosylase.

Under the assay conditions of high enzyme and low glycogen levels the degradation of the outer tiers proceeds as a first-order reaction at least until the reaction is 75 or 80% complete (Fig. 2). This is a surprise, since with high glycogen and relatively low enzyme levels the rate falls off as limit dextrin is approached (14).

Kinetics of Glucosidase-Transglucosylase (15, 16)

The two enzyme activities of the debrancher complex have not been separated; consequently a kinetic study of either would be difficult. Nevertheless it is of practical importance to know the relationship between over-all activity and substrate concentration. Under the assay

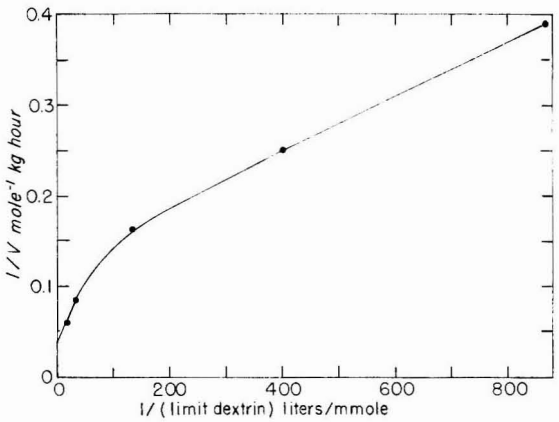

FIG. 3. Reciprocal plot of initial velocity of glucose-1-P formation vs. limit dextrin concentration. Limit dextrin was prepared by diluting glycogen to about 1 m*M* concentration with 0.025 *M* imidazole-HCl, pH 7.0, containing 5 m*M* K_2HPO_4, 0.1 m*M* 5′-AMP, and 10 μg/ml 6-times crystallized muscle phosphorylase *a*. After 30 min at 25°, HCl was added to a concentration of 0.035 *N*. The solution was heated 10 min at 100° to hydrolyze the glucose-1-P, then neutralized with NaOH. Measurements were made in the fluorometer with the regular analytical reagent, which was enriched with 2.5 μg/ml 6-times crystallized phosphorylase. The debrancher complex was provided by crude phosphorylase and velocities (*V*) are here referred to the total protein of the preparation. Limit dextrin concentration is recorded as total glucosyl units that can be converted to glucose-1-P debrancher complex and phosphorylase.

conditions a reciprocal plot of limit dextrin concentration vs. initial velocity is nonlinear (Fig. 3). Low levels of substrate are attacked more rapidly than expected from rates at higher levels. It is for this reason that modest amounts of the debrancher complex combined with phosphorylase are able to degrade low levels of glycogen completely in a reasonable time.

Consider, for example, a mixture of phosphorylase and debrancher complex in which phosphorylase predominates 100 to 1 when expressed as maximum velocities. At a glycogen level (as glucosyl units) of 50 μM (30 μM limit dextrin) the ratio of activities would be 90 to 1; with 2 μM glycogen the ratio would be reduced to 25 to 1.

In spite of the complexity of the kinetic situation, when a low level of limit dextrin is degraded completely the reaction follows almost a first-order time course (Fig. 2).

Preparation of Tissues for Analysis

Because of the specificity of the enzymic procedure, whole tissue homogenates (prepared in weak acid or alkali to prevent loss of glycogen by tissue enzymes) can be analyzed directly without removal of any tissue constituents. However, low results are obtained unless such homogenates are heated prior to analysis. Possibly some disaggregation is necessary, as in the case described above for stored stock solutions, or it may be necessary to denature adherent protein. The routine procedure adopted has been to homogenize in 0.03 N HCl and heat 10 min at 100°. The increase on heating with brain is about 30%, with liver and muscle 10% or less. Although this heat treatment solubilizes most of the glycogen in many tissues, it is recommended that assays be made without removing insoluble material.

Special preparative procedures may be desirable in special cases. An example may be given of a satisfactory procedure for brain when it is desired to use the same sample for labile acid-soluble constituents.[3] The quick-frozen tissue is placed in a tube at —20° with 2 or 3 vol of 0.1 N HCl in methanol and thoroughly dispersed with a glass rod. When no ice remains, the sample is brought to 0° and sufficient 0.3 M $HClO_4$ is added to dilute the methanol to 15% or less. The sample is centrifuged at no less than 5000 g for at least 10 min. The precipitate, which contains practically all the glycogen, is dispersed in 1.3 ml of 5 N KOH per gram of tissue (NaOH would presumably do as well) and heated for several min at 60° with gentle mixing until the precipitate is dissolved or finely dispersed. This is now diluted and analyzed directly with due allowance for the volume of precipitate and entrapped fluid. Curiously, heating $HClO_4$ precipitates of brain with 0.03 N HCl instead of alkali has given erroneously low glycogen values. Although brain glycogen is almost completely precipitated by $HClO_4$ in this procedure, other tissues would need to be tested separately. Acid precipitants ordinarily precipitate only part

[3] This is a procedure devised by Dr. Stanley Nelson. For extraction of water from frozen tissues in the first step he found that among a variety of organic solvents methanol was the most satisfactory.

of the glycogen from liver, heart, and muscle, and Roe *et al.* (17) demonstrated that with sufficiently violent homogenization almost none of the glycogen in these three tissues is precipitable by trichloroacetic acid.

Sensitivity and Reproducibility

Reproducibility is satisfactory over a wide range of glycogen concentration. In the fluorometer the optimal range of concentration is from 10^{-5} to 10^{-6} *M* (as glucosyl units), but with care to keep the reagent fluorescence as low as possible satisfactory measurements can be made down to 2×10^{-7} *M* (2×10^{-10} mole) or even less (Table 1). Since the analytical end product is TPNH, when greater sensitivity is required the excess TPN^+ can be destroyed with alkali and the TPNH measured by enzymic cycling (18). Completely satisfactory analyses have been made with

TABLE 1

Proportionality and Reproducibility of Glycogen Measurement

Measurements were made fluorometrically with 1 ml of reagent. The results have been calculated on the basis of 87% average yield of glucose-1-P in the experiment of columns 1 and 2 and an 85% yield in the experiment of columns 3 and 4. The amounts of glycogen are given as glucosyl units. (The theoretical yield would be about 92%.) Expressed as weight the range would be from 0.01 to 1 μg.

Calc	Obs.	Calc.	Obs.
10^{-11} mole		10^{-11} mole	
606	598	74.7	76.3
	591		77.3
	603		76.9
	604		70.3
311	315	38.3	38.1
	312		38.3
	318		38.6
	310		38.5
160	159	16.0	14.9
	161		16.0
	161		15.3
	159		17.2
74.	78	6.7	6.7
	76		6.5
	76		6.2
	83		6.0
38.	40		
	39		
	39		
	38		

tissue samples containing 10^{-12} mole (0.000,16 μg) or less of glycogen. However, this is beyond the scope of the present paper.

Comparison with Pflüger-Type Methods

Liver, muscle, and brain were analyzed for glycogen by precipitation with alcoholic KOH followed by acid hydrolysis and measurement of the liberated glucose (Table 2). In the case of liver and muscle, agreement is

TABLE 2

Comparison of Enzymic Method with Modified Pflüger Procedures

For the modified Pflüger procedure 1 gm of liver or muscle from an anesthetized rabbit was heated for 30 min in a boiling H_2O bath with 2 ml of 30% KOH. The glycogen was precipitated with 3.5 ml of 95% ethanol and hydrolyzed by heating 2 hr in a sealed tube with 2 ml of 2 *N* HCl. For brain the glycogen isolation was adapted from Kerr (19). Brain was obtained from mice that had been quickly frozen in Freon-12 chilled to −150°. The frozen brains were powdered and pooled. About 100 mg of powder was heated 15 min at 80° with 1 ml of 20% KOH in 60% ethanol. The precipitate was washed in the centrifuge with 1 ml of 4:1 methanol-chloroform mixture. The precipitate was hydrolyzed by heating 2 hr in a sealed tube with 1 ml of 2 *N* HCl. For all three tissues the glucose liberated by hydrolysis was measured by the TPNH formed with hexokinase, ATP, glucose-6-P dehydrogenase, and TPN^+. For the enzymic glycogen method 1 gm samples of liver or muscle or 100 mg samples of frozen brain powder were homogenized at 2° with 10 vol of 0.03 *N* HCl. The homogenates were heated 3 min at 100°, then analyzed by the phosphorylase procedure without removal of insoluble material. A and B represent separate tissue samples. Values are expressed as glucosyl units (162 gm/mole).

Method	Liver		Muscle		Brain	
	A	B	A	B	A	B
	mmoles/kg wet weight					
Pflüger	168	182	33	34	2.60	2.45
Enzymic	189	196	35	35	2.98	3.31
KOH-enzymic[a]	160	160	29	33		

[a] In this case the KOH-ethanol precipitates were analyzed by the phosphorylase method.

satisfactory between the values for glycogen measured in this manner and those obtained by the proposed phosphorylase method. With brain, however, which contains much lower levels of glycogen, the enzymic procedure consistently gave higher, and presumably more nearly valid, results. The difference is ascribed to the difficulty of precipitating all the glycogen and possibly some destruction during the heating in alkali.

SUMMARY

An enzymic method for measuring glycogen has been described in detail. Glycogen plus inorganic phosphate and TPN^+ are converted in one analytical step to 6-P-gluconolactone and TPNH. The TPNH is measured by

its fluorescence or ultraviolet absorption. The method uses commercially available enzymes: phosphorylase *a*, P-glucomutase, and glucose-6-P dehydrogenase. It takes advantage of the fact that most phosphorylase preparations contain sufficient transglucosylase and glucosidase to permit complete degradation of glycogen. The specificity is such that whole tissue can be analyzed directly. The sensitivity is sufficient to measure 0.05 μg of glycogen with precision.

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ANALYTICAL BIOCHEMISTRY 19, 327–337 (1967)

Determination of Dipicolinic Acid in Bacterial Spores by Ultraviolet Spectrometry of the Calcium Chelate[1]

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The occurrence of substantial amounts of dipicolinic acid (DPA) in bacterial spores, the idea that DPA is related in some yet-to-be-explained way to the extraordinary heat stability of these cells, and the lack of knowledge of any function for this biologically unusual compound make a rapid accurate determination of DPA useful. The sensitivity and the convenience of the absorbance near 270 mμ has been used for comparative assays of crude extracts (1–3). Conditions for quantitative extraction of DPA from spores (1) and for spectrometry as the acid (4) have been reported. This communication presents a more specific extraction procedure, a more specific spectrum for measurement, and a method of calculation that largely eliminates the effects of moderate blanks. The method is about 5 times more sensitive than the widely used Fe(II) complex absorbance at 440 mμ (5).

EXPERIMENTAL

Apparatus

Spectra were recorded with a Cary model 14 spectrophotometer. Photometric accuracy was verified with alkaline potassium chromate solutions (6). Mercury and cadmium emission lines were used for wavelength calibration. Matched fused-quartz stoppered cells were used. The optical path usually was 1.000 ± 0.001 cm. The temperature was 25.0°C except as noted. Spectra were recorded, usually from 390 to 220 mμ. For the more precise data, the maxima and minima at about 278, 274, 270, and 265 mμ were scanned repeatedly at 0.05 mμ/sec with a 5 sec period until the absorbances at the peaks could be estimated to 0.001.

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

Reagents and Standards

Dipicolinic Acid and Calcium Chelate. 2,6-Pyridinedicarboxylic acid (DPA) was obtained from Aldrich Chemical Company. The acid was recrystallized successively from 0.1 *N* HCl, from 95% ethanol, and from water. The calcium chelate, $Ca \cdot C_5H_3NO_4 \cdot nH_2O$, was prepared by addition of the equivalent amount of CaO and crystallized from aqueous solution. All materials were dried under vacuum at 90°C before use as standards.

Throughout this paper, DPA refers to the dipicolinate moiety without reference to ionic (or chelational) state, or in a stoichiometric context to $C_7H_5NO_4$, molecular weight 167.12.

Aqueous Ethanol-Acetic Acid Extractant. The recommended extractant is prepared by mixing 3 vol of 95.6% ethanol with 1 vol of deionized water and 0.04 vol of glacial acetic acid.

Recommended Procedure

Weighing Spores. Bacterial spores exhibit water adsorption isotherms similar to those shown by wool, with a sharp rise at low and high relative humidities and a gradual and fairly linear rise between about 20 and 80% RH. At 40% RH several lots and species of spores equilibrated slowly by adsorption to 5 to 11% water content. The equilibrium water content varied with RH by 1 to 2 water per cent per 10 RH per cent. For precise work it is necessary to weigh specially dried spores or spores equilibrated to a determined moisture content, or to use aliquots from a spore suspension.

Extraction of Spores. Dry a sample of spores estimated to contain about 120 μg DPA (usually 1 to 2 mg) and transfer to a glass ampoule (7 mm i.d. × 14 cm is convenient). Add 2 ml of extractant, and seal by torch without evacuation. Immerse in warm water to check for leakage. Heat at 101°C (flowing steam) for 1 hr. Cool, open, centrifuge, and decant carefully into a 12 ml conically pointed screw-top glass centrifuge tube. Suspend the residue in 2 ml of 80% ethanol, centrifuge, and decant into the 12 ml tube. Evaporate to dryness at room temperature by means of a fine stream of dry filtered air directed at the liquid surface. Add 5 ml of 0.005 *M* $Ca(OH)_2$; close the tube with a polytetrafluoroethylene-lined cap; mix and centrifuge to give a clear solution for spectrometry.

Calculations. Calculate DPA by one of the functions listed in Table 3. Choice of a function is discussed later. For extracts of dormant spores obtained by the procedure just described the function *N* is preferred. $N = 1$ for 0.0002065 *M* DPA or a content of 33.8 μg DPA per milliliter.

RESULTS AND DISCUSSION

DPA Standard. The nuclear magnetic resonance spectrum of the free acid as obtained from Aldrich was consistent with identity and purity. Molar absorptivities at 25.0°C in 0.005 *M* $Ca(OH)_2$ in water (Table 1) agreed well among the several recrystallizations. Spectra of the mother liquors in water and in 0.005 *M* $Ca(OH)_2$ indicated little or no non-DPA absorbance in the original DPA.

TABLE 1
Molar Absorptivity (ϵ) and Temperature Coefficients of Ca·DPA in 0.005 *M* $Ca(OH)_2$ in Water at 25.0°C

Wavelength of maximum or minimum, mμ	277.8	274.5	269.8	264.8	263.3	244
ϵ, liter/mole-cm	4888	3453	5362	3759	3814	1485
Temperature coefficient[a] of ϵ, %, 1° C	−0.21	+0.09	−0.13	−0.01	−0.05	+0.04

[a] Not corrected for expansion of water, which contributes −0.025%/1°C at 25°C.

Anhydrous Ca·DPA is hygroscopic; the acid is not. Standard solutions of the acid or of the calcium chelate in water (0.003 *M*) were stable for months in diffuse light. The solutions diluted in 0.005 *M* $Ca(OH)_2$ were reasonably stable.

Spectra. Ca·DPA was recognized by Powell (7) to have a distinctive double maximum near 278 and 270 mμ with a particularly deep trough. If a sufficient excess of cation is used to repress the dissociation of the chelate, somewhat similar spectra may be obtained with Mg(II), Sr(II), Ba(II), and Mn(II) (8). In the absence of chelating metals distinct spectra (Fig. 1) are observed (8) as the pH is varied through the pK_a's 4.7 and 2.1 (9). No attempt was made to optimize the expression of the ionic forms $[H\cdot DPA]^{-1}$ and $[H_2\cdot DPA]^{\circ}$. Some protonation of the pyridine N is evident in 1 *N* HCl.

The association constant for the equilibrium:

$$Ca^{+2} + [DPA]^{-2} \rightleftarrows [Ca\cdot DPA]^{\circ}$$

in water at 25°C is sufficiently small—log $K = 5.4$ (8)—that, at 0.00015 *M*, a concentration that gives $A_{270} = 0.83$, about 33% of the DPA gives the $[DPA]^{-2}$ spectrum. The spectrum of a solution of Ca·DPA in water confirmed this value approximately. This difficulty is avoided by the addition of a high concentration of Ca(II) with an inert anion. For 0.00001 *M* DPA and a log *K* of 5.4, 0.0004 *M* Ca(II) is required for 99.9% conversion to the $[Ca\cdot DPA]^{\circ}$ spectrum. With higher ionic strength the

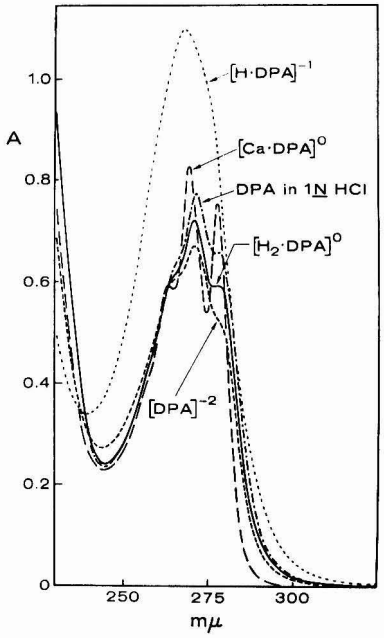

FIG. 1. Ultraviolet spectra of ionic forms of dipicolinate, 0.000155 *M* in water. $[H\cdot DPA]^{-1}$ is $H_2\cdot DPA$ in water. $[DPA]^{-2}$ is $H_2\cdot DPA$ in 0.0004 or 0.01 *M* NaOH or 0.1 *M* Na acetate. $[H_2\cdot DPA]^{\circ}$ is $H_2\cdot DPA$ in 0.1 *N* HCl; another ionic form becomes apparent in 1 *N* HCl. $[Ca\cdot DPA]^{\circ}$ is $H_2\cdot DPA$ in 0.005 *M* $Ca(OH)_2$ or 0.1 *M* $CaCl_2$, or other combinations (see text).

association constant is smaller, and higher concentrations of Ca(II) are required. Substances that compete effectively for Ca(II) will interfere with complete expression of the $[Ca\cdot DPA]^{\circ}$ spectrum.

Sodium acetate (0.1 *M*) plus $Ca(OH)_2$ (0.005 *M*), $CaCl_2$ (0.1 *M*) alone or plus $Ca(OH)_2$ (0.005 *M*), or $Ca(OH)_2$ (0.005 *M*) alone gave spectra that agreed closely. The identity of spectra near neutrality (0.1 *M* $CaCl_2$) and at high pH (0.005 *M* $Ca(OH)_2$) demonstrates that the pK_a for dissociation of a proton from the $Ca\cdot DPA\cdot nH_2O$ complex must lie well above 12.

$Ca(OH)_2$ was chosen for the recommended procedure to eliminate possible interference by metals that chelate with DPA more firmly than Ca(II) but have relatively insoluble hydroxides. Mn(II), which often occurs in spores in high concentration, is eliminated in this way: Ba(II) and Sr(II), which have highly soluble hydroxides, are not normally found in spores in amounts sufficient to compete with 0.005 *M* $Ca(OH)_2$. To check on the possibility of an inconveniently slow exchange of previously chelated metal with added Ca(II), a stoichiometric amount of $NiCl_2\cdot$

$6H_2O$ was dissolved in 0.00030 *M* Na_2·DPA, after which the solution was diluted with an equal volume of 0.01 *M* $Ca(OH)_2$. The spectrum recorded immediately agreed with that of [Ca·DPA]°; the spectrum of Ni·DPA is markedly different.

The [Ca·DPA]° spectrum varies with temperature. Measurements at 10.0 to 30.0°C (at intervals of 5.0°) showed the dependence at any wavelength to be almost linear. Molar absorptivities at 25.0° are given in Table 1 together with the wavelengths of maxima and minima and the temperature coefficients at each wavelength. The temperature coefficient for the function *N* is −0.64%/1°C; thus, the temperature for absorbance measurements must be controlled. Although lower temperature gives the more favorable spectrum, 25.0° was chosen for the recommended procedure for convenience.

Ca·DPA·$3H_2O$ crystals are composed of Ca·DPA dimers in which the shortest Ca—O bond length is between the Ca ion associated with one DPA and an oxygen of the other DPA (10). Because of the possibility of a dimerization equilibrium in solution, spectra were measured with 10 to 0.0113 cm path lengths for 0.000014, 0.00014, 0.0014, and 0.014 *M* Ca·DPA in 0.005 *M* $Ca(OH)_2$, and for a saturated solution of Ca·DPA in water (0.015 *M*). No alterations of molar absorptivity were found.

An attempt was made to further sharpen the fine structure of the [Ca·DPA]° spectrum by the use of alcoholic solutions. However, the absorbances at the peaks and the magnitude of the *N* function were reduced (Table 2). For this reason the ethanol used for extraction of spores was removed before preparation of the extracts for spectrometry.

TABLE 2
Molar Absorptivity (ϵ) of Ca·DPA in Alcoholic Solutions[a]

Solvent	Approx. wavelength (mμ) of max. or min.						N^b = 1 for concentration (in μg DPA/ml) of:
	277	274	269	265	263	244	
Methanol (100%)	4460	3560	5140	3820	3850	1530	44.2
Methanol (80% in water)	4560	3570	5240	3860	3890	1570	41.4
Ethanol (95.6% in water)	4050	3260	4680	3520	3530	1450	49.6
Ethanol (76.5% in water)	4080	3170	4650	3410	3440	1360	46.0

[a] 25.1 mg $C_7H_5NO_4$, 20 mg NaOH, and 555 mg $CaCl_2$ were dissolved in 500 ml solvent.
[b] See Table 3.

Calculations. The existence of maxima at 277.8 and 269.8 mμ and minima at 274.5 and 264.8 mμ permits a choice of methods for calculating the DPA content of extracts. (The maximum at 263.3 mμ differs so little from the minimum at 264.8 mμ that its use offers no advantage.) Some functions that have been evaluated are listed in Table 3.

TABLE 3
Functions for the Calculation of DPA Content

Function	Magnitude 1 given by μg DPA/ml	Rel. S.D.[a] for σ_A = 0.001, %	Temp. coef., %/1°C	Relative contribution by blank, %		Usefulness
				Nucleic acid[b] type	Linearly[c] rising	
$A_{269.8}$	31.2	0.17	−0.13	41		Low spectrometric accuracy; blank determined by independent means
$A_{277.8}$	34.2	0.19	−0.21	31		
$K = A_{277.8} - 2A_{274.4} + A_{269.8}$	50.0	0.48	−0.52	3.5	1.8	Constant blank
$L = -A_{274.4} + 2A_{269.8} - A_{264.8}$	47.6	0.46	−0.34	7.7	0.6	Linearly-rising blank
$M = A_{277.8} - 3A_{274.4} + 3A_{269.8} - A_{264.8}$	24.4	0.43	−0.86	5.7	0.6	Linearly-rising blank
$N = A_{277.8} - 2A_{274.4} + 2A_{269.8} - A_{264.8}$	33.8	0.42	−0.64	−0.5	−3.9	Nucleic-acid-type blank

[a] The ratio of the assumed standard deviation of the DPA determination to the magnitude of the function for 25 μg DPA/ml. σ = 0.001 means an assumed standard deviation of 0.001 for each measured absorbance, A, and of the zero setting of the spectrophotometer in the case of the first two functions.

[b] Based on an arbitrarily selected DPA-free spectrum obtained by autoclaving spores of *B. stearothermophilus* (previously extracted under the recommended condition) at 121°C for 20 min, making to 0.005 M $Ca(OH)_2$ and 0.50 mg spores per ml, and centrifuging. The figures give the percentage by which the true DPA content would be changed. The contribution in a spore extract obtained by the recommended procedure was only about 1/7 as large (cf. Table 5).

[c] A linear blank rising 0.100 from 277 to 265 mμ is assumed.

For spectrometrically pure DPA use of the more complex functions offers no advantage over use of the absorbance at a single peak provided that the blank can be determined independently. Where the blank is significant, the use of an instrument capable of measuring absorbance to 0.001 and the use of a complex function can give an advantage in specificity at the cost of a moderate loss of precision and convenience. For less accurate instruments the choice must be considered in the light of the application. The function N is recommended for spore extracts, which exhibit a nucleic acid type of blank. Where the blank rises linearly, as where turbidity is marked, the ternary function L or the quaternary function M may be preferred. The complex functions are more sensitive to temperature variations than the simple absorbances at 277.8 and 269.8 mμ.

Extraction of Spores. Inasmuch as the release of other ultraviolet-absorbing components has been the main obstacle to the use of ultraviolet spectrometry for DPA in spores, extraction methods were investigated. Heating in water with temperatures and durations somewhat more severe than required to kill bacterial spores releases the DPA (11), but it also releases absorbers with a peak near 260 mμ (nucleic acid components). Traces of Ca·DPA can be observed readily on the shoulder of this peak. Spectra of extracts obtained by autoclaving spore suspensions at 121°C for 30 min were used to evaluate the completeness of removal of DPA by prior milder treatments of the spores. Such milder treatments were sought.

Rode and Foster reported (2) that 2 hr at 56° in 80% ethanol sufficed to release DPA from dormant spores of *Bacillus megaterium*, and that this solvent mixture was much more effective than 95% or 60% aqueous ethanol, 80% aqueous methanol, or 80% aqueous acetone. For the present investigation the optimum composition of aqueous ethanol was evaluated more closely, the effects of certain additives were tested, and the temperatures and durations required for a wide variety of spores were determined. Complete release of DPA and minimization of the ultraviolet blank were the main criteria for this search.

Various contributions to blanks were noted: too severe heating or a poor choice of solvent gave a nucleic acid type of absorption as mentioned above; long exposure of extracts in contact with the extracted spores in water, even cold, gave an absorbance with a maximum near 280 mμ (possibly proteinaceous); and heating the extracts in air to speed evaporation gave a pale yellow tint on re-solution that raised the blank in the near ultraviolet without forming sharp peaks. To avoid autolysis, extracts were stored when storage was necessary, before removal of ethanol, or after drying, or after drying and re-solution in 0.005 M $Ca(OH)_2$. The extraction solvent was evaporated rapidly near room temperature in flow-

TABLE 4
Rate of Release of DPA from Spores of *Bacillus* Species in 80% Aqueous Ethanol at 90°C

Spores	DPA found, %			
	Initial 10 min extract	1 hr re-extract	Autoclaved residue	Total[a]
B. cereus t	7.9	0.3	0.0	8.2
B. megaterium B938	10.1	0.1	0.0	10.2
B. subtilis 15U	7.6	2.2	0.0	9.8
B. coagulans 43P	8.4	0.3	0.0	8.7
B. stearothermophilus 1518	3.3	6.0	2.2	11.5

[a] Uncorrected for water content of the lyophilized spores.

ing air. Spectra were recorded for 0.005 M $Ca(OH)_2$ solutions of the extracts after centrifugation to minimize turbidity.

An initial trial of rate of release of DPA by 80% aqueous ethanol from five species of spores is summarized in Table 4. Three successive extractions were made on each lot of spores; the final extraction by heating in water for 60 min at 121°C. The release rate varied widely between the lots of spores. The more resistant type, *Bacillus stearothermophilus*, was picked for optimizing the extraction conditions. The results of such an experiment are shown in Table 5. The lower ethanol concentrations (70 and 75%) and the addition of acetic acid (1% of the total volume) favored minimum retention of DPA in the spore residue. The ready release from "cation-stripped" spores (12) also is noteworthy. A mixture of

TABLE 5
Release of DPA from Spores of *B. stearothermophilus* by Aqueous Ethanol at 97°C

Ethanol concentration, %	Additive	Time, min	DPA found, %[a]		
			In extract	In autoclaved residue	Total
85		30	8.0	2.2	10.2
80		10	5.6	3.5	9.1
80		30	9.0	1.8	10.8
80		90	10.3	0.3	10.6
75		30	10.5	1.0	11.5
70		30	10.3	1.2	11.5
80	1% acetic acid	30	11.1	0.1	11.2
80	0.5% CTAB[b]	30	7.3	3.0	10.3
80	"Cation-stripped" spores[c]	30	10.8	0.0	10.8

[a] By function N.

[b] Cetyltrimethylammonium bromide.

[c] Another lot of spores, exposed to 0.1 N HCl at 18°C overnight, followed by washing and lyophilization (12).

3 parts of 95.6% ethanol plus 1 part of water plus 1% (of the total volume) glacial acetic acid was chosen as the standard extractant for further trials directed at the magnitude of the blank. Cetyltrimethylammonium bromide, a substance that induces release of DPA in aqueous solution (13), was ineffective.

In the experiment just described, spores of *Bacillus subtilis* also were extracted for 30 min with 80% ethanol. The DPA in the extract corresponded to 9.53% of the spore weight, and no DPA was found in the autoclaved spore residue.

Blanks were evaluated by subtracting the contribution of [Ca·DPA]° at 350, 300, 278, 274, 270, 263, and 244 mμ. The contribution of DPA was estimated for each wavelength from the assay based on the function N.

Extracts of dormant spores generally gave a minor constant absorbance from 390 to below 350 mμ that presumably arose from turbidity. Estimations of blanks were corrected (usually by less than 0.01) on the assumption that $A_{350} = 0.000$. The 263 mμ maximum for [Ca·DPA]° is close to the nucleic acid maximum, so that the non-DPA contribution to A_{263} proved a useful measure of the blank incurred by various modifications of extractant and procedure. This index is defined by:

$$B = \frac{(A_{263\ \text{total}} - A_{263(\text{DPA by } N)})}{A_{263(\text{DPA by } N)}}$$

Table 6 presents a comparison of 1% acetic acid and 0.05 N HCl in the 71.8% ethanol extractant on the index B for heat treatments of *B. stearothermophilus* spores. The index B is about 7× larger with 0.05 N HCl in the extractant than with 1% acetic acid in the extractant. The index B also was larger for extractant without acid than for extractant with 1% acetic acid.

The index B for the spores of Table 4 extracted with the standard extractant for 1 hr at 101°C varied from 0.04 to 0.07. Thus, the more severe treatment required for spores of *B. stearothermophilus* did not give bad blanks with the less resistant spores.

Table 6 shows that both acidic extractants give the same DPA content when function N is used for the calculation. The scatter of the data was somewhat greater for 0.05 N HCl in the extractant than for 1% acetic acid. The function K gave results for the former extractant that were a few per cent too high. No DPA could be detected in any of the residues.

A computation was made of the error in DPA content that arises from use of the actual maxima and minima recorded rather than absorbances at the wavelengths of the [Ca·DPA]° standard spectrum. The slight wavelength displacements caused by the blank gave a relative error of

TABLE 6
Blanks in the Assay of DPA in Extracts of *B. stearothermophilus* Spores with 71.8% Ethanol plus 1% Acetic Acid or plus 0.05 *N* HCl

Temperature, °C	Time, hr	DPA, %[a] Extractant with: Acetic acid by *N*	Acetic acid by *K*	HCl by *N*	HCl by *K*	Index of blank, B[b] Extractant with: Acetic acid	HCl
97	1	11.6	11.6	12.0	12.3	0.05	0.39
97	2	11.6	11.7	11.4	11.6	0.06	0.43
110	0.5	11.7	11.7	11.9	12.1	0.08	0.47
110	1	11.8	11.8	11.8	12.0	0.07[c]	0.48
	Av.	*11.7*	*11.7*	*11.8*	*12.0*	*0.065*	*0.44*

[a] By function *N* or *K*. In no case was as much as 0.01% DPA observed in the residues heated at 117°C in water.

[b] See text.

[c] The index B for extraction with 71.8% ethanol (no acid added) was 0.15.

about 0.4% of the total DPA content for the blank with 0.05 *N* HCl in the extractant, and a negligible error for the recommended extractant.

The spores remained bright by positive contrast phase microscopy (American Optical Company, dark M 97× objective) after the recommended extraction.

SUMMARY

Dipicolinic acid is determined by spectrometry of the calcium chelate in aqueous 0.005 *M* $Ca(OH)_2$. Molar absorptivities at 25.0°C are:

277.8 mμ	4888 liter/mole-cm
274.4	3453
269.8	5362
264.8	3759
263.3	3814
244	1485

The equation:

$$c = 33.8\ (A_{277.8} - 2A_{274.4} + 2A_{269.8} - A_{264.8})$$

where c is the concentration of dipicolinic acid (MW 167.12) in μg/ml and A is the absorbance for 1 cm cell length and 25.0°, gives minimal interference by other components of extracts of bacterial spores. The temperature coefficient for c is −0.22 μg/ml/1°C, or −0.64%/1°C. Dipicolinic acid is extracted from spores with much less contamination by other ultraviolet absorbance, by heating for 1 hr at 101° in an aqueous solution containing 71.8% ethanol and 1% acetic acid than by heating in water.

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A Paper Chromatographic Method for Identification and Estimation of Pyrrolidonecarboxylic Acid

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Pyrrolidonecarboxylic acid (PCA), which is readily formed by cyclization of glutamine and other γ-glutamyl derivatives, and from glutamic acid under more drastic conditions (1, 2), has recently received recognition in enzymic reactions involving glutamic acid (3–6). For identification of this compound on paper chromatograms, the procedure of Rydon and Smith (7) for acylamino acids is generally employed (3). Apart from being nonspecific, this method is also unsuitable for quantitative estimation of PCA. An interesting chemical property of PCA—its capacity to undergo quantitative conversion into γ-glutamyl hydroxamate under suitable conditions (8)—has now been utilized for visual identification of this compound on paper chromatograms as well as for its quantitative estimation.

MATERIALS

L-Glutamine (Light Chemicals).

L-Glutamic acid (E. Merck).

Hydroxylamine hydrochloride (British Drug Houses).

Methanol (B. D. H).

Ferric chloride·$6H_2O$ (B. D. H).

Trichloroacetic acid (E. Merck).

DL-Pyrrolidonecarboxylic acid was prepared by heating L-glutamic acid over an oil bath maintained at 200°C for 4 hr. The crude material so obtained was dissolved in a small quantity of warm water and boiled with charcoal for 2–5 min and the hot solution was rapidly filtered. The solid which separated out on cooling was recrystallized once; m.p. 181°C.

Reagents: Hydroxylamine reagent—25 gm hydroxylamine hydrochloride is dissolved in 100 ml 50% methanolic 2 *N* NaOH. *Ferric chloride spray reagent*—100 gm ferric chloride and 50 gm trichloroacetic acid are dissolved in distilled ethanol and to this solution is added 65 ml concen-

trated hydrochloride acid (A.R.), after which the volume is made up to one liter with ethanol.

PROCEDURE

Aliquots (10–50 μl) containing 2–10 μmoles of PCA and/or glutamine were chromatographed by the ascending technique on Whatman filter paper (3 mm) for approximately 16 hr, using ethanol/ammonia/water (8/1/1) as solvent system. After air-drying the chromatogram, it was moistened with 25% hydroxylamine reagent in a shallow dish. Due care must be exercised to see that the chromatogram is just moist with the reagent and that the reagent does not drip from the paper. This was accomplished by holding the two ends of the chromatogram and carefully dipping one edge of it in the reagent and moving the paper toward the other edge so that the entire area of the paper *gradually* absorbs the reagent. If excess reagent is applied to the paper, spots have a tendency to diffuse and spread. In any case, the reagent should not be applied by means of a pipet. Compact and intense spots are obtained only by such careful treatment with the reagent. Next, the chromatogram was air-dried for 10 min and heated in an oven maintained at 110°C for 15 min. The chromatogram was then carefully moistened with the ferric chloride reagent just as described for the hydroxylamine reagent. Dark brown spots on a yellow background appeared immediately.

Elution and Estimation. After spraying the chromatogram with the ferric chloride reagent, the paper is air-dried for 30 min; spots containing the γ-glutamyl hydroxamate (which correspond to PCA or glutamine) were cut out and eluted with 8 ml of ethanol. Gentle shaking elutes the color rapidly and it is measured in a Klett-Summerson colorimeter using filter No. 54.

RESULTS AND DISCUSSION

Specificity and Color Development

Glutamine, PCA, asparagine, and glutathione can be made visible on paper chromatograms by this procedure while a number of acylamino acids, glutamic acid, and aspartic acid do not react in this manner. As shown in Figure 1, both PCA and glutamine gave spots of the same color and almost the same intensity for a given concentration. They can be separated and made visible in this manner in a number of solvents. Asparagine and glutathione gave less intense spots compared to glutamine and PCA, while *N*-acetyl-DL-phenylalanine gave a barely visible color at very high concentrations (30–50 μmoles).

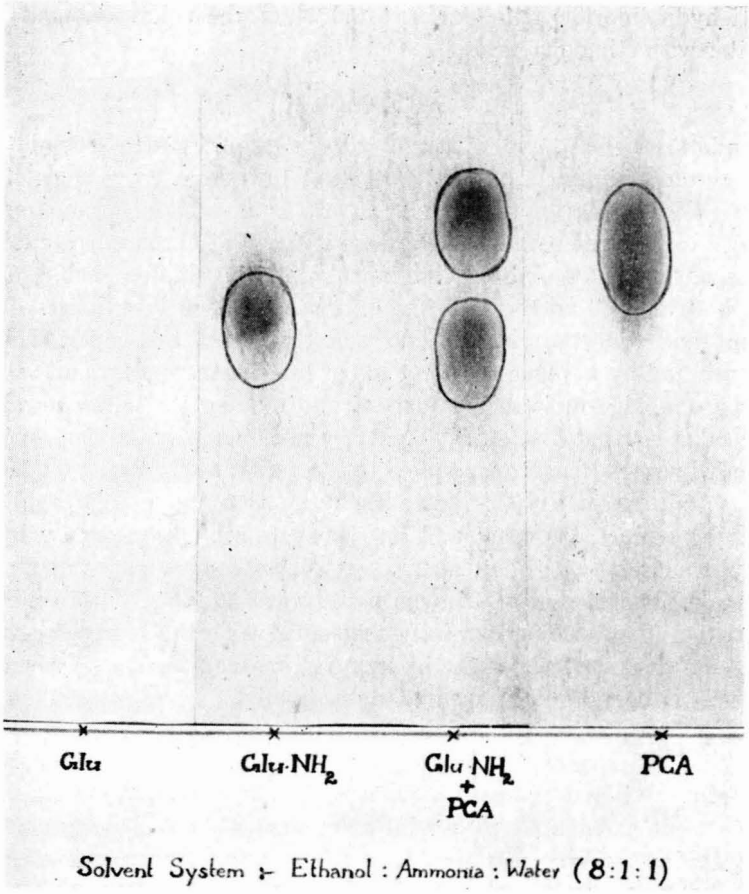

FIG. 1. Visual identification of pyrrolidonecarboxylic acid and glutamine as γ-glutamyl hydroxamate-ferric chloride complex on paper chromatograms. Solvent system: Ethanol/ammonia/water (8/1/1).

Separation of Glutamine, Glutamic Acid, and PCA

Pyrrolidonecarboxylic acid is closely related in enzymic and metabolic reactions to glutamic acid and glutamine (3–6, 3). While these compounds can be separated either by paper chromatography or by high-voltage electrophoresis (3), PCA cannot be made visible on the chromatograms or electrophoregrams by ninhydrin spray. The procedure of Rydon and Smith (7) as applied to PCA presents difficulties on account of residual chlorine, which is adsorbed on the paper. Furthermore, in solvent systems in which the R_f values of glutamine and glutamic acid are close to each other, it is difficult to distinguish the two compounds by ninhydrin spray

TABLE 1
Separation and Identification of Glutamic Acid, Glutamine, and PCA

Compound	$R_f \times 100$	
	n-Butanol/acetic acid/water (4/1/1)[a]	Ethanol/ammonia/ water (8/1/1)
Glutamic acid[b]	24	24
Glutamine[c]	18	38
Pyrrolidone carboxylic acid[c]	52	48

[a] In this solvent, PCA gave an additional faint spot with R_f ($\times 100$) value of 38.
[b] Detected by ninhydrin spray.
[c] Detected as hydroxamate-ferric chloride complex.

(Table 1). By the new technique, both glutamine and PCA can be made visible as γ-glutamyl hydroxamate, while glutamic acid does not respond to this test. It is thus possible to identify PCA, glutamine, and glutamic acid in mixtures by employing a combination of the ninhydrin test and the new technique on separate chromatographic strips.

Quantitative Estimation of PCA and Glutamine

The ferric chloride-hydroxamic acid complex formed on paper from PCA and glutamine could be conveniently eluted into methanol and this was found to have an absorption maximum at 535 mμ. The eluted color was stable up to 1 hr and decreased gradually thereafter. It was found that there was a linear relationship between color intensity and concentration from 2–10 μmoles of both PCA and glutamine and the color intensity values for both these compounds was nearly the same at these concentrations. The small difference observed between glutamine and PCA may be because of a slight and spontaneous cyclization of glutamine to PCA which is well recognized (1). This was confirmed by the observation that glutamine invariably showed an additional and very faint hydroxamate spot in th area of PCA. Zamir and Lichtenstein (8) described a procedure for the estimation of glutamic acid in proteins by its conversion to PCA. By this procedure, the concentration of glutamic acid that could be measured was in the range of 10–50 μmoles. The paper chromatographic procedure described here can be used to estimate PCA and glutamine in the range of 2–10 μmoles.

Recovery of PCA from a Biological Sample

To samples of 20 ml human urine, 0.5, 1.0, and 1.5 mmoles of PCA were added and passed through columns (2×10 cm) of Dowex 50 (H^+) resin. Columns were washed with water until the washings were free of acid. The breakthrough fluid and the washings in each case were pooled

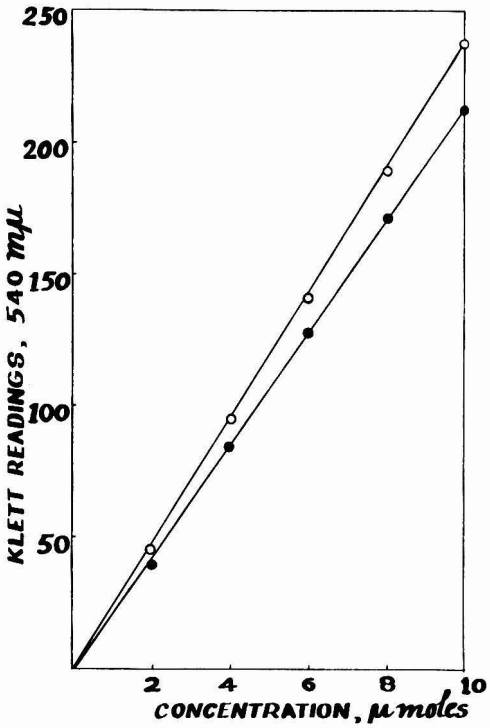

FIG. 2. Calibration curves for pyrrolidonecarboxylic acid and glutamine: (○) pyrrolidonecarboxylic acid, (●) glutamine.

and concentrated to dryness with a rotary flash evaporator. The residues were taken in 2 ml of water. Aliquots of 20 and 40 μl in duplicate were chromatographed along with authentic standards of PCA and estimated by the procedure described above. It is evident from the recovery values given in Table 2 that this procedure can be satisfactorily employed to measure PCA in biological samples like urine. If the levels of PCA present in any source or material are very low, concentration of the material, or

TABLE 2

Recovery of Added Pyrrolidonecarboxylic Acid from Human Urine[a]

Pyrrolidonecarboxylic acid added to urine, mmoles	Pyrrolidonecarboxylic acid recovered from urine, mmoles	Recovery, %
0.0	0.020	—
0.5	0.484	92.8
1.0	1.006	98.6
1.5	1.484	97.6

[a] Average values of three experiments. In no case did the recovery vary by more than ±10%.

of the extracts from it, to small volumes can bring the level of PCA within the range of accurate estimation by the present technique.

SUMMARY

A paper chromatographic method is described for identification and semimicro estimation of pyrrolidonecarboxylic acid (PCA). When heated with hydroxylamine, PCA undergoes quantitative conversion to γ-glutamyl hydroxamate, which on treatment with ferric chloride gives a brown spot on a yellow background on paper. Glutamine, asparagine, and glutathione also respond to this test, while glutamic acid, aspartic acid, and acylamino acids do not. PCA and glutamine in the range of 2–10 μmoles can be estimated by elution of this color into methanol and measurement of absorption at 535 mμ. Satisfactory recovery of pyrrolidonecarboxylic acid from biological samples like urine can be obtained.

Note added in proof: Pyrrolidone, the decarboxylation product of Pyrrolidonecarboxylic acid can also be made visible and quantitatively determined by the technique described. In the ethanol/ammonia/water solvent, it moves with a $R_f \times 100$ value of 82.

ACKNOWLEDGMENT

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ANALYTICAL BIOCHEMISTRY 19, 344–350 (1967)

Qualitative and Quantitative Determination of Mixtures of Amino Acids Using 2,4,6-Trinitrobenzenesulfonic Acid[1]

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Satake and his colleagues have described the use of 2,4,6-trinitrobenzene-1-sulfonic acid (TNBS) for the qualitative and quantitative assay of amino acids and peptides (1, 2). They have resolved these mixtures either on paper (3) or with automatic amino acid analyzers (4) and determined the components colorimetrically after reaction with TNBS rather than ninhydrin.

In working with poly-γ-D-glutamic acid (5) and with peptides of glutamic acid (6), TNBS was employed in this laboratory for the estimation of molecular weight and for the quantitative determination of amino groups, using glutamic acid as a standard. TNBS reacts only with primary amino groups and has several distinct advantages in comparison to 1-fluoro-2,4-dinitrobenzene (FDNB). The TNBS reagent is water soluble, which results in homogeneous reaction mixtures. At the pH of the reaction (8.0–8.5) it exists as an anion in solution, which prevents it from reacting with hydroxide ion to form picric acid. On the other hand, the FDNB reaction suffers from the formation of a large excess of dinitrophenol, which is often difficult to remove completely. However, excellent separations using paper and thin-layer chromatography have been achieved with DNP-amino acid and peptide derivatives (7).

In the course of these investigations, it was noted that a mixture of TNP-amino acids could be resolved remarkably well by thin-layer chromatography. This communication describes the qualitative and quantitative analyses of derivatized amino acids with that technique.

[1] This investigation was supported by grants from the National Institutes of Health (AI 05664), United States Public Health Service (Training Grant #1T1 A1 299), and the Committee on Research of the University of California.

MATERIALS

Mann Assayed amino acids were used, purchased from Mann Research Laboratories. Thin-layer chromatography plates were coated with silica gel G and air-dried for at least 18 hr before use. TNBS was purchased from Nutritional Biochemicals Corporation and was used without purification.

METHODS AND RESULTS

Determination of Glutamic Acid

A series of quantitative determinations of glutamic acid was performed by the method of Satake *et al.* (3, 4). One milliliter of the sample solutions containing 0.01–0.5 μmole/ml of glutamic acid combined with 1 ml of 4% sodium bicarbonate and 1 ml of 0.1% TNBS were kept in the dark at 37°C. After 2 hr the mixture was treated with 1 ml of 1 *N* hydrochloric acid. The optical density values were obtained by direct spectrophotometric readings of the reaction mixtures at 340 mμ, corrected for blank absorption. Optical density rose linearly with concentration. The molar extinction coefficient for TNP-glutamic acid was 3×10^3.

Qualitative Separations

One milliliter of an aqueous solution containing a mixture of amino acids in concentrations of 10 μmoles/ml was mixed with 1 ml of 4% sodium bicarbonate solution and 1 ml of 1% freshly prepared TNBS

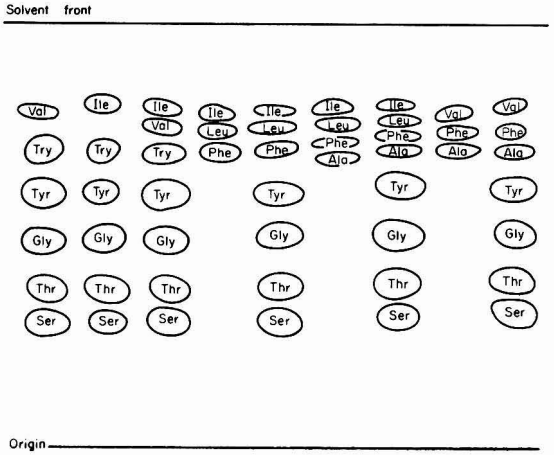

FIG. 1. Separations of various TNP-amino acids by thin-layer chromatography on silica gel G in isopropyl ether/$CHCl_3$/AcOH (6/3/1).

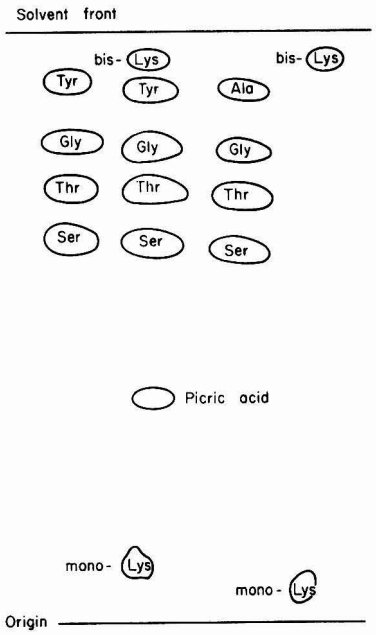

FIG. 2. Separation of various TNP-amino acids in $CHCl_3$/*n*-BuOH/AcOH (6/3/1).

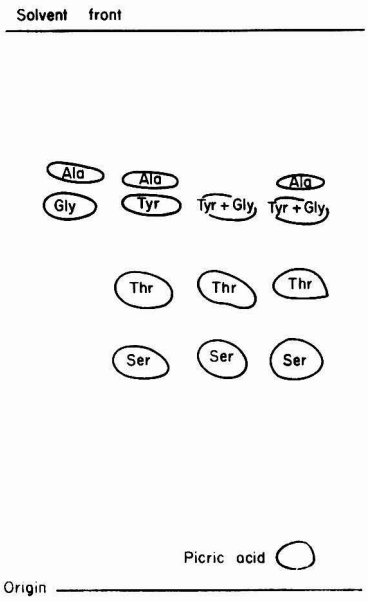

FIG. 3. Separations of various TNP-amino acids in $CHCl_3$/ethyl acetate/AcOH (6/3/1).

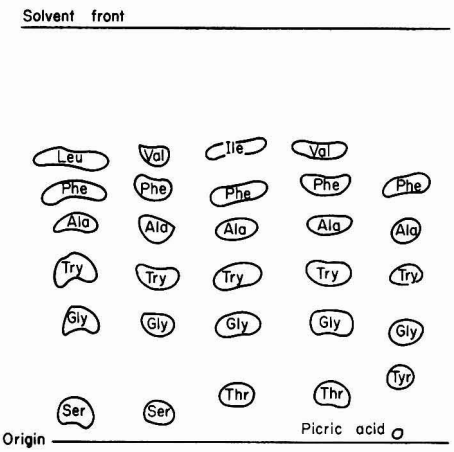

FIG. 4. Separations of various TNP-amino acids in $CHCl_3$/AcOH (9/1).

solution in water. The mixture was kept in the dark for 2 hr at 37–40°C and then acidified with 1 ml of 1 *N* hydrochloric acid. Since most TNP-amino acids are not soluble at these concentrations, they were extracted into ethyl acetate or ether. The extracts were spotted on silica gel plates

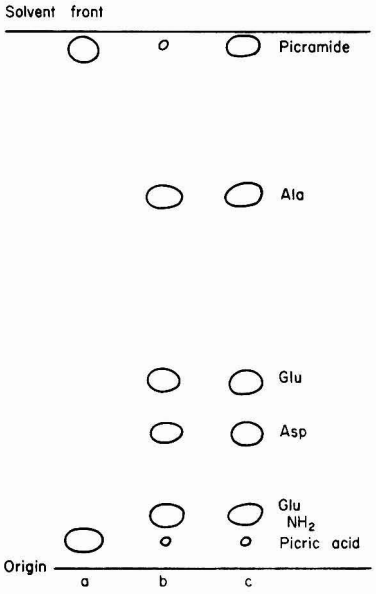

FIG. 5. Separations in $CHCl_3$/AcOH (5/1): (a) mixture of picric acid and picramide, (b) reaction mixture of Ala, Glu, Asp, and Glu(NH_2) with TNBS, (c) reaction mixture of (b) and ammonia with TNBS.

and developed in appropriate solvent systems. If the concentration of amino acids in the starting solutions does not exceed 1 μmole/ml, the extraction is usually unnecessary and the reaction mixture can be directly applied to the plates. In almost all experiments a small amount of picric acid was produced, which in most of the developing solvent systems remained near the origin. The chromatograms of various TNP-amino acid mixtures are shown in Figures 1–5.

Reactions involving glutamine (Fig. 5) invariably produced some picramide, probably from the hydrolysis of glutamine. This was verified both by chromatographing an authentic sample of picramide and by the addition of ammonia to the reaction mixture (Fig. 5).

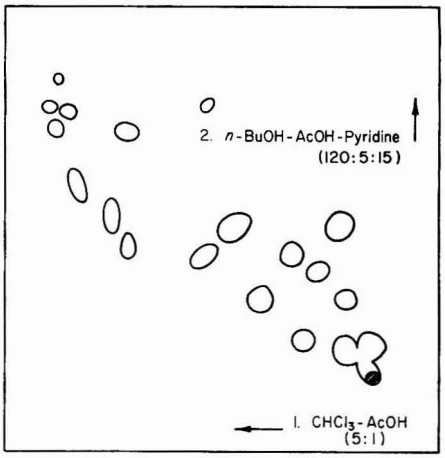

FIG. 6. Two-dimensional separation of a mixture of TNP derivatives of eighteen amino acids (Ala, Asp, Arg, CysCys, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, Val, and Hypro). The plate was developed with $CHCl_3$/AcOH (5/1), dried, turned 90°, and developed with *n*-BuOH/AcOH/pyridine (120/5/15).

Basic TNP-derivatives of His,[2] Arg, and mono-TNP-Lys are not extracted from acid solution and these reaction mixtures were spotted directly on the plates. Lysine invariably gave two derivatives, bis-substituted and probably ϵ-mono-substituted (1). In at least one instance two products were observed for arginine and histidine as well. The separations of TNP derivatives of the basic amino acids were generally less satisfactory than those of the neutral and acidic compounds.

A mixture of eighteen common amino acids was treated with TNBS as described above and subjected to two-dimensional chromatography on

[2] The abbreviations used are those suggested in "Peptides: Proceedings of the Fifth European Symposium, Oxford, September 1962" (G. T. Young, ed.), p. 261, Pergamon Press, New York, 1963.

silica gel as shown in Figure 6. Although the positions of each of the components of the mixture was not determined, it can be readily seen that good resolution was achieved. Chromatography of a mixture of the unsubstituted amino acids under similar conditions resulted in little or no movement of the components from the origin.

Quantitative Determinations

Solutions containing 10 μmoles/ml of Ala, Gly, Phe, Try, Tyr, and Val were prepared in 4% sodium bicarbonate. A mixture of 1 ml of each of the above solutions was combined with 6 ml of water and 6 ml of a 1% solution of TNBS. The reaction was kept in the dark at 50°C for 2 hr and then acidified with 6 ml of 1 *N* hydrochloric acid. The reaction mixture was extracted 5 times with 4.5 ml of ethyl acetate and the combined extracts were adjusted to 25 ml. This solution was stable in the refrigerator for many days. Varying volumes (0.2–1.0 ml) were spotted or streaked on TLC plates and developed with a chloroform/acetic acid (9/1) solvent. No drying stream of air was used during the application, as some TNP-amino acids are volatile. The use of these derivatives in gas chromatography is currently being investigated. The developed plates were dried in a ventilated hood for a few minutes. Individual spots were rapidly scraped off the inverted plate with the sharp edge of a polyethylene spatula onto squares of weighing paper. The collected powder was poured into a small

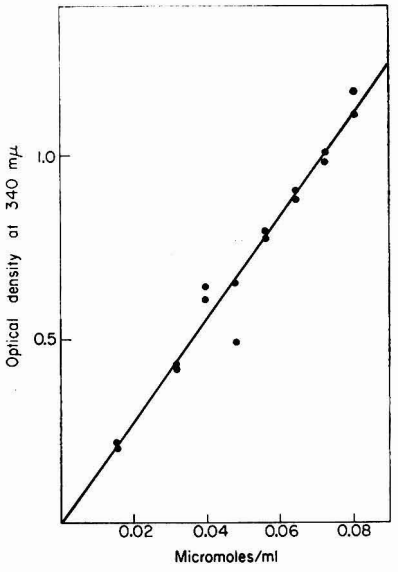

FIG. 7. Quantitative determination of TNP-phenylalanine in a mixture of Phe, Val, Ala, Try, Gly, and Tyr treated with TNBS and separated by TLC.

eluting column (commercially available disposable Pasteur pipets with a flared wide end and a small cotton plug in the constriction were used) and washed directly into 5 ml volumetric flasks with methanol. The volumes were adjusted to 5 ml and the resulting solutions containing 0.016–0.08 μmole/ml were immediately measured at 340 mμ.

Care was taken to shield all these operations from direct sunlight or strong artificial lights. It is also important to remove the TNP derivatives from the TL plates as rapidly as possible since the optical density drops sharply with time when these derivatives remain on silica gel. Typical results are shown in Figure 7 for TNP-phenylalanine determined in the mixture of amino acids. Other amino acids gave similar results. Glycine and tyrosine were somewhat more erratic, the first probably due to its volatility and the second possibly because the TNP-tyrosine spot lies rather close to that of picric acid and the area to be removed is hence somewhat less certain.

SUMMARY

Mixtures of TNP-amino acids may be rapidly resolved by chromatography on thin-layer plates of silica gel. The colored derivatives can be quantitated spectrophotometrically following elution from the plates. The advantages of this method lie in its rapidity and the modest equipment needed.

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ANALYTICAL BIOCHEMISTRY 19, 351–356 (1967)

Analysis of Cyclitols in Cycad Plant Tissues

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Cycad plants contain, besides the acutely toxic azoxyglycosides (1), quantities of simple sugars, principally glucose and fructose, together with much smaller amounts of sucrose and the cyclitols sequoyitol (2, 3), pinitol (3, 4), and myoinositol (4). For quantitative analysis of the cyclitols, gas chromatography of the trimethylsilyl (TMS) ethers, following the pioneering work (5) on the separation of a wide variety of carbohydrates as TMS ethers, appeared promising and has been previously used for myoinositol (6–8). On most column substrates myoinositol TMS ether forms a well-resolved band whose retention time is considerably greater than those of other common simple polyols and much less than those of higher polyols such as sucrose, whereas the TMS ethers of pinitol and sequoyitol are not resolved completely from those of fructose and α- and β-glucose on a variety of substrates at temperatures in the range 150–250°. Treatment of extracts containing myoinositol with hot barium hydroxide solution followed by deionization on ion-exchange resins has been previously used (9, 10) to remove reducing sugars although its efficacy appears not to have been tested. Vigorous acid treatment is precluded because it may be expected to demethylate pinitol and sequoyitol (11).

METHOD

The following procedure gave results reproducible within about 5% over-all.

The plant tissue sample (0.5–2 gm, dry weight) was blended with water (20–50 ml), and the mixture was boiled for 20–30 min. Starch and protein were then precipitated by addition of 4 vol of acetone and, after 12–16 hr, the mixture was filtered with the aid of a little Celite, the residue being washed with acetone (10 ml). The combined filtrate and washings were evaporated to a gum on a rotary evaporator. A solution of the residue in water or an aliquot (5–10 ml, corresponding to 0.2–0.5 gm of dry plant tissue) was heated with barium hydroxide solution (final pH $>$ 12; 1–2 ml of cold, saturated solution was usually added) at 100° for 1 hr, then

the cooled mixture was passed successively through columns (5 mm bore) containing Dowex 50-X4 (H^+ form; 1–2 ml of wet suspension) and Dowex 1-X8 (OH^- form; 1–2 ml of wet suspension), followed by water (20 ml). The eluate was evaporated on a rotary evaporator and made to 10 ml. An aliquot (5 ml) was reserved for addition of internal standard as below. The remainder, not accurately measured, was evaporated to dryness on the rotary evaporator, then in a vacuum desiccator over concentrated sulfuric acid and potassium hydroxide pellets for several hours. The residue was treated with trimethylsilylating reagent (0.3–1.0 ml of a mixture, anhydrous pyridine/hexamethyldisilazane/chlorotrimethylsilane = 7/2/1). After at least 3 hr, samples (0.5–2 μl) were injected into the gas chromatograph for determination of relative peak areas and approximate concentrations. Then to the aliquot reserved above was added a known amount of myoinositol (1–4 times the amount already present; an aqueous solution containing 0.5 mg/ml was convenient), and the evaporation, drying, trimethylsilylation, and gas chromatographic determination of relative peak areas were repeated.

The chromatograph, an Aerograph Hy-fi Model 600-D fitted with a $5' \times 1/8''$ stainless-steel column containing 5% SE30 on Chromosorb W, 60–80 mesh, and hydrogen flame-ionization detector, was conveniently operated at 190° with nitrogen as carrier gas at an inlet pressure of 15 psi (flow rate about 30 ml/min). Under these conditions, the pinitol, sequoyitol, and myoinositol peaks appeared at approximately 5.1, 8.3, and 13.5 min, respectively, the pinitol peak being on the tail of the pyridine peak. No change in operating conditions caused improvement, and evaporation of the pyridine in a vacuum and replacement with carbon disulfide caused selective loss of the pinitol derivative. Peak areas were measured (in arbitrary units) as the product of retention times (assumed proportional to peak widths at half height) and peak heights above an artificial baseline. Provided the sample injected did not exceed 2 μl and contained at least 0.3 μg of pinitol as TMS ether, at the sensitivities used (range 1, attenuation factors 1–8) relative peak areas were reproducible to within 2%. Use of a disc integrator was less convenient because of corrections required by tailing of the pyridine peak, and was no more precise.

Let, respectively, x_p, x_s, and x_m be the percentage weights of pinitol, sequoyitol, and myoinositol in the sample, a_p, a_s, and a_m the peak areas in the absence of standard, a'_p, a'_s, and a'_m those produced when w mg of myoinositol is added to a sample of weight W mg, and r_p, r_s, and r_m the response factors in area per unit weight. Only relative areas and response factors are required. It is convenient to define relative areas as

$$A_m = a_m/a_p; \qquad A_s = a_s/a_p; \qquad A'_m = a'_m/a'_p; \qquad A'_s = a'_s/a'_p$$

(where A'_s should be equal to A_s), and reciprocal relative response factors as

$$k_p = r_m/r_p; \qquad k_s = r_m/r_s$$

Then:

$$x_m = (100wA_m)/[W(A'_m - A_m)]$$
$$x_p = x_m k_p/A_m$$
$$x_s = x_m k_s A_s/A_m$$

Relative response factors were determined on a mixture of similar composition to that found in the plant samples analyzed. Under the given conditions, $k_p = 1.33$ and $k_s = 1.49$.

Initially, extractions of cycad seed were made with 70% aqueous ethanol. The chromatograms then showed a peak at 7.2 min not completely resolved from the sequoyitol peak and of comparable size. It was not produced if the extract freed of ethanol was heated with 1 *N* hydrochloric acid at 100° for 1 hr before the alkali treatment, if the extraction was made with 70% aqueous acetone or with water alone, or if the previously dried and ground seed was added to boiling 70% aqueous ethanol. When pieces of unground fresh seed were boiled in 70% aqueous ethanol for 30 min before blending, the peak was still present but of considerably reduced size. The retention time was identical with that from an authentic sample of ethyl β-D-glucopyranoside. Formation of this compound in these extracts is probably enzymic since no trace of ethyl α-D-glucopyranoside appeared on the chromatograms. Cycad emulsin is known to bring about transglycosylation (12); it is apparently relatively resistant to heat and not inactivated by 70% ethanol. Interference from this source was avoided by the procedure described. Some samples gave minor unidentified peaks in this region partly overlapping the sequoyitol peak (Table 1, footnote 6); they did not correspond to any of the reference cyclitols or sugar alcohols available.

After barium hydroxide treatment and deionization, solutions of glucose and fructose gave chromatograms showing residual components as only minor irregularities in the baseline. Larger amounts of barium hydroxide than that given were not necessary even when heavy precipitates (protein ?) appeared in the treated extract. To recover cyclitols (and sugar alcohols) quantitatively, it was important to elute the ion-exchange columns slowly with at least 10 bed-volumes of water. Up to 10% of the myoinositol was not recovered when deionization and elution were conducted rapidly in short, wide-bore columns.

Use of an internal standard is a well-known method of avoiding difficulties of reproducing volumes of sample injected. Usually the standard chosen gives a well-resolved peak in a region of the chromatogram within

the range of retention times of the components to be analyzed. The present method of adding a known amount of one of the components already present is of course limited to samples yielding at least one other peak whose area then measures the volume of sample injected. It does not require that the chromatogram show a clear "window" nor that a suitable auxiliary standard be sought. In the present case, scylloinositol (retention time of TMS ether, 10.5 min under the conditions used) would in fact have been suitable, but a pure sample in sufficient quantities is difficultly accessible.

RESULTS AND DISCUSSION

Results on some representative samples of cycad plant parts and common forage plants are shown in Table 1.

TABLE 1
Cyclitol Content[1] of Some Plant Species

Species	Part	Pinitol	Sequoyitol	Myoinositol
Cycas circinalis L.	Seed[2]	0.63	0.28	0.12
	Seed[3]	0.62	0.42	0.13
Cycas revoluta Thunb.	Seed[4]	0.97	0.04[6]	0.28
	Husk[5]	0.66	0.04[6]	0.06
Macrozamia communis L. Johnson[7]	Seed[8]	0.43	0.26	0.04
	Leaf[8]	0.57	0.02[6]	0.06
Zamia debilis en Ait.	Leaf[9]	0.45	0.34	0.11
Medicago sativa L.	Tops[10]	1.26	0	0.09
Lotus corniculatus L.	Tops[10]	1.49	0	0.16
Phleum pratense L.	Tops[10]	0	0	0.1[11]

[1] Values expressed as per cent dry weight.

[2] Ground dried kernel from Guam, provided by Dr. H. Matsumoto, University of Hawaii.

[3] Kernel of fresh whole seed from Guam, provided by Dr. J. Keresztezy, National Institutes of Health. Sample dried in air at 40–50° for 7 days, lost 40.2% of its fresh weight.

[4] Ground dried kernel from Japan, provided by Drs. O. Mickelsen and M. Yang, Michigan State University.

[5] Ground dried outer flesh and seed casing, provided by Drs. Mickelsen and Yang.

[6] The sequoyitol peak was partly overlapped by peaks due to unidentified components. This value is therefore an upper limit.

[7] Formerly *M. spiralis*.

[8] Unground dried kernel and leaf provided by Mr. P. Healy, University of Sydney.

[9] Ground dried sample provided by Dr. J. Keresztezy, National Institutes of Health.

[10] Ground dried whole tops of "vernal" alfalfa, "Empire" birdsfoot trefoil, and "verdant" Timothy grass, respectively, collected in June, 1966, provided by the Department of Agronomy, University of Wisconsin. Alfalfa and trefoil each showed unidentified components (0.05–0.15%) at 6.2 and 8.0 min. Addition of sequoyitol gave a fifth partly resolved peak at 8.3 min.

[11] Estimate based on peak area only. No other peak appeared in the chromatogram.

The occurrence of pinitol in cycad plant parts (3, 4), the "loco-weeds" of the American midwest (13), and the New South Wales stock poison, Darling pea (*Swainsona* spp.) (14), and the common ability of these plants to induce locomotory disturbances in sheep or cattle that graze upon them (for a review of cycad toxicity, see reference 15; "pea-strike" is described in reference 16) suggest a possible causal connection. Healy (17) has observed abnormalities in mice kept on a diet containing pinitol; the effects are reversed by feeding sufficient myoinositol and are attributed to antagonism of myoinositol metabolism by pinitol. These effects are not those seen in the field. Moreover, since in alfalfa and trefoil, common forage crops upon which animals may feed with relative impunity, the content of pinitol and its ratio to myoinositol are about twice those for cycad plant parts, it seems unlikely that pinitol is implicated in cycad toxicity. Samples of cycad flour prepared for human consumption on Guam by grinding and washing with water showed only residual traces of cyclitols (about 5% of the concentrations in the untreated seeds) when analyzed by the method described. This of course estimates only the free cyclitols or those in forms readily hydrolyzed by base and, to this extent and because unknown amounts are ingested in other items of diet, the content or ratio may not be valid criteria.

SUMMARY

Cyclitols (and sugar alcohols) in extracts of plant tissues may be analyzed by gas chromatography as trimethylsilyl ethers after removal of reducing sugars by treatment with barium hydroxide followed by deionization. A known amount of one of the components already present may be used as an internal standard. The method was applied to various samples of cycad plant tissue and some common forage crops. Neither the content of pinitol nor its ratio to myoinositol appears likely to be implicated in cycad toxicity.

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The Use of 2,4-Dinitro-5-fluoroaniline in the Determination of Protein Structure on a Micro Scale[1]

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Sanger's DNP method for the determination of *N*-terminal groups in proteins and peptides (1) has been widely used since its introduction, but in order to improve the sensitivity, E. Bergmann (2) developed a new reagent, 2,4-dinitro-5-fluoroaniline. This compound reacts with free amino groups in proteins, peptides, and amino acids to yield *N*-2,4-dinitro-5-aminophenyl (DNAP) derivatives. These compounds are yellow like the corresponding DNP derivatives but their absorption maximum is at 410 mμ (2) compared with 350 mμ (3) for the DNP derivatives. Since this is further into the region of the spectrum visible to the human eye, the compounds are more readily seen (e.g., on chromatograms). Also, the derivatives contain free aromatic amino groups which can be diazotized and coupled with phenols, etc. to form azo dyes that absorb light strongly at 425 and 610 mμ (2) and are thus even more visible.

This paper reports some work aimed at the exploitation of 2,4-dinitro-5-fluoroaniline as a reagent for the determination of *N*-terminal groups in proteins and peptides on an ultramicro scale.

MATERIALS

Amino acids were purchased from Nutritional Biochemicals Corp. 2,4-Dinitro-5-fluoroaniline was purchased from Pierce Chemical Co. Dimethyl sulfoxide was a gift from the Crown Zellerbach Corporation. Silica gel G, prepared by E. Merck, was obtained from Brinkmann Instruments. Nitrogen dioxide was obtained in steel cylinders from Air Products and Chemicals, Inc. Insulin was purchased from Mann Research Labora-

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tories. All solvents were analytical grade and were used without further processing.

METHODS

Preparation of DNAP-Amino Acids. A mixture of 2 mmole of amino acid, 2 mmole of 2,4-dinitro-5-fluoroaniline, 6 mmole of sodium bicarbonate, and 25 ml of 50% aqueous ethanol was boiled under reflux for 30 min. The mixture was cooled, diluted with 25 ml of water, and boiled gently until the odor of ethanol became very faint. After cooling again, any solid or cloudiness was filtered out and discarded. The solution was acidified to pH 2 with dilute hydrochloric acid. The DNAP amino acid came out of solution as a yellow precipitate. It was filtered off and recrystallized repeatedly to a constant melting point. The yields were 10 to 70% for different amino acids. *N*-α-Mono-DNAP-histidine was prepared directly from histidine in this way. *O*-DNAP-tyrosine was prepared by acid hydrolysis of the *N*-acetyl derivative which in turn was prepared from *N*-acetyltyrosine.

When sodium carbonate was substituted for sodium bicarbonate, the yields were about the same but the products were generally not as pure, as indicated by chromatography. Excellent yields of quite pure DNAP amino acids were obtained when dimethyl sulfoxide was used as the solvent. The reaction mixture was heated at 90° for 30 min instead of being refluxed.

Preparation of DNAP Proteins. A solution of 10 mg of protein, 10 mg of 2,4-dinitro-5-fluoroaniline, and 15 mg of sodium bicarbonate in 1 ml of dimethyl sulfoxide was heated at 90° for 30 min. After the red mixture was cooled, 2 ml of water and enough dilute hydrochloric acid were added to bring the pH to 2. The yellow precipitate was centrifuged off and washed several times with acetone until the washes were colorless. The product (a yellow powder) was dried in a current of nitrogen. The yield varied from 5 to 20 mg depending on the protein used.

Hydrolysis of DNAP Proteins. 0.5 mg of DNAP protein was heated with 0.1 ml of 4 *N* perchloric acid in a sealed tube at 110° for 18 hr. The yellow solution was diluted with 0.2 ml of water and extracted several times with ethyl acetate. The extracts were shaken with a saturated solution of potassium chloride, spotted on a thin-layer plate, and chromatographed.

Preparation and Hydrolysis of DNAP Insulin on an Ultramicro Scale. Insulin (0.1 mg), 2,4-dinitro-5-fluoroaniline (0.1 mg), sodium bicarbonate (0.15 mg), and dimethyl sulfoxide (0.1 ml) were mixed in an improvised test tube made from about 13 cm of 6 mm o.d. glass tubing sealed at one end. The tube was capped with aluminum foil and placed in a hot water

bath at 90° for about 40 min. The bright red mixture was cooled and diluted with 0.3 ml of water and then acidified with 1 drop of 4 *N* perchloric acid. A voluminous yellow precipitate formed and was centrifuged at high speed. The precipitate was almost entirely dissolved in acetone to leave a small amount of insoluble DNAP-insulin, which was washed twice with acetone, centrifuging each time. It was finally dried in a current of nitrogen.

Then 0.1 ml of 4 *N* perchloric acid was added to the DNAP insulin and the tube was sealed shut. The hydrolysis mixture was heated at 110° for 17.5 hr. The tube was broken open and the mixture was drawn into a 1 ml tuberculin syringe through a Teflon "needle" (Hamilton Co., Whittier, Calif.). Water (0.3 ml) was then drawn into the syringe to dilute the mixture. Using the syringe as a separatory funnel, the mixture was extracted three times with 0.3 ml portions of ethyl acetate. Previous experiments had shown that perchloric acid extracted into the ethyl acetate and disturbed the chromatographic separation; it was removed by shaking the extract with saturated aqueous potassium chloride which forms insoluble potassium perchlorate. The extract (which was virtually colorless) was evaporated to about 35 μl in a stream of nitrogen and spotted, 1 μl at a time, on an activated Eastman Chromagram sheet in a nitrogen atmosphere. The chromatogram was run 10 cm in solvent A (chloroform/*n*-propanol/acetic acid, 70/30/1). The spots were intensified by diazotization as described below.

Thin-Layer Chromatography. 2 × 20 cm glass plates were coated with silica gel G in the usual manner using the RsCo spreader. Just before use, they were activated by heating at 110° for 30 min. In later work, Eastman Chromagram sheets were used occasionally. The R_f values were the same as on silica gel G plates but the solvents ran more slowly and the spots tended to diffuse.

In screening various solvent systems, 1 μl samples containing about 1 μg of the DNAP amino acids were spotted on the plates. When working with hydrolyzates, either the whole sample was spotted 1 μl at a time or enough was spotted to produce a clearly visible spot. Normally, spotting was done in the open but with protein hydrolyzates (particularly of insulin) it was found necessary to spot in a nitrogen atmosphere using the Desaga model DB application box. The solvent flow on the plates was limited to 10 cm by scribing a front on the plate.

Intensification of DNAP Amino Acid Spots on Thin-Layer Chromatograms. After a chromatogram had been dried, it was exposed briefly to nitrogen dioxide fumes to diazotize the free aromatic amino group of the DNAP amino acid (4). The exposure should be brief (about 15 sec) and the gas should be dilute, since silica gel adsorbs nitrogen dioxide. The

adsorbed nitrogen dioxide reacts with the β-naphthol solution used in a later step to produce a strong yellow background. After exposing the plate to nitrogen dioxide, it is aired out for about 10 min and then sprayed with a solution of 0.14 gm of β-naphthol and 0.14 ml of triethylamine in 10 ml of benzene. When the chromatograms are run on Eastman Chromagram sheets, the developed chromatograms can be preserved from further color change by spraying them with clear acrylic lacquer. A thin coat will not change the appearance of the chromatogram but will harden the layer so that it can be written upon and stored in a notebook.

RESULTS

DNAP Amino Acids. The properties of the DNAP amino acids prepared in this work are summarized in Tables 1. Table 1a covers the compounds prepared in a high state of purity and analyzed for carbon, hydrogen, and nitrogen; 1b covers all other compounds. In the case of arginine, two products were obtained: one was apparently a sodium salt according to its solubility behavior and analysis; the other one was

TABLE 1a
2,4-Dinitro-5-aminophenyl Derivatives of Amino Acids

Amino acid	M.p.[a]	Recryst. from	Yield, %	Carbon, %		Hydrogen, %		Nitrogen, %	
				Calc.	Found	Ca'c.	Found	Calc.	Found
DL-Alanine[b]	209	H_2O	73	37.51	37.82	4.19	4.15	19.44	19.40
DL-Asparagine	199	MeOH	28	38.22	38.14	3.21	3.47	17.83	17.56
Glycine	221	MeOH-H_2O	41	37.51	37.76	3.15	3.20	21.87	21.51
L-Histidine[c]	205	H_2O	29	40.68	40.65	3.98	4.14	23.72	23.49
DL-Leucine	148	H_2O	13	46.15	46.17	5.13	5.32	17.95	17.73
DL-Valine	198	MeOH-H_2O	61	44.29	44.56	4.73	5.16	18.79	19.02

[a] Melting points are corrected.
[b] Monohydrate.
[c] *N*-α-Monosubstituted, monohydrate.

obtained in very low yield and is apparently a weak acid, but otherwise nothing else is known about it. An attempt was made to prepare the DNAP derivative of histidine substituted only on the imidazole ring (im-DNAP-histidine) by starting with *N*-α-acetylhistidine. im-DNAP-*N*-α-acetylhistidine was obtained but the acetyl group could not be removed by acid hydrolysis. ε-DNAP-lysine was prepared by a method similar to the one used by Sanger and Porter (5) for the DNP derivative but only an impure aqueous solution was obtained. The derivatives of proline and tyrosine were chromatographically pure but the analyses were unsatisfactory.

DNAP Proteins. All of the free amino groups in a protein should be

reactive with 2,4-dinitro-5-fluoroaniline. Thus insulin, which has two *N*-terminal groups (phenylalanine and glycine) and one lysine residue should react to form a tris-DNAP derivative. The actual degree of substitution in DNAP insulin was determined by a spectrophotometric method and it was found that there were 2.8 DNAP groups per molecule, which is 93% of the theoretical value.

TABLE 1b
2,4-Dinitro-5-aminophenyl Derivatives of Amino Acids

Amino acid	M.p.[a]	Recryst. from	Yield, %	Notes[b]
β-Alanine	197	MeOH-H_2O	42	P
L-Arginine	231	MeOH-H_2O	40	P
DL-Aspartic acid	227	MeOH	63	P
L-Cystine	184	H_2O	8	I
α,ε-Diaminopimelic acid	239	EtOH	48	I
L-Glutamic acid	182	MeOH-H_2O	37	P[c], Lit. m.p. (2), 102°
DL-Isoleucine	195	MeOH-H_2O	44	Lit. m.p. (2), 204°
DL-Lysine	184	H_2O	22	
DL-Methionine	207	MeOH-H_2O	46	Lit. m.p. (2), 216°
DL-Norleucine	185	MeOH-H_2O	67	P
DL-Norvaline	211	MeOH-H_2O	47	P
DL-Phenylalanine	225	MeOH-H_2O	53	Lit. m.p. (2), 235°
L-Proline	182	H_2O	65	P
DL-Serine	195	EtOH-C_6H_6	69	I, Lit. m.p. (2), 209°
DL-Threonine	212	$CHCl_3$	67	P[d]
DL-Tryptophan	134	MeOH	50	P
DL-Tyrosine	257	EtOH	—	P
DL-Tyrosine[d]	Dec.	—	51	P
3-Amino-4,6-dinitrophenol	230	MeOH	61	P, Lit. m.p. (9), 226°
4,6-Dinitro-*m*-phenylenediamine	299[e]	EtOH	49	Lit. m.p. (8), 297°

[a] Melting points are corrected.

[b] P = chromatographically pure. I = slightly impure, shown by chromatography.

[c] A small quantity was purified by extraction with chloroform in a Soxhlet extractor. The substance crystallized from the extract.

[d] *O*-Monosubstituted.

[e] Uncorrected.

Chromatography of DNAP Amino Acids. Several adsorbents including cellulose, Florisil, kieselguhr, and silica gel were studied. Silica gel G was found to be the best. Three solvent systems (chloroform/*n*-propanol/acetic acid, chloroform/*tert*-amyl alcohol/acetic acid, and benzene/pyridine/acetic acid) were studied in detail. Systematic variation in the composition of the solvents showed the following to be most effective in separating DNAP amino acids:

A. Chloroform/*n*-propanol/acetic acid (70/30/1, v/v/v).
B. Chloroform/*tert*-amyl alcohol/acetic acid (50/50/3, v/v/v).
C. Benzene/pyridine/acetic acid (60/13/27, v/v/v).

Table 2 records the R_f's of all the DNAP amino acids tested.

TABLE 2
R_f Values of DNAP Amino Acids on Silica Gel G Thin Layers with Various Solvents

Amino acid	Solvent[a]		
	A	B	C
Alanine	0.33	0.58	0.64
β-Alanine	.57	.73	.67
Arginine	.00	.00	.12
Aspartic acid	.02	.09	.53
Glutamic acid	.09	.39	.40
Glycine	.17	.36	.49
Histidine[b]	.00	.00	.10
Isoleucine	.70	.87	.86
Leucine	.65	.84	.83
Methionine	.47	.69	.74
Norleucine	.67	.86	.84
Norvaline	.59	.82	.92
Phenylalanine	.50	.76	.88
Proline	.38	.60	.83
Serine	.04	.12	.39
Threonine	.09	.20	.37
Tryptophan	.46	.69	.52
Tyrosine	.29	.36	.75
O-Mono DNAP tyrosine	.00	.00	.15
Valine	.60	.84	.92
OH[c]	.80	.92	1.00
NH_2[d]	Streaks	Streaks	.86

[a] See text for the compositions of the solvents.
[b] *N*-α-DNAP histidine.
[c] 3-Amino-4,6-dinitrophenol.
[d] 4,6-Dinitro-*m*-phenylenediamine.

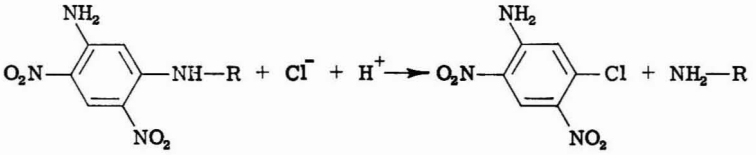
Application to End Group Determination of Insulin. Bovine insulin was chosen to test the applicability of dinitrofluoroaniline as a reagent to detect *N*-terminal amino acids because it is readily available and of known structure. Insulin has two *N*-terminal groups, glycine and phenylalanine. Hydrolysis of DNAP insulin as described under methods gave distinct spots on the chromatogram (solvent B) at R_f's corresponding to glycine and phenylalanine. Another spot corresponding to dinitroaminophenol also appeared as well as some substances of low R_f that probably correspond to partial hydrolysis products of the DNAP protein.

DISCUSSION

The intensification of the color of DNAP amino acids with nitrogen dioxide and β-naphthol is based on the knowledge that NO_2 can diazotize aromatic amines (6). It was tried after it was found that the aqueous diazotizing reagent suggested by Bergmann (1) proved to be unsatisfactory on silica gel thin-layer chromatograms because the strongly acid nitrous acid solution caused elution and diffusion of the spots. The use of a toluene solution of nitrous acid (or dinitrogen trioxide) suggested by Ratney (7) avoided this problem but it has proved somewhat unreliable. The nitrogen dioxide method introduced here is more reliable when done correctly and is very much more convenient.

On chromatograms which have not been treated with nitrogen dioxide, the faintest visible spot contains about 0.2 μg. Diazotization with NO_2 and coupling with β-naphthol does not increase visibility very much (about 0.1 μg is visible) but the spots are turned from yellow to brown by the treatment and become much clearer and better defined.

In experiments on the hydrolysis of DNAP proteins and peptides, it was found that hydrochloric acid almost always produced chromatographic artifacts which are probably due to removal of the DNAP group from the labeled amino acids by reaction with chloride ion:



Sulfuric and perchloric acids produced only small amounts of artifacts and this may be due to the sulfate and perchlorate ions being less nucleophilic. Perchloric acid was finally chosen for the hydrolysis because it produced the cleanest chromatograms with the fewest artifacts on them. However, it was noticed that the perchloric acid was extracted into the ethyl acetate and caused the formation of crescent-shaped spots with higher than normal R_f values. When the extracts were shaken vigorously with saturated potassium chloride solution, insoluble potassium perchlorate formed, thus effectively removing the acid interference.

In the chromatography of DNAP insulin hydrolyzates, it was noticed that brown materials were produced that ran close to the solvent front; they were eliminated by spotting the plates in a nitrogen atmosphere. Apparently, the brown substances are oxidation products.

The extent of incorporation of dinitroaminophenyl groups into the insulin molecule was measured spectrophotometrically. It was assumed,

as a working approximation, that the DNAP groups in insulin absorb at the same wavelength and with the same molar extinction coefficient as DNAP phenylalanine and that each chromophoric group absorbs light independently of the others. The extent of incorporation of DNAP groups can then be calculated from the equation:

$$n = \frac{dvM_0}{\epsilon_0 lw - dvM'}$$

where n = degree of substitution,
d = observed optical density,
v = volume of solution substance is dissolved in,
M_0 = molecular weight of unsubstituted protein,
ϵ_0 = molar extinction coefficient of chromophoric group (27,460 for the DNAP group),
l = path length of spectrophotometer cell,
w = weight of substance taken, and
M' = molecular weight increment of chromophoric group (182 for the DNAP group).

The hydrolysis of DNAP insulin clearly showed the presence of glycine and phenylalanine as *N*-terminal groups, thus proving the effectiveness of dinitrofluoroaniline as a reagent for their determination. In most experiments, 500 μg of DNAP insulin was used but only a part of the solution of DNAP amino acids obtained was spotted on the thin-layer chromatograms. By use of the ultramicro method described above, 100 μg of insulin yielded clearly visible spots of DNAP alanine and DNAP glycine; satisfactory results could probably be obtained with about 50 μg. With a more typical protein such as hemoglobin, which has a molecular weight more than ten times that of insulin, it would probably require at least 500 μg to obtain a reasonable amount of labeled *N*-terminal amino acid.

It was found that several DNAP substituted materials of low R_f are formed during the hydrolysis of DNAP insulin. These are probably DNAP peptides formed by partial hydrolysis. Preliminary experiments showed that more stringent hydrolysis conditions (longer time, more concentrated acid) decreased the amounts of these partial hydrolysis products but did not correspondingly increase the amounts of DNAP amino acids formed because they underwent partial destruction.

SUMMARY

2,4-Dinitro-5-fluoroaniline was reacted with various amino acids and proteins. The *N*-2,4-dinitro-5-aminophenyl (DNAP) derivatives of twenty amino acids were prepared and three solvent systems which can effect

their separation on thin-layer chromatograms were developed. DNAP insulin was prepared and it was shown that *N*-terminal amino acids can be determined starting with 50 μg of this derivative or 100 μg of insulin. The color of DNAP amino acids can be intensified on thin-layer chromatograms by first exposing the chromatograms to nitrogen dioxide fumes and then spraying with a solution of β-naphthol and triethylamine in benzene; this permits the detection of as little as 0.1 μg (about one nanomole) of DNAP amino acid.

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ANALYTICAL BIOCHEMISTRY 19, 366–373 (1967)

Automated Ion-Exchange Chromatography of α- and β-Aspartyl Oligopeptides

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The presence of several β-aspartyl di- and tripeptides in human urine (1) and in enzymic hydrolyzates of protein (2) can be effectively studied only when specific analytical methods are available, not only for these compounds, but also for the α-isomers from which the β-peptides may in part derive. Because of the structural and ionic similarity of aspartyl peptides they are difficult to separate. The buffer elution systems used by others (3, 4) to separate amino acids and other constituents of physiological fluids cause the β-aspartyl peptides to separate poorly.[1] The low pK_1 range of these peptides relative to most amino acids made it likely that buffers of lower pH might improve resolution on cation-exchange resins. Of several buffers with pH ranges below that of citrate buffer, one utilizing the primary dissociation of phosphoric acid has proved most useful.

Although it has not been possible to resolve all of the 15 urinary β-aspartyl peptides with a single analytical chromatogram, enough of them emerge singly on a cation-exchange column to have permitted a useful study of their urinary excretion pattern (5) and of the content of β-aspartylglycine in enzymic hydrolyzates of several proteins (2).

This paper gives elution times on cationic and anionic exchange resins as well as calibration constants for the synthetic β-L-aspartyl di- and tripeptides corresponding to those found in urine, for the synthetic α-L-aspartyl isomers, and for several amino acids which, if present, would emerge in the elution patterns. A scheme is shown for the complete separation of the β-aspartyl peptides by sequential use of cation- and anion-exchange columns.

[1] F. E. Dorer, E. E. Haley and D. L. Buchanan, unpublished observations (1965).

MATERIALS AND METHODS

Materials. Synthetic α- and β-L-aspartyl peptides were prepared in this laboratory (6). Synthetic α- and β-L-aspartylglycyl-L-glutamine were prepared in the same way to yield amorphous, hygroscopic products, homogeneous by paper and column chromatography. The calibration constants for these peptides were obtained from analyses of the three amino acids produced on acid hydrolysis in equimolar amounts. Peroxide-free methyl Cellosolve, ninhydrin, and hydrindantin were from Pierce Chemical Company, and prepurified grade nitrogen was obtained from Matheson Company. The components of the amino acid analyzer were purchased from Technicon Chromatography Corporation.

Ninhydrin Reagent. Ninhydrin reagent was prepared by dissolving 15 gm of ninhydrin and 1.125 gm of hydrindantin in 488 ml of methyl Cellosolve. To this were added 263 ml of 4 *M* sodium acetate buffer, pH 5.5, and finally 3 liters of 50% (v/v) methyl Cellosolve. Before and after each addition all liquids were vigorously gassed with nitrogen for 10 min, and the final solution was gassed for 1 hr.

Procedure. The cation-exchange columns packed with Technicon Chromobeads, Type A, or Dowex 50-X8 graded by flotation (7) to 25 $\pm$ 5 μ spheres were 0.624 $\times$ 128 cm and kept at 60°. The eluent, sodium phosphate buffer, pH 1.82 and 0.5 *M* with respect to sodium, was made up with 0.25 mole of $Na_2HPO_4 \cdot 7H_2O$ and 0.75 mole of H_3PO_4 per liter. The column was pumped at 0.50 ml/min for elution, for stripping after each analysis with 50 ml of 0.2 *N* NaOH, and for regeneration with the phosphate buffer for 5 to 6 hours.

Columns of Dowex 1-X4 graded by flotation (7) to 20 to 30 μ spheres were 0.624 $\times$ 134 cm and were kept at room temperature. They were operated either in the acetate or the formate form, using 0.5 *M* acetic acid or 0.1 *M* formic acid at a flow rate of 0.50 ml/min.

Samples typically contained 0.4 to 0.8 μmole of each compound dissolved in water and were applied to the column with air pressure. During elution approximately one-fifth of the column effluent entered the analytical system diagrammed in Figure 1. The remainder went to a fraction collector set to index at 5 or 10 min intervals. The fractions from the cation-exchange columns were desalted on Dowex 50 by displacement with pyridine (8), those from the anion-exchange columns were evaporated to dryness *in vacuo* to remove the volatile solvent. The material so obtained was used to establish the identity of peaks in question by paper chromatography.

Descending paper chromatography was carried out on Whatman No. 1 paper with 1-butanol/acetic acid/water (4/1/1) and pyridine/acetic acid/

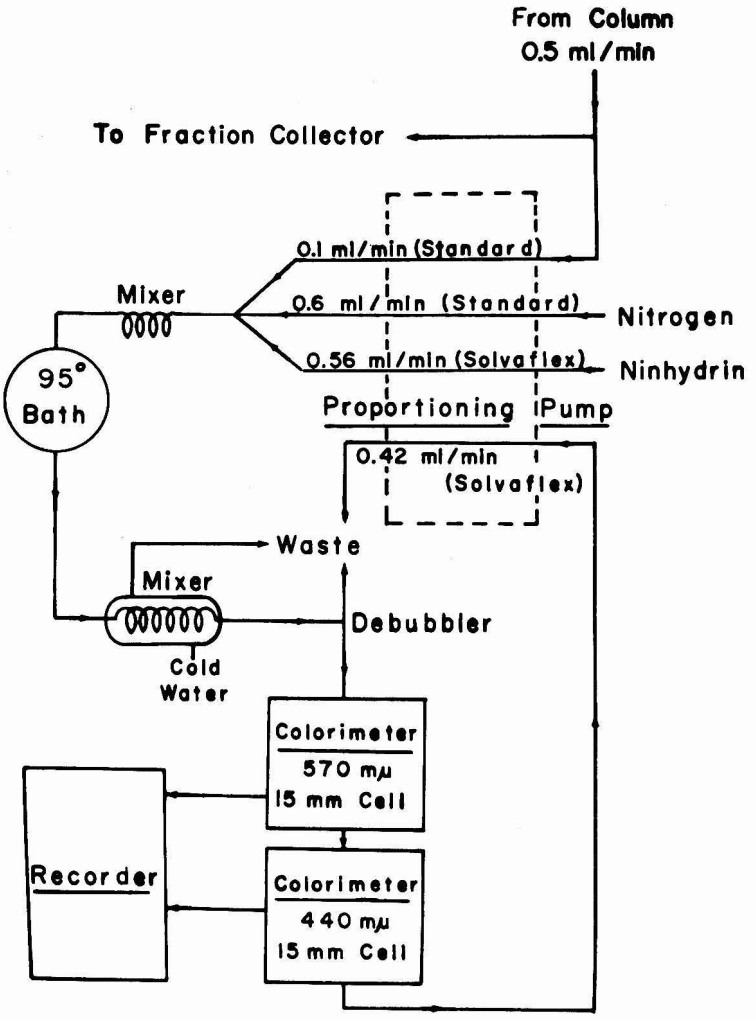

FIG. 1. Schematic diagram of proportioning pump and analytical system of Technicon amino acid analyzer.

water (10/7/3). With peptides of low mobility the solvent was allowed to run off the end of the paper for 2 or 3 days. The dried paper was sprayed with 0.3% ninhydrin in 95% ethanol and heated to 120° for 3 min. As noted previously (1) all of the β-aspartyl peptides gave characteristic blue colors.

L-Leucine (1.0×10^{-4} *M* in water) was used as an external standard before and after each analysis. The optical density plateau corresponding to the 15 min when the solution was being drawn through the sample tube

was found to be linear with leucine concentration and was used to correct the areas of all peaks for variations in the flow rates of proportioning pump tubes, ninhydrin reactivity, and colorimeter response. The mean optical density of the leucine plateau before and after each run was divided by 0.486, the mean optical density of the leucine standard for the first run. This factor was then multiplied by the area of each peak divided by the amount (in μmoles) of compound put on the column to give the calibration constant. Peak areas were determined by the height times width method (3).

RESULTS AND DISCUSSION

Cation-Exchange Columns. Several sodium phosphate and sodium chloride buffers and mixtures thereof were tested. Each had a pH between 1.6 and 3.0 and a sodium concentration between 0.05 and 1.0 M. The buffer described met the primary criterion established, namely, the complete resolution of β-L-aspartylglycine, and also resolved several but not all of the other physiological β-aspartyl peptides of urine.

Pumping the columns at 1.0 ml/min, using pH and concentration gradients in elution, and lowering the temperature below 60° all failed to improve the resolution. At 60° some of the α-aspartyl peptides showed evidence of slight decomposition with elevation of the baseline on one or both sides of the peak. The loss in peak area was estimated to be always less than 10% and was fully compensated by the method of calibrating the color yield.

Table 1 gives the elution times of 30 peptides, the protein amino acids that emerge within 16 hr, and ammonia. A comparison of the performance of the Dowex 50 and Chromobead columns showed only minor variations in elution times of compounds that were run on both. Although of equal length, the Dowex 50 column seems to have slightly greater exchange capacity. In no case was there a significant reversal of sequence between the two columns. During the first 300 min satisfactory resolution is achieved if adjacent peaks are separated by 10 min; later in the run 20 min is needed.

The dipeptides come off in the same order as their C-terminal amino acids in citrate buffer elution (3, 4). Consistent with their lower pK_1 (8, 9), the β-aspartyl peptides emerge earlier than their α-isomers. Ammonia, which comes off with the basic amino acids in elution with citrate buffers (3, 4), here emerges near alanine as it does with HCl elution (10).

As anticipated from the position of ammonia during elution, an ammonia plateau begins about 7 hr after regeneration is begun. With buffer prepared as described it increases the baseline absorbance by about 0.02. When the buffer is prepared from monosodium phosphate, even recrystal-

TABLE 1
Elution Times and Calibration Constants

Compound	Elution Time (Minutes)				Calibration Constant
	Chromobead	Dowex 50	Dowex 1 (Formate)	Dowex 1 (Acetate)	
β-Asp-Gly-Asn	161	167	375	785	13.7
β-Asp-Gly-Gln	161		335	690	12.2
β-Asp-Ser	168	176	410	930	18.1
β-Asp-Thr	174		315	690	15.3
β-Asp-Gln	176		350	745	17.4
β-Asp-Asn	178		375	825	21.0
β-Asp-Gly-Gly	190	196	315	690	14.0
β-Asp-Gly-Ala	204		270	560	15.2
β-Asp-Gly	215	222	345	775	20.2
α-Asp-Gly-Gln	230				36.0
α-Asp-Gly-Asn		235			38.9
α-Asp-Ala	237	242	295	635	21.5
β-Asp-Thr		260			31.7
α-Asp-Asn		261			33.4
Aspartic Acid	249	266	230	525	28.9
Serine	249	266			29.8
α-Asp-Ser	255	265			39.4
β-Asp-Gly-Pro	265	265	320		17.4
β-Asp-Gly-Val	265	270	300		14.9
Threonine	265	278			28.2
α-Asp-Gln	273				35.5
α-Asp-Gly-Gly	292				42.6
α-Asp-Gly-Ala		310			40.1
α-Asp-Gly	303	316	144	290	41.7
β-Asp-Val	335		280		14.7
Glutamic Acid	336	353	120	220	31.0
Glycine	336	360	70	75	31.5
α-Asp-Ala	362	380			38.4
α-Asp-Gly-Pro		395			40.8
α-Asp-Gly-Val		415			39.7
Alanine	407	424			32.0
NH_4Cl	407	436			19.6
Proline	429	446			7.0[a]
β-Asp-Ile	503		315		15.1
β-Asp-Leu	548		355	750	20.2
α-Asp-Val		573			39.3
Valine	643	656			30.0
α-Asp-Ile		895			39.1
α-Asp-Leu		943			40.1

[a] Read at 440 mμ.

lized several times, the plateau is considerably higher. If regeneration is continued for 6 hr before the sample is applied, the baseline increase precedes the first peptide peak, and the baseline will then remain constant.

Anion-Exchange Columns. The anion-exchange columns generally showed poorer resolution than the cation-exchange columns. Increasing the temperature and changing the flow rate and acid concentration did not improve the resolution. Nevertheless, these columns proved useful in their ability to separate those β-aspartyl peptides which were confluent on the cation-exchange columns. Consistent with their higher pK_2 values (8, 9), the α-aspartyl peptides emerge earlier than their β-isomers. Since ammonia comes off the columns at the start of the elution, the baseline remains stable throughout the entire analysis. There are slight but significant differences in the relative positions of some of the β-aspartyl peptides on the acetate and formate forms of the Dowex 1 columns (Table 1).

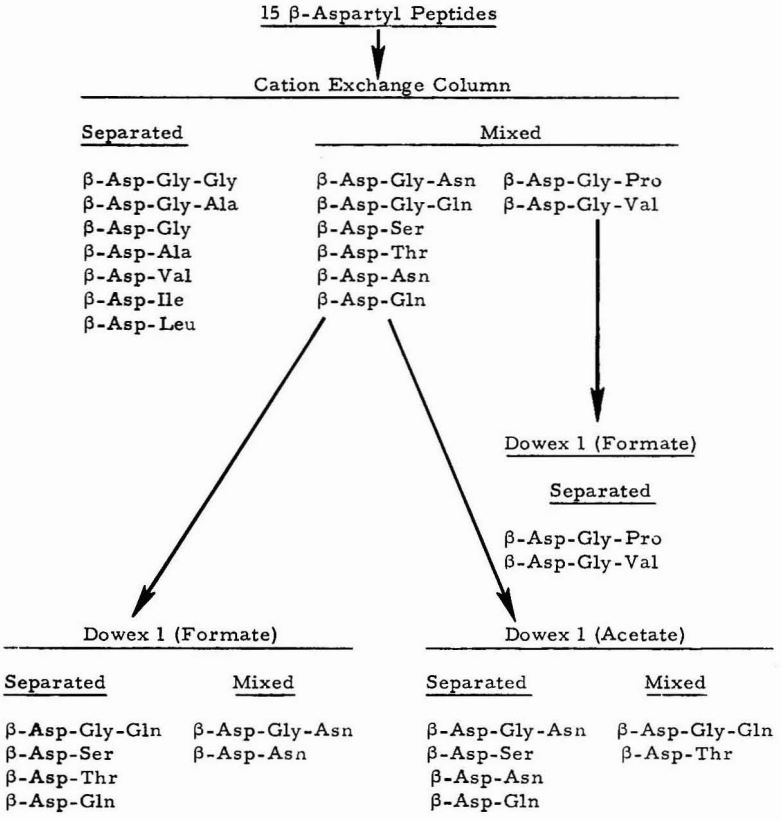

FIG. 2. Scheme for separation of fifteen β-aspartyl peptides.

Complete Separation of β-Aspartyl Peptides. A scheme for the complete separation and individual measurement of the β-aspartyl peptides in this study is shown in Figure 2. Of the fifteen β-aspartyl peptides, seven may be measured directly in the analysis on the cation exchange column. The remaining eight emerge from the column in two groups, one of 6, and the other of 2 peptides. Each of these groups is further analyzed by pooling of the relevant tubes in the fraction collector, desalting on Dowex 50, and rechromatography of the fraction on an anion exchange column. Thus a total of four analyses is required for a sample containing all fifteen β-aspartyl peptides. If interest is centered on a smaller number of peptides, fewer analyses are needed.

Calibration Constants. No significant difference in color yield was noted when the same compound was analyzed on a cation- or anion-exchange column. Because with some of these compounds slight isomerization, cyclization, or hydrolysis may occur under acid conditions, the calibration constants given are deemed more reliable than relative color values obtained by drawing standard solutions through the analytical system.

It is noteworthy that on an equimolar basis the β-aspartyl peptides all gave colors less intense than those produced with free amino acids, while the color values of α-aspartyl peptides were considerably higher. The ratio of color yields between α- and β-isomers was highest with the tripeptides. These findings indicate extensive breakdown of α-aspartyl linkages during the ninhydrin reaction at 95° and are consistent with previous findings on the relative ease of hydrolysis of α- and β-L-aspartylglycines (11).

SUMMARY

Useful resolutions have been achieved in the automated chromatography of 30 α- and β-L-aspartyl di- and tripeptides by sequential use of analytical columns of cation- and anion-exchange resins. The effluent is analyzed with the Technicon amino acid analyzer system. In this way all of the β-aspartyl peptides being studied may be separated from each other. Elution times and calibration constants are given for the peptides and for several amino acids for comparison.

ACKNOWLEDGMENTS

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Some Characteristics of the Fluorescence of Quinine

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Quinine in dilute acid solutions is quite stable and has long enjoyed favor as a fluorescence standard (see, for example, reference 1). It has become commonplace to express analytical results in terms of "quinine units," i.e., the fluorescence observed from quinine solutions of stated composition under a given set of experimental conditions. In addition, Melhuish has studied the emission spectrum and quantum yield of quinine in dilute sulfuric acid and has proposed that the spectrum be used as a standard for calibrating spectrofluorometers (2), and that quinine be used as a standard for determining fluorescence efficiencies by the comparative method (3, 4). Melhuish obtained a quantum yield of 0.55 for quinine in 0.1 N H_2SO_4 (3, 4), and this value has been used in a number of laboratories (e.g., 5–9). Using quinine as a standard, Parker and Rees (7) obtained quantum yields for several compounds which were in agreement with literature values obtained by other methods.

However, recent communications (10, 11) have pointed out that the excitation spectrum of acidic quinine does not match the absorption spectrum, a fact which would imply that the quantum yield is dependent on the excitation wavelength. This finding would be contrary to the known behavior of the vast majority of fluorescent organic compounds (12). Furthermore, one recent report (13) disputes the quantum yield figure obtained by Melhuish (3) as being too high. Because of the wide use of quinine in fluorometry, it seemed important to reinvestigate the emission properties of this compound. The data presented in this communication, including fluorescence polarization measurements and observations on the fluorescence of 6-methoxyquinoline, the parent compound of quinine, show that the quantum efficiency of quinine in dilute acid is indeed dependent on the excitation wavelength and that the fluorescence probably arises from more than one excited singlet state. Thus, the fluorescence characteristics of quinine are among the most unusual so far observed for any compound in solution.

MATERIALS AND METHODS

Fluorescence was measured with the Aminco-Bowman spectrophotofluorometer fitted with an off-axis ellipsoidal mirror accessory for the 150 W Hanovia high-pressure xenon arc lamp. An RCA 4472 photomultiplier tube was the detector used in conjunction with an emission monochromator grating blazed for maximum response at 500 mμ. Excitation and emission spectra were corrected (unless otherwise stated) by applying calibration factors for the excitation source and detector response, determined as previously described (14). All measurements were made in a short path length microcell in order to eliminate artifacts due to inner-filter effects (15). The spectrophotofluorometer was fitted with four continuously variable slits placed as follows: (*1*) At the entrance to the excitation monochromator. (*2*) On the excitation face of the cuvet holder, about 1 cm from the cuvet surface—slit position #3 as shown diagrammatically by Price *et al.* (16). (*3*) On the adjacent (emission) face of the cuvet holder, also about 1 cm from the cuvet—slit position #6 of reference 16. (*4*) At the exit of the emission monochromator. In addition, there were two fixed slits 1 mm in width, placed between the cuvet and the second and third variable slits. The function of the fixed slits was to limit the width of the excitation and emission rays to avoid production of signal from the microcuvet walls. The two variable excitation slits were kept the same, as were the two corresponding slits on the emission side. Under these conditions, the dispersion was found to be 12 mμ/mm slit width.

Fluorescence quantum yield was measured by the comparative method (7, 14). The relative quantum yields of two substances (or the same substance excited at two different wavelengths) was determined from the formula (17):

$$\frac{Q_1}{Q_2} = \frac{\phi_2}{\phi_1} \times \frac{F_1}{F_2} \times \frac{A_2}{A_1}$$

where Q is the quantum yield, F is the area under the corrected emission spectrum, and ϕ and A are the relative photon output of the light source and optical density of the given solution at the wavelength of excitation.

Fluorescence polarization spectra were obtained with glycerol solutions maintained at 8°. The polarizers and method of determining polarization have been described (18). The polarization (P) was determined from the formula (19):

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}}$$

where I is the observed intensity, the first and second subscripts refer to the orientation of the polarizer and analyzer, and G is the grating correction factor, given by $G = I_{HV}/I_{HH}$.

Optical density and absorption spectra were determined with Beckman DU and Cary model 11 spectrophotometers.

Quinine hydrochloride (crystalline) was a product of Merck & Co., Inc., Rahway, N. J. Elemental analysis[1] for $C_{20}H_{24}O_2N_2 \cdot HCl \cdot 2H_2O$: C 61.5, H 8.0, N 7.3 (calculated: C 60.5, H 7.3, N 7.1). Quinine was also obtained from Calbiochem and recrystallized twice from ethanol-water mixtures. Both samples yielded only a single fluorescent spot when chromatographed on Eastman silica gel thin-layer film in the following systems: (*1*) methanol (20), (*2*) *n*-propanol/formic acid (80/20) (21), (*3*) chloroform/methanol/acetic acid (75/20/5) (21). 6-Methoxyquinoline (Eastman) was extracted with dilute alkali, dissolved in dilute sulfuric acid, and treated with activated charcoal. The resultant solution was colorless. On thin-layer chromatography in *n*-propanol/formic acid (85/15), only one fluorescent spot with an R_f of 0.5 was noted. Fluorescein and rhodamine B were obtained from National Aniline Division of Allied Chemical Co. Fluorescein was purified by conversion to the diacetate and recrystallization from acetic anhydride (22); it was hydrolyzed with dilute KOH prior to use. Rhodamine B was recrystallized from ether/benzene. Crystalline bovine serum albumin, lysozyme, and ovalbumin were obtained from Armour Laboratories. 1-Dimethylaminonaphthalene-5-sulfonic acid was prepared by the method of Laurance (23). 8-Anilinonaphthalene-1-sulfonic acid was purchased from J. T. Baker Chemical Co. and recrystallized as the magnesium salt (24). Spectro-quality glycerol obtained from Matheson, Coleman & Bell did not exhibit fluorescent impurities.

RESULTS

Absorption and Excitation Spectra. The extinction coefficient of quinine in 0.1 N H_2SO_4 at the 345 mμ peak was found to be 5.02×10^3, and the absorption spectrum is given in Figure 1. The corrected excitation spectrum is superimposed on the absorption spectrum and shows that there are deviations in the long-wavelength region, in confirmation of the report by Børresen (11). The quantum yield is clearly higher at the low energy edge of the long-wavelength band than at shorter wavelengths. Below about 340 mμ, however, no significant difference between absorption and excitation spectra were noted.

Emission Spectra. It was observed that the spectrum of quinine emission was dependent on the wavelength of the exciting light (Fig. 2). The dif-

[1] Elemental analysis by J. Alicino, Metuchen, N. J.

ference in emission spectra obtained with 340 and 390 mμ excitation can easily be seen with the uncorrected spectra of Figure 2*A*. Here, approximately 30-fold greater amplifier sensitivity was required to normalize the spectrum elicited by 390 mμ excitation. The 8 mμ shift of the spectrum toward longer wavelengths obtained with 390 mμ light cannot be explained by nonlinearity of phototube response, because the same results can be

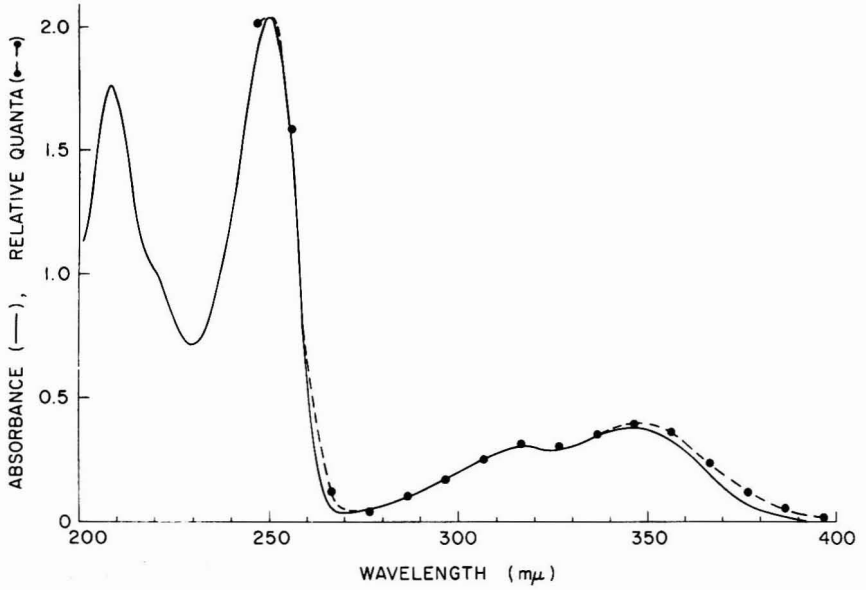

FIG. 1. Quinine absorption and excitation spectra in 0.1 *N* H_2SO_4. Concentrations used were 10^{-4} *M* for the absorption spectrum and 10^{-6} *M* for the excitation spectrum. Emission was observed at 450 mμ. Excitation band width, 6 mμ.

obtained when the emission intensities are kept constant and the input energy is changed by variation of the excitation slits. The corrected emission spectra are shown in Figure 2*B* and indicate that the peak of 390 mμ excited emission is at 466 mμ as compared with 458 mμ obtained with 340 mμ input. The spectra obtained with excitation at wavelengths shorter than 350 mμ (such as into the 250 mμ absorption band) did not differ from the 340 mμ excited spectrum.

Since quinine is a natural product, there was still the possibility of impurities being present despite the chromatographic evidence of homogeneity; therefore it was decided to investigate the emission of 6-methoxyquinoline, the parent compound. In 0.1 *N* H_2SO_4, this compound exhibited a brilliant blue fluorescence, and the absorption spectrum had some similarities with that of quinine. The spectral parameters are listed in

Table 1. It should be noted that, whereas the spectrum of quinine has a shoulder at 315 mμ and a maximum at 345 mμ, the 313 mμ absorption band appears not as a shoulder but as a peak which is of slightly greater intensity than the peak at 337 mμ. As in the case of quinine, it was possible to obtain different emission spectra from 6-methoxyquinoline in

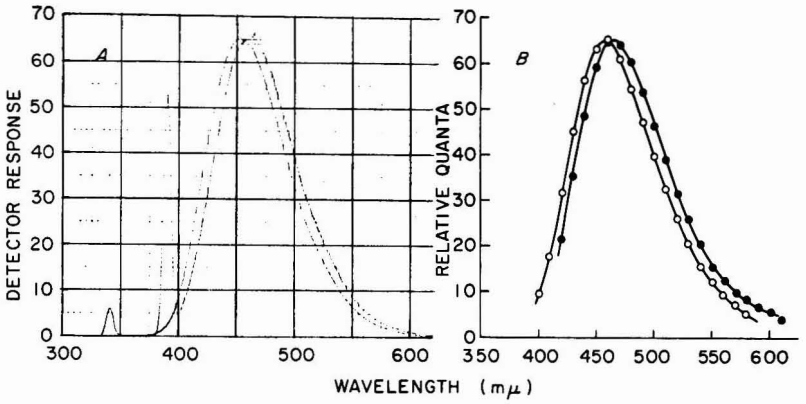

FIG. 2. Variation in emission spectrum of quinine. (*A*) Direct tracing of uncorrected emission spectra obtained by exciting at 340 and 390 mμ. As shown by the scatter peak, the band width at half maximum was 8 mμ. Normalization of the spectra was accomplished by raising the amplifier gain about 30-fold. (*B*) Corrected spectra corresponding to those of *A*. Conditions as in Figure 1.

0.1 *N* H_2SO_4 by exciting at 337 mμ (or lower wavelengths) and at 390 mμ (Table 1). One can thus feel confident that the changes in emission spectra in quinine are due to properties inherent in the electronic structure of the 6-methoxyquinoline nucleus rather than due to impurities or even of the substituent at the 4-position.

The change in quinine emission was also observed in the following concentrations (*N*) of sulfuric acid: 0.01, 0.05, 0.50, 1.0, 5.0, 10.0, and 20.0. The presence of more than one species with different ionization states is

TABLE 1

Spectral Properties of 6-Methoxyquinoline in 0.1 *N* H_2SO_4 at 23°

Measurement	λ_{max}	ϵ
Absorption	248 mμ	3.10×10^4
	313	4.29×10^3
	337	3.97×10^3
Emission	442 mμ	when exciting at 337 mμ
	450	when exciting at 390 mμ

The emission data were obtained with 8 mμ band width in both monochromators and refer to corrected emission spectra (quanta vs. wavelength).

thus ruled out, as would be expected since the pK_a of the nuclear nitrogen is 4.7 (25). The phenomenon of excited-state ionization studied by Weller (26) is by now well known and could provide a mechanism for the appearance of two different species in the excited state even when there is only one species in the ground state. However, blocking of any effect exhibited by a group having an excited-state pK_a as low as — 1.3 should easily have been detected in 20 N H_2SO_4. It is difficult to see how the excited-state ionization mechanism could account for any of the observations found here for quinine in any case, since it has been observed in this laboratory that the fluorescence of quinine, when the proton is lost from the ring nitrogen, has a maximum below 400 mμ.

Polarization of the Absorption and Emission Bands. The relative orientations of the absorption oscillators in a molecule can be studied by measurement of the degree of fluorescence polarization in rigid solutions in which Brownian motion is negligible (27). The excitation and emission spectra of quinine in glycerol containing 0.1 N H_2SO_4 are shown in Figure 3*A*. Clearly, the observation that the emission spectrum is

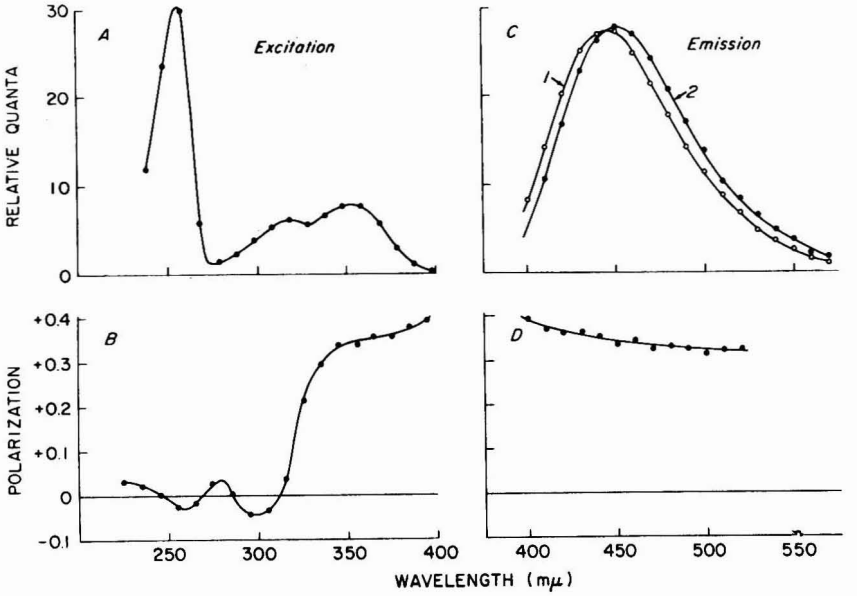

FIG. 3. Fluorescence and polarization of fluorescence spectra in glycerol containing 0.1 N H_2SO_4 at 8°. (*A*) Excitation spectrum. (*B*) Fluorescence polarization spectrum obtained by maintaining the emission monochromator at 450 mμ while varying the excitation wavelength. (*C*) Emission spectra obtained by exciting at 340 mμ (curve *1*) and 390 mμ (curve *2*). (*D*) Polarization in the emission band, with excitation constant at 340 mμ. The polarization spectra were obtained with a band width of 8 mμ in each monochromator.

dependent on the wavelength of excitation is valid for glycerol as well as aqueous solutions. With the emission monochromator set at 460 mμ, the polarization of fluorescence obtained at different excitation wavelengths was measured and is shown in Figure 3*B*. The spectrum is similar to that published by Feofilov (28), although he did not show any points below 310 mμ. The polarization spectrum indicates clearly that there are at least two and probably three electronic transitions in the long-wavelength region (including the 315 mμ shoulder of the absorption spectrum); if there were only a single transition in this region, the fluorescence polarization would be high (near 0.5) and constant. Instead, the data show that the polarization decreases rapidly in the region of the 315 mμ shoulder, and increases at the long-wavelength edge of the 345 mμ band. At the peak of that band the polarization is only 0.34; a possible explanation of these observations is given below.

When the wavelength of the excitation is kept constant and the emission monochromator is varied, the degree of polarization of the emission band can be measured; and the results for quinine in glycerol are shown in Figure 3*D*. The high polarization in the short-wavelength part of the emission is probably real rather than due to scattered light reaching the detector, in view of the good resolution of the instrument under the conditions employed. The shown decrease in polarization within the main portion of the emission band must also be real because the polarization measurements could be made with a precision of $\pm$ 0.002 in P at the peak of the emission band and at least $\pm$0.02 at any point shown (18).

Polarization of the Emission Bands of Other Fluorescent Compounds. According to the classical view of fluorescence (e.g., 12), the emission of light arises only from the first excited singlet state. For this reason, there is generally thought to be only one emission oscillator although several electronic transitions may take part in the absorption of light. Thus, the emission band should have a constant degree of fluorescence polarization; but this was clearly not the finding in the case of quinine, as shown in the previous section. Some workers (29) who reported variations in the degree of fluorescence polarization of the emission band may have been in error due to instrumental artifacts, as pointed out in a critical article by Klochkov and Neporent (30). If the present results on quinine emission polarization shown in Figure 3*D* had been due to such instrumental artifacts, such effects should also distort the fluorescence polarization spectra of other compounds. To test this possibility, the polarization in the emission bands of fluorescein and rhodamine B were examined, since it is known that the polarization should be constant in both cases (30). The results are shown in Figure 4, which also gives polarization data for the emission bands of 8-anilinonaphthalene 1-sulfonate and 1-dimethyl-

aminonaphthalene 5-sulfonate, as well as for a complex of 8-anilinonaphthalene 1-sulfonate and serum albumin in water. In each case, the fluorescence polarization is constant throughout the emission band. The wavelength region covered by these measurements (Fig. 4) is 440–600 mμ,

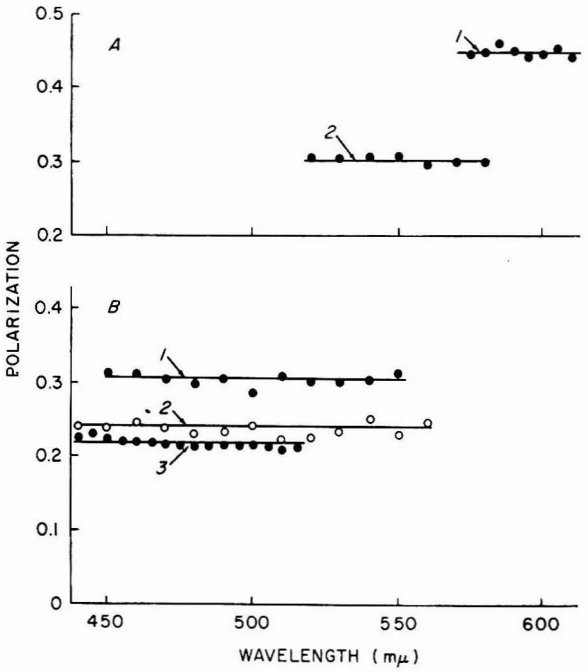

FIG. 4. Polarization of the emission band (substances having constant degrees of fluorescence polarization). (*A*) Glycerol solutions of rhodamine B (curve *1*) excited at 510 mμ and fluorescein (curve *2*) excited at 490 mμ at 8°; concentrations, 1×10^{-6} *M*. (*B*) Glycerol solutions of 1-dimethylaminonaphthalene 5-sulfonate (curve *1*) excited at 330 mμ and 8-anilinonaphthalene 1-sulfonate (curve *2*) excited at 350 mμ at 8°; curve *3* was obtained with a 10^{-5} *M* solution of 8-anilinonaphthalene 1-sulfonate containing 1.5×10^{-4} *M* bovine serum albumin in 0.1 *M* Tris-Cl^- buffer, pH 7.0, at 25°.

a span encompassing most of the emission of quinine in glycerol. Such data, therefore, strongly indicate that instrumental artifacts did not influence the results in the case of quinine.

It should be noted that low-temperature studies on aromatic compounds by Zimmermann and Joop (31–33) have shown that the polarization in the emission band may indeed be inconstant. In most cases the inconstancies can be attributed to vibronic coupling (34), but the variation of polarization in the emission of indole was attributed to emission from two singlet states (31). Studies by Konev *et al.* (35) showed that there are variations

in the polarization of the emission spectra of indole-containing substances such as tryptophan and proteins. Indeed, the only other substances examined in the author's laboratory which have variations in the polarization in the emission band have been proteins and tryptophan, as illustrated in Figure 5. The results essentially confirm those of Konev *et al.* (35),

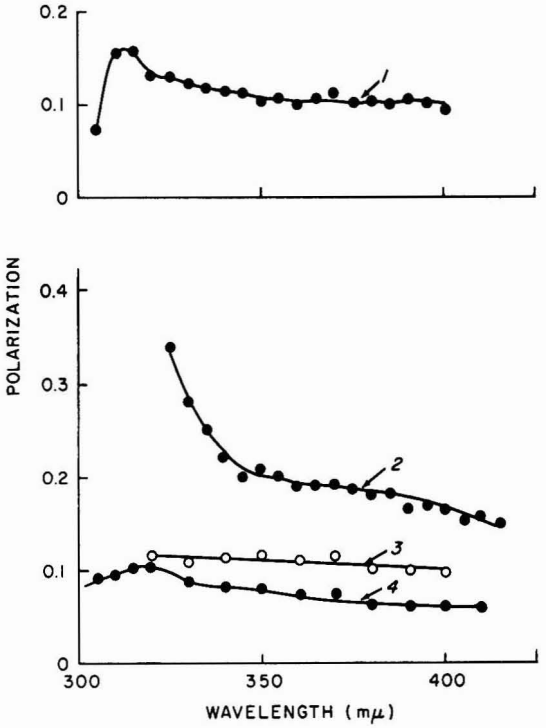

FIG. 5. Polarization of emission bands of substances having wavelength-dependent polarizations: (*1*) ovalbumin, (*2*) bovine serum albumin, (*3*) tryptophan, 10^{-5} *M* in glycerol, (*4*) lysozyme. Protein concentrations were all 1.0 mg/ml in 0.1 *M* Tris-Cl^- buffer, pH 7.0. Curves *1* and *4* were obtained at 25°; curves *2* and *3* were obtained at 8°. Excitation, 280 mμ. Band width, 6 mμ for excitation and 12 mμ for emission.

although the variation in tryptophan polarization is considerably smaller than would be suggested by their results.

Relative Quantum Yields of Quinine Sulfate with Different Excitation Wavelengths. Although the relative fluorescence quantum yields of quinine excited at different wavelengths could in theory be calculated from the normalized absorption and corrected excitation spectra (Fig. 1), more precise measurements were made on a relatively concentrated solution (7.42×10^{-5} *M*) using several excitation wavelengths and taking into

account the changes in emission spectra. This high concentration was chosen so that the optical density and fluorescence measurements could be carried out on the same solution. The results are shown in Table 2 along with the factors that had to be applied to correct for absorption of incident light. These correction factors are simply the antilogarithms of the products of the absorbancies for a path length of 1.0 cm $\times$ 0.145, the effective path length of the microcuvet.

TABLE 2
Relative Quantum Yields of Quinine in 0.1 N H_2SO_4 at 25°

Excitation wavelength, mμ	Factor[a]	Relative Q[b]
250	1.07	1.02
313	1.10	[1.00]
345	1.13	0.98
348	1.13	0.99
366	1.07	1.09
380	1.02	1.20
390	1.01	1.23

Measurements were made on a 7.42×10^{-5} M solution of quinine in each case except 250 mμ, when a 7.42×10^{-6} M solution was used. The total error is thought to be 3% (see text).

[a] Correction applied for absorption of incident light.

[b] The yield from 313 mμ excitation is set equal to 1.00.

Since the determinations were made on the same solution, errors due to refilling of the cuvet or other sample handling were avoided; measurements in triplicate of the relative quantum yields (with reference to the value obtained by exciting at 313 mμ) agreed to ± 0.01. However, the accuracy of the measurements depends also on knowing the photon output of the energy system (lamp plus monochromator) at each wavelength; and it is estimated that the accuracy of the calibration is no better than 2%. Still, it is obvious from Table 2 that the quantum yields obtained by exciting at wavelengths $\leqslant$ 348 mμ are nearly equal. At longer wavelengths, however, the quantum yield is as much as 23% higher.

DISCUSSION

It appears that Moss (10) may have been the first to notice the discrepancy between the excitation and absorption spectra of quinine in acid. Børresen (11) also found such a difference and estimated the ratio of quantum yields obtained with excitation at 29,000 cm^{-1} (345 mμ) and 40,000 cm^{-1} (250 mμ) to be 1.47. This particular result is not in accord with data of the present study, which shows that, whereas there are discrepancies in the excitation and absorption spectra, the quantum yields

obtained by exciting at 250 and 345 mμ are, within experimental limits, identical.

Like other workers (5, 7–9), I have employed Melhuish's value of 0.55 as the quantum yield of quinine in 0.1 N H_2SO_4 as a standard for quantum yield determinations (6, 14). Since those studies employed excitation at 348 mμ rather than 366 mμ as used by Melhuish (3), the assumed value of $Q = 0.55$ might have been about 9% too high. (If $Q = 0.55$ at 366 mμ, Q should be about 0.50 when exciting at 348 mμ.) The quantum yield figure given by Melhuish appears to be at least approximately correct, since reasonable quantum yield figures for other substances have been obtained by the comparative method (7). The note by Drobnik and Yearghers (13) stating that the quantum yield of quinine must be no higher than 0.4 is suspect, because of the dearth of experimental details, because of the statement that the quantum yield of quinine was independent of the excitation wavelength up to 380 mμ, a result not in accord with data of Moss (10), Børresen (11), or the present study, and because the extinction coefficient of their quinine sample at 313 mμ was said to be 8×10^3, a value which is about twice the extinction of samples used here or in the study of Parker and Rees (7).

A further note of confusion is contributed by Melhuish's subsequent report (36) that the quantum yield of quinine was the same whether excited at 366 or at 313 mμ! Until such discrepancies are resolved, probably by a consensus of individual reports from different laboratories, the value of 0.55 obtained by 366 mμ excitation should be taken to involve an uncertainty of at least 10%.

In addition to the confusion concerning quinine fluorescence efficiency there are some peculiar characteristics of the fluorescence itself. Before discussing the results of the present study, some pertinent data concerning the fluorescence lifetime of quinine should be recalled. Birks and Dyson (5) and Ware and Baldwin (37) made direct measurements of the fluorescence decay time of quinine in dilute sulfuric acid with a view toward correlating the quantum yield with the "natural" lifetime (the lifetime in the absence of competing quenching processes) as determined by calculations based on absorption intensity measurements. The measured lifetimes of 19–20 nsec were longer by about 25% than would have been predicted by the formula of Strickler and Berg (38). Birks and Dyson (5) suggested that this discrepancy was due to a change in nuclear configuration between ground and excited states—a change which is not considered in the formula. Such configurational change, or relaxation, may also explain the wide separation of the absorption and emission bands of quinine in acid; and indeed Bowen and Seaman (39) postulated some sort of relaxation process to account for the wide separation of absorption and emission

bands in naphthylamine derivatives. Ware and Baldwin (37) chose to attribute the discrepancy between calculated and observed lifetimes to inadequacy of the formulas used for calculation of the natural lifetimes.

On the other hand, the formulas commonly employed to calculate fluorescence lifetimes such as those of Förster (40) or Strickler and Berg (38) require that the lowest singlet absorption band be adequately defined. Berlman and Steingraber (41) were able to utilize the large discrepancy between the calculated lifetime of biphenyl and its observed lifetime, which was about 50-fold larger, as evidence that a hidden singlet transition exists in the long-wavelength absorption band.

While the discrepancy between calculated and observed lifetimes is smaller for the case of quinine than for biphenyl, at least part of the difficulty may be due to the presence of overlapping transitions in the long-wavelength absorption region as shown by the polarization data. Not only is the shoulder at 315 mμ matched by a steep slope in the polarization of the excitation spectrum, but the polarization appears to rise in the long-wavelength region (above 370 mμ). A weak transition centering at perhaps 370 mμ seems possible, although the exact position of the pure transition would be difficult to determine experimentally due to its weakness and the extensive overlap.

Several observations suggest that the emission arises from two different states simultaneously. First, the difference in emission spectrum depending on excitation wavelength is hard to explain any other way if the chemical evidence for purity of both quinine and 6-methoxyquinoline can be believed. Second, the polarization of the emission spectrum is not constant, indicating that emission arises from at least two oscillators and that the oscillators are not mutually parallel. Third, the polarization observed in the bulk of the absorption band of quinine in glycerol is low—only 0.34—compared with the theoretical maximum of 0.50 for the case in which a single anisotropic absorption oscillator is parallel to a single linear emission oscillator. This last result can be explained if emission arises from two singlet states simultaneously, as postulated for the case of indole (31).

The hypothesis that the absorption spectrum of quinine in acid contains a weak hidden transition at long wavelength, and that the emission arises from two states, probably the two lowest singlet states S_2 and S_1, would account for all the observations made in this study. Such a hypothesis would also offer an alternate explanation of the discrepancies noted between calculated and observed fluorescence decay times of quinine (5, 37).

If the fluorescence of quinine arises from both the first and second singlet states, the question arises as to why the internal conversion be-

tween the two states does not occur with complete quantum efficiency. In the case of azulene, it has been postulated (42) that the energy of S_1 is only slightly higher than that of the ground state, S_0. The $S_2 \rightarrow S_1$ transition therefore occurs with little greater probability than the $S_2 \rightarrow S_0$ transition. Apparently, the lack of fluorescence from S_1 is due to efficient intersystem crossing from that state (42). However, in quinine, the energy difference between the postulated S_1 and S_0 seems to be large, and the incomplete internal conversion between S_2 and S_1 requires some other explanation, such as the lack of overlapping vibrational levels in the two states. Furthermore, since the quantum yield is higher when S_1 is reached directly rather than through S_2, a mechanism for radiationless conversion from S_2 to S_0 is needed. Resolution of the theoretical basis of the anomalies of quinine emission will require low-temperature luminescence studies and energy level calculations.

Quinine is one of the most widely used fluorescent test substances, and for this reason it seems particularly ironic that it should have such unusual emission properties. However, if the wavelength dependence of its quantum yield and emission spectrum is recognized and taken into account, quinine should continue to serve as a useful fluorescence standard.

SUMMARY

The fluorescence of quinine has some anomalous features: (*1*) Quinine in acid solution has an excitation spectrum which fails to coincide with its absorption spectrum at long wavelengths. (*2*) The quantum yield is 23% higher with 390 mμ excitation than it is when 313 mμ excitation is used. (*3*) The emission spectra of both quinine and its parent compound, 6-methoxyquinoline, are shifted toward the red when they are excited at 390 mμ rather than near 340 mμ. (*4*) The fluorescence polarization in the emission band of quinine in rigid medium is inconstant, suggesting that emission arises from two singlet states simultaneously. (*5*) The polarization of the excitation spectrum suggests the presence of a hidden electronic transition in the long-wavelength absorption band. These unusual optical features should be considered when using quinine as either a fluorescence test substance or a quantum yield standard.

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ANALYTICAL BIOCHEMISTRY 19 (1967)

SHORT COMMUNICATIONS

A Rapid and Simple Assay of Serotonin Sulfokinase Activity

Although a great deal of information has been presented on the metabolism and the physiological action of serotonin, little is known about its esterification by sulfate, which is thought to be an important metabolic pathway of this hormone. The possibility of the formation of serotonin *O*-sulfate both *in vivo* and *in vitro* was reported in 1960 (1) and the enzymic sulfation of serotonin to serotonin *O*-sulfate was recently ascertained by demonstrating the formation of S^{35}-labeled serotonin *O*-sulfate (2). To study the purification of this enzyme an assay method was needed. Thus, a rapid and sensitive assay of the activity of serotonin *O*-sulfate forming enzyme, serotonin sulfokinase, was devised and is described in this communication.

Materials: Serotonin creatinine sulfate and adenosine triphosphate (ATP) were purchased from Sigma. All other chemicals were obtained from commercial sources. The solution of ATP or of cysteine was freshly prepared and neutralized with 1 *N* NaOH before use. Serotonin *O*-sulfate was synthesized according to Kishimoto *et al.* (3). A 3′-phosphoadenosine-5′-phosphosulfate generating system was prepared from yeast according to Lipmann *et al.* (4). Serotonin sulfokinase was purified from rabbit liver (5).

Method: Incubation mixtures contained 100 μmoles of imidazole-HCl buffer, pH 7.0, 12.5 μmoles of magnesium chloride, 15 μmoles of potassium sulfate, 10 μmoles of cysteine hydrochloride, 10 μmoles of ATP, 1.5 μmoles of serotonin, 1 mg of the sulfokinase, and 1 mg of the yeast enzyme. Total volume was made up to 1.0 ml with water. Reaction was started by addition of the sulfokinase. The reaction mixture without the sulfokinase or serotonin was incubated and used as blank. Incubation was carried out at 37°C. After incubation, the mixture was heated in boiling water for 2 min and mixed with 3 ml of water. Denatured protein was centrifuged off and the supernatant was passed through a small column of IR-CG-120 (H^+)

(0.5 × 3 cm) in order to remove unesterified serotonin. The column was then washed with 10 ml of water and the total effluent (14 ml) was collected. To avoid interference by ATP, which fluoresces at 350 mμ with excitation at 285 mμ in acid solution, the effluent was adjusted to pH 4–8 with 0.2 ml of 2 *N* NaOH, and then 0.8 ml of 1 *M* potassium phosphate buffer (pH 7.0) was added. The fluorescence of this solution was measured

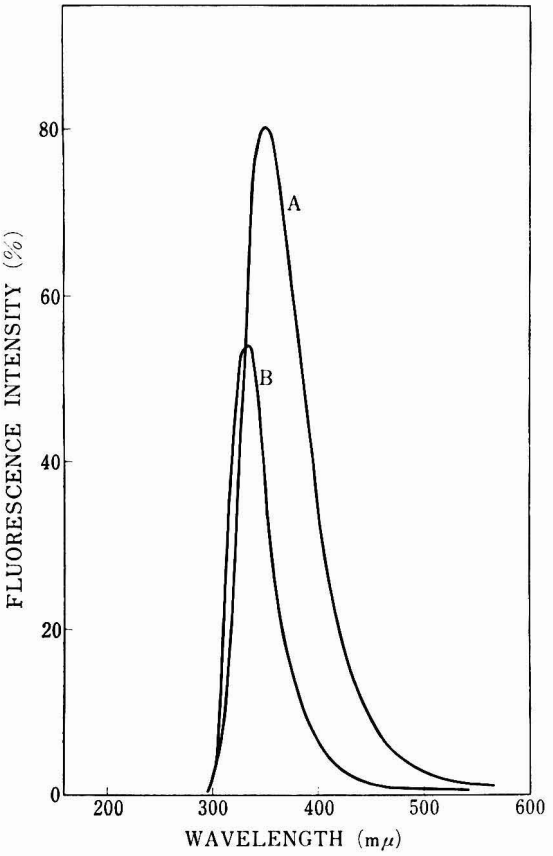

FIG. 1. Fluorescence spectra of synthesized serotonin *O*-sulfate (A) and serotonin (B) in 0.1 *M* potassium phosphate buffer, pH 7.0 (excited at 285 mμ).

at 350 mμ with the exciting light at 285 mμ. The instrument used was an Aminco-Bowman spectrophotofluorometer. When authentic serotonin *O*-sulfate was added to the blank, the recovery was found to be 80–90%. Since a large quantity of ATP in the incubation mixture inhibits the fluorescence of serotonin *O*-sulfate, 25 mμmoles of authentic serotonin *O*-sulfate was used as an internal standard. The fluorescence spectrum of

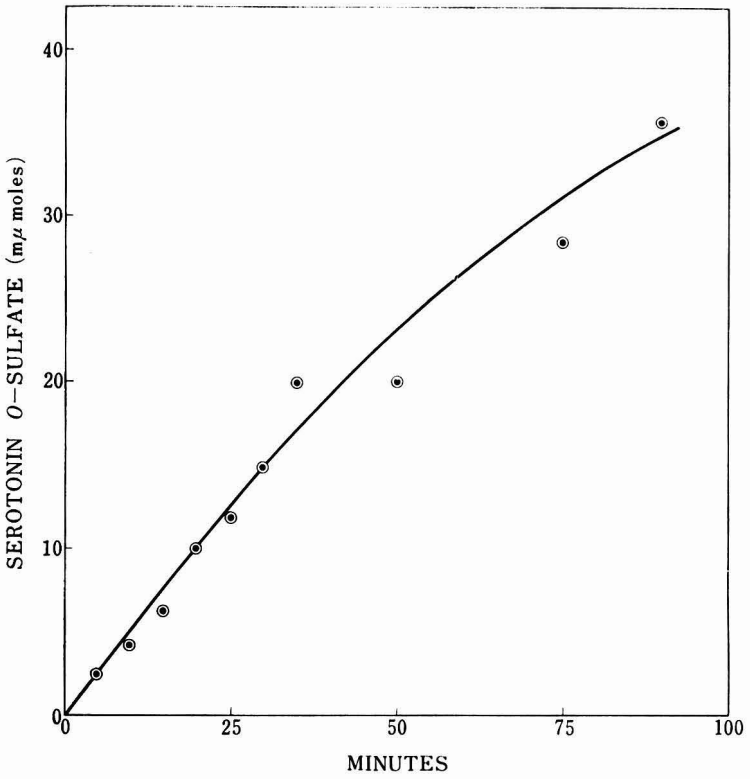

FIG. 2. Enzymic formation of serotonin *O*-sulfate as a function of time.

synthesized serotonin *O*-sulfate is shown in Figure 1. The peak of the excitation spectrum of serotonin *O*-sulfate was found at 285 mμ, and that of the fluorescence spectrum of serotonin *O*-sulfate at 350 mμ. The peak of the fluorescence spectrum of serotonin *O*-sulfate shifted slightly to a longer wavelength as compared with that of free serotonin. The rate of enzymic formation of serotonin *O*-sulfate and its dependence on the enzyme concentration are shown in Figures 2 and 3.

Stoichiometry: By measuring the fluorescence of the column effluent at 350 mμ with excitation at 285 mμ, 15 ml of this solution was found to contain 70 mμmoles of serotonin *O*-sulfate. Then it was mixed with 5 ml of 12 *N* HCl and hydrolyzed at 60°C for 60 min. The amount of serotonin in the hydrolyzate was measured by the usual fluorometry at 540 mμ with excitation at 295 mμ. Decomposition of serotonin liberated during the hydrolysis was corrected by using 60 mμmoles of serotonin as standard. As shown in Figure 4, the recovery of serotonin from serotonin *O*-sulfate

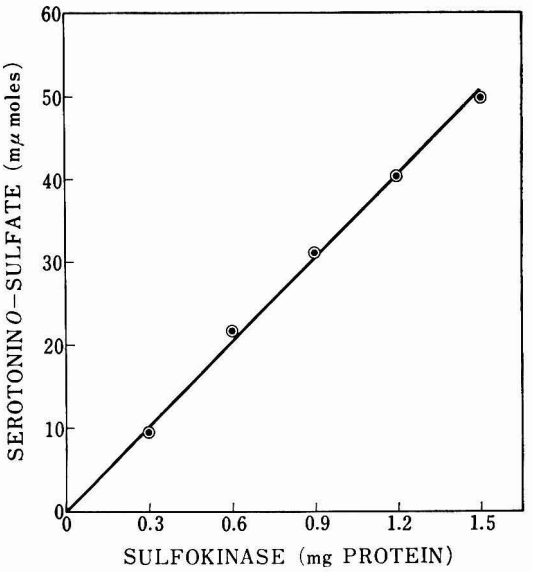

FIG. 3. Enzymic formation of serotonin *O*-sulfate as a function of enzyme concentration.

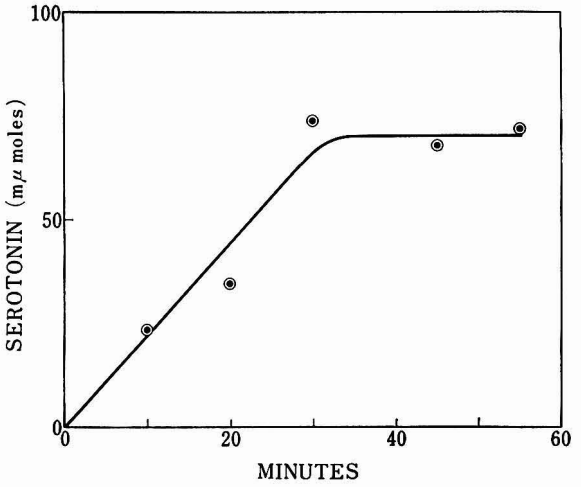

FIG. 4. Hydrolysis of the enzymically formed serotonin *O*-sulfate. Column effluent containing 70 mμmoles of serotonin *O*-sulfate was hydrolyzed in 3 *N* HCl at 60°C, and liberated serotonin was measured (see text).

was found to be almost quantitative, showing the validity of this assay procedure.

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Gas-Liquid Chromatography of Mevalonic Acid and β-Hydroxy-β-Methylglutaric Acid[1]

Following the discovery of mevalonic acid (MVA) in 1956 (1, 2), several methods for the identification and quantitative determination of this substance were established. Methods for identification and quantitation of β-hydroxy-β-methylglutaric acid (HMG) soon followed.

The detection of MVA has been achieved by column chromatography (3–5), paper chromatography (6, 7) and by formation of derivatives (6), and its quantitation by colorimetric methods (5, 7) but mostly by isotopic methods (3, 6, 8, 9). Column chromatography (4, 10) and formation of derivatives (3–5) have also been used for the determination of HMG.

Recently two methods were published for the identification of MVA by gas-liquid chromatography: Siperstein's gas chromatographic method (11) for the identification of MVA and HMG as their methyl esters, and

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Guchhait and Porter's method (12) for the determination of mevalonolactone. In both papers isotopic methods were used for the quantitation of the products. This paper describes a new gas-liquid chromatographic method for the identification of MVA as its lactone and HMG as its methyl ester and their quantitation by the same method using diethyl phenylmalonate and diethyl pentylmalonate as internal standards.

Materials. Mevalonolactone (M.W. 130–131), chromatographically pure (paper) was purchased from Sigma Chemical Co. and β-hydroxy-β-methylglutaric acid (m.p. 101–104) chromatographically pure from Mann Research Laboratories Inc.

N-Methyl *N*-nitrosotoluenesulfonamide (Diazald) (m.p. 100–102) was purchased from Aldrich Research Chemicals, 2-(2-ethoxyethoxy)ethanol (practical) from Eastman Organic Chemicals, diethyl phenylmalonate 97.2% (m.p. 16–18) and diethyl pentylmalonate 96.8% (practical) from Matheson, Coleman & Bell Co.

Chloroform, methanol, and ethyl ether were of "Fisher certified anhydrous reagent" grade—ethyl ether was stored over ferrous sulfate before use and methanol was dried by treatment with magnesium turnings and iodine followed by a reflux of 1 hr and a distillation; 20% polyethylene glycolglutarate on 60–70 mesh Anakrom U was purchased from Analabs Inc.

Instrument. An F & M model 402 high-efficiency gas chromatograph equipped with a flame ionization detector was used. The columns were of glass, U-shaped, 4 ft long, and packed with 5% diethyleneglycol succinate (LAC-728) on 80–100 mesh Diatoport S.

Chromatographic conditions. Injection chamber temperature for MVA 240°, for HMG 220°. *Oven temperature* for MVA 180°, for HMG 150°. *Detector temperature* for MVA 200°, for HMG 180°. *Gas flow:*

Air (to detector)	300 ml/min
Hydrogen (to detector)	35 ml/min
Helium (the carrier gas)	60 ml/min

Chart speed for MVA 0.5 in./min, for HMG 2.0 in./min. *Sensitivity:* range, from 10^{-2} to 1; attenuation, from 1/128 to 1/2.

Methods. Methylation of HMG: Amounts of HMG varying from 5 to 20 mg were introduced in 12.5 × 1.3 cm test tubes equipped with a side arm and 3 ml of 10% methanol in ethyl ether was added. The tubes were agitated till all the HMG was dissolved. HMG was then methylated by the method of Schlenk and Gellerman (13).

When gas chromatographic analyses were to be performed, volumes of chloroform varying from 300 to 1000 λ were added in the test tubes. Small amounts, 2–3, were injected in the gas chromatograph and the

sensitivity (range and attenuation) set to obtain a maximum peak on the recorder chart. Then from 50 to 200 λ of 2% diethyl pentylmalonate in chloroform was added into the solution in order to obtain two peaks of approximately the same height on the recorder chart. Amounts of diethyl pentylmalonate equal to those added into the HMG methyl ester solution were chromatographed alone.

Volumes of the HMG methyl ester solution corresponding to amounts of HMG methyl ester ranging from 0.25 to 175 μg were injected for chromatography. The samples were kept in ice-cold water between injections.

Mevalonolactone: Amounts of mevalonolactone varying from 10 to 20 mg were introduced into a test tube with a side arm and 300–1000 λ of chloroform was added as for HMG methyl ester. From 2 to 3 λ of this solution was chromatographed, the sensitivity was set, and 50–200 λ of 2% diethyl phenylmalonate was added into the solution to obtain two appropriate peaks on the recorder chart. Volumes of solutions corresponding to amounts of mevalonolactone ranging from 0.125 to 100

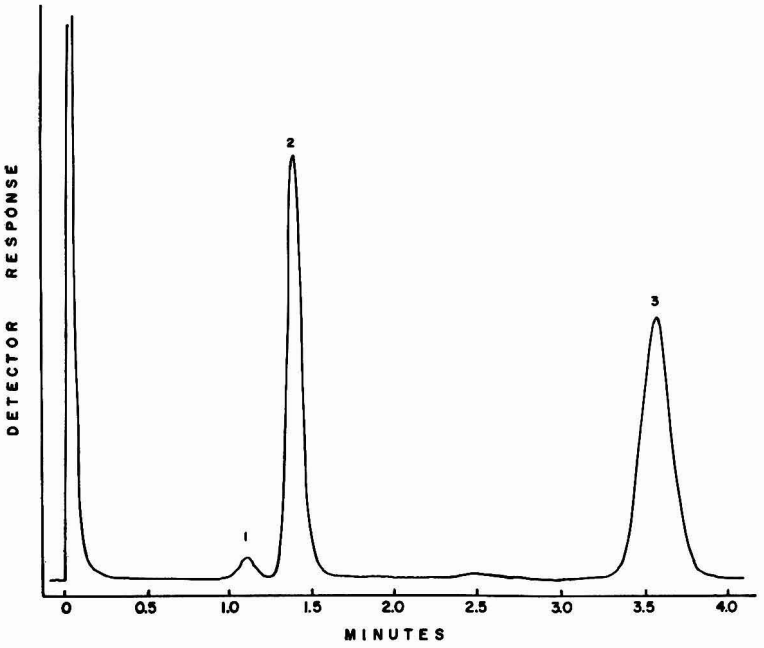

FIG. 1. Typical chromatogram obtained when HMG dimethyl ester and diethyl pentylmalonate are injected simultaneously into the gas chromatograph containing a 4 ft, U-shaped glass column packed with 5% LAC-728 on 80–100 mesh Diatoport S. Oven temperature was 150° and helium flow was adjusted to 60 ml/min.

μg were injected for chromatography. The test tubes were also kept in ice-cold water between injections.

Results and discussion: The chromatogram (Fig. 1) shows that when HMG methyl ester and diethyl pentylmalonate were simultaneously injected into the gas chromatograph, three peaks were registered on the recorder chart. The first peak corresponded to an unknown substance contained in the diethyl pentylmalonate. This was confirmed by chromatography of diethyl pentylmalonate alone, which was of a "practical" grade, and this compound proved to be 96.3–97.2% pure.

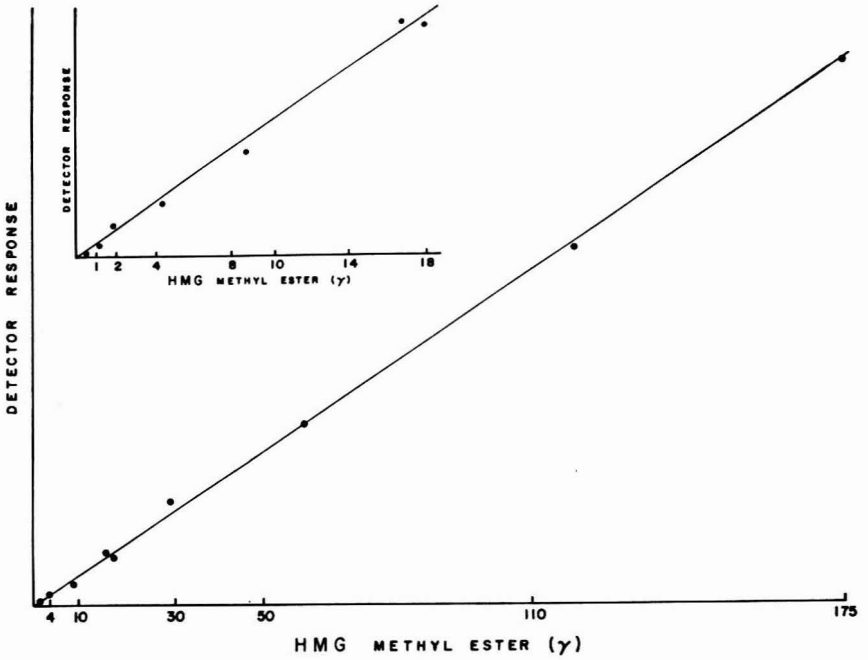

FIG. 2. Degree of response of the detector to variable amounts of HMG dimethyl ester.

The second peak recorded corresponded to the diethyl pentylmalonate. This substance, chromatographed in the conditions described above, has a retention time of 1.4 min. The third peak to appear corresponded to HMG methyl ester, which has a retention time of 3.5 min. In order to compare these results with those obtained by Siperstein (11), a 4 ft U-shaped glass column was packed with 20% polyethyleneglycol glutarate on 60–70 mesh Anaktrom U. Under the experimental conditions described, we observed that HMG methyl ester had a greater retention time when 20% PEGG on 60–70 mesh Anakrom U was used as the stationary phase than on 5% DEGS, 80–100 mesh Diatoport S.

The number of theorical plates per foot of column calculated for diethyl pentylmalonate and HMG methyl ester were 988 and 1354, respectively.

The behavior of the detection system when different amounts of HMG dimethyl ester were chromatographed was studied. Figure 2 illustrates that the detector response was linear for amounts of HMG dimethyl ester ranging from at least 0.5 to 175 μg. Siperstein previously observed

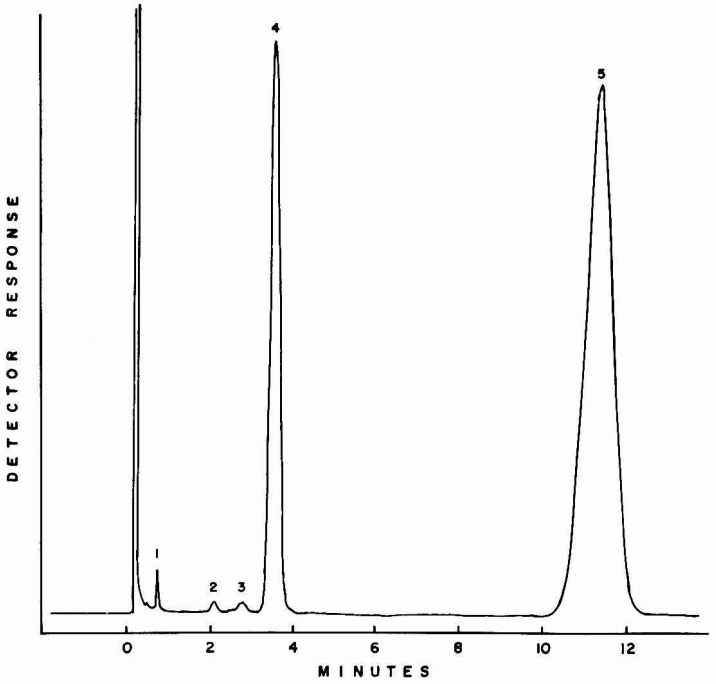

FIG. 3. Typical chromatogram obtained when mevalonolactone and diethyl phenylmalonate were injected simultaneously into the gas chromatograph containing a 4 ft, U-shaped glass column packed with 5% LAC-728 on 80–100 mesh Diatoport S. Oven temperature was 180° and helium flow was adjusted to 60 ml/min.

such a linear response for amounts of HMG dimethyl ester ranging from approximately 30 to 100 μg. Results obtained with amounts of HMG dimethyl ester smaller than 0.5 μg were discarded because of the instability of the baseline on the chart, making the estimation of the peak areas rather difficult.

The chromatogram illustrated in Figure 3 shows that, when mevalonolactone and diethyl phenylmalonate were simultaneously injected in the chromatograph, five peaks were recorded on the recorder paper chart. Chromatography of diethyl phenylmalonate alone showed that the first and the third peaks corresponded to contaminants of the diethyl phenyl-

malonate. The purity of this compound was found to be of the order of 96.8–97.8%. The second peak appeared only when the two substances where chromatographed simultaneously and proved to be a contaminant of mevalonolactone. The impurity represented less than 1% of the total substance injected. The fourth peak corresponded to diethyl phenylmalonate. This substance, chromatographed under the conditions mentioned under in the paragraph on chromatographic conditions above, has a retention time of 3.9 min. The fifth peak corresponded to mevalonolactone, which showed a retention time of 11.3 min. Compared to the results obtained by Guchhait and Porter (12), we noted that even with an oven temperature 10 to 15 degrees lower and a gas flow of 40 ml/min lower, the retention time was 11 min instead of 45 as previously obtained by Guchhait. Considering the fact that Guchhait used a 6 ft column, it still seems that mevalonolactone is less retained by 5% DEGS on 80–100 mesh Diatoport S than by 20% butanediol succinate polyester on 60–80 mesh Chromosorb W. We did not, however, try Guchhait's column with this substance.

The number of theorical plates per foot of column calculated for mevalonolactone and diethyl phenylmalonate were 1756 and 1381, respectively. These results were estimated satisfactory and appear to correspond to those obtained by Guchhait (12).

Figure 4 illustrates that the detector response was linear for amounts of mevalonolactone ranging from 0.25 to 100 μg. These results are in agreement with the results of Siperstein (11), even if he chromatographed MVA as its methyl ester. Results obtained with amounts of mevalonolactone smaller than 0.25 μg were discarded because of the instability of the baseline, resulting from the high sensitivity that had to be applied to the detector.

The volume of solution injected into the gas chromatograph never exceeded 5 μl. For small amounts of substances, a few micrograms or less, volumes of solution smaller than 1 μl were injected in order to allow the pen to return promptly to its baseline on the recorder chart.

The advantages of using gas-liquid chromatography for the identification of mevalonic acid and β-hydroxy-β-methylglutaric acid has already been discussed by Siperstein (11) and Guchhait (12) and will not be discussed here. This method has the advantage of allowing the quantitation of the products (MVA and HMG) by gas-liquid chromatography only, without having recourse the isotopes.

Summary. A new gas-liquid chromatographic method was developed for the identification of mevalonic acid in its lactone form and of β-hydroxyl-β-methylglutaric acid as it's dimethyl ester derivative. Both substances were quantitated using diethyl pentylmalonate and diethyl phenylmalonate as internal standards.

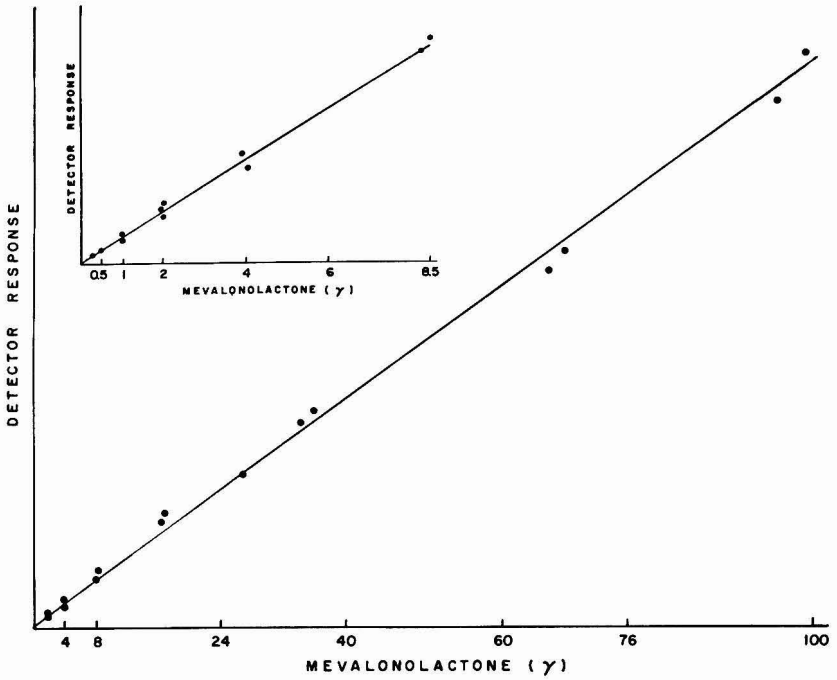

FIG. 4. Degree of response of detector to variable amounts of mevalonolactone.

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Acrylamide Gel Slab Electrophoresis in a Simple Glass Cell for Improved Resolution and Comparison of Serum Proteins

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Acrylamide gel slab electrophoresis was developed by Raymond (1, 2) using cells similar to those of the earlier starch gel methods; he discusses the advantages of acrylamide gels compared to starch, and describes a rather complex and expensive water-cooled acrylic cell for vertical slab electrophoresis. Tombs (3) described a simple tray, 14 $\times$ 8 cm, for horizontal electrophoresis of slabs 2 mm thick with four 1.5 mm deep sample slots preformed in the gel. However, we always obtained rather better separations of milk or blood serum proteins by electrophoresis in 60 $\times$ 5 mm acrylamide gel rods (4, 5) than in gel slabs in horizontal or vertical acrylic cells. The minor protein zones appeared sharper and resolved better in the rods. A disadvantage of individual rods compared to the slabs, however, is that the migration distances of a particular protein may differ by a few millimeters in rods of the same experiment. This variation is possibly due to slightly different gel lengths in the tubes or slightly different gel compositions at the top ends caused by disturbance when overlaying the distilled water onto the acrylamide monomer solution. Thus, close comparisons and certain identity of migration of an unknown with a known protein sample cannot always be made. Also, subsequent handling, labeling, and recording of results by photography or densitometry is more laborious with rods than slabs if large numbers of samples are being examined routinely.

The better resolution of the rod technique may be due to the better adherence of acrylamide gel to the glass walls of the tubes rather than to the acrylic walls holding the vertical slabs, preventing any possibility of sample seepage down the outside of the gel. Also, the sample solution covers the whole of the top surface of the gel rod, giving completely uniform material and electrical field conditions along any

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section at right angles to the direction of migration. Gel slabs as made by Raymond (2) or Tombs (3), however, contain slits or sample slots or acrylamide gel separator sections for application of separate samples to the same gel; correct filling of slots in a horizontal gel slab is difficult, and all these methods suffer from the objection of nonuniform material and electrical field conditions at the sample application position which may give rise to blurred or distorted protein zones. Thermal gradients are symmetrical in the rods and this, together with their short length (5–6 cm) compared to most slab cells, permits higher voltage gradients and shorter runs without need of elaborate cooling metods.

The simple slab technique described below overcomes all these objections, giving resolution of serum proteins equal to that obtained in gel rods and allowing close comparison of the genetic variants of many samples. The gel and the experimental conditions are designed to reproduce as nearly as possible in the two-dimensional slab form the conditions appertaining in the one-dimensional acrylamide gel rod disc electrophoresis technique (4, 5). This slab technique gives the same advantages of simple apparatus, small sample requirements (e.g., 5 μl of 2% protein solution), rapid (40–200 min) separations, and excellent resolution. Pig and human blood sera were examined and methods of choosing the best acrylamide concentration and buffers are discussed.

METHODS

1. Construction of Glass Cells

Two rectangular gel spacer strips of 2 mm thick glass (7.5 $\times$1.0 cm) are cemented by epoxy resin (e.g., Araldite) onto the ends of a larger sheet (23 $\times$ 7.5 cm) of 3 mm thick clear glass to form the base plate of the cell. A loose cover plate of glass of the same size is held in position by means of strong spring clips (Fig. 1). This simple cell, with open top

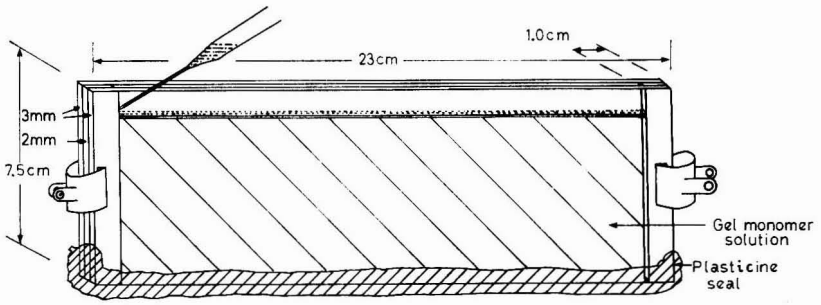

FIG. 1. Assembled simple glass cell: overlayering distilled water from pipet on top of gel monomer solution.

and bottom, can be used to separate blood serum proteins in 100 min electrophoresis at 80 mA (Fig. 2).

A cell made from two water-cooled plates can be used in place of the simple sheets of glass for heat-sensitive proteins, or to achieve faster migration rates by use of currents up to 150 mA. These composite plates are easily made by cementing internal narrow (1 cm wide) spacer strips of 3 mm thick glass or plastic round the edges of two sheets of 2 mm thick glass to form a hollow, watertight box (23 × 7.5 × 0.7 cm).

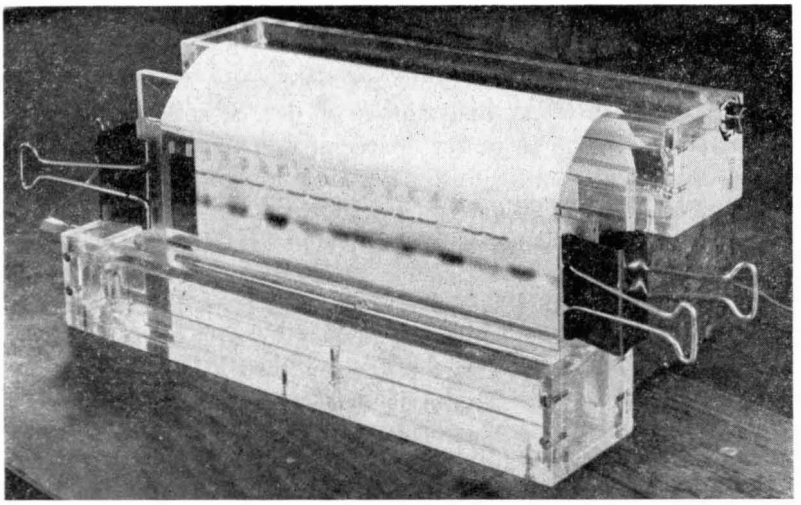

FIG. 2. Simple cell during electrophoresis. The rubber tubing sample separators have been left in place along the top edge of the gel. The fast-migrating bromophenol blue tracker dye and the slower, stained albumin zones are visible in the gel.

An adequate volume of cooling water can be passed through flexible polyvinyl chloride tubing (4 cm × 2 mm bore) cemented in place at both top corners of each composite plate; internal baffles to direct the flow of cooling water were not found to be necessary. A thin layer of epoxy resin applied to all the exposed edges of the plate, and particularly where the two pieces of tubing enter, ensures that the plate is watertight.

2. Electrode Tanks

The cell stands in the lower electrode tank. The upper electrode tank is placed behind the top of the cell and is connected to it by means of a filter paper wick (Fig. 2). Rectangular plastic storage boxes can be used as tanks, or a commercial paper electrophoresis apparatus can be used as the lower tank. Special tanks to hold a minimum volume of buffer solution can be made from 6 mm thick acrylic sheet cemented to

form a box with internal dimensions 24 cm long $\times$ 3.5 cm high $\times$ 4.5 cm wide. The electrode is a single strand of platinum wire 24 cm long, placed in a straight line on the tank floor 0.7 cm from one wall. To minimize pH changes in the tank, an internal weir (24 $\times$ 2.5 $\times$ 0.6 cm acrylic sheet) is placed to form a 1.5 cm wide electrode compartment and 2.4 cm wide buffer compartment. The two compartments are connected by either filling with buffer solution to a depth 1 mm over the top of the weir or by a long bridging wick of several layers of thick filter paper.

3. Preparation of Gel Slab

The surfaces of the glass cell are scrubbed clean with hot detergent, rinsed with distilled water, and allowed to dry. A little petroleum jelly is smeared onto the faces of the spacer strips; then the cover plate is laid in position and held firmly by strong spring clips. The open bottom edge of the cell must now be temporarily sealed so that the cell can be filled with the gel monomer solution. We do this by gently pushing a 25 $\times$ 3 $\times$ 0.3 cm strip of Plasticine modeling clay symmetrically against the cell bottom and ends, pressing the excess strip firmly against the external walls of the cell with the fingers to give a watertight seal (Fig. 1). The acrylamide monomer solution with just-added ammonium persulfate catalyst is poured into the cell, clamped vertically, to within 1.0 cm from the top. Distilled water is then immediately overlayered on top of the gel monomer solution to a depth of about 5 mm. This excludes air, which inhibits gelation, and gives a flat, meniscus-free top edge to the gel where the samples will be applied. The overlayering should be completed within 5 min of adding the catalyst to the acrylamide monomer solution, as gelation should be completed in 10–15 min. The distilled water is added slowly and carefully, with the tip of the pipet or syringe touching the internal wall of the cell at one end a few millimeters above the top of the monomer solution. The outflow of water is controlled so that no mixing occurs, until about one-third of the monomer surface is overlayered. This is then repeated at the other end of the cell until the whole gel monomer surface is uniformly covered. The glass walls must be very clean so that they are wetted easily.

If required, a 5 mm depth of large-pore "spacer" gel can now be polymerized on top of the main gel to follow the complete disc electrophoresis method of Ornstein and Davis (4, 5), but good resolutions can often be obtained without this complication (2, 6). A 7.0% acrylamide gel can be prepared by dissolving 2.1 gm Cyanogum 41 (mixture of 95 parts acrylamide and 5 parts *N,N'*-methylenebisacrylamide; British Drug Houses Ltd., Poole, Dorset, U. K.) in the desired gel buffer (30

ml). After adding 100 μl of *N,N,N',N'*-tetramethylethylenediamine or dimethylaminopropionitrile as accelerator, the solution is filtered and 0.15 ml of freshly prepared 10% ammonium persulfate catalyst solution added. The amount of accelerator and catalyst can be adjusted within a wide range if necessary to cause gelation to occur within the required 10–15 min (5).

After 20 min the Plasticine is removed to expose the flat bottom edge (21 $\times$ 0.2 cm) of the gel slab. The distilled water above the gel is poured off and the top edge washed with 3 changes of water. The cell is now mounted vertically with the bottom edge 5 mm from the floor of the buffer compartment of the lower electrode tank. Buffer solution is poured into the tank to a depth 1 mm above the weir, and any air bubbles under the gel are removed. Distilled water is pipetted to fill the cell above the top edge of the slab, and the cell adjusted to ensure that the top edge of the gel is horizontal.

4. Sample Application and Electrophoresis

Temporary sample compartments are now formed on the flat top edge of the gel by inserting small separators; up to 20 can be used on a 21 cm long slab. Soft rubber tubing (e.g., Escorubber translucent silicone rubber tubing, 1.5 mm bore $\times$ 0.5 mm wall, from Esco (Rubber) Ltd., 35, Seething Lane, London, E.C.3) in square-cut 5 mm lengths with outside diameter 0.5–1.0 mm greater than the gel thickness forms convenient separators. They are pushed through the overlaying water with a long needle into position about every 8 mm along the top edge of the gel. The end of each separator must firmly touch the gel surface to prevent sideways diffusion of the applied sample, but it must not distort the gel surface by being pushed too far into the gel (Fig. 2). The top of each separator should be about 5 mm from the top of the cell, to allow the wick to be inserted. The elasticity of the squashed tubing prevents it from floating upward, and so each sample compartment is adequately separated by about 3 mm from the next. Care must be taken not to prize apart the plates during this operation as buffer and sample could then leak down between the slab and plates. With a little practice, 20 separators can be correctly positioned in less than 5 min.

The sample solutions are preferably made denser to allow easy application to the gel surface through the overlaying distilled water. To achieve this we dilute blood serum with an equal volume of 20% w/v aqueous sucrose solution containing a trace of bromophenol blue as tracker dye. We then apply 10 μl of mixture by micropipet with its tip 5–8 mm above the gel surface in the appropriate sample compartment. The dense sample falls through the water and uniformly covers

the flat gel surface in the compartment. As soon as all samples have been applied, the small compartment above the sample separators is connected to the upper electrode tank buffer compartment by a long wick made of three layers of Whatman 3 MM filter paper enclosed between two pieces of thin polythene sheet to prevent evaporation where the wick is in the air. The wick is soaked with tank buffer solution and then one end is carefully placed just inside along the full length of the compartment above the gel, taking care not to disturb the samples or separators.

A current of 20 mA is then passed for 10 min until the tracker dye and proteins have concentrated at the interface and just entered the gel as a series of very narrow, sharp zones. The current is then switched off, the wick is removed, and all the separators are taken out with a needle. This leaves the gel slab unobstructed and completely uniform; since the wick is as wide as the gel, the electrical field is uniform and parallel, and all the protein zones subsequently migrate as flat, horizontal bands. The wick is replaced so that it terminates 1 mm above the top edge of the gel and the free space above the gel is kept filled with buffer solution. Electrophoresis using constant current conditions is recommended. The time required depends on current, buffer composition and pH, gel slab temperature, and acrylamide concentration, but is normally 40–200 min. When using pH 7.0 T-E-B continuous buffer (1.0% Tris + 0.1% Na_2-EDTA + 2.7% boric acid), at 100 mA constant current the voltage shown on the power pack was 440 V and the actual voltage measured across the gel was 230 V. Thus the paper wick caused a loss of 210 V, and the voltage gradient through the gel was 35 V/cm.

5. Staining, Recording, and Storage of Gel Slabs

On completion of electrophoresis, when the albumin zone is about 1 cm from the bottom of the gel, the cover plate is slid off and the first sample position marked on the slab by making a small hole at the bottom of the gel with a cork borer. The whole slab can be transferred, supported by a thin sheet of plastic, to the stain bath. Immersion for 30 min in amido black (0.5% dye dissolved in 7% aqueous acetic acid) followed by 16–24 hr washing with several changes of very dilute acetic acid is a satisfactory general protein stain.

Comparative selective staining can be performed if replicate sample applications were made to the gel; after electrophoresis the slab is cut into strips which are separately stained in different baths so that, for instance, the benzidine or oil-red O or Schiff-reagent positive bands can be matched on the amido black pattern of the same sample run under exactly the same conditions in the same gel.

The gel slabs are stored, still wetted with dilute acetic acid, in transparent, vaportight plastic bags. They can be photographed in the bags by transmitted light, or directly evaluated by transmitted light in a scanning microdensitometer after cutting the slab into strips containing one sample per strip.

RESULTS AND DISCUSSION

Some pig and human blood serum separations obtained using the glass cells are shown in Figures 3–5. Often 15–20 sharp bands can be distinguished on the gels. The uniform, parallel migration along the whole length of the slab enables easy comparison of genetic variants.

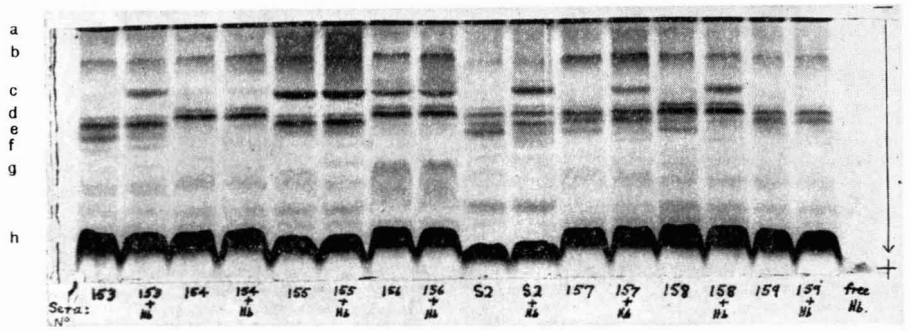

FIG. 3. Pig sera proteins amido-black stained electrophoresis patterns in 7.0% acrylamide gel slab (21 × 6.5 × 0.2 cm). Continuous pH 7.0 T-E-B buffer system (10.0 gm Tris + 1.0 gm disodium salt of ethylenediaminetetraacetic acid + 27.0 gm boric acid + water to 1 liter) used for gel and electrode tanks. Current of 80 mA for 120 min. Two consecutive applications made of each serum, the left-hand pattern showing serum as obtained and right-hand pattern showing same serum with 0.1% pig hemoglobin added to complex with free haptoglobins and hemopexins. *Key:* (a) sample application position; (b) 7S-globulin region; (c) haptoglobin-hemoglobin complex; (d) transferrin B variant; (e) transferrin A variant; (f) haptoglobin; (g) hemopexin region; (h) albumin.

The albumin band is generally narrower and the separation pattern cleaner and sharper than is obtained by the more laborious starch gel methods.

Thin glass is preferred to the more usual plastic sheet for construction of the cell because acrylamide gel bonds to glass more firmly, preventing sample seepage down the faces of the slab. Other advantages of glass are complete transparency and ease of scrubbing clean without scratching the surface; also the greater rigidity and better thermal conductance allow higher currents to be used for rapid electrophoresis. The thickness of the gel slab should be 1.5–2.0 mm; thinner slabs are more difficult to handle, and thicker ones besides requiring a greater current

may overheat and give distorted bands, since a protein in the warm center of the slab will migrate faster than at the cooler face of the slab. The height of the slab should be only 5–8 cm to give rapid migration rates without overheating; the length of the slab is governed by available electrode tanks, the number of samples to be compared, and the power supply (about 3–6 mA/cm length of 2 mm thick gel is needed).

Preparation of the slabs and sample application takes only a short time; electrophoresis can be commenced within an hour of mixing the

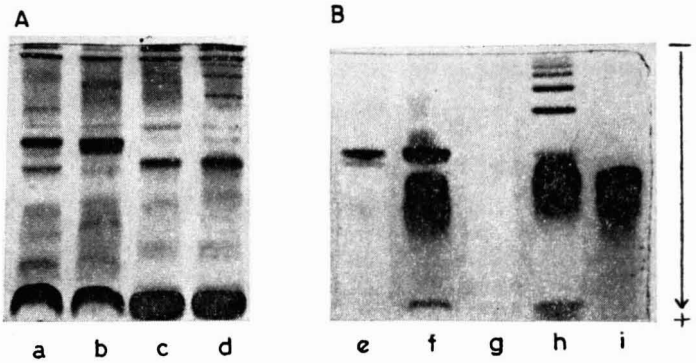

FIG. 4. Pig and human sera electrophoresis patterns in 7.0% acrylamide gel slab. Continuous pH 8.35 T-E-B buffer system (10.8 gm Tris + 0.93 gm disodium salt of ethylenediamine tetraacetic acid + 5.5 gm boric acid + water to 1 liter) used for gel and electrode tanks. Current of 80 mA for 100 min. Slab then cut and left-hand piece (A) (10.5 × 6.5 × 0.2 cm) stained in amido black bath, and replicate piece (B) stained in benzidine bath to locate free and bound hemoglobin. *Key:* (a and e) pig serum No. 48, with free haptoglobin (H_p) migrating faster than transferrin B; (b and f) pig serum No. 48 with added hemoglobin, showing H_p–H_b complex overlapping transferrin B; (c and g) human serum, with many fine, slow-migrating H_p (type 2,1) bands visible on original gel; (d and h) same serum as c but with H_b added to form H_p–H_b complexes; (i) free hemoglobin (H_b). Thus although this pH 8.35 system gives good separation of human serum H_p–H_b bands, the pH 7.0 system (see Fig. 3) is preferable for pig serum proteins.

gel monomer and one operator can prepare and run several gels in the course of a day. Thus the method is very suitable for routine examination of large numbers of samples.

Any of the discontinuous or continuous buffer systems described in the literature can be used: thus, the gel can be prepared in 0.37 *M* Tris-HCl, pH 8.9, buffer and 0.039 *M* glycine-Tris, pH 8.4, buffer solution used in the electrode tanks (4, 5). Another frequently satisfactory system is a 0.076 *M* Tris-citrate, pH 8.6, gel buffer used in conjunction with a 0.3 *M* boric acid-NaOH tank buffer. Often, however,

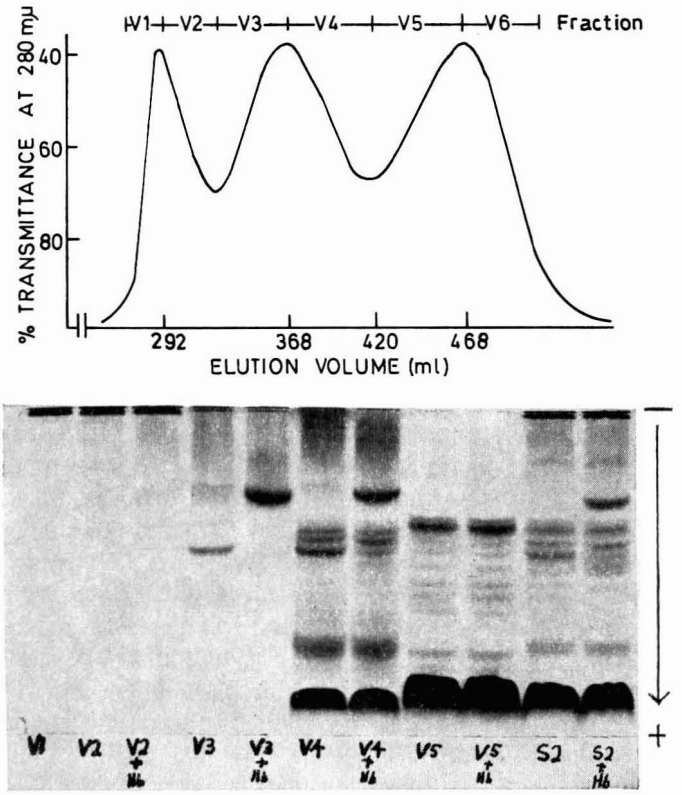

FIG. 5. Gel filtration and then electrophoretic separation of pig serum proteins. Serum No. S2 (7.0 ml; substantially hemolysis-free) applied to 3.0 × 100 cm column of Sephadex G-150 beads kept at 4°C and eluted with 0.1 *M* Tris-HCl, pH 8.0, buffer containing 1 *M* NaCl at 20 ml/hr flow rate. The eluate was continuously monitored at 280 mμ, then divided into the six fractions indicated, concentrated, and examined by gel slab electrophoresis (same conditions as in Fig. 3). High molecular weight proteins such as macroglobulin and β_1-lipoprotein are present in fractions V1 and V2. Fraction V3 contains γ-globulin and haptoglobin; the disappearance of the free haptoglobin and appearance of the slower migrating complex on addition of hemoglobin to V3 is clearly seen. Fraction V4 contains some components of both V3 and V5, while V5 contains transferrin, albumin, and many postalbumin bands.

equally good results can be obtained by using the same buffer solution for both gel and tanks (2, 6). In this case it is possible to "prerun" the gel slab by electrophoresis at 50 mA for 15 min before any samples are applied, in order to remove any ionic impurities from the gel. Tris-EDTA-borate (T-E-B) continuous buffers often give good separations.

When blood serum proteins are examined, it is desirable that the main

bands of interest, such as transferrins, free haptoglobins, free hemoglobin, and the various haptoglobin-hemoglobin complexes, are separated as far as possible on the gel and are easily identifiable without need of multiple staining procedures. In many starch and acrylamide systems all the above proteins intermingle and overlap in a fairly narrow zone of gel about midway between the sample application point and the fast-migrating albumin.

For determining the best acrylamide concentration/buffer system it is helpful to separate a sample of the serum by gel filtration through a long column of Sephadex G-150 or G-200 polydextran beads (7, 8). Six main

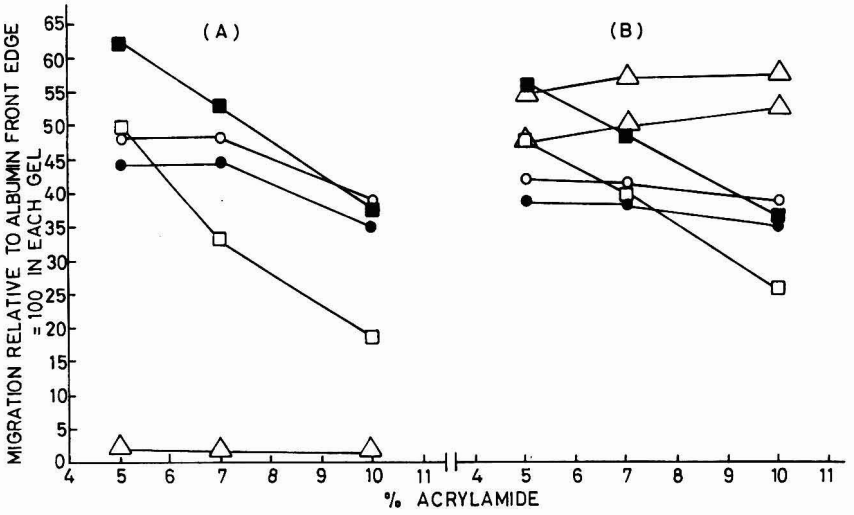

FIG. 6. Migrations of various pig serum proteins, relative to albumin front = 100 in each gel, in 5, 7, and 10% acrylamide gels: (A) pH 7.0 continuous T-E-B buffer system as described in Fig. 3; (B) pH 8.35 continuous T-E-B buffer system as described in Fig. 4. *Key:* (■) free haptoglobin; (□) haptoglobin-hemoglobin complex; (△) free hemoglobin; (○) transferrin A; (●) transferrin B variant.

fractions can be collected, concentrated by ultrafiltration in ¼″ diameter Visking cellophane tubing and then examined by electrophoresis (Fig. 5). The first eluting fraction from the column contains the highest molecular weight proteins such as β_1-lipoprotein; the third fraction contains γ-globulins, haptoglobins, and any haptoglobin-hemoglobin complexes, while the remaining fractions of lower molecular weight proteins contain transferrins, postalbumins, and albumin. These fractions, without and with added hemoglobin, are then examined by electrophoresis in 5, 7, and 10% acrylamide gels using the same buffer system each time. In 10% acrylamide gels there is a strong molecular

sieving effect, so that, in alkaline buffers, free hemoglobin tends to migrate fastest, followed by transferrins, then haptoglobins and finally the haptoglobin-hemoglobin complexes. In 5% gels the sieving effect is less pronounced and the molecular charge at the gel pH is more important than molecular size for the middle and last fractions. The first fractions from the G-200 column can migrate further and resolve better in 5% than in 10% gels. The same fractions are then examined in 5, 7, and 10% gels at another buffer pH, so that the effect of pH on migration rate can be distinguished from the molecular sieving effect.

Thus Figure 6 shows graphically for pig serum the relative separating powers of 5, 7, and 10% acrylamide gel slabs in T-E-B continuous buffer systems of pH 7.0 and 8.35. After electrophoresis of the pig serum gel-filtration fractions, the migration of each protein band was measured as the percentage migration relative to the migration of the front edge of the albumin band ($= R_a$ value) in that gel. It is clear that optimum separation of the major proteins of pig serum occurs in 7% acrylamide gel using the pH 7.0 T-E-B buffer (cf. Figs. 3 and 4). This system has the advantage that free hemoglobin does not migrate because it is at its isoelectric point; thus any excess of the hemoglobin added to serum to find the free haptoglobins and hemopexins does not interfere with the electrophoresis pattern.

Probably no single gel/buffer system will give complete separation of all protein variants without some intermingling and overlap, and each serum, without and with added hemoglobin, should be examined in at least two gel/buffer systems in order to obtain maximum information about its component proteins.

SUMMARY

The excellent, rapid resolution of blood serum proteins obtained by disc electrophoresis in acrylamide gel rods can be reproduced in thin acrylamide gel slabs by the technique described. The simple cell is quickly made from four rectangular pieces of thin glass, and the gel slab (21 cm long, 6.5 cm high, and 2 mm thick) formed inside the cell, filling it to within 1 cm of the top. The cell is mounted vertically between electrode tanks, and 16–20 temporary sample compartments are formed along the top edge of the gel by inserting short pieces of thin rubber tubing as separators. Rapid separations can be obtained by using currents up to 80 mA, or up to 150 mA if water-cooled glass plates are employed.

The technique is particularly suitable for examining protein polymorphism patterns in sera from many animals, or any application requiring the close comparison of large numbers of samples. Methods of choos-

ing the best acrylamide concentration and buffer system for separating blood serum proteins are discussed.

ACKNOWLEDGMENTS

I thank Mr. C. J. Schram for helpful discussions and the suggested use of glass instead of acrylate for the cell construction, and Miss D. H. Davis, Mr. M. Lowe, and Mr. M. Rogers for expert technical assistance.

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Effect of Functional Group Substituents on the Paper Chromatography of Purines and Pyrimidines

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In previous work, Berger and Hedrick (1) introduced the use of acetonitrile solvent systems to separate closely related purines and pyrimidines. A portion of that work indicated that pH effects were not as important as was the solvent composition. There was some indication that the partition mechanism of the solutes consisted in a competition between the hydroxyl groups in the cellulose stationary phase of the paper support and the hydroxyl groups (represented by water) in the acetonitrile-buffer mixtures used as the mobile phases. It might be presumptuous, however, to consider a pure hydrogen-bonding mechanism as the explanation of the observed phenomena. Pimentel (2) has reviewed many approaches to the measurement of hydrogen bonding in complex compounds. Work in which chromatography is used as a measurement technique has been restricted to adsorption chromatography, and the numerous variables associated with that technique have led to inconclusive results. In this work, we have restricted the variables in the paper chromatographic method only to the composition of the mobile phase. Acetonitrile does not undergo hydrogen bonding to a significant degree, while its high dielectric constant permits ion dissociation and efficient buffering action. In our procedure, solutes are allowed to migrate in different compositions of acetonitrile and water, and R_f-water content curves are obtained. By measuring the water content of the mobile phase necessary to bring each solute to an R_f (arbitrary) of 0.30, a measure of the hydroxyl affinity (or polarity) of each solute can be obtained under conditions that are as nearly identical as possible for each solute. It was discovered that, if the mobile phase compositions (referred to as MPC) are expressed in terms of the mole fractions of water necessary to bring each solute to $R_f = 0.30$, a simple equation can be constructed such that the MPC of each solute is predicted if the functional groups present on

the purine or pyrimidine nucleus are known. The equation is of further value in that the relative effect of important functional groups such as the amino, hydroxyl, and methyl groups can be ascertained at a given pH and ionic strength.

METHODS

The same general procedure is followed as was recommended earlier (1), with the restriction that only the pH 10 (ammoniacal) system is used, and that at least 5% volume increments are employed in preparing different concentrations of water in acetonitrile. The solvent systems are prepared by combining fixed volumes of the reagents, and ignoring volume changes after mixture. This procedure permits the later calculation of mole fraction data from volume per cent data. Paper chromatographic runs were made in duplicate, and in general great care was taken to obtain data on each solute under comparable conditions. Some problems in streaking of solute were found to be due to insufficient drying of solute spots before chromatography.

RESULTS AND DISCUSSION

Although hydrogen-bonding studies of paper have been reported, the use of paper chromatography to obtain a measure of the hydrogen-bonding capability of solutes has been restricted due to the many variables inherent in the technique (3). For example, the stationary phase in paper is complex and indeterminate. This stationary phase changes with mobile phase composition. The rate of migration of the mobile phase is not constant in the paper, and the migration rate changes with mobile phase composition. The effect of salts, temperature, pH, and initial water content of the paper must also be considered. In this work, as many variables as possible are held constant. The pH is fixed at 10 using dilute ammoniacal water. There are no salts in the system.

The Mobile Phase. Acetonitrile is a useful solvent in the sense that it combines a relatively high dielectric constant (37.5 at 20°C) with a low hydrogen-bonding capability. Acetonitrile has a low viscosity and a boiling point that is also quite low (80–82°C) considering the fact that it is nearly three times as massive as water. These facts indicate low self-association of the acetonitrile molecules in spite of a high dipole moment, and hence support the statements regarding the low hydrogen-bonding capability of acetonitrile. Water on the other hand is an excellent hydrogen-bonding solvent, as indicated by its high boiling point and extensive self-association in the liquid state. When acetonitrile and water are mixed, the solution decreases in temperature. This indicates that little association occurs between water and acetonitrile in the mixed solvents.

Rather, it appears that the acetonitrile destroys the liquid structure of the water, while contributing no ordering processes itself. Such a solvent system should be ideal for constructing a series of mixtures which change only in the apparent concentration of hydroxyl content (due to the water) and hence in the effective hydrogen-bonding capability of the solvent system.

The Stationary Phase. One flaw in the above reasoning is the inability to predict the stationary phase composition of the paper. The hydrogen-bonding capability of a solute is estimated by the competition for solute retention between a constant stationary phase and a variable mobile phase. We are assuming that the cellulose support and its associated stationary phase maintains an essentially fixed composition even though the volume percent of the water changes by as much as 35%, and the mole fraction of water changes up to 0.60. We present our results with the reservations expressed above.

Procedure Used to Establish Relative Solute Polarity. The variable represented by the change in mobile phase velocity is eliminated by selecting a fixed R_f value, and measuring the mobile phase composition necessary to bring the solute to the stated R_f. We chose an R_f of 0.30 because the R_f–MPC curves were nearly linear at this R_f, and because most of the solutes fell in this R_f region. Actually, the MPC is obtained only after the R_f–MPC curves are plotted for each solute. An example of the technique is illustrated in Figure 1 for several purines and pyrimidines. In this figure, the data are seen to be precise, as far as permitted by paper chromatography. Nevertheless, a certain amount of smoothing was necessary, as may be observed in the curve for uracil.

Discussion of Results. If the data of Figure 1 and similar plots are accumulated into a table, such as Table 1, regularities appear among the solutes. In general, it can be said that a relatively polar solute requires more water to move the solute a given distance on a polar support than does a nonpolar solute. Another way of stating this conclusion is that the polar solute will have a greater affinity for a polar stationary phase. It will therefore require a mobile phase which is equal to or more polar than the stationary phase to move the solute to a given R_f. Essentially, the mobile phase composition is being changed to permit all solutes to migrate at the same rate. The absolute polarity of the mobile phase necessary to perform this function depends upon the R_f to which the solute must move (or the common rate of migration decided upon).

Table 1 can provide a measure of the relative hydroxyl affinity or polarity of various solutes under certain conditions of pH and ionic strength. What is perhaps more interesting is the fact that this ordering depends upon the functional groups present on the purine or pyrimidine

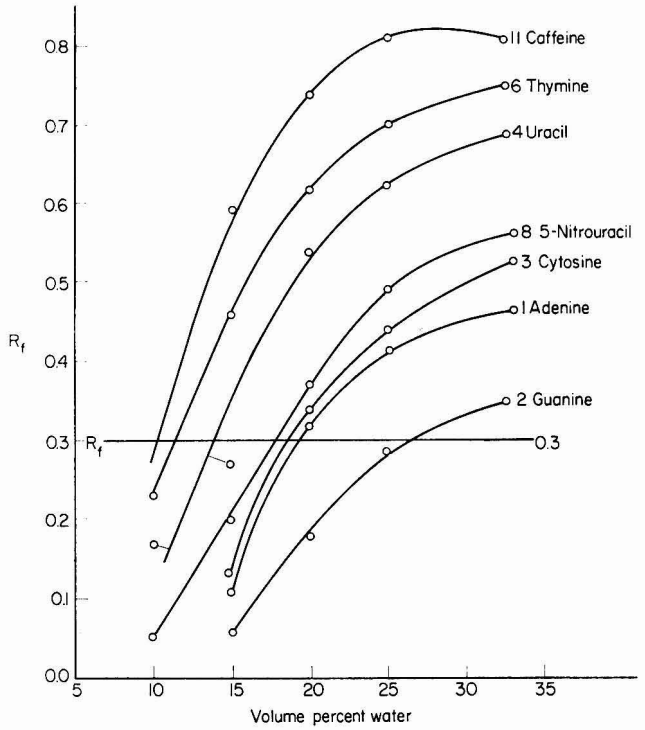

FIG. 1. R_f of several purines and pyrimidines as a function of mobile phase water content.

TABLE 1

Mobile Phase Water Content Necessary to Obtain a Solute R_f of 0.30

Solute	Volume % H_2O	Mole fraction H_2O	Functions[a]
Xanthine	31.3	0.57	2O
Guanine	26.5	0.51	O, NH_2
6-Aminouracil	25.0	0.495	2O, NH_2
Hypoxanthine	22.4	0.46	O
Isocytosine	20.0	0.42	O, NH_2
Adenine	19.4	0.415	NH_2
Cytosine	18.6	0.40	O, NH_2
5-Nitrouracil	17.5	0.38	2O, NO_2
Purine	17.4	0.38	—
Uracil	15.4	0.35	2O
Theophylline	15.0	0.34	2O, 2Me
6-Methyluracil	11.4	0.275	2O, Me
Thymine	11.2	0.27	2O, Me
Caffeine	10.2	0.25	2O, 3Me
Theobromine	5.0	0.13	2O, 2Me

[a] Letter notations represent functions substituted on purine and pyrimidine rings, including oxygen (O), amino nitrogen (NH_2), and methyl (Me).

nucleus. If the partition mechanism is indeed some form of hydrogen bonding, or at least hydroxyl affinity, then the structure of the solute will play an important role in establishing the magnitude of the competition between the cellulose stationary phase and the increasing molar concentration of water hydroxyl in the mobile phase. In Table 1 we have included the mole fraction of water calculated for each volume percent value of the MPC at $R_f = 0.30$. We also list the functional groups present in either the purine or pyrimidine nucleus. A study of Table 1 shows that purines with methyl group functions have smaller MPC values than purines with either oxygen or amino group functions. This trend is also present in the case of the pyrimidines. If the MPC of purine is taken as a standard, then it should be possible to construct an equation such that MPC values of other solutes can be predicted by combining a knowledge of the numbers of each functional group in the nucleus and an appropriate "functional group factor," referred to as an FG factor, or FGF. The FGF should vary with the conditions under which the solutes are allowed to migrate, but should permit an estimate of the relative effect of each functional group under the chosen conditions of pH, etc.

In this work, the equation developed for pH 10, ionic strength 0.0, is:

$$X = 0.38[1 + 0.094\ (NH_2) + 0.240\ (O) - 0.293\ (Me)] \qquad (1)$$

In this equation, X represents the mole fraction of water in the mobile phase, 0.38 represents the mole fraction of water necessary to bring the solute purine to an R_f of 0.30, and (NH_2), (O), and (Me) represent the numbers of amino, oxygen, and methyl groups substituted on the purine or pyrimidine ring. The other numerical values represent the FG factors.

The equation outlined above is tested in Table 2. The predicted MPC in terms of the mole fraction of water is listed in the second column and the results actually found are in the third column. Although only three unknowns are solved in the equation, six compounds are successfully fitted to the data. Theobromine does not fit, possibly because one of the methyl groups in the molecule is positioned on the five-membered ring. Such is not the case for theophylline, its isomer.

A similar test could not be performed on the pyrimidine derivatives because pyrimidine itself is a volatile liquid, and difficulties are associated with paper chromatography of the substance. However, a test of the precision of the equation can be made by attempting to predict the MPC of pyrimidine itself had it actually been submitted to the procedure outlined in this work. Again referring to Table 2, it is seen that, indeed, a good precision (14%) is obtained by using individual pyrimidines to calculate the expected MPC of the parent compound. While the absolute accuracy of the equation cannot therefore be tested, we feel that the data

TABLE 2
Prediction of Mobile Phase Water Content

Solute	Mole fraction predicted[a]	Mole fraction found	Deviation %
Xanthine	0.56	0.57	−1.8
Guanine	0.51	0.51	±0.0
Hypoxanthine	0.47	0.46	+2.2
Adenine	0.415	0.415	±0.0
Purine	—	(0.38)[b]	—
Theophylline	0.34	0.34	±0.0
Caffeine	0.23	0.25	−8.0
Theobromine	0.34	0.13	+260
Pyrimidine			
from 6-aminouracil	0.315		
from cytosine	0.300		
from uracil	0.236		
from 6-methyluracil	0.232		
from thymine	0.228		
average	*0.271*		*±14.3*

[a] For purines, $[X_{H_2O}] = 0.38[1 + 0.094\ (NH_2) + 0.240\ (O) - 0.293\ (Me)]$. For pyrimidine, MPC of pyrimidine = MPC of substituted pyrimidine divided by the equation in brackets, above.

[b] Standard reference of mobile phase water content.

presented in Table 2 certainly verify its consistency over a significant variety of purine and pyrimidine derivatives.

Present work involves an attempt to obtain MPC and FGF values at several different hydrogen ion concentrations. Since we pointed out earlier (1) that pH had only a small effect on the R_f–water content curves, it is expected that the functional group factors will change with pH, and that these changes will be compensating. For example, as the acidity of the mobile phase increases, the relative effect of the amino groups should increase, while that of the oxygen should decrease, and the effect of the methyl groups should remain constant.

SUMMARY

Curves relating the R_f of purines and pyrimidines on paper to the water content of acetonitrile-water mobile phases are used to predict the relative polarity of the solutes and the relative effect of functional groups in establishing these relative polarities. The water content of the mobile phase necessary to cause the solute to migrate to a given R_f value is a measure of the polarity of the solute. Oxygen and amino groups enhance the polarity of purine, while methyl groups strongly decrease the polarity. At pH 10, oxygen is 2.5 times as effective as the amino group in establishing differences in polarity among the solutes.

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ANALYTICAL BIOCHEMISTRY 19, 418–425 (1967)

A Mass Spectrometric Procedure for Rapid Identification of Trace Volatile Metabolites[1]

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Detection of trace amounts (10^{-7} to 10^{-12} mole) of volatile metabolites evolved by actively respiring animal tissue preparations is being investigated in this laboratory. Ethylene has been reported to evolve from normal rats by Kokonov (1), and from isolated rat liver mitochondria and rat intestinal mucosa by Ram Chandra and Spencer (2). One of the methods of ethylene detection requires trapping in $Hg(ClO_4)_2$, regeneration by LiCl addition, and detection by gas chromatography (3, 4). Although that method is specific and sensitive for ethylene, other components, which may be present in the incubation flask atmosphere, escape detection. Described here, is a method whereby the respiratory atmosphere contained in an isolated incubation flask is examined directly for volatile compounds by means of the mass spectrometer.

METHODS AND MATERIALS

Mass Spectrometer

A Bendix time of flight, model-14-101, was utilized for all sample analyses. Operation theory of this instrument has been described (5, 6). Our model 14 is equipped with a 170 cm flight tube, a 14-107 electron bombardment ion source (0.005 in tungsten filament), five analog output systems, a total current monitor, and a recording system consisting of a Honeywell 1508 Visicorder oscillograph and a Tektronix 543A oscillo-

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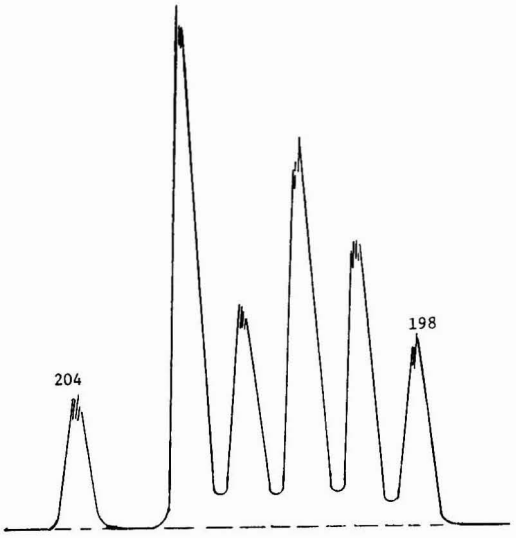

FIG. 1. Resolution of mercury isotope peaks (M/e 198 through 202, and 204) at NOC.

scope. The total current monitor measures half of the total multiplier current, since it receives an input signal on every other spectrometer cycle. The ionizing electron beam operates in a continuous mode, while ion withdrawal remains pulsed. Due to increased ionization, the noise level of the electron multiplier, when operated at 1500 V applied to the dynode strip input end, is high, but lowering the potential to 1100 V results in a better signal-to-noise ratio. The total current, due to specific M/e values,[3] and as a result of continuous electron beam generation, is increased 100-fold over a pulsed electron beam operation at identical instrument settings. A detection limit, of approximately 2.7×10^{-11} torr,[4] is imposed at NOC of 2.9 A filament current, 0.125 mμA trap current, continuous electron beam of 50 eV,[5] dynode strip input end voltage of 1100 V, and pressure of 3×10^{-8} torr (BA gage). The pumping system consists of a Welch 1402 rotary pump, 6 in. diameter mercury

[3] Abbrevations: M/e, mass-to-charge ratio; NOC, normal operating conditions; A, amperes; mμA, millimicroamperes; EV, elevtron volts; BA, Bayard-Alpert.

[4] The smallest amount of current detectable is 8.6×10^{-12} A; total current is 9.5×10^{-9} A at a pressure of 3×10^{-8} torr. Hence:

$$\frac{8.6 \times 10^{-12} \times 3 \times 10^{-8}}{9.5 \times 10^{-9}} = 2.7 \times 10^{-11} \text{ torr}$$

[5] The electron beam voltage is monitored via a vacuum tube voltmeter, 10 megohm input impedence, tapped into the voltage supply line to the ion source filament leads.

diffusion pump (600 liter/sec), Freon cooled baffle, and a liquid nitrogen cooled baffle (5 liter capacity). The ultimate vacuum attainable is 2.0 $\times 10^{-8}$ torr as measured by the hot-filament BA gage located approximately 40 cm from the ion source region. Resolution with the instrument is such that the mercury isotopes are clearly distinguishable and resolved almost to the baseline (Fig. 1.) Sample pressure in the ion source is kept between 3×10^{-8} and 3×10^{-7} torr (BA gage).

Sample Inlet System

Volatile components were introduced into the mass spectrometer ion source via an ambient-temperature, glass-metal sample inlet system

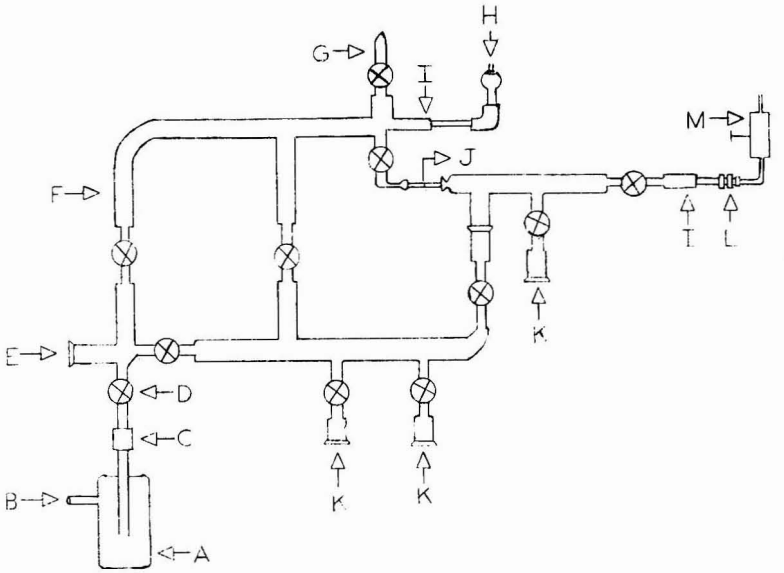

FIG. 2. Schematic of the sample inlet system: A, glass trap immersed in liquid nitrogen; B, to auxiliary pumping unit; C, suction rubber tubing; D, hollow-bore high-vacuum stopcocks; E, connection to preparative sample system; F, glass 15 ml Pyrex tubing; G, attachment for 5 liter reservoir; H, thermocouple gage tube DV-1 M plug to readout system; I, glass-Kovar seals; J, porous frit for molecular leak (not normally used); K, sample ports of ground-glass ball joints; L, Swagelok 3/8 to 1/4″ reducing union; M, Hoke valve 2 RB280, 1/16″ orifice, 20 turns, connecting ion source with inlet pipe 1/8″.

shown in Figure 2. Its total volume[6] is 175 ml and it contains an auxiliary pumping unit (Welch rotary pump, oil diffusion pump, and a glass trap immersed in liquid nitrogen) capable of attaining vacuums of 10^{-7} torr.

[6] Expansion of a gas at known volume and pressure into the sample inlet system at a measured pressure and constant temperature.

Apiezon L stopcock grease[7] was used to seal all stopcocks and ground-glass joints, and small leaks arising from repeated use of the Swagelok fittings were sealed by Fisher Sealit.

Preparative Sample System

Samples usually were rat liver mitochondrial preparations, positioned for mass spectrometric analysis on the all-glass system illustrated in Figure 3. The preparative sample system is attached to the sample inlet

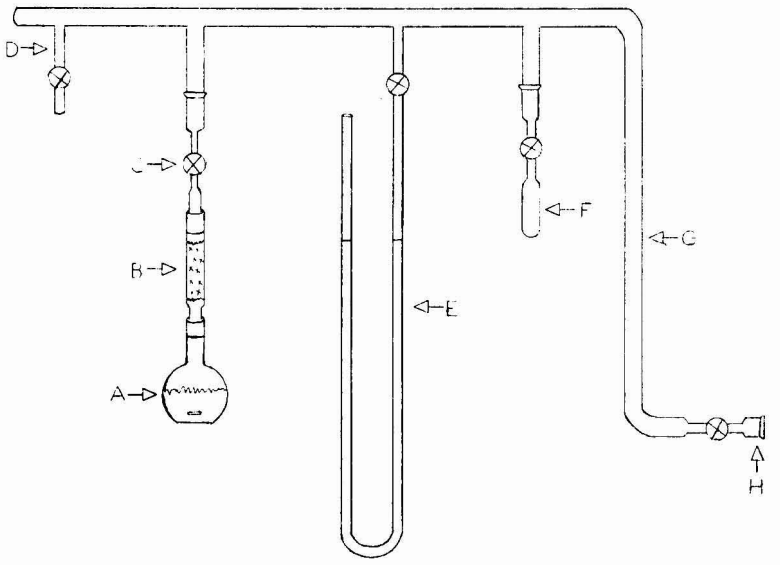

FIG. 3. Shematic of preparative sample system: A, incubation flask (50, 100, or 250 ml) containing a magnetic bar for stirring and incubation medium; B, CO_2 absorber tube containing Mallcosorb with glass wool plugs at each end; C, stopcock; D, stopcock port to introduce gas via Teflon tube connection; E, mercury U-tube manometer; F, collection tube (20 ml capacity stopcock closed) detachable by ball-and-socket joint; G, 15 mm Pyrex tubing; H, ball joint connection to sample inlet system.

system via ball-and-socket ground-glass joints, so its contents may flow to the mass spectrometer via the sample inlet system or to the auxiliary pumping unit described above.

Mass Spectrometric Analysis

After incubation, the tissue-containing flask (usually 100–250 ml), fitted with a carbon dioxide absorber tube containing 25 gm of ab-

[7] Apiezon N grease exhibited higher background levels than L at room temperature.

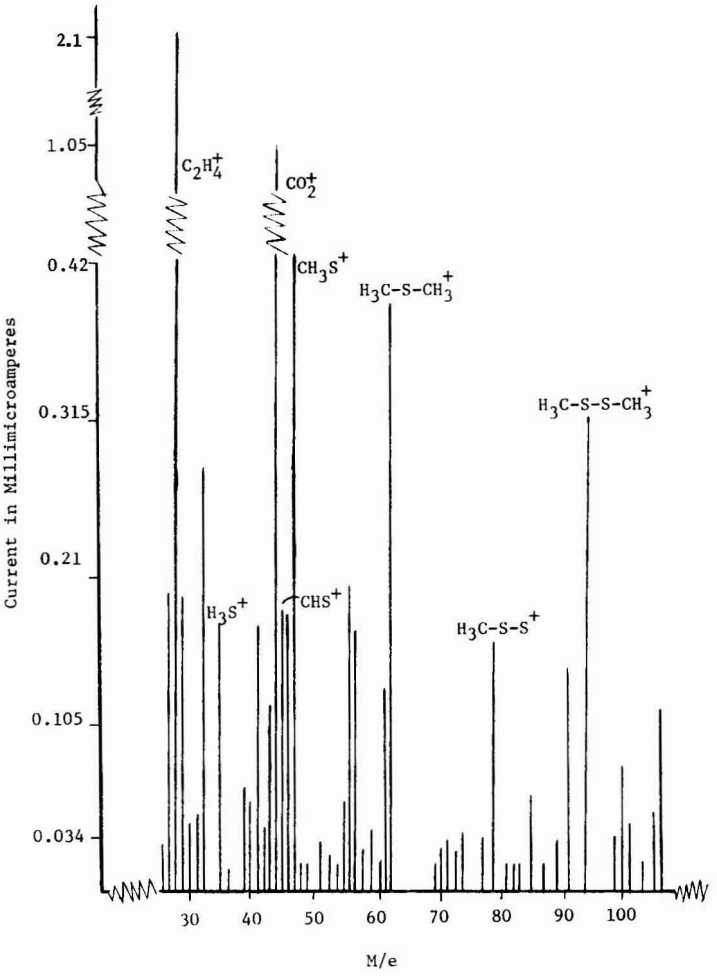

FIG. 4. A composite representation of typical mass-to-charge ratio intensities due to trace volatile metabolites present in effluent from incubating system.

sorber,[8] was attached to the preparative sample system via the ground-glass ball-and-socket joints. Without disturbing the incubation flask atmosphere, the preparative sample system was evacuated to 10^{-7} torr and isolated from the pumping units (auxiliary and instrument). After the collection tube (20 ml) was immersed in liquid nitrogen, the contents of the incubation flask were allowed to enter the preparative sample system and condense in the cold collection tube for 3–5 min. The stopcocks on the absorber tube and collection tube were closed,

[8] Mallcosorb 30–50 mesh, Mallinckrodt.

isolating those segments from the rest of the system. The collection tube, while immersad in liquid nitrogen, was removed and secured to the port of the sample inlet system. In the same manner, subsequent aliquots could be taken from the incubation flask without contaminating its contents with room atmosphere. With the sample inlet system closed to the mass spectrometer, excess air in the collection tube was pumped away via the auxiliary pumping unit, while the collection tube remained immersed in liquid nitrogen. On reaching a vacuum of 1 μ, the sample inlet system was closed to the auxiliary pumping unit and opened to the mass spectrometer (inlet valve maximum orifice opening). After approximately 5 min, the vacuum in the collection tube and the sample inlet system was 3×10^{-8} torr (BA gage). At that point, the liquid nitrogen was removed, and the collection tube allowed to warm to room temperature. Materials coming from the warmed collection tube were detected by noting the increase in current readings on the total current monitor, and cracking patterns (Fig. 4) were recorded at NOC.

RESULTS AND DISCUSSION

Background Contribution

Before the contents of the incubation flask atmosphere are allowed to flow into the evacuated preparative sample system, a sample serving as a control is collected. At NOC, the background of the combined sample inlet system and mass spectrometer is low and arises chiefly from air, (N_2^+ and O_2^+), mercury, and hydrocarbons (*M/e* 27, 29, 41, 43, 45, 56, 57, 91, 100, and 106). A glass trap immersed in liquid nitrogen, and placed in the vacuum line between the forepump and the diffusion pump, virtually eliminated hydrocarbon background levels, presumably originating from back diffusion of forepump oil vapor.

Acetone and xylene may be present when the empty incubation flask, fitted with the glass carbon dioxide absorber tube, is sampled after 2 hr at 37°C. Xylene originates from Apiezon L grease, and acetone presumably is a Mallcosorb contaminant.

The sample inlet and preparative sample systems must be detached and cleaned frequently, since many compounds adsorb to the Apiezon L grease and degas slowly, complicating background problems.

Compound Indentification

To determine small amounts of material contained in the incubation flask atmosphere, it is essential that gross quantities of N_2, O_2, CO_2, and H_2O be removed before being introduced into the mass spectrometer

ion source, since multiplier saturation occurs, making it insensitive to other incoming signals. The N_2 and O_2 are essentially removed by pumping on the collection tube while it is immersed in liquid nitrogen. Mallcosorb removes CO_2 by absorption, and H_2O is not a problem until the collection tube (liquid N_2 temperature) has been at room temperature for 3 min. Then *M/e* 18 produces current levels which saturate the multiplier; however, within 3 min most volatile compounds have been withdrawn from the collection tube.

To ensure completeness of transfer of volatile metabolites from the incubation flask to collection tube, two or three subsequent collections are taken so further aliquots contain only trace amounts (0.009 mμA) of material.

The mixture components may be partially separated when the collection tube begins warming from liquid nitrogen temperature to room temperature. Thus, incubation of mitochondria, freshly prepared from 28 gm wet weight of rat liver and suspended in 100 ml of a buffered medium containing 1.8 mmole sodium pyruvate and 0.2 mmole L-methionine produced a compound, 10 sec after removal of the collection tube from liquid nitrogen, whose molecular ion (M^+) at *M/e* 28 was observed at 10 eV, producing at NOC a current of 2.1 mμA and identified as ethene. A second compound, liberated from the collection tube 60 sec following its removal from liquid nitrogen, giving a M^+ at *M/e* 62 at 12 eV and a current of 0.42 mμA was identified as 2-thiapropane. Upon further warming (120 sec) of the collection tube, a cracking pattern and M^+ at *M/e* 94 (12 eV) matching that of 2,3-dithiabutane was observed, producing a current of 0.31 mμA (Fig. 4). Separation of components, although aiding in identification of the mixture, is not essential. If components of the mixture evolve from the collection tube at the same time, they could still be identified by comparing recorded intensities of major *M/e* values with literature values (7), and by interpreting recorded mixture cracking pattern taken at decreasing electron energies illustrating the ease of fragmentation characteristic for a specific compound or class of compounds (8). Moreover, molecular ions (M^+) may be determined (the *M/e* value at M^+ represents the approximate molecular weight) at low electron energies (sufficient for molecular ionization but not for ionization resulting in fragmentation processes).

It is possible to determine mass spectrometrically the number of moles of each component contained in the incubation flask atmosphere if the conditions stated by Beynon are fulfilled (9).

Due to the sensitivity of this method, changes in minute amounts of

ethylene[9] can be observed, providing information that may be pertinent to the mechanism of ethylene evolution from animal tissue preparations. Moreover, the procedure permits identification of other volatile compounds evolved by normal metabolic processes, which could be overlooked when more selective sampling or isolation procedures are employed.

SUMMARY

Mass spectrometric techniques permit detection and identification of minute amounts of volatile components (contained in an incubation flask) after the mixture (contained in a closed system) is passed through CO_2 absorber, condensed at liquid nitrogen temperature (excess N_2 and O_2 pumped away), and warmed to room temperature over a 2–3 min period.

ACKNOWLEDGMENT

Mr. M. Ohno blew the glass for the sample inlet system.

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[9] Gibson (10) reported 1.8×10^{-9} mole of ethylene produced from mitochondria isolated from approximately 83 gm wet weight of beef heart.

ANALYTICAL BIOCHEMISTRY 19, 426–433 (1967)

A Convenient Method for Isolating the Disaccharide and the Tetrasaccharide in Muramidase Digests of *Micrococcus lysodeikticus* Cell Walls[1]

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We describe a simple method for preparative-scale isolation of the disaccharide and the tetrasaccharide[3] in muramidase digests of *Micrococcus lysodeikticus* cell walls. This procedure, developed to meet a need in this laboratory for gram amounts of the tetrasaccharide, consists of only two steps: (*1*) removing basic and, possibly, certain neutral substances from dialyzates of 12 to 24 hr muramidase digests of the cell walls on the hydrogen ion form of a sulfonated polystyrene resin (Amberlite CG-120) and (*2*) fractionating the substances that are not absorbed on this resin by gel filtration through Sephadex G-15, monitoring the effluent in this step by measuring its absorbance at 230 mμ in a spectrometer equipped with flow cell and recorder.

METHODS

Preparation of the Cell Walls. Dried *Micrococcus lysodeikticus* cells (American Type Culture Collection No. 4698, purchased from Sigma Chemical Co.) were broken and the cell walls separated from the resulting mixture as described by Sharon and Jeanloz (1), except that the trypsin digestion step of this procedure was omitted since the trypsin treatment did not improve the yields obtained in the present isolation procedure and it appeared to cause occasional contamination of the disaccharide fraction with peptide material.

[1] Supported by a grant (GM 05524) from the National Institutes of Health.

[2] National Institutes of Health Predoctoral Fellow.

[3] Jeanloz and co-workers' structural analysis of the disaccharide (2) showed that this substance probably is *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-2-acetamido-2-deoxy-3-*O*-(D-1-carboxyethyl)-D-glucose. The recent investigation of the *Micrococcus lysodeikticus* cell wall glycan by Leyh-Bouille *et al.* (7) has provided corroborative evidence for this structure and has indicated that the cell wall tetrasaccharide is composed of two residues of this disaccharide linked β-(1 → 4).

Muramidase-Catalyzed Hydrolysis of the Cell Walls. The conditions for these digestions were similar to those reported by Sharon *et al.* (2). In a typical experiment, 2.24 gm of the freeze-dried cell walls was suspended in 100 ml of 0.05 *M* ammonium acetate solution; 5 mg of 3 times crystallized hen egg muramidase (Pentex, Inc.) and several drops of toluene were added to this mixture, which then was held at 37° for 12 hr.

Dialysis of the Muramidase Digests. The mixture from the preceding step was transferred to a dialysis sac approximately 50 cm long, prepared from 18/32 in. cellulose tubing (Union Carbide Corp., Food Products Division; this cellulose tubing is a sausage casing grade having a sufficiently small average pore size to retain substances of molecular weight greater than about 3500). The dialysis sac was suspended in a length of glass tubing (i.d. 21 mm) which was fitted with inlet and outlet tubes for continuous flow. The dialysis then was carried out by passing 600 ml of toluene-saturated water through the system at room temperature (23°) over a periood of 12 hr. Concentration of the dialyzate at reduced pressure, followed by freeze-drying, gave 450 mg of yellowish white solid.

Treatment of the Dialyzate with Amberlite CG-120. The resin was a fraction derived from the product designated several years ago as Amberlite CG-120, Type II (200 mesh and finer). Amberlite CG-120 is currently available in the 200 to 400 mesh size from Mallinckrodt Chemical Works. The fraction used in this step was the material that did not flow at a wash velocity of 590 ml/min in Hamilton's procedure (3) for classifying the resin for use in quantitative amino acid chromatography. This is the coarse residue obtained in this method, having a particle size of about 70 μ and larger. The dimensions of the resin bed were 3.5 $\times$ 20 cm. The resin was converted into the acid form and the residue from the dialyzate, dissolved in 5 ml of water, was placed on the column. The column then was washed with 500 ml of water. The effluent from the column yielded 210 mg of colorless, amorphous solid on concentration at reduced pressure, followed by freeze-drying.

Gel Filtration on Sephadex G-15. The dimensions of the Sephadex bed were 2.5 $\times$ 97 cm. A Milton Roy Minipump (Milton Roy Co.) was used to pump water through this column at a precisely controlled rate. The resistance to flow was regulated with a variable-bore stopcock at the bottom of the column. This stopcock was connected by means of 1 mm polyethylene tubing to the 1 cm flow cell of a Beckman (model DB) spectrometer equipped with a Sargent (model SRL) recorder. An event marker on the recorder was connected in parallel with the fraction collector motor. In a typical run a 100 mg sample of the neutral and acidic

material in the effluent from the Amberlite 120 column was dissolved in 2 ml of water and placed on the column. This is a conservative load—considerably lower than the maximal—for a Sephadex G-15 column of this size. Boiled water then was pumped through the column at a rate of 8 ml/hr, collecting 3 ml fractions. Although this step was carried out at room temperature, growth of microorganisms was not a serious problem. The columns occasionally were repacked, however, and the packing autoclaved. Coating the inside of the column tubing with dichlorodimethylsilane seemed to improve the resolution of these columns.

Paper Chromatography of the Oligosaccharides. Descending chromatography was carried out for 50 hr on Whatman No. 1 paper, using the upper phase of an *n*-butyl alcohol/acetic acid/water mixture (25/6/25 by volume, respectively). The reference compound was 2-acetamido-2-deoxy-D-glucose (Sigma Chemical Co.). The cell wall oligosaccharide spots and those containing the reference compound were located by the method of Sharon and Seifter (4), which consists of making them fluorescent by dipping the chromatogram in 0.5 *M* sodium hydroxide in ethyl alcohol/*n*-propyl alcohol mixture (6/4 by volume, respectively) and then heating the paper in an oven at 120° for 5 min.

Amino Acid Analysis. A Beckman-Spinco (model 120) amino acid analyzer was used for these determinations. All samples were hydrolyzed in constant-boiling hydrochloric acid in evacuated, sealed ampoules at 105° for 24 hr. Corrections for glucosamine and muramic acid losses incurred during hydrolysis were approximated by subjecting glucosamine (Pfanstiehl Chemical Co.) and muramic acid (Sigma Chemical Co.) to these hydrolysis conditions and determining recoveries with the aid of the amino acid analyzer.

Electrophoresis. A Gilson Medical Electronics (model D) electrophoresis apparatus was used for high-voltage paper electrophoresis of Fraction 1 from the gel filtration setp. The buffer was 0.05 *M*, pH 2.7 ammonium formate, and the papers were Whatman Nos. 1 and 3 MM. Electrophoresis was carried out for 40 min at a potential gradient of 98 V/cm. Bands containing cell wall oligosaccharide material were made fluorescent by the same procedure that was used for locating these spots on the paper chromatograms. Peptide-containing bands were located by the chlorination procedure of Rydon and Smith (5).

RESULTS

Figure 1 indicates the progress of a typical separation on a 100 cm Sephadex G-15 column in this fractionation scheme. Table 1 gives the weights of the residues from the first three fractions (these were the only fractions yielding significant amounts of residue on evaporation),

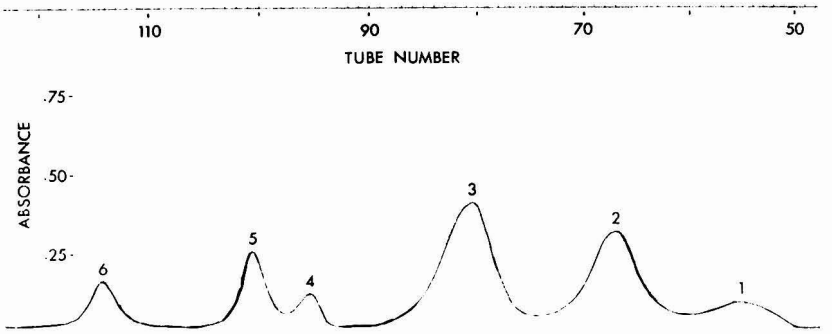

FIG. 1. Fractionation by gel filtration through a column of Sephadex G-15 (2.5 × 97 cm) as indicated by the recorder trace of the 230 mμ absorbance of the effluent. The line at the top of the figure is that drawn by the event marker of the recorder.

the volumes taken for these fractions, and the volumes corresponding to the different peaks.

The residues from Fractions 2 and 3 were identified as the cell wall tetrasaccharide and disaccharide, respectively, by paper chromatography, quantitative amino acid analysis, and digestion of the material from Fraction 2 with muramidase. Thus, hydrolyzates of these products contained muramic acid and glucosamine in equal concentrations. Both resi-

TABLE 1
Typical Data for the First Three Fractions from the Gel Filtration through Sephadex G-15

Fraction	Peak volume, ml	Fraction volume, ml	Residue weight, mg
1	36	27	7.1
2	36	18	27.5
3	45	24	44.7

dues gave single spots on paper chromatography, the R(2-acetamido-2-deoxy-D-glucose) values for the tetrasaccharide fraction ranging from 0.47 to 0.54 and those for the disaccharide from 0.96 to 1.0. Paper chromatography of a muramidase digest of the tetrasaccharide fraction gave a single spot having an R(2-acetamido-2-deoxy-D-glucose) value of 0.96.

Elemental analysis of the tetrasaccharide and determination of water of hydration removable by drying *in vacuo* over phosphorus pentoxide at 78.5° indicated that the freeze-dried product had a composition that is nearly that of an octahydrate. The calculated composition of

$C_{38}H_{62}N_4O_{25} \cdot 8H_2O$ is: C 40.79%, H 7.03%, N 5.01%; weight loss on dehydration, 12.88%. The values found are: C 40.97%, H 6.86%, N 4.91%; residue, 1.16%,[4] and weight loss on dehydration, 13%.

Amino acid analysis of hydrolyzates of the residue from Fraction 1 indicated that this product contained peptide material as well as a fragment, or fragments, of the cell wall polysaccharide. As shown in

TABLE 2
Substances Determined in Fraction 1 Hydrolyzates (μmoles/mg fraction 1)

Expt.	Muramic acid[a]	Glucosamine[a]	Lys	His[b]	Glu	Gly	Ala
317	1.8	1.7	0.21	0.01	0.25	0.25	0.50
391	1.7	1.5	0.11	0.07	0.14	0.15	0.30
396	1.8	1.7	0.24	0.04	0.27	0.28	0.58

[a] Corrected for loss during 24 hr hydrolysis at 105° in constant-boiling hydrochloric acid.

[b] The presence of histidine indicates a certain amount of contamination of the cell walls.

Table 2, the amino acid composition of the peptide-containing product(s) was, disregarding the small amount of histidine present, approximately that of the recently described *Micrococcus lysodeikticus* cell well pentapeptide (6):

$$\text{L-Ala} \rightarrow \text{D-Glu} \rightarrow \text{L-Lys} \rightarrow \text{D-Ala}$$
$$\downarrow$$
$$\text{Gly}$$

and the ratio of muramic acid to glucosamine was 1; yet the ratio of peptide to carbohydrate varied. High-voltage electrophoresis of a Fraction 1 residue separated it into a band containing both peptide and cell wall oligosaccharide material, a minor peptide band, and a band that was composed mostly of cell wall oligosaccharide material—as determined by qualitative tests and by quantitative amino acid analysis of hydrolyzates of material eluted from these bands. Quantitative amino acid determinations have not as yet been carried beyond the point of a single analysis on a small sample from each of the major bands. These data for the material from the oligosaccharide-peptide band, expressed in μmoles, are as follows: serine 0.01, glutamic acid 0.05, glycine 0.06, alanine 0.11, lysine 0.04 (excepting the serine value, these results correspond roughly to the composition of the cell wall pentapeptide); muramic acid 0.07; glucosamine 0.07. Values obtained for the oligosac-

[4] Microanalysis of this substance was performed by Micro-Tech Laboratories, Skokie, Illinois.

charide band are: serine 0.02; glutamic acid 0.02; glycine 0.02; alanine 0.02; lysine 0.14; muramic acid 0.38; glucosamine 0.27.

The weights of the residues from Fractions 4, 5, and 6 were very small. Nevertheless, the absorbance of the substances in the residues accounted for most of the 230 mμ absorbance of these fractions. The character of the ultraviolet spectrum of each residue was that of end absorption. Quantitative amino acid analysis of the hydrolyzates of the residues revealed the presence of significant amounts of ammonia, but of only traces of muramic acid, glucosamine, and amino acids.

DISCUSSION

The present method for isolating the *Micrococcus lysodeikticus* cell wall disaccharide and tetrasaccharide is somewhat simpler and more convenient than are previous procedures (2, 7) since it does not require operations such as elution with salt solutions or gradients and does not utilize chemical tests for determining the progress of fractionation. The monitoring system of the present scheme has proved to be especially satisfactory and has been useful also for following fractionation of oligomer mixtures from the partial hydrolysis of chitin; however, in the latter application the method gives better results if its sensitivity is increased by measuring the absorbance at 225 mμ, since for some unknown reason the extinction of the chitin oligomers in this region is lower than that of the cell wall oligosaccharides.

The disaccharide yields in the present experiments (3.5 to 4% of the cell wall dry weight) are about the same as that Sharon *et al.* (2) reported, but they are somewhat higher than that Leyh-Bouille *et al.* (7) indicated. The latter workers expressed their disaccharide yield as 4.5% of the hexosamine content of the cell walls; on the basis of their hexosamine value (0.93 μmole/mg of the cell walls, which is lower by a factor of 2 than that found by quantitative amino acid analysis in this laboratory) this yield is around 0.9% of the cell wall dry weight. The tetrasaccharide yields in the present work (2 to 2.5%) are higher than that Sharon *et al.* (2) reported and are about the same as observed by Leyh-Bouille *et al.* (7). The ratios of disaccharide to tetrasaccharide yields obtained in the three different laboratories appear to parallel roughly the lengths of the muramidase digestion periods—as they should.

The desirability of dialyzing the muramidase digests in the preparation of these cell wall oligosaccharides deserves some discussion. This step was not included in the first experiments that had to do with development of the present method, and it is not a part of the very recently reported scheme of Leyh-Bouille *et al.* (7), which is similar to the one described here in that it utilizes chromatography on the protonated form

of carboxymethylcellulose and gel filtration through Sephadex G-25 for fractionating the cell wall hydrolysis mixture. The principal reason for dialyzing the muramidase digests as described in the present procedure—in a flowing system, using a membrane that retains substances of molecular weight greater than about 3500—is that it affords a possible means of controlling the extent of higher oligosaccharide hydrolysis without stopping the digestion. A second reason for including a dialysis step such as this is that there may be less risk of fouling the ion-exchange resin, which is regenerated and used again in the present method, when high molecular weight substances are removed prior to separating the mixture on this resin.

The identities of substances in Fractions 1, 4, 5, and 6 from the gel filtration step have not been established. Fractions 4, 5, and 6 are of little importance for the nonce since they contain insignificant amounts of material and no cell wall oligosaccharide or peptide. Fraction 1, on the other hand, is more interesting. It is clearly a mixture, and the position of its peak in the elution pattern indicates that it may be composed of substances having molecular weights of about 1500. The molecular weight of the hypothetical cell wall hexasaccharide is 1453.36; that of a peptidoglycan made up of one tetrasaccharide residue and one residue of the cell wall pentapeptide that Petit *et al.* (6) have described is 1431.40. It is tempting to speculate that the hexasaccharide and a peptidoglycan such as this are the major components of Fraction 1, but the complete identification of the substances in this mixture will require more data than that obtained in the preliminary experiments described here.

SUMMARY

This communication describes a simple, preparative-scale procedure for isolating the *Micrococcus lysodeikticus* cell wall disaccharide and tetrasaccharide from muramidase digests of the cell walls. In this scheme dialyzates of the digests are passed through a column of the hydrogen ion form of Amberlite CG-120 and substances in the effluent from this operation are fractionated by gel filtration through Sephadex G-15. It has been found that the progress of fractionation during gel filtration may be followed most conveniently by measuring the absorbance of the effluent at 230 mμ. Also described are experiments that have partially characterized four other fractions from the gel filtration step.

ACKNOWLEDGMENT

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ANALYTICAL BIOCHEMISTRY 19, 434–440 (1967)

Quantitative Determination of Soluble Cellular Proteins by Radial Diffusion in Agar Gels Containing Antibodies[1]

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A sensitive and rapid method for quantitative estimation of immunoglobulin proteins that relies on their radial diffusion from a central well through gels containing antibodies specific for these proteins was recently described by Fahey and McKelvey (1). A circular precipitin zone forms that is rendered visible for quantitation by direct observation with dark field illumination. A similar method had been described previously by Mancini *et al.* (2) but it requires five or more days for a complete assay, whereas the method of Fahey and McKelvey requires only 24 hr for quantitative estimation of the immunoglobulin fractions. Both methods are very sensitive and can measure below 0.1 μg protein per assay.

Biochemical studies in this laboratory require a sensitive and rapid determination of the concentration of individual plant and animal proteins in crude homogenates and in partially purified preparations. This report describes the modification of the radial diffusion method of Fahey and McKelvey (1) for use in quantitating these smaller soluble proteins in contrast to the large immunoglobulins for which the method was originally developed.

MATERIALS

Five times crystallized chymotrypsin inhibitor from potatoes (PI)[2] was prepared by the method of Balls and Ryan (3). Antiserum was prepared in rabbits by weekly subcutaneous or intramuscular injections of 1 or 2 mg of the purified protein emulsified in complete Freund's adjuvant. The antiserum reacted to give a single precipitin line when challenged with its specific antigen using the method of Ouchterloney (4).

[1] College of Agriculture Scientific paper 2912. Supported by U. S. Public Health Career Development Award Program Grant 1K3-GM-17059 and U. S. Public Health Service Grant GM-12505.

[2] Abbreviation used: PI, chymotrypsin inhibitor from potato.

PREPARATION OF AGAR

Agar gels were prepared using 2% Noble agar (Difco) in 0.1 *M* sodium Veronal, 0.9% NaCl, pH 8.2, and 1:10,000 thimerosal (5). The agar was dissolved in buffer over a steam bath and cooled to 45–50°C. A drop of trypan blue was added to enhance the dark field illumination of the precipitin zones. The desired antiserum was added to give the optimal final dilution (see below) and mixed thoroughly. The agar-antibody solution was poured into plastic 100 × 15 mm Petri dishes to a depth of about 2 mm (15 ml agar per plate) and allowed to cool and solidify. Plates prepared in this manner could be stored at 2°C for three days without noticeable deterioration.

Using a thin-walled hollow cylinder (4.3 mm o.d.), six to eight wells were punched in the gels at 2 cm intervals. Smaller holes may be used to conserve materials. The center agar of the wells was removed by gentle

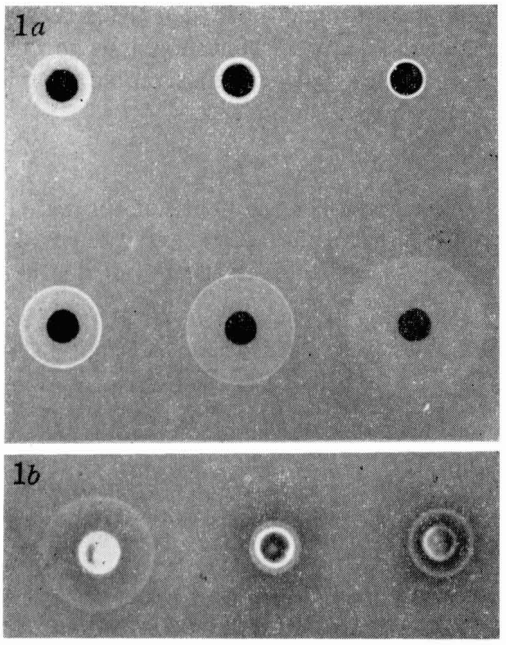

FIG. 1a. Radial diffusion zones of potato inhibitor in agar gels containing anti-potato inhibitor serum (1:100 dilution). Wells, 4.3 mm diameter, contained, counter-clockwise from upper right, 12.5, 25, 50, 100, 200, and 400 μg/ml potato inhibitor. Diffusion time was 44 hr at room temperature.

FIG. 1b. Radial diffusion zones of potato juice from tissues of a year-old Russet potato tuber. From left to right; juice from apical cortex, center pith, and stem end cortex. Conditions identical to Figure 1a.

suction using a Pasteur pipet. The wells were filled with the test solutions. The Petri dishes were covered and incubated in a humid atmosphere at room temperature. The radial diffusion of the antigen through the antibody impregnated agar resulted in the formation of radial precipitin zones. At the appropriate time the diameter of each zone was carefully measured under dark field illumination using a vernier caliper precise to 0.1 mm, aided by a 12 cm diameter 1.5 × magnifier. Parallax must be carefully considered during measurements. Figure 1 shows some typical diffusion zones using anti-PI serum in agar.

RESULTS

Selection of Agar-Antiserum Dilutions

Suitable dilutions of antiserum in agar were determined by testing serial dilutions ranging from 1:25 to 1:200 with known standards. The size of radial diffusion zones at any given concentration of antigen is a function of the antiserum-to-agar ratio (2). Thus the dilution used will depend upon the range of protein concentrations to be tested. For this study, dilutions were chosen that gave measurable precipitin zones in the range of 10 to 1000 μg protein per milliliter.

Effect of Well Depths

In order to determine whether the depth of the well (i.e., agar thickness) affected radial diffusion diameters, a standard PI solution of 270 μg/ml was added to a series of increasing well depths and allowed to diffuse for 40 hr. The final diffusion diameter was independent of the depth of agar (Table 1). Therefore, the volume of agar medium added to different plates need not be carefully controlled.

TABLE 1
Effect of Agar-Antibody Gel Thickness on Diffusion Diameter[a]

Agar thickness, mm	Radial diffusion diameter, mm
0.6	15.1
0.9	15.2
1.2	15.4
2.0	15.4
2.5	15.2

[a] Wells, 4.3 mm in diameter, were filled with a standard solution of 270/μgml of potato inhibitor. Antiserum: agar ratio was 1:100. Diffusion time was 40 hr at room temperature.

Effects of Time and Antigen Concentration on Diameter of Radial Zones

Figure 2 shows the time-dependent effect of PI concentration on the diameters of radial diffusion zones in gels containing anti-PI serum at

1:100 dilution. The zone diameters increased rapidly in the first 20 hr of incubation. At lower antigen concentrations the maximum diameters were achieved during this time. However, at the higher concentrations radial diffusion continued throughout the entire term of the experiments (55 hr). At 4.5 hr diffusion a semilog plot of PI concentration vs. diffusion-zone diameter was linear. However, at longer times the semilog plots

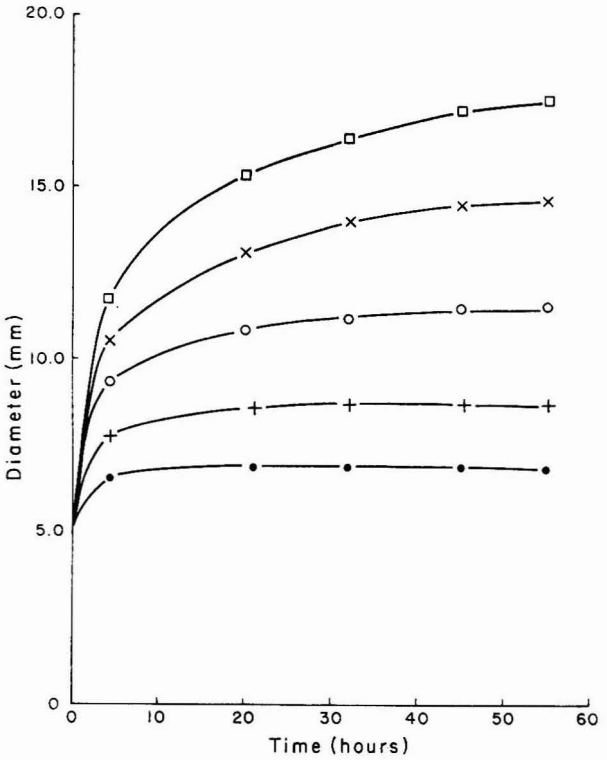

FIG. 2. Radial migration of potato inhibitor in agar gels containing anti-potato inhibitor serum (1:100 dilution). Wells, 4.3 mm diameter, contained standards of potato inhibitor of: (●) 33.8, (+) 67.5, (○) 135, (×) 270, and (□) 540 μg/ml.

exhibited a pronounced progressive curvature. For periods greater than 30 hr it was found that PI concentration plotted against diffusion diameter on a log-log scale was linear, as shown in Figure 3. At the shorter time periods of 4 to 7 hr, with careful measurements, a semilog plot could be used to quantitate purified antigens. However, when using crude homogenates the zones are not always easily discernible. Therefore, in practice, the plates were usually read at about 40 hr and quantitated using a standard log-log plot. In order to conserve both material and time, smaller diameter wells may be used, and the radial zones are

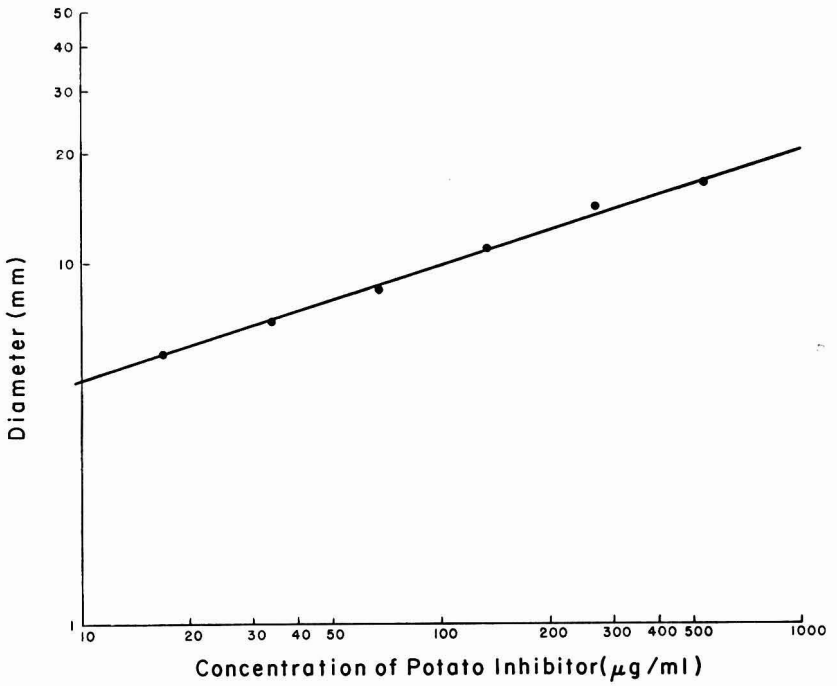

FIG. 3. Log-log plot of diameters of radial diffusion zones vs. PI concentration at room temperature from the 44 hr data of Figure 2.

concomitantly smaller. For example, in separate experiments not described in detail here, using 2.3 mm diameter wells, the log-log plot of the radial diffusion zones vs. concentration was linear within a 20 hr incubation time.

Determination of Chymotrypsin Inhibitor in Potato Juice Extracts

The estimation of PI in potato juice was made using a sample of juice that had been diluted 1:2 with water and with known standards of PI. Table 2 shows the results of a typical experiment. The data are representative of the range of error with this method. The endogenous PI with added purified PI was additive within an error of $\pm 12\%$.

In experiments not reported here, the method has proved useful in determining PI concentrations in various potato plant tissues (e.g., see Fig. 1b) and in determining yields in potato tuber extracts during purification. The latter application has been valuable because the tuber juice contains several different proteins that inhibit chymotrypsin. Exact yields of the specific PI inhibitor in crude extracts and in ammonium sulfate fractions and column eluates were determined with this method.

The method was applied to the determination of some other protein antigens using their specific antibodies in agar gels. Antiserum[3] to the proteins α-chymotrypsin and a derivative, phenylmethylsulfonylchymo-

TABLE 2
Additive Effect of Potato Juice with Known Standards of Potato Inhibitor

Additions of potato inhibitor to potato tuber juice, μg	Potato inhibitor found, μg	Calculated, μg
None	20	—
12.5	30	32
25	40	45
50	67	70
125	145	145

trypsin (6), were successfully used to quantitate a cross-reacting protein, chymotrypsinogen A (7). The method is not without its limitations; specific antiserum to an avian pancreatic protein (8) did not give radial diffusion zones dense enough for precise quantitation.

DISCUSSION

The radial-diffusion technique can be applied to the quantitative determination of specific proteins from tissue extracts. However, as pointed out by Fahey and McKelvey (1), the antiserum must be highly specific for the antigen if radial precipitin zones are to be used for quantitative measurements. When this requirement is fulfilled, the method can easily estimate protein concentrations at levels equivalent to, or lower than, methods generally employed for enzymic analysis (0.1–10 μg/assay). The method is potentially useful for quantitating purified proteins in tissue homogenates when reliable quantitation cannot be obtained by enzyme analysis, for quantitating nonenzymatic proteins or inactive forms of enzymes, and for quantitating a single protein among several proteins having similar assayable properties. Interference of colored substances in reading the radial zones was minimal. If crude homogenates or their extracts contained highly colored substances of low molecular weight, these substances could usually be removed after diffusion was complete by washing the agar overnight with water. This treatment did not affect subsequent readings of the precipitin zones.

The optimal time for measurement of the radial-zone diameters for quantitation must be determined initially for each protein and antiserum. It was found that a linear semilog plot of the diameters of the radial

[3] Antisera to chymotrypsin and phenylmethylsulfonylchymotrypsin were gifts of Dr. R. J. Foster.

precipitin zones vs. antigen concentration was obtained only in the first few hours of diffusion at room temperature. After longer diffusion times the data became linear when plotted on a log-log scale. The time required to obtain a linear log-log plot can be shortened by using smaller diameter wells.

SUMMARY

Radial diffusion of protein antigens through gels containing antibodies has been used to quantitate proteins derived from cellular origins. For quantitation, the diameters of radial precipitin zones formed by tissue extracts were compared with zones formed by known concentrations of the standard proteins. The method employs immunological features of specific proteins to measure concentrations within the range of sensitivity usually obtained only with enzymic analyses.

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ANALYTICAL BIOCHEMISTRY 19, 441–447 (1967)

Collection of $C^{14}O_2$ for Scintillation Counting by a Modification of the Van Slyke Procedure

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Previously reported methods (1–3) for collecting carbon dioxide for scintillation counting are time consuming and involve a transfer of the sample to a collecting apparatus after the quantitative determination. This report describes a method by which the measurement of carbon dioxide and its subsequent collection for scintillation counting can be performed quickly within the same apparatus.

APPARATUS

The apparatus used for the collection of CO_2 is an accessory unit of the standard Van Slyke-Neill blood gas apparatus, preferably the newer model provided with a magnetic stirrer. It is connected to the Van Slyke reaction chamber through the side arm, which is provided with a ℱ 10/30 male joint. Except for this modification no changes in the Van Slyke machine are necessary.

The absorption unit (Fig. 1) consists of a 3-way capillary stopcock, one arm of which connects it to the side arm, the second to the collection compartment, and the third serves to evacuate the system. The dimensions are such that the assembled unit fits into the space between the reaction chamber and the manometer. Because it is made of heavy-walled glass it requires no permanent support. The collection compartment normally rests on a small, flat magnetic stirrer.[2]

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[2] Because of its simplicity and appropriate dimensions a magnetic stirrer, #61/0507, Chemical Rubber Company, 2310 Superior Street, Cleveland, Ohio, is recommended. It is easily attached to the front panel by means of a clip.

The collection compartment accommodates a counting vial which is inserted and removed by disconnecting the receptacle of the T 40/35 joint. This operation is facilitated by rotating the whole unit forward around the smaller joint connecting it to the Van Slyke apparatus. The i.d. of the receptacle is just large enough to admit a standard counting

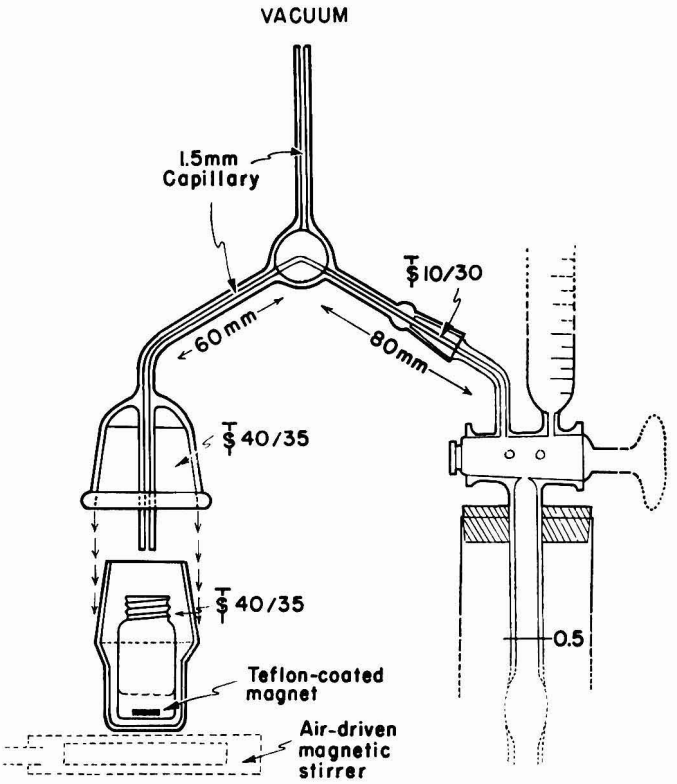

FIG. 1. The absorption unit. The dimensions of the device are such that it fits into the space between the reaction chamber and the manometer of the standard Van Slyke-Neill blood gas apparatus. It is connected to the reaction chamber (right) through the side arm provided with a standard tape. The receptacle (left) accommodates a standard counting vial, and provision is made for agitation of the absorbing agent during transfer of the gas. The system can be evacuated through the 3-way stopcock.

vial, thus keeping dead space to a minimum. The capillary within the collection compartment extends beyond the female joint so that during the transfer the stream of gas is directed toward the surface of the absorbing solution. By means of the 3-way stopcock the capillary connection and the collection compartment can be evacuated at will.

PROCEDURE

The carbon dioxide determinations are performed according to the procedures described by Peters and Van Slyke (4), but the directions as given apply to a Van Slyke-Neill chamber with a magnetic stirrer. Following the recording of the manometer reading "P_2," the carbon dioxide is liberated for collection. This is accomplished by lowering the mercury meniscus to the 50 ml mark and adding 1.0 ml of 25% sulfuric acid from the cup. The stopcock is then sealed with mercury and the mixture stirred for 5 min. During this time, the absorption unit is made ready. The counting vial containing the absorbent and a small Teflon-coated magnet is placed in the receptacle. With the receptacle in place the magnetic stirrer is set in motion and its speed adjusted so that the solution is agitated slowly. Using the 3-way stopcock, the collection compartment and the capillary are then evacuated. When running several samples in succession it is convenient to evacuate the capillary to the side arm while changing the counting vial.

At the end of 5 min stirring, the reaction chamber is connected to the collection compartment. Controlled by means of the stopcock to the leveling bulb, the aqueous solution is allowed to rise until it reaches the capillary of the stopcock above the reaction chamber. Then, by lowering the bulb the mercury meniscus is lowered to the 50 ml mark, the mixture stirred for 30 sec, and the solution allowed to rise again. The stirring is repeated once more for 30 sec, after which the solution is allowed to rise up to, but not into, the capillary of the stopcock. The stopcock is closed, and the leveling bulb is placed in the upper position. The aqueous solution is now expelled into the cup and the capillary to the side arm sealed with mercury. If the original Van Slyke-Neill apparatus is used, the absorption unit must be disconnected during the two final 30 sec stirrings.

To assure complete absorption it is advisable to allow the counting vial to remain in position for 15 or 20 min with the stirrer in operation. Meanwhile the next quantitative determination may be carried out in the Van Slyke apparatus. In order to remove the counting vial from the unit, air is slowly introduced via the vacuum line. The counting vial is replaced and the system evacuated, and the unit is now ready for the absorption of the next sample.

APPLICATION

Carbon-14 carrier solutions of bicarbonate, lactate, and urea were prepared by adding the labeled compounds to nonisotopic solutions of the species. The bicarbonate solution contained about 22 meq/liter, and

the lactate and urea solutions were adjusted to concentrations approximating those expected in Folin-Wu blood filtrates (4). Quantitative determinations were performed according to the techniques of Peters and Van Slyke (4) with the following minor modifications: For bicarbonate samples of 0.5 ml and less, 0.1 *N* lactic acid was used in place of the 0.01 *N* lactic acid, and 1.0 ml of 1.0 *N* sodium hydroxide was substituted for the 0.2 ml of 5 *N* sodium hydroxide recommended by Peters and Van Slyke. For the 2.0 ml bicarbonate samples, the volumes of the reagents used were twice the volumes used in the analysis of 1.0 ml samples. In the determination of urea, urease Type III[3] was used in place of the crude jackbean extract normally employed for this purpose. If whole blood or serum is to be analyzed the use of 2.0 ml of 5 *N* lactic acid instead of 25% sulfuric acid is recommended. This prevents excessive denaturation of the proteins and facilitates the washing of the reaction chamber.

The liberated CO_2 was absorbed in Hyamine[4] or phenethylamine[5] in two controlled series in which the size of the sample was varied from 0.1 to 2.0 ml. In the Hyamine series 2.0 ml of the commercial methanol solution was used. In the phenethylamine series the absorbing agent was a mixture similar to that described by Woeller (5). It contained phenethylamine, methanol, and toluene in a ratio of 1:1:2. Two milliliters of this mixture were used.

After the collection of the carbon dioxide, 15 ml of Liquifluor[6] solution diluted with toluene to contain 4 gm PPO and 50 mg POPOP per liter was added to each vial. The vials were counted in a Nuclear-Chicago 720 series, liquid scintillation system at 4°C over a background of 22 cpm. The results obtained as counts per minute were corrected for counting efficiency using a series of quenched standards and the results expressed as disintegrations per minute per millimole (dpm/mmole).

RESULTS AND DISCUSSION

The results are given in Table 1. The analyses for acid volatile CO_2 (bicarbonate) were carried out to test the advantages of the absorbents and the influence of the volume of sample used. The counting efficiencies of Hyamine and phenethylamine were found to be about 40 and 60%,

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

[4] Trade name of Rohm and Haas Company for *p*-(diisolbutylcresoxyethoxyethyl)-dimethylbenzyl ammonium hydroxide. The solution used was hydroxide of Hyamine-10-X, 1 *M* in methanol, Packard Instrument Company, Inc., Downers Grove, Illinois.

[5] 1-Amino-2-phenylethane, Packard Instrument Company, Inc., Downers Grove, Illinois.

[6] Trade name of Pilot Chemical Company, distributed by Nuclear-Chicago Corporation, Des Plaines, Illinois.

respectively. The studies on lactate and urea were intended to demonstrate the applicability of the technique to any situation in which labeled carbon dioxide may be quantitatively liberated by chemical or enzymic means.

TABLE 1

Analytical Values for Varying Samples of Bicarbonate, Lactate, and Urea (Each value represents the mean of 10 determinations ± standard deviation. The bicarbonate series was used to test the efficiency of the absorbents, Hyamine and phenethylamine.)

Series	mmoles/liter	dpm	dpm/mmole × 10^6
Bicarbonate:			
	0.1 ml sample		
Hyamine	22.68 ± 0.55	11,001 ± 508	4.85 ± 0.27
Phenethylamine	22.95 ± 0.59	10,268 ± 348	4.48 ± 0.20
	0.2 ml sample		
Hyamine	22.27 ± 0.16	20,263 ± 208	4.55 ± 0.04
Phenethylamine	22.45 ± 0.46	19,747 ± 313	4.41 ± 0.12
	0.5 ml sample		
Hyamine	22.49 ± 0.36	51,011 ± 943	4.54 ± 0.11
Phenethylamine	22.59 ± 0.32	50,148 ± 671	4.46 ± 0.07
	1.0 ml sample		
Hyamine	22.57 ± 0.28	99,979 ± 706	4.43 ± 0.05
Phenethylamine	22.22 ± 0.17	98,797 ± 1,636	4.45 ± 0.07
	2.0 ml sample		
Hyamine	22.71 ± 0.38	200,393 ± 8,221	4.41 ± 0.12
Phenethylamine	22.27 ± 0.23	188,625 ± 5,832	4.23 ± 0.13
Lactate:			
	0.5 ml sample		
Phenethylamine	0.1075 ± 0.61	13,923 ± 342	25.9 ± 0.12
Urea:			
	0.5 ml sample		
Phenethylamine	4.41 ± 0.09	78,807 ± 2,227	3.58 ± 0.13

Since the carrier solutions were prepared from labeled compounds with extremely high specific activity only an estimate of the absolute recovery of radioactivity in the carbon dioxide can be given. The carrier was prepared by dissolving 8.4 mg of sodium bicarbonate with a specific activity of 10 mc/mmole in 50.0 ml saline; 0.250 ml of this solution, which contained 0.04 mc/ml, was added to 200 ml of carrier bicarbonate containing 22.6 meq/liter or 22.6/5 = 4.52 meq. The specific activity in the carrier can then be estimated as

$$(0.25)\,\frac{(0.04)(2.2)(10^9)}{4.52} = 4.86 \times 10^6 \text{ dpm/mmole}$$

which represents a recovery of at least 90%. The corresponding values for the urea and lactic acid carriers were 92 and 96%, respectively.

For most biological investigations only relative values are needed or desirable; hence precision rather than accuracy is significant in the application. The data reported for the bicarbonate series were collected for the purpose of statistical analysis to test the precision of the method. The findings summarized in Table 1 indicate that the greatest irregularities occur with 0.1 and 2.0 ml samples. The large standard deviations of the 0.1 ml samples can be attributed to difficulties in the introduction of the sample, and as the volume is increased this error becomes less significant. The low specific activity at the 2.0 ml level is especially noticeable in the phenethylamine series. It is doubtful that this is due to retention of carbon dioxide by the acid solution in the reaction chamber during the final liberation. Peters and Van Slyke (4) report that 99.9% of the dissolved carbon dioxide is liberated following triple extractions in the manner employed in this procedure. There is, therefore, every reason to believe that the values obtained with the 2.0 ml samples should be satisfactory, whereas the use of 0.1 ml samples is not recommended.

For the Hyamine series the means obtained for 0.2, 0.5, 1.0, and 2.0 ml volumes show a significant difference by analysis of variance, and Bartlett's test indicates that the standard deviations (of these means) also differ significantly from each other.

The means obtained at 0.2, 0.5, 1.0, and 2.0 ml volumes with phenethylamine as the absorbent also differ significantly from each other. Further testing indicates that the means at 0.2, 0.5, and 1.0 ml may be the same but differ from that at 2.0 ml.

Bartlett's test shows that the standard deviations of these four sample volumes in the phenethylamine series do not differ significantly from each other. They can, therefore, be combined to give 0.101 as the pooled standard deviation based on 36 degrees of freedom. The confidence-interval estimate for the true mean of ten measurements at each volume can then be expressed as

$$\bar{X} \pm KS / \sqrt{N}$$

where k is the t value at 36 degrees of freedom for the desired probability and $N = 10$, i.e., the number of trials in the set. At each volume this confidence-interval at the 99% level is $(\bar{X} \pm 0.09) \times 10^6$.

The technique described has been developed as an analytical routine in transfer studies of metabolites where time–activity curves are constructed for mathematical treatment. In a typical experiment the specific activity changes have a range of 1 to 1000. Frequently there is sufficient

sample for only one measurement. In that event, the 99% confidence-interval or prediction-interval becomes:

$$\bar{X} \pm ks\sqrt{1 + 1/N} \quad \text{or} \quad (\bar{X} \pm 0.30) \times 10^n \text{ dpm/mmole}$$

The quantities of the absorbent solutions used are well within the limits suggested by Woeller (5), and one would not expect a difference in their absorption efficiency. The data indicate that phenethylamine is superior to Hyamine for two reasons: (*1*) the constancy of the variability as assessed by Bartlett's test and (*2*) the higher counting efficiency of phenethylamine may have distinct advantages in situations in which the isotope concentration is expected to be low.

SUMMARY

A method for the collection of CO_2 for scintillation counting is described in which a simple absorption unit is used as an accessory to the Van Slyke-Neill blood gas apparatus. Carbon dioxide is absorbed directly in the scintillation vial immediately after the determination of the total CO_2 content of the samples. The procedure was tested for varying sizes of samples and absorbents. Phenethylamine is superior to Hyamine as shown by statistical analyses of the data and the higher counting efficiency. The technique is applicable to standard procedures for blood and blood filtrates where labeled carbon dioxide may be liberated by chemical or enzymic means.

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Tryptophan Resolution with the Technicon AutoAnalyzer

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Although the amino acid composition of many proteins has been reported, estimates of the number of tryptophan residues has often been lacking. This situation is a reflection of the difficulty associated with quantitative determination of tryptophan residues in a protein since acid hydrolysis results in tryptophan destruction and alkaline hydrolysis may not release this amino acid quantitatively.

Our interest in resolving tryptophan from lysine and histidine in the Technicon standard 20 hr system is threefold. First, we wish to estimate tryptophan (and other amino acids) released from proteins and peptides by enzymic hydrolysis. Second, there is a requirement for the determination of the levels of free amino acids in biological extracts. Third, we are using alkaline hydrolysis of proteins as one approach to the determination of tryptophan in these proteins.

The results presented here show that tryptophan can be readily resolved from lysine and histidine by increasing the sodium chloride concentration of the pH 5.00 buffer of the Technicon 20 hr Autograd system.

MATERIALS AND METHODS

Standard Amino Acid Mixture. This was made up from Mann Assayed L-amino acids (Mann Research Laboratories) to contain all amino acids commonly found in proteins. In addition, cysteic acid, norleucine and ammonium sulfate were included. All the components were present at a concentration of 0.2 μmole/ml in a solution of 0.1 *N* HCl, 12.5% (w/v) sucrose.

Chromatographic Procedure. The Technicon 20 hr system was used employing a 130 cm column (type A Chromobeads) held at 60°C throughout the run. Samples of 1.0 ml of the standard amino acid mixture were loaded. Elution through the column was at 0.5 ml/min.

Composition of Autograd Buffers. The pH 2.875 and 3.80 buffers required for the Technicon 20 hr system were of standard composition

(Technicon AutoAnalyzer Manual). The pH 5.00 buffers used were also of standard composition except with respect to the concentration of sodium chloride. Buffer A (Table 1) contained the recommend concentration of sodium chloride (35.07 gm/liter) and the other buffers B to D contained increasing concentrations of salt up to 53 gm/liter (see Table 1). The four chromatographic runs in which the four different pH 5.00 buffers were used are referred to subsequently as A, B, C, or D.

TABLE 1
Composition of pH 5.00 Autograd Buffers

Buffer	NaCl added, gm/liter	Conductivity (mmho) at 22°C
A	35.07	42.1
B	41.0	48.0
C	47.0	53.0
D	53.0	57.0

RESULTS

1. Elution Times of Amino Acids with Increasing NaCl Concentration

The increase in sodium chloride concentration of the pH 5.00 buffers had virtually no effect on the elution volumes of amino acids in the first half of the chromatogram, but there was a marked effect on the elution of the basic amino acids. The position of cysteic acid was used as a reference point to measure the times of elution of the relevant amino acids in each of the four chromatograms since the elution time of this acidic amino acid was constant (48–50 min). The results in Table 2 show the times of elution and the integration constants (per millimole) for lysine, histidine, tryptophan, and arginine, respectively for chromatograms A, B, C, and D. The elution times indicated were measured from cysteic acid to the centers of the amino acid peaks.

The relative position of tryptophan with respect to lysine and histidine is shown in Figure 1. It can be seen that the increases in sodium chloride concentration of the pH 5.00 buffers apparently result in delaying the emergence of tryptophan (compare Fig. 1A with D). In fact the elution of tryptophan is relatively insensitive to salt concentration whereas the elution volumes of lysine, histidine, and arginine are markedly decreased as the salt concentration increases (see Table 2).

2. Resolution of Amino Acids with Increasing NaCl Concentration

The considerable increase in salt concentration through the series of pH 5.00 buffers A to D (Table 1) had very little effect on the resolution

TABLE 2
Elution Time and Integration Constants for Tryptophan and Basic Amino Acids with Increasing NaCl Concentration of pH 5.00 Buffers

Buffer	R_f with respect to cysteic acid								Integration constants/mmole[a]			
	Lys		His		Trp		Arg					
	hr	min	hr	min	hr	min	hr	min	Lys	His	Trp	Arg
A	15	27	16	18	16	2	19	36	65.2	66.6	42.7	62.8
B	15	5	15	52	16[b]	approx.	18	50	65.7	[b]	[b]	59.9
C	14	41	15	25	15	50	18	10	67.4	62.2	41.9	60.4
D	14	28	15	9	15	52	17	45	65.8	63.3	40.7	61.2

[a] Corrected to a common norleucine value.
[b] Position indeterminate—see Figure 1B.

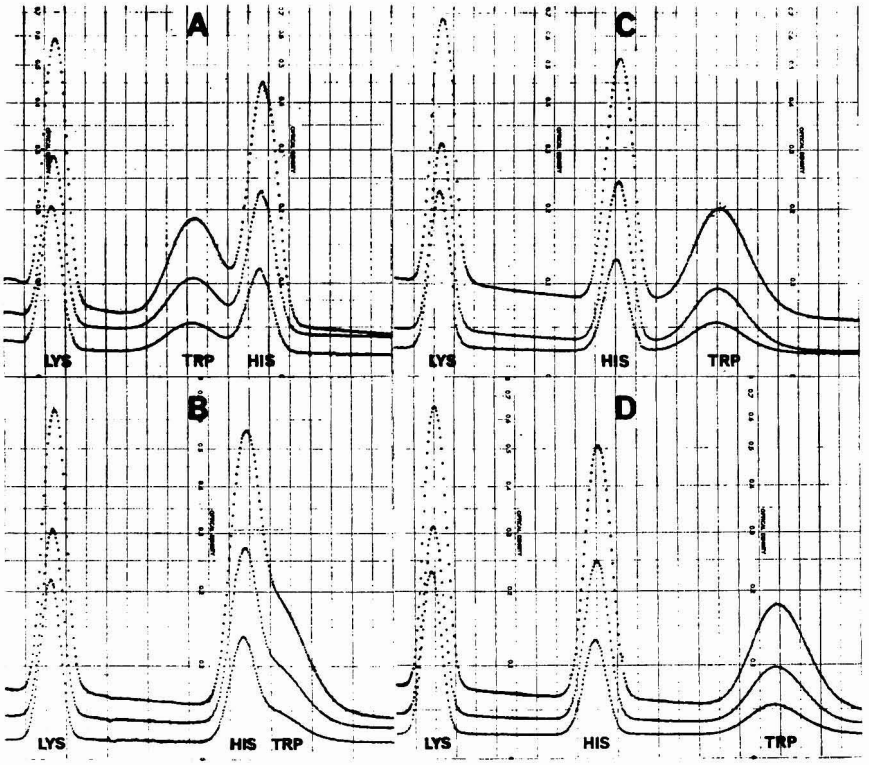

FIG. 1. Relative positions of lysine, histidine, and tryptophan with respect to concentration of sodium chloride in pH 5.00 buffer of the Technicon 20 hr system. Buffers A to D contained, respectively, 35.07, 41.0, 47.0, and 53.0 gm/liter of NaCl.

of amino acids. As indicated before, the composition of pH 5.00 buffers had virtually no effect on the elution volumes of amino acids in the first half of the chromatograms, and, as shown in Table 3 there was little loss in resolution of amino acids toward the end of the chromatogram. The

TABLE 3
Elution Times between Adjacent Amino Acids with Increasing Salt Concentration of pH 5.00 Buffers

Buffer	Separation of adjacent amino acids, Min					
	Ileu-Leu	Leu-norleu	Tyr-Phe	Lys-His	His-Trp	Trp-Arg
A	26	25	23	51	−16	214
B	26	25	23	47	0 approx.	180 approx.
C	25	24	24	44	24	141
D	25	24	23	41	43	113

elution times given in Table 3 refer to the times between the centers of adjacent amino acid peaks.

DISCUSSION

The possibility of decreasing the over-all time required for a complete amino acid analysis was not the primary object of this study, as Autograd systems for fast chromatograms have already been described (see *Fourth Amino Acid Colloquium,* Technicon Instruments Co., 1966, p. 17). However, it is a useful adjunct to the resolution of tryptophan that an increase in salt concentration from 35.07 to 53.0 gm/liter of the pH 5.00 buffer decreases the time required for an analysis by some 1 hr 51 min (Table 2). This is due largely to the marked effect of salt concentration on the elution of arginine.

The relative positions of lysine, histidine, and tryptophan with increasing salt concentrations of the pH 5.00 buffers (A to D) are shown in Figure 1. Although tryptophan appears to be retarded through the series, the results in Table 2 show that the elution of this amino acid is almost completely insensitive to the range of salt concentrations used here. A comparison of the position of tryptophan using the standard Autograd system (pH 5.00 buffer A) and pH 5.00 buffer D shows that the increase in sodium chloride concentration of the latter system resulted in a decrease of only 10 min in the elution time of this amino acid. The corresponding decreases in elution times for lysine and histidine are 59 and 69 min, respectively.

Since the increase in salt concentration of the pH 5.00 buffer to a value of 53 gm/liter has permitted adequate separation of tryptophan from lysine and histidine without loss of resolution of other amino acids (see Table 3), it may be possible to increase the salt concentration even further and thereby decrease the analysis time. However, any drastic increase in salt concentration would result in considerable shrinkage of the resin and undesirable increase in column pump pressure.

It is of interest to note the position of tryptophan in a modified Autograd system suggested by van Stekelenburg (*Fourth Amino Acid Colloquium,* p. 20). In this 12.5 hr chromatogram, the last four chambers of the Autograd contain pH 5.00 buffer with 1.0 *M* sodium chloride. This salt concentration is slightly higher than that of buffer D (Table 1) and results in excellent resolution of tryptophan from lysine and histidine.

SUMMARY

Tryptophan can be resolved completely from lysine and histidine in the Technicon 20 hr system simply by increasing the sodium chloride concentration of the pH 5.00 buffer to a value of 53 gm/liter. Resolution of

other amino acids is virtually unchanged. The increase in salt concentration decreases the analysis time by nearly two hours.

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Analysis by Reflectance Spectroscopy of Nucleotides Resolved on Chromatoplates

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Thin-layer chromatography affords several distinct advantages in the separation of nucleic acid derivatives. The resolution of nucleotides on chromatoplates not only can be achieved in but a fraction of the time required in paper chromatography (1–3), but the quality of the resolution attained with the use of thin layers of cellulose and silica gel G has been found to be superior to that attained with the use of paper (1, 4). Randerath (2) has reported that the spots obtained on cellulose thin plates are smaller and more discrete than those obtained on paper under identical conditions.

Reliable methods have been devised for the analysis by means of spectral reflectance of the components of mixtures of dyes (5) and of amino acids (6–8) resolved on thin plates. An extension of the technique to the ultraviolet portion of the spectrum has also made it possible to analyze aspirin-salicylic acid mixtures (9) and some vitamins of the B group (10) following their resolution on chromatoplates. In many cases the resolved substances could be identified by means of their reflectance spectra and without recourse to R_f values; in all cases it was possible to carry out their determination without first eluting the species of interest from the adsorbent. In view of the obvious advantages afforded by this technique, it was decided to apply spectral reflectance to the analysis of some nucleotides resolved on thin-layer plates.

METHODS

The nucleotides were employed in the form of aqueous stock solutions. A 2.0 mg/ml solution of each nucleotide was prepared by dissolving appropriate amounts of adenosine-3′(2′)-phosphoric acid monohydrate (3′- + 2′-AMP), disodium guanosine-3′(2′)-phosphate monohydrate (3′- + 2′-GMP), uridine-3′(2′)-phosphoric acid (3′- + 2′-UMP), cytidine-2′(3′)-phosphate (2′- + 3′-CMP), and adenosine-5′-diphosphate (5′-ADP) in distilled water. A few drops of concentrated ammonium hydroxide

were added where necessary to achieve solution. The first three nucleotides, which were mixtures of the 3′ and 2′ isomers, were of California Corporation for Biochemical Research grade A quality. The remaining two were from Nutritional Biochemical Corporation of Cleveland, Ohio. The stock solutions were applied as spots by means of 5 and 10 μl micropipets.

A Desaga-Brinkman model SII applicator was used to coat $20 \times 20 \times 0.35$ cm plates with a 15:85 MN-cellulose powder 300 (Macherey, Nagel and Co., 516 Düren, Germany)-water mixture. The height of the applicator gate was set at 0.25 mm for plates employed for qualitative analysis and at 0.50 mm for plates used for quantitative work. After the plates had been allowed to "set" for 15 min, they were dried at 90° for 30 min in a mechanical convection oven.

After spotting, the plates were dried at 85° for 15 min and then developed by the ascending technique with the use of an 80:18:2 saturated ammonium sulfate, 1 *M* sodium acetate, and isopropyl alcohol mixture according to a procedure devised by Randerath (2, 11). The developed plates were dried at 85° for 60 min. Since the resolved compounds appeared as light to dark blue spots under ultraviolet light (maximum at 254 mμ), no difficulty was experienced in locating them on the plates. For qualitative analysis, each spot was excised and then centered in a windowless reflectance cell atop a uniformly thick layer of 40 mg of cellulose powder that had been packed in the cell with the use of a fitted tamp made of an aluminum planchet affixed to a cork stopper (10). The ultraviolet reflectance spectra of these samples were recorded with the use of a Beckman model DK-2 spectrophotometer fitted with a standard reflectance attachment. For quantitative work, enough cellulose powder from the plate under investigation was added to the excised spot to make up an analytical sample weighing 60 ± 0.2 mg. This was ground in an agate mortar for seven periods of thirty strokes each to ensure homogeneity and relatively uniform particle size and then packed into a windowless reflectance cell with the use of the tamp. The reflectance of these samples was measured with a Beckman model DU spectrophotometer fitted with the appropriate reflectance attachment. The reflectance standard in all of the cases discussed above consisted of adsorbent from the plate under examination, with quantitative standards being prepared in the same manner as analytical samples.

RESULTS AND DISCUSSION

Qualitative Analysis. Although only one spot was obtained for the 3′- + 2′-UMP mixture and 2′- + 3′-CMP, the 3′- + 2′-AMP and the

3′- + 2′-GMP mixtures were resolved into their component isomers. As indicated in Table 1, the R_f values obtained for the various nucleotides were in general agreement with those reported by Randerath (2, 11). Differences in the procedures followed in the preparation of the chromatoplates and the fact that the MN-cellulose employed by Randerath, unlike that used in this study, included a binder are probably responsible for whatever variations were observed.

TABLE 1
R_f Values and Absorption Maxima of Nucleotides Adsorbed on MN-Cellulose, and Absorption Maxima of Nucleotides in Aqueous Solution

Nucleotide	Isomer	R_f value		Absorption maximum. mμ	
		MN-300	MN-300G (2,11)	Reflectance	Transmittance
AMP	2′	0.35	0.35	260	260
AMP	3′	0.21	0.28	260	260
ADP	5′	0.45	0.48	259	259
CMP	2′ + 3′	0.73	0.73	281	280
GMP	2′	0.51	0.58	256	252
GMP	3′	0.39	0.49	256	252
UMP	3′ + 2′	0.68	0.73	264	262

Except for AMP and ADP, the reflectance spectra of the various nucleotides adsorbed on cellulose, which are depicted in Figure 1, were such as to permit the ready identification of the adsorbed species. It was also not possible to distinguish between the isomers of AMP and the isomers of GMP solely on the basis of spectral data, as identical spectra were obtained for the members of each isomer pair. By making use of both spectral data and R_f values, however, all the resolved nucleotides could be identified unequivocally. It is noteworthy that in most cases the absorption maximum of the reflectance spectrum coincided closely with the absorption maximum of the corresponding transmittance spectrum obtained with the use of an aqueous solution of the nucleotide. This would seem to indicate that these data, which are listed in Table 1, can be used interchangeably for most purposes.

Quantitative Analysis. Calibration plots for the nucleotides adsorbed on MN-cellulose-300 were prepared by applying different volumes of the stock solutions to thin-layer plates, which were then developed, dried, and readied for the measurement of reflectance according to the procedure outlined above. Because the isomers of AMP and of GMP had identical spectra, it was decided to include only the 2′ isomer of the former and the 3′ isomer of the latter in this particular study.

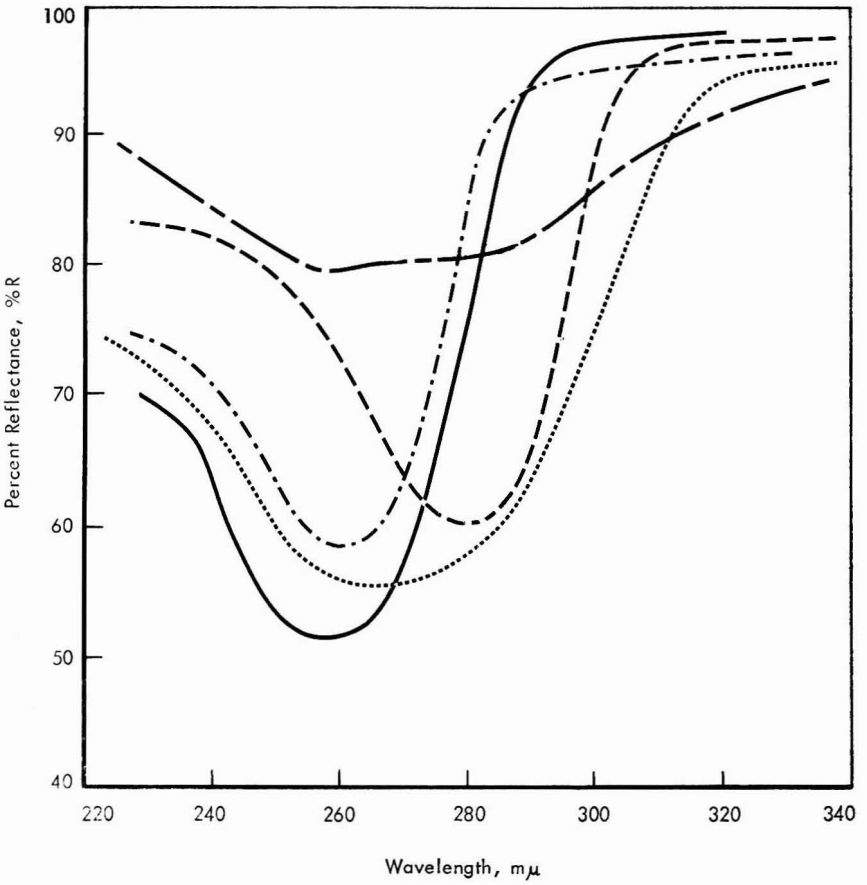

FIG. 1. Reflectance spectra of: 3′-GMP and 2′-GMP (— - —); 2′- + 3′-CMP (——); 3′-AMP and 2′-AMP (-•-); 3′- + 2′-UMP (• • •); and 5′-ADP (unbroken curve) adsorbed on MN-cellulose 300.

When the percent reflectance at the absorption maxima of the various nucleotides was plotted as a function of the logarithms of their concentrations, the curves depicted in Figure 2 resulted. This mode of presentation has the advantage of emphasizing the optimum concentration range for maximum accuracy, which corresponds to the straight-line portion of the curve where the slope is greatest. By employing this slope in conjunction with the equation $\%\ \text{error} = 2.303d\ (\log\ C)/dR$, it is also possible to estimate the percent error in the analysis that would derive from an error amounting to 1% R (12). The optimum range and the probable percent error that were deduced in this manner for the analysis of the various nucleotides adsorbed on cellulose are listed in Table 2.

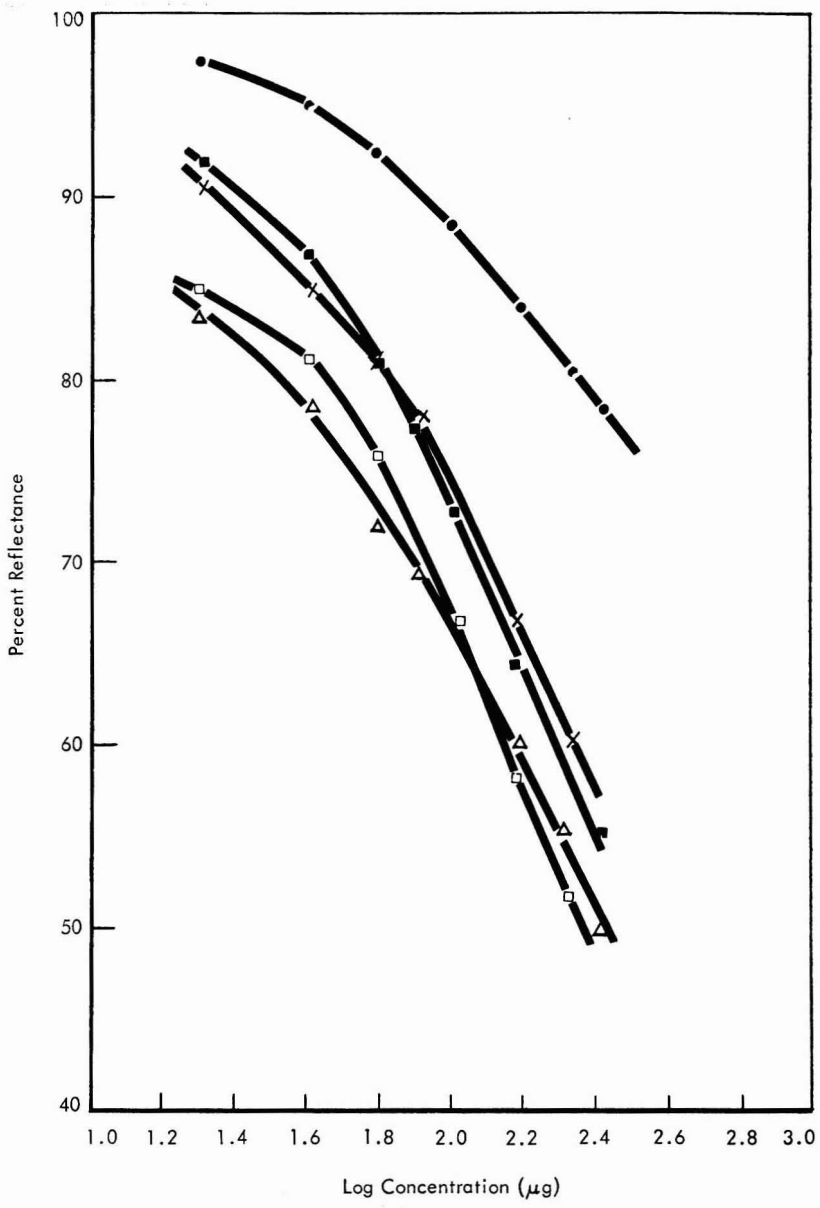

FIG. 2. Percent reflectance of 2′-AMP at 260 mμ (■), 5′-ADP at 259 mμ (□), 2′- + 3′-CMP at 281 mμ (×), 3′-GMP at 256 mμ (●), and 3′- + 2′-UMP at 264 mμ (△) adsorbed on MN-300 cellulose as a function of the logarithm of concentration.

TABLE 2
Optimum Range and Probable Percent Error in the Analysis of Nucleotides Adsorbed on MN-Cellulose 300

Nucleotide	Optimum range		% analysis error/ error of 1% R
	Reflectance (% R)	Concn. (μg/60 mg cellulose)	
2′-AMP	55–80	65–250	−5
5′-ADP	50–80	50–200	−5
2′- + 3′-CMP	55–80	65–250	−5
3′-GMP	75–90	80–250	−9
3′- + 2′-UMP	50–75	50–250	−6

As is shown there, a probable percent error of −5 to −6% was indicated for all of the nucleotides save 3′-GMP, for which a figure of −9% was obtained. This anomaly may be ascribed to the fact that the 3′-GMP did not absorb as strongly in the ultraviolet as the other nucleotides when adsorbed on cellulose.

The precision that can be achieved when the above procedure is employed for the assay of nucleotides was determined by running through the analyses with replicate samples of each nucleotide. As may be seen in Table 3, which summarizes the results of this study, the reflectance

TABLE 3
Reproducibility of Percent Reflectance Obtained for Replicate Samples of Nucleotides Adsorbed on MN-Cellulose 300 at Their Absorption Maxima

Sample	Concn. (μg/60 mg cellulose)	Reflectance (% R) of samples				Mean value	S.D.	% deviation in concn.
		1	2	3	4			
2′-AMP	70	78.8	79.2	80.4	79.2	79.4	±0.78	4
5′-ADP	100	66.3	66.2	67.2		66.6	±0.59	3
2′- + 3′-CMP	130	68.8	68.8	68.2	68.0	68.5	±0.39	2
3′-GMP	150	83.0	84.0	83.2	84.5	83.7	±0.70	6
3′- + 2′-UMP	50	74.2	74.8	73.5		74.2	±0.65	4

readings obtained for samples of the various nucleotides adsorbed on cellulose were found to have standard deviations ranging from 0.39 to 0.78 reflectance unit. If use is made of the analysis error data included in Table 2, deviations of this order in the reflectance readings are found to correspond to deviations of 2–4% in the measurement of the concentration of all of the adsorbed nucleotides except 3′-GMP. Deviations to be expected in the determination of this particular nucleotide were estimated to be 6%.

SUMMARY

A procedure has been devised whereby five nucleotides—adenosine-3′-(2′)-phosphoric acid, adenosine 5′-diphosphate, cytidine-2′(3′)-phosphate, disodium guanosine-3′(2′)-phosphoric acid—resolved on cellulose thin-layer plates can be analyzed by means of ultraviolet reflectance spectroscopy. The procedure makes it possible to determine the concentration of the adsorbed nucleotides, which can also be identified by using reflectance spectra in conjunction with R_f values, with a precision of 2–6%.

ACKNOWLEDGMENTS

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ANALYTICAL BIOCHEMISTRY 19, 461–467 (1967)

A Simple and Rapid Method for the Quantitative Determination of α-Tocopherol (Vitamin E) in Leaves

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The method described here is essentially a modification of the method of Dilley and Crane (1) but has the advantages of greater speed and simplicity and gives higher recoveries.

MATERIALS

Benzene, petroleum ether (60–80), diethyl ether, absolute ethanol, and acetone were all analytical reagent grade. Chloroform was redistilled commercial grade, and commercial grade 95% ethanol was refluxed over KOH and $KMnO_4$ (2 gm and 1 gm per liter, respectively) for 30 min before collecting the fraction distilling at 77–78°C. Sodium borohydride, ferric chloride (A.R.) and 2,2-dipyridyl (A.R.) were obtained from British Drug Houses, Ltd. Standard α-tocopherol was prepared by preparative thin-layer chromatography from DL-α-tocopherol (C-Grade, Calbiochem, Los Angeles).

METHODS

Extraction of the leaves was based on the method of Booth (2). Leaf material (0.3 to 0.5 gm) was ground with 5 ml of acetone in a Tenbroeck glass tissue grinder (Kontes Glass Co., Vineland, New Jersey). Petroleum ether (10 ml) was then mixed in and the supernatant solution decanted into a glass filter funnel fitted with a solvent-washed glass-wool plug. This acetone/petroleum ether extraction was repeated as often as was necessary to remove all of the green color from the leaf residue and grinder (2). The combined filtrates were collected in a 100 ml flask and the solvent was removed on a rotary evaporator. About 5 ml of absolute ethanol/benzene (90/10 v/v) was added to the flask and then removed on the rotary evaporator to remove traces of water in the lipid residue. The dry leaf lipid was then transferred to a tapered, glass centrifuge tube in 4×1 ml washings with petroleum ether. The petroleum ether was

removed under a stream of nitrogen and the residue redissolved in 0.2 ml of chloroform.

α-Tocopherol in the extract was separated from other lipids by a single run on a thin layer (250 μ) of silica gel G (Merck) using benzene as the developing solvent. The chloroform solution was applied to the 20 × 20 cm layer as a 12 cm streak using an applicator of the type designed by Achaval and Ellefson (3). Petroleum ether (0.2 ml) was used to rinse out the centrifuge tube, pipet, and applicator and this was also applied to the layer. Standard α-tocopherol spots were applied on both sides of the streak.

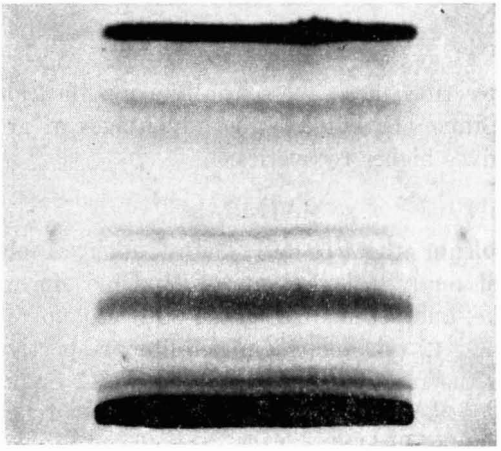

FIG. 1. Thin-layer chromatogram showing separation of α-tocopherol from the lipid extract from 0.5 gm fresh weight of white clover leaves. Layer; Silica gel G layer; developing solvent benzene. Stained with iodine vapor. α-Tocopherol standard spots were applied on either side of the extract.

Thin-layer plates had been activated at 120°C for 30 min immediately before use and were always developed at 90° to the direction of spreading. The solvent was allowed to ascend to a height of 14–15 cm from the origin (approximately 30 min), the plate was air dried for 5 min, and the separated bands were detected by exposure to iodine vapor. A few crystals of iodine in the bottom of a T.L.[1] tank or similar receptacle will soon saturate its atmosphere with iodine vapor, particularly if the tank is placed in a warm place, e.g., on top of a hot-air oven. The band corresponding to α-tocopherol was usually the first to appear and was quite easily recognized (Fig. 1). It was carefully outlined with a needle and the iodine allowed to evaporate before scraping the adsorbent into a glass filter funnel fitted with a solvent-washed, glass-wool plug. Elution of the

[1] Thin layer.

α-tocopherylquinone, formed by the iodine oxidation of α-tocopherol, was accomplished by washing the adsorbent with 4 × 2 ml of diethyl ether. These washings were collected in a graduated centrifuge tube, the ether was removed under a stream of nitrogen, and the residue was redissolved in 3 ml of 95% ethanol. Centrifugation of the ethanolic solution may be necessary if traces of adsorbent pass through the filter. The absorbance of this solution was measured at 262 mμ before and after the addition of a few small crystals (<0.05 mg) of sodium borohydride. Dilley and Crane give $\Delta A^{1\%}_{m} = 414$ for calculating α-tocopherol concentration in this system, so we have routinely multiplied $\Delta A \times 72.5$ to give the amount of α-tocopherol (in μg) in the chromatographed extract. A complete UV spectrum (320 to 230 mμ) before and after reduction with borohydride can be done as a quick check on the purity of the preparation. A modification (4) of the classical dipyridyl/ferric chloride assay (5), in which α-tocopherol reduces ferric to ferrous iron, which in turn reacts with 2,2-dipyridyl to give a red complex, was used to check results and to test the efficiency of the iodine oxidation.

RESULTS

α-Tocopherol is quantitatively oxidized to its quinone by iodine. When 30 μg of α-tocopherol was chromatographed, stained with iodine, and eluted with diethyl ether the recovery was zero as measured by the dipyridyl/ferric chloride assay and 97% as measured by the method of Dilley and Crane. Similarly when the equivalent of 0.5 gm of white clover leaflets was chromatographed and the chromatogram exposed to iodine vapor, no dipyridyl/ferric chloride reacting substance could be eluted from the region between the α-tocopherol standards. The eluate did give a near-perfect UV absorption curve for α-tocopherylquinone (Fig. 2) and showed the characteristic change upon reduction with borohydride. This held for all the plant species for which analytical figures are given below (Table 3). The oxidation of the tocopherol to its quinone was completed by the time the band became visible and it was never possible to recover partially oxidized material from even a very lightly stained band. Very light staining was employed except where it became necessary to differentiate the α-tocopherol band from another closely following band. Exposure times of 10 to 20 sec have always been sufficient in a chamber saturated with iodine vapor at 20°C. A comparison of the recoveries of standard α-tocopherol as measured by the proposed method and by the dipyridyl/ferric chloride assay (4) is given in Table 1. In this instance two 4 cm streaks, each containing 15 μg of standard, were applied to a 20 cm^2 layer. After development and evaporation of the benzene, one-half of the layer was covered with a 20 × 10 cm glass plate

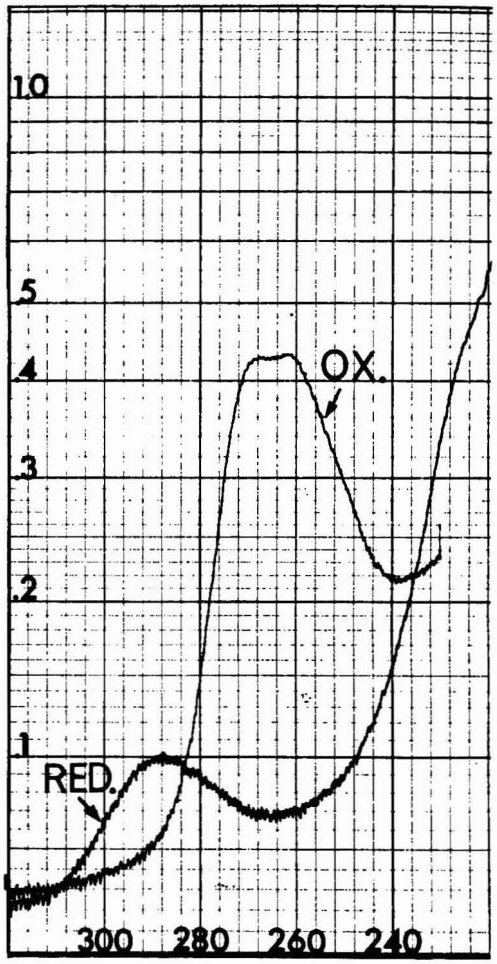

FIG. 2. UV absorption spectrum of α-tocopherylquinone (OX.) formed by iodine oxidation of α-tocopherol and of the hydroquinone (RED.) formed by reduction of the quinone with sodium borohydride. α-Tocopherol was separated from an extract of 0.5 gm fresh weight of white clover leaflets by a single run on silica gel G with benzene as developing solvent. α-Tocopherol content of leaflets = 52.5 μg/gm fresh weight.

and the remainder exposed to iodine vapor. The stained zone was marked, as was a complementary but fairly liberal area on the unstained portion of the layer. Both zones were scraped up, eluted, and finally made up to 3 ml in ethanol. A blank zone was also removed from the unstained area of the plate, carried through the same procedure, and used as the blank in the dipyridyl/ferric chloride assay.

TABLE 1
Recoveries of Standard α-Tocopherol after TLC as Measured by the Dipyridyl/Ferric Chloride Assay and the Proposed Iodine Oxidation Assay[a]

Method	α-Tocopherol, μg		% recovery
	Chromatographed	Recovered	
Dipyridyl/ferric chloride	15	15	100
Iodine oxidation	15	14.5	97

[a] See text for details.

When an extract of 0.6 gm of white clover leaves was divided into two equal portions, 15 μg of α-tocopherol added to one portion and then both parts were analyzed by the method outlined; recovery of the added α-tocopherol was in the range 95–98%. In agreement with Booth (2) we find that the extraction method is quantitative because no further α-tocopherol could be extracted from the leaf residue by a 24 hr exposure to acetone at 2–4°C.

Since we have found that the α-tocopherol content of leaves varied considerably (see also reference 6), a single extract of 10 gm of white clover leaves in 10 ml of petroleum ether was repeatedly analyzed to test the reproducibility of the assay. The results presented in Table 2 show that

TABLE 2
Reproducibility of α-Tocopherol Assay[a]

Date (1966)	Number of determinations	Tocopherol, μg	
		Mean	S.D.
Oct. 18	6	17.41	0.01
Oct. 20	6	13.67	0.01

[a] Aliquots of a single extract (= 10 gm white clover leaves in 10 ml petroleum ether) were repeatedly analyzed on the dates indicated.

the reproducibility was good. Chromatography of the extract should be carried out as soon as possible, however, since about 20% of the α-tocopherol in the extract disappeared over 48 hr even though the extract had been stored at 2–4°C.

When a white clover extract (≡0.5 gm fresh weight of leaves) was chromatographed, the recovered α-tocopherylquinone divided into two, one half analyzed and the other half rechromatographed on silica gel H using benzene as the developing solvent, analysis of the rechromatographed quinone showed a 94–96% recovery compared with the original analysis. Considering the different chromatographic mobilities of the chromanol and its quinone, we conclude that, in white clover at least, there is no interference with the assay, after a single T.L. run in benzene.

TABLE 3
α-Tocopherol Content of Some Leaves

Species	Month	α-Tocopherol, μg/gm fresh wt.
Trifolium repens[a] (white clover)	June–July	37.5–52.0
	Oct.	22.0–35.5
Xanthium orientale[b] (Oriental cocklebur)	Oct.	76.0
Cucurbita pepo[b] (var. butternut)	Oct.	55.0
Glycine soja (Biloxi soybean)	Oct.	55.0
Lolium perenne[a] (perennial ryegrass)	Oct.	38.5
Trifolium pratense[a] (red clover)	Oct.	42.0
Dactylis glomerata[c] (orchard grass)	Oct.	55.0

[a] Growing outside in natural daylength (= 10 hrs in June–July and 13 hrs in October).
[b] Growing in glasshouses in constant light.
[c] Growing in glasshouses in natural daylength.

The equivalent of 1.0 gm fresh weight of white clover leaves has been successfully separated on one 250 $\mu \times 20$ cm^2 layer in this system.

α-Tocopherol levels found in a range of species by the present method are given in Table 3.

DISCUSSION

It is considered that this procedure has three major advantages over that of Dilley and Crane. First, the extraction is much more rapid and an individual analysis can therefore be completed in 90 min or less. Second, the iodine visualization of the separated tocopherol eliminates the possibility of error due to incorrect marking of the α-tocopherol region on the thin layer. This can happen for instance when the bands run in a wavy manner through some fault of the layer. In these cases recovering the zone between the α-tocopherol standard will not give a quantitative recovery of the α-tocopherol from the extract. The major advantage, however, is that the oxidation of α-tocopherylquinone is accomplished with such ease and efficiency. Dilley and Crane claim 87% recovery of α-tocopherol in their assay, but most of that loss could be attributed to the ethanolic gold chloride oxidation step with its accompanying isooctane

extraction of the quinone. We have consistantly observed recoveries of pure α-tocopherol of greater than 95% in our thin-layer/iodine oxidation method.

SUMMARY

A modification of the method of Dilley and Crane for the analysis of α-tocopherol in leaves is described. Detection of the α-tocopherol band separated by TLC and oxidation of α-tocopherol to its quinone are performed simultaneously with iodine vapor. An individual analysis can be completed in 90 min or less and recoveries are of the order of 95–98%.

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ANALYTICAL BIOCHEMISTRY 19, 468–480 (1967)

Isolation of Krebs Cycle Acids from Tissues for Gas Chromatography[1]

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Successful application (2–7) of gas chromatography to the separation of standard mixtures of Krebs cycle acids has prompted interest in the exploitation of this technique in the resolution and quantitative measurement of organic acids in biological materials. Satisfactory gas chromatograms, however, have thus far been reported (3–6) only with plant materials, which are relatively rich in these acids. Before gas chromatography can be effectively utilized in the separation of organic acids from animal products, extensive concentration and purification of the extracts is necessary. This has been recognized in the work of Alcock (8), which describes a simple but efficient method for recovering model mixtures of standard organic acids from aqueous systems in a concentrated form.

The present study uses gas chromatography to compare the recoveries of common organic acid mixtures from animal and plant sources obtained by a variety of techniques generally employed in the determination of these acids by conventional methods. It is shown that a preliminary purification and segregation of the natural acids by means of chromatography on thin layers of silica gel greatly facilitates their separation, identification, and quantitative measurement by gas chromatography.

MATERIALS AND METHODS

The sources of the standard organic acids have been described (2) except for lactic (85–90%) and pyruvic acids, which were obtained from Fisher Scientific Company and were of reagent-grade quality. The animal tissues were from Sprague-Dawley rats and were obtained under chloro-

[1] Supported by grants from the Medical Research Council of Canada and the Ontario Heart Foundation. A preliminary report has appeared (1).

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form anesthesia. The plant tissues were from fresh ripe produce obtained in a local market.

The ion-exchange resins were obtained from J. T. Baker Chemical Company. Dowex 1 formate was prepared by washing Dowex 1-X2 (100–200 mesh spheres), Cl form, with 2 *N* sodium formate until free of chloride. The excess formate was removed by washing with distilled water. Dowex 50-X2 (200–400 mesh), H^+ form, was washed with 2 *N* formic acid and distilled water before use.

All chemicals and solvents were of reagent-grade quality and were used without further purification except for diethyl ether and methanol, which were distilled over sodium hydroxide to remove organic acids.

Extraction of Acids

The procedures described below have yielded satisfactory results in the isolation of organic acids from the test materials. Further modifications or alternative techniques may be necessary for other applications.

(*1*) *Animal Tissues.* Fresh tissue (1–5 gm) of rat heart, kidney, liver, or skeletal muscle was weighed and homogenized in a Waring Blendor (micro) with 1.3 ml of 0.6 *N* perchloric acid per gram of wet material (9). The homogenate was centrifuged to remove protein and the supernatant neutralized to Congo red with 2 *N* potassium hydroxide. The solution was refrigerated and the precipitated potassium perchlorate centrifuged off. The supernatant was evaporated to small volume in a rotatory evaporator and saved for subsequent purification.

(*2*) *Plant Tissues.* Fresh fruit tissue or juice (1–5 gm) of tomato, orange, lemon, celery, apple, and bean cotyledons was weighed and homogenized with 5 to 10 ml of 75% ethanol per gram of wet material. The suspension was heated on a steam bath to flocculate the precipitate and centrifuged. The supernatant was evaporated to dryness in a rotatory evaporator and the residue treated with 0.33 *N* perchloric acid as described for animal tissues.

Purification of Extracts

The following methods of purification of the crude acid extracts were effective in removing contaminating substances and reducing the background in the gas chromatograms.

(*1*) *Ion-Exchange Chromatography.* An aqueous solution of the crude acid extract was absorbed on a column (10 × 1 cm) of Dowex 1 formate and washed with 5 volumes of distilled water. The organic acids were then eluted with 5 column volumes of 6 *N* formic acid (9). The formic acid was evaporated to dryness in a rotatory evaporator at 40°C. The residue was taken up in distilled water and passed through a column

(10 × 1 cm) of Dowex 50-hydrogen, and the column washed with 4 volumes of distilled water. The eluate and the washings were combined and again evaporated to dryness in the rotatory evaporator. The dry residue was methylated either with diazomethane or with boron trifluoride/methanol.

(*2*) *Silica Gel Partition.* The mixed acid sample was acidified to Congo red with 9 *N* sulfuric acid and mixed thoroughly with dry silicic acid (2–3 gm/ml of solution). The moist silicic acid was transferred quantitatively to a small column (10 × 1 cm diameter) and the organic acids were eluted from the silicic acid by allowing diethyl ether to run through the column (10). The eluate was usually evaporated to dryness at low temperature and the dry residue methylated. In some experiments an acidic alumina column of the type described by Alcock (8) was introduced for the adsorption and one-step methylation-elution of the acids.

(*3*) *Thin-Layer Chromatography.* Mixed organic acids or their salts (1–50 mg) dissolved in small amounts of methanol or water were applied as a band to silica gel G (Merck & Co.) plates (20 × 20 cm, 250 mμ thick) previously washed with formic acid/methanol (25/75, v/v) and reactivated at 105°C for 45 min. The plates were developed with a mixture of benzene/dioxane/acetic acid (90/25/4, v/v/v) or a mixture of benzene/methanol/acetic acid (45/8/4, v/v/v) for about 30 min (11). After drying the plates, the bands were located by exposing the plate for 10–20 min to iodine vapor. The position of the bands was marked with a needle and the silica gel containing the desired components was collected in screw-cap vials. The acids were converted into the methyl esters by treating the scrapings with enough boron trifluoride/methanol to cover them completely.

(*4*) *Lead Salt Formation.* The crude organic acid extract was treated with a saturated solution of lead acetate until no further precipitation took place (4). The precipitate was sedimented by centrifugation and the treatment and sedimentation repeated if necessary. The supernatant was decanted and the precipitate washed with distilled water and centrifuged. The new supernatant was again decanted and the residue suspended in 50% methanol. The free acids were regenerated by bubbling hydrogen sulfide into the suspension. The lead sulfide formed was removed by filtration and the filtrate evaporated to dryness. Traces of moisture were eliminated by distillation with benzene and the dry residue methylated.

(*5*) *Formation of Hydrazones.* The 2,4-dinitrophenylhydrazones of α-keto acids were prepared by the method of Rosan and Nieland (12). The *N,N*-dimethylhydrazones of the keto acids were obtained by dissolving several milligrams of the keto acid mixture in 0.3–0.5 ml of *N,N*-

dimethylhydrazine and allowing to stand at room temperature overnight (13). After evaporation of the dimethylhydrazine, the sample was diazomethylated.

Methylations

The methyl esters of the organic acids were prepared by diazomethylation or a treatment with boron trifluoride/methanol at room temperature as previously described (2). The reaction mixtures were directly injected into the gas chromatograph. Satisfactory methylations were also obtained with an excess of boron trifluoride/methanol and the organic acids bound to the silica gel G as well as the ammonium salts of these acids. The reaction was usually complete after an overnight (16 hr) standing at room temperature.

Gas Chromatography

The methyl esters were separated and quantitatively estimated by the gas chromatographic methods outlined earlier (2). In addition to the Teflon column, two other columns (stainless-steel tubes, ⅛ in. o.d. × 6 ft) containing siliconized Chromosorb W (70–80 mesh) coated with 4% Carbowax 20 M and 6% diethylene glycol succinate (DEGS) were found to be satisfactory. After an initial equilibration at 200°C overnight, these columns were operated isothermally at 150° and 180°, respectively. The 4% Carbowax column allowed the elution of trimethyl citrate as a sharp peak in about 25 min at 180°, as did the DEGS column. The Carbowax-coated Teflon column described previously (2) was temperature programmed in these experiments from 50 to 200°C. Temperature programming was also used to operate an SE-30 column (stainless-steel tube, ⅛ in. o.d. × 5 ft, packed with 5% SE-30 on siliconized Chromosorb W, 70–80 mesh), which gave separations comparable to those obtained on the Teflon column but produced sharper peaks. The latter column gave particularly well-defined peaks with the dimethylhydrazones of the few α-keto acids examined. The Carbowax-coated Teflon powder was stored in a freezer and was used for filling columns in the frozen state.

Quantitation

The recoveries of the various organic acids by the different extraction and purification schemes were estimated on the basis of the behavior of either individual known acids or their standard mixtures. Comparisons of corrected area recoveries provided a satisfactory indication of losses of any volatile components (lactate, pyruvate, succinate) in comparison to the less volatile ones (citrate, aconitate). An additional check was

provided by the use of dimethyl glutarate as an internal standard. It was added in a known amount to the final methylated acid mixture just before injection into the gas chromatograph.

The methods of peak area calculations and of the determination of correction factors for differences in the detector response have been discussed (2). The corrections derived for the more polar acids on the Chromosorb W supports were somewhat higher than those characterizing the Teflon columns and suggested that the siliconizing process had not produced a completely inert surface. In these cases it was convenient to read the concentrations of the acids from a response curve determined previously. All of the gas-liquid chromatography (GLC) systems, however, allowed effective quantitations of standard esters in the range of 0.1 to 10 μg.

The usefulness of the low-efficiency Teflon column (150 theoretical plates per foot) for quantitative measurements was greatly increased by a preliminary segregation of the acid mixture on the thin-layer plate. Furthermore, the Teflon support remained unaffected by the continued injection of the complete reaction mixtures into the gas chromatograph, while there was evidence of a gradual destruction of the inorganic supports resulting in decreasing recoveries of the more polar esters.

RESULTS AND DISCUSSION

Recovery of Standard Acids from Tissues

Table 1 shows the recoveries of various mixtures of standard acids during different isolation procedures. When solvent evaporations were avoided after methylation, nearly complete recoveries were obtained for all acids in the working ranges tested (10 to 50 mg total acid per 5 gm of tissue or fluid). Although minor differences were noted in the gas chromatographic elution patterns recorded for the recovered acid mixtures, the additional peaks and shoulders appearing were not large enough to alter significantly the acid proportions. Utilization of very dilute acid mixtures requiring concentration of the methylated material resulted in losses of the more volatile components.

Thus evaporation to dryness on the steam bath brought about a nearly complete loss of the methyl esters of lactic, pyruvic, and succinic acids. Even when the solvent volume was only partially reduced there were noticeable losses of the volatile esters. Losses of both lactic and pyruvic acids also occurred when the solvent evaporations were performed with the free acid mixtures and during the drying (room temperature) of the developed thin-layer plate. Solvent evaporations could be carried out safely, however, with the acids in the ammonium salt form. These losses

TABLE 1
Recoveries of Various Standard Mixtures of Organic Acids during Different Isolation Procedures[a]

Acid mixture	Composition of added acids		Estimated composition[b] of recovered acids: Ion exchange		Silica gel partition		Lead salt formation	
	mg	%	mg	%	mg	%	mg	%
From distilled water								
Lactic	3.8	25.2	3.7	24.6	3.8	25.0		
Succinic	3.6	23.8	3.5	23.0	3.6	24.0		
Ketoglutaric	4.0	26.4	4.1	27.2	3.9	26.1		
Citric	3.7	24.6	3.8	25.2	3.8	24.9		
From liver homogenate								
Lactic	2.5	25.2			2.4	24.5		
Succinic	2.4	23.8			2.4	23.1		
Ketoglutaric	2.6	26.4			2.5	26.8		
Citric	2.4	24.1			2.5	25.6		
From lemon juice								
Fumaric	3.8	12.6	3.6	12.0	3.6	12.5	3.5	11.8
Succinic	3.6	11.9	3.5	11.5	3.6	12.0	3.5	12.0
Ketoglutaric	4.0	13.2	4.1	13.6	4.1	13.3	4.1	13.3
Tartaric	7.4	24.8	7.4	24.5	7.5	25.0	7.4	24.8
Citric	3.7	12.3	3.9	12.9	3.9	12.9	3.9	12.9
Isocitric	7.7	25.2	7.7	25.5	7.8	26.1	7.9	25.4
From urine								
Pyruvic	9.3	18.5	8.3	16.5	8.5	16.9		
Fumaric	8.8	17.5	9.9	19.7	9.7	19.3		
Malic	7.2	14.3	7.0	14.0	7.1	14.1		
Ketoglutaric	10.2	20.3	11.0	22.0	11.0	21.9		
Aconitic	14.7	29.4	13.9	27.8	13.9	27.8		
From skeletal muscle homogenate								
Lactic	1.6	15.8	1.5	15.6	1.5	15.0		
Pyruvic	1.6	16.5	1.7	16.7	1.5	16.1		
Fumaric	1.4	13.5	1.3	13.7	1.4	14.3		
Aconitic	2.1	20.7	2.0	20.5	2.0	20.1		
Citric	3.5	34.5	1.7	34.6	3.6	35.7		

[a] Following methylation, solvent evaporations were avoided. Amounts of total acid added varied from 10 to 50 mg/5 gm tissue or fluid.

[b] Absolute recoveries were estimated using dimethyl glutarate as internal standard. The weights represent mean values from 2 to 3 separate isolations. Standard error of the mean varied from 5 to 10%. Proportional recoveries were computed from the means.

were true losses of volatilization and were not due to incompleteness of methylation, although the type of solvent to be removed following the esterification step made a difference in the conditions required for rapid evaporation and consequently in the amount of the volatile esters lost.

The proportionally correct recoveries of the organic acids in the absence of solvent evaporations indicated that the methods of methylation (diazomethylation and boron trifluoride/methanol treatment) were adequate for the complete conversion of these carboxylic acids into the ester form. Side reactions have been noted with the keto acids (2–4) as have been the isomerizations of fumaric and *cis*-aconitic acids (2, 3). Isomerization of the latter acids takes also place during methylations with hot boron trifluoride/methanol (2, 4, 8) and with hot methanolic hydrogen chloride (14). High yields of methyl ester were obtained for all acids when the reaction mixture was kept at room temperature (25°C) overnight.

Despite the reduced yields due to isomerizations, side reactions, and evaporation, most acids were recovered in the correct proportions provided more than 1 mg of each acid was added per 5 gm of tissue. With lower acid additions (less than 1 mg each) the method of acid extraction and isolation became of great importance. Both over-all and specific losses became proportionally greater and resulted in distorted proportions of the recovered acids. This was apparently due to the rather constant losses of the material on the adsorbant surfaces, vessel walls, and filter paper and to some isomerization and destruction. The losses were greatest with the keto acids. It was concluded that most of the methods described in the literature for the extraction and isolation of the Krebs cycle acids give fairly representative samples of the true acid mixtures present.

Decreased recoveries of the more polar compounds were most prominent when working with small amounts of the material on the silica gel plates. Despite the formic acid wash, all active sites were not completely inactivated, and some of the acids became bound. Although this technique was the most promising means of Krebs cycle acid purification, it was apparent that a careful choice of the adsorbent was crucial if these losses were to be completely avoided. Table 2 gives the recoveries of organic acids from the acid-washed silica gel G plates. The advantages of the technique rest in the ability to use the nonvolatile carboxylic acid salts for spotting of the material and in the segregation of the acids on the basis of the number and type of functional groups. This was seen to simplify greatly the task of gas chromatographic separation and identification of the complex organic acid mixtures. In addition the thin-layer chromatography (TLC) method surpassed all the other techniques

in simplicity and speed of acid purification. Although the ion-exchange and partition chromatographic systems can also provide groups of carboxylic acids containing the same types and numbers of functional groups when eluents are properly selected, these separations are time consuming and inferior to those obtained by TLC. Furthermore, the column techniques are noteworthy for their inability to cope with mixtures of acids

TABLE 2
Recovery of Organic Acids from Thin-Layer Plates[a]

Acid	Amount of acid plated, mg	Acid recoveries (average of 2 runs each)			
		Methyl esters		Methyl ester hydrazones[b]	
		mg	%	mg	%
Lactic	5.0	4.7	95		
Pyruvic	10.0	9.9	99	10.1	101
Succinic	5.5	5.4	98		
Fumaric	5.5	5.5	100		
Oxalacetic[c]	15.0	14.2	95	14.9	100
L-Malic	30.0	29.1	97		
Tartaric	10.5	9.3	90		
α-Ketoglutaric[c]	15.0	15.1	100	14.7	99
Aconitic[c]	5.0	4.7	95		
Citric	15.0	15.1	101		
Isocitric	12.5	11.9	95		

[a] Recoveries of the acids determined by comparing the areas obtained on GLC for the methyl esters or the methyl ester hydrazones before and after thin-layer chromatography. Dimethyl glutarate was used as an internal standard.

[b] These acids were applied to the thin-layer plates as their *N,N*-dimethylhydrazones.

[c] Since these acids gave double peaks, the sum of the two areas was used in each case.

containing major and minor components. Table 3 gives the approximate R_f values of some of the organic acids plated and indicates the locations of the cuts made for the collection of suitable groups of acids. It should be noted that a direct methylation of the ammonium salts of the carboxylic acids by boron trifluoride/methanol may be possible in the presence of the silica gel scrapings. This would allow the utilization of the volatile ammonia/alcohol systems for the TLC of the ammonium salts and would greatly facilitate the location of the acid bands and the removal of the developing solvent.

The conversion of the keto acids into their hydrazone methyl esters was investigated as a means of simplifying the multiplicity of peaks occasionally obtained when chromatographing the methyl esters of the keto acids. The appearance of the *cis* and *trans* derivatives of the keto acids, which became resolved to different degrees in the chromatographic systems, prohibited the realization of a clear-cut advantage over the

TABLE 3
R_f Values[a] of Carboxylic Acids on Silica Gel G Layers

Section[b]	Acid	R_f value	
		Benzene/MeOH/AcOH (45/8/4, v/v/v)	Benzene/dioxane/AcOH (90/25/4, v/v/v)
Solvent front	Fatty acids hydrazones	1.00	1.00
0.65–0.90	Pyruvic acid	0.80 (streaking)	
	Lactic acid	0.70 (streaking)	
0.40–0.65	Fumaric acid	0.60	0.25
	Suberic acid	0.50	0.45
0.15–0.40	Maleic acid	0.26	0.05
	Succinic acid	0.25	
	α-Ketoglutaric acid	0.21 (major spot)	0.11
	trans-Aconitic acid	0.20	0.05
0.05–0.15	Malic acid	0.10	0.02
	Malonic acid	0.05	0.05
	cis-Aconitic acid	0.05	0.05
	Citric acid	0.05	0.05
Origin	Tartaric acid	0.00	0.00
	Oxalic acid	0.00	0.00

[a] The locations of the acids on the plate were determined by iodine staining and were confirmed by a gas chromatographic examination of 1 cm wide sections over the entire length of the developed plate.

[b] Sections of the TLC plate from which scrapings were routinely collected during the examination of an unknown organic acid mixture.

simple methyl esters. The formation of the phenylhydrazones, however, allowed an effective removal of the keto acids from the total acid mixture.

Determination of Krebs Cycle Acids in Natural Products

When working with concentrated solutions of organic acids (>5 gm tissue) the values found for the various acids of the rat tissues by gas chromatography (Table 4) agreed in general with those reported by Frohman *et al.* (15) on the basis of partition chromatographic studies. With smaller acid concentrations (<5 gm tissue) the necessary solvent evaporations following esterification brought about significant losses of the more volatile esters. Figure 1 shows the elution pattern recorded for the organic acids of normal rat heart tissue. The acids were isolated using the perchlorate/ion-exchange chromatographic system. The methyl ester peaks were tentatively identified by cochromatography of the unknown ester mixture with appropriate standards. Comparable elution patterns were also recorded for the liver, kidney, and skeletal muscle

TABLE 4
Average Concentrations of Organic Acids in Selected Animal and Plant Tissues (mg/100 gm tissue or juice)[a]

Acid	Rat tissue			Lemon	
	Heart	Muscle	Liver	Peel	Juice
Pyruvic[b]	10.0	3.0	2.1		
Succinic	8.5	0.1	2.0	5.1	100
Fumaric	11.0	1.5	5.5	1.0	200
Oxalacetic[b]	1.0	0.1	0.5		
Oxalic				50	300
Lactic[b]	6.9	5.5	12.5		
Malic	3.5	0.5	2.5	70	800
Ketoglutaric	2.1	0.1	1.5	5	500
Aconitic	1.0	0.1	0.1	7	300
Citric	4.0	0.5	3.0	30	4800
Isocitric	0.6	0.1	0.5	1.5	

[a] Average of at least three separate extractions and determinations.
[b] For these acids the highest values only have been recorded.

preparations. When compared to the data of Frohman *et al.* (15) the proportions of the lactic and pyruvic acids were low. Furthermore, the estimated levels of these acids were found to vary greatly from one preparation to the next, with the lowest recoveries being recorded from

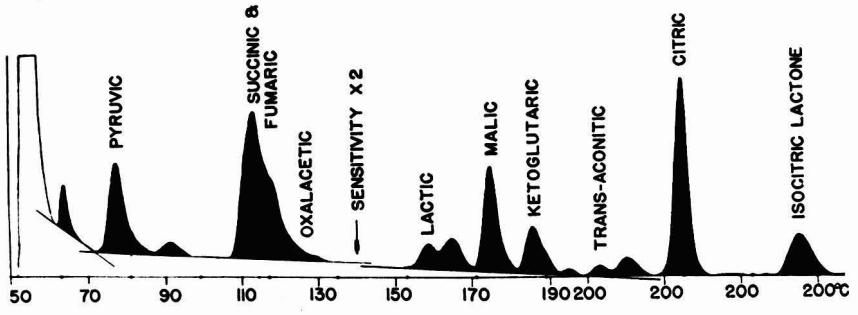

FIG. 1. Gas chromatographic elution pattern recorded for methyl esters of the organic acids of rat heart tissue. Tentative peak identities as given in the figure. *Chromatography conditions:* Column, 8 ft × ⅛ in. o.d. stainless-steel tube packed with Teflon 6 powder, 90–100 mesh, containing 3% (w/w) Carbowax 20 M. Instrument, Aerograph Hy-Fi 600C (Wilkens Instrument & Research, Inc.) equipped with an F & M Model 420 linear temperature programmer (F & M Scientific Corporation). Temperature program, as given in the figure. Carrier gas, N_2, 50 ml/min.

preparations requiring extensive concentration of the final ester mixture. Significant, but considerably lower were the volatilization losses noted for the dimethyl esters of succinic and fumaric acids. As noted earlier (2), in this gas chromatographic system the fumaric and succinic acid

peaks are incompletely separated and double peaks are recorded for *cis*-aconitic and keto acids. In the estimation of the acid proportions the appropriate areas were added.

The suitability of the lead salt precipitation technique for the isolation of the di- and tri-carboxylic acids from plant tissues is illustrated in Figure 2. It shows the elution pattern recorded for the methyl esters of the organic acids of lemon juice. In the order of emergence, peaks are recognized for fumaric and succinic, oxalacetic, malic, α-ketoglutaric, aconitic, and citric acids. The large proportion of citric acid in this juice is clearly obvious. Other plant juices and extracts were shown to contain oxalic, malonic, fumaric, succinic, malic, α-ketoglutaric, tartaric, aconitic,

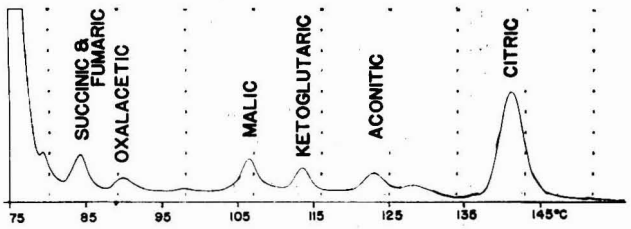

FIG. 2. Gas chromatographic elution pattern recorded for methyl esters of the organic acids of lemon juice. Tentative peak identities as given in the figure *Chromatography conditions:* as in Figure 1 except that a 5 ft column was used.

citric, and isocitric acids. The tentative identifications of the acids were based on thin-layer chromatography and cochromatography of the unknows with appropriate standards in the gas chromatograph. The analytical data obtained with the plant materials (Table 4) approximated the values recorded by Bonner (16). A more complete analytical account of the organic acid content of plant and animal tissues will be presented elsewhere using better defined acid sources.

In addition to the peaks attributable to known organic acids, there were others the identity of which remains to be established. None of them was due to the impurities in the solvents or in the chromatographic media. The present survey of the isolation and purification techniques has demonstrated that most of the organic acids of the tissues can be recovered and determined by GLC, and that the results obtained are representative of the types and proportions of the acids actually present.

The extensive purification of the acid samples required prior to GLC introduces an element of inconvenience in the otherwise swift analytical system. The adoption of the thin-layer or open-column technique to the purification of the acid extracts by adsorption chromatography provides a convenient shortcut in the sample preparation. Similar thin-layer tech-

niques (17) of partition and ion-exchange chromatography are likely to yield comparable results, although as yet they have not been sufficiently refined for routine use.

The problem of loss of the more volatile methyl esters during concentration remains, as does the inconvenience of multiple derivative formation during methylation of the α-keto acids. Although the yield of specific derivatives can be favored by selecting the reaction conditions, and single peaks obtained also for the keto acids (see also reference 7), further work is necessary to resolve this difficulty completely. When dealing with natural acid mixtures it is therefore best to remove the keto acids as the hydrazones and examine them separately. In order to avoid losses on evaporation the acids should be concentrated while in the salt form, and serious consideration should be given to preparing derivatives less volatile than the methyl esters for the GLC analysis of all acids.

Despite the shortcomings, a GLC system of analysis of organic acids still has the advantages of extremely short analytical runs, a high sensitivity, and general applicability that cannot be approximated at the present time at least by other chromatographic systems which might handle cruder extracts and might not isomerize the keto acids.

SUMMARY

Modifications of several techniques commonly employed for quantitative isolation and partition or ion-exchange chromatographic separation of organic acids were investigated for their suitability in providing Krebs cycle acids for methylation and gas chromatography. Of the systems examined, the one based on the perchlorate/Dowex 1 formate anion-exchange method of Busch *et al.* (9) was the most satisfactory for the isolation of these acids as a group. For the examination of complex mixtures of organic acids, a preliminary segregation of the acids on the basis of molecular weight and the number and type of functional groups by means of thin-layer chromatography proved useful. The free acids or their salts were effectively esterified by boron trifluoride/methanol at room temperature. The methyl esters were separated and quantitated using previously described gas chromatographic techniques.

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Use of Water-Soluble Polymers for the Isolation and Purification of Human Immunoglobulins[1]

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The separation and isolation in pure form of individual protein components of serum pose a constant challenge to the biochemist. Investigators have used a number of different techniques to achieve this aim with varying degrees of success. Most of the methods for the large-scale fractionation of serum proteins reported in the literature are adaptations of one or more of the inclusive schemes devised by Méhu (1) and Cohn *et al.* (2), in which proteins are serially precipitated in the reverse order of their molecular weights and molecular volumes by the use of inorganic salts and/or nonaqueous solvents. Since then, procedures based on ion exchange (3) or molecular sieve chromatography (4) were developed.

There have been some recent reports of the use of water-soluble polymers for the separation of macromolecules. A combination of polyethyleneglycol and dextran was employed for the partition of nucleic acids (5, 6). Polyethyleneglycol and ammonium sulfate were used in the fractionation of serum components (7). Such studies indicated that high molecular weight compounds were effective precipitating agents for serum proteins. The purpose of the present report is to describe a convenient method for the isolation of pure immunoglobulins[3] from either whole serum or commercial preparations of Cohn fraction II with antibody activity, by a simple precipitation technique using a single, water-soluble, nonionic polymer.

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[3] The recommendations of the World Health Organization Committee on Nomenclature for Human Immunoglobulins (8) were used in this text, whereby γ_G (I_gG) is synonymous with 7 S, γ_2, or γ_{ss}; γ_M (I_gM) with 19 S, γ_1M, or β_2M, and γ_A (I_gA) with γ_1A or β_2A.

MATERIALS AND METHODS

Precipitating Agents—Polyglycols.[4] The water-soluble, nonionic polymers which may be used with this technique are the polyethyleneglycols, PEG-2000, PEG-4000, and PEG-5700 (General Biochemicals); aldosperse ML-14 (Glyco Chemicals, Inc.); 1,4-nonylphenylethylene oxide (NPEO) NP-14 (J. T. Baker Chemical Co.); and the polypropyleneglycol, PPG-400 (J. T. Baker Chemical Co.). Sodium dextran sulfate (molecular weight 2000) was obtained from Pharmacia Fine Chemicals.

In the present report, experiments using only one of these agents, PEG, are described. Polyethyleneglycol, PEG-5700, was further purified for these studies by dissolving in acetone and then precipitating with ether. This procedure, described by Albertsson (9), was effective in removing from commercially available polymers impurities that absorb light in the ultraviolet region.

The desired concentrations of PEG were achieved by using a stock solution prepared by dissolving 50% (w/v) of the PEG-5700 (5700 denotes molecular weight) in distilled water at 37°C.

Serum Proteins. Solutions of the following Cohn fraction II preparations were maintained at 4°C until use: immune serum globulin 172–292 (Pitman-Moore Co.), immune globulins G-099461-A (Parke, Davis & Co.), polio immune globulins 2175-491D (Lederle Laboratories), and 372-1 (E. R. Squibb & Sons).

Mixtures of solutions of human serum albumin (Pentex, Inc., Cohn fraction I), γ-globulins (Squibb, Cohn fraction II), and α-globulins (Armour, Cohn fraction IV) were used as a synthetic model for serum for the purification of each component with polyglycols.

Pooled whole human serum was obtained from the University of Florida Blood Bank. The serum was exhaustively dialyzed on a rotating dialyzer against 0.055 *M* phosphate buffer, pH 7.0, with changes every 3 hr for 48 hr. A slight precipitate which formed after equilibration of the sample with this buffer was removed by centrifugation at 35,000 *g* for 20 min and discarded.

Gel Diffusion and Immunoelectrophoresis. Immunodiffusion analysis and immunoelectrophoresis were carried out in 3.0 mm layers of 1.0% Ionagar dissolved in 0.05 *M* Veronal buffer, pH 8.6. Goat anti-human γ_G (G_p-965A) and goat anti-human γ_A (G_p-1164) were obtained from Hyland Laboratories. Rabbit anti-human γ_G sera were obtained from immune rabbit serum prepared by injection of human γ_G, which was

[4] The following abbreviations are used in this paper: PEG, polyethyleneglycol; PPG, polypropyleneglycol; NPEO, 1,4-nonylphenylethylene oxide; RAH, rabbit anti-human serum.

purified by DEAE-cellulose column chromatography of Levy and Sober (10). The rabbit anti-human whole serum was provided by the Department of Pediatrics, University of Florida.

All other chemical reagents used in these experiments were the best commercially available grades and were used without further purification. Distilled deionized water was used for all solutions.

Protein Concentration. Absorption at 280 mμ ($E^{1\%}_{280\,m\mu} = 14.9$ dl/gm) (11) and the method of Lowry *et al.* (12) were used for determination of immunoglobulin concentration.

Ultracentrifugation. A Spinco model E ultracentrifuge equipped with phase plate, schlieren diaphragm, and RTIC unit was used. The temperature of the rotor was controlled at 20°C. Measurements of the ultracentrifuge schlieren patterns were made by conventional methods (13). The sedimentation boundary analyses were carried out by a modification of the procedures described by Baldwin (14) and Chun and Gehrke (15).

EXPERIMENTAL PROCEDURE

Preliminary Precipitations of Serum Proteins. To determine the range of concentrations of PEG required for protein precipitation, a 1% solution of human serum albumin in phosphate-KCl buffer, pH 7.0, $\mu = 1.1$, was prepared; 10 ml of the solution was placed in each of a series of test tubes, to which was added sufficient PEG stock solution to obtain concentrations between 0 and 34% in 2% steps. The solutions were mixed and allowed to stand for 15 min, then centrifuged at 35,000 *g* for 30 min. The precipitates were redissolved in 5 ml of phosphate buffer before analysis for protein content by Lowry's method. A similar procedure was followed for Cohn fractions I, II, and IV.

Purification of Immunoglobulins from Cohn Fraction II. Based on the results of the preliminary experiments, the following procedure was devised: 100 mg of Cohn fraction II (Lot No. 1615, Squibb) was dissolved in 10 ml of 0.055 *M* phosphate buffer, pH 7.0, $\mu = 0.1$, and then precipitated with PEG-5700 at a final polymer concentration of 35%. The precipitate was dissolved in 10 ml of phosphate buffer and further purification steps were carried out according to the flow chart in Figure 1. Each precipitate was redissolved in phosphate buffer and twice reprecipitated with 35% $(NH_4)_2SO_4$. These precipitates were redissolved in 0.01 *M* phosphate buffer, pH 7.0, and added to a suspension of DEAE-cellulose. Polyethyleneglycol remains unabsorbed to the anion exchanger under these conditions and is readily removed from the resin by washing with 0.01 *M* phosphate buffer. The absorbed immunoglobulins were recovered by eluting with 0.1 *M* KCl. These solutions were dialyzed against water and lyophilized. An alternate, and more rapid, method for

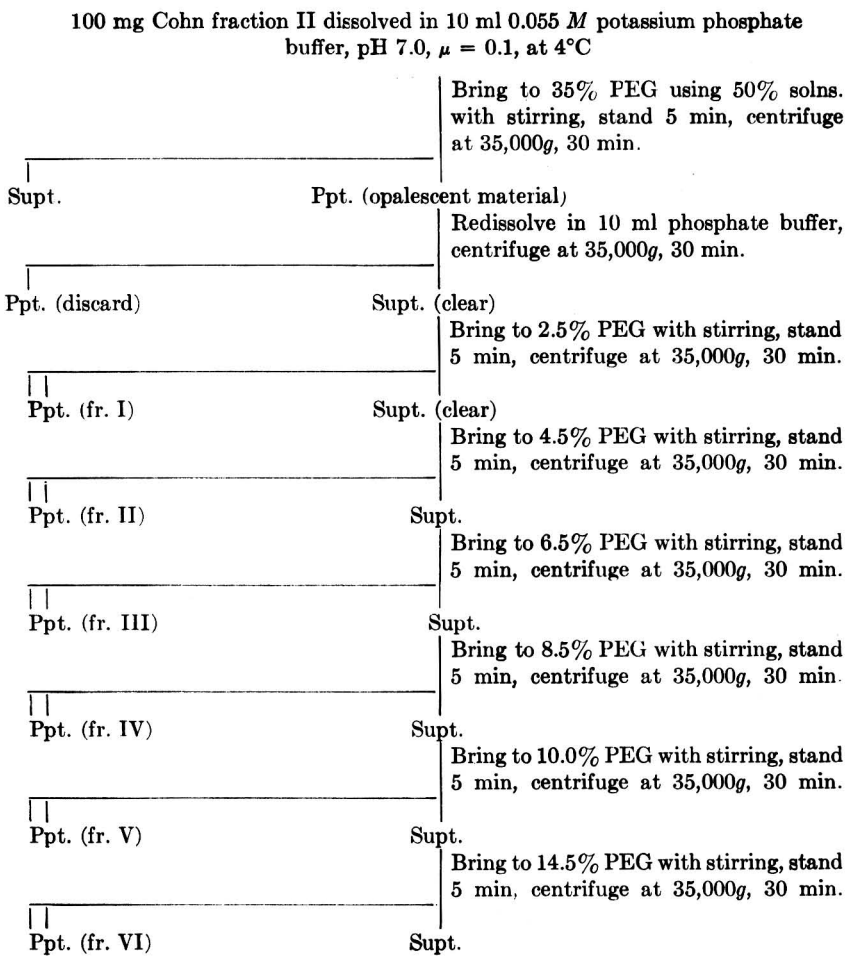

FIG. 1. Isolation of immunoglobulins from Cohn fraction II. Each precipitate was suspended in 10 ml of 0.055 *M* potassium phosphate buffer and was twice precipitated with 35% $(NH_4)_2SO_4$. The precipitates were redissolved in buffer and dialyzed against H_2O. The solutions were freed of remaining PEG as described in the text.

removing PEG was to precipitate the immunoglobulins with cold 20% ethanol, filter and redissolve in buffer, dialyze, and lyophilize.

Isolation of Immunoglobulins from Whole Human Serum. The following procedure was developed on the basis of the results obtained in the preliminary experiments with the individual proteins and with synthetic mixtures of Cohn fractions I, II, III, and V: 5 ml of whole serum diluted with 5 ml of 0.055 *M* phosphate buffer was dialyzed against the same

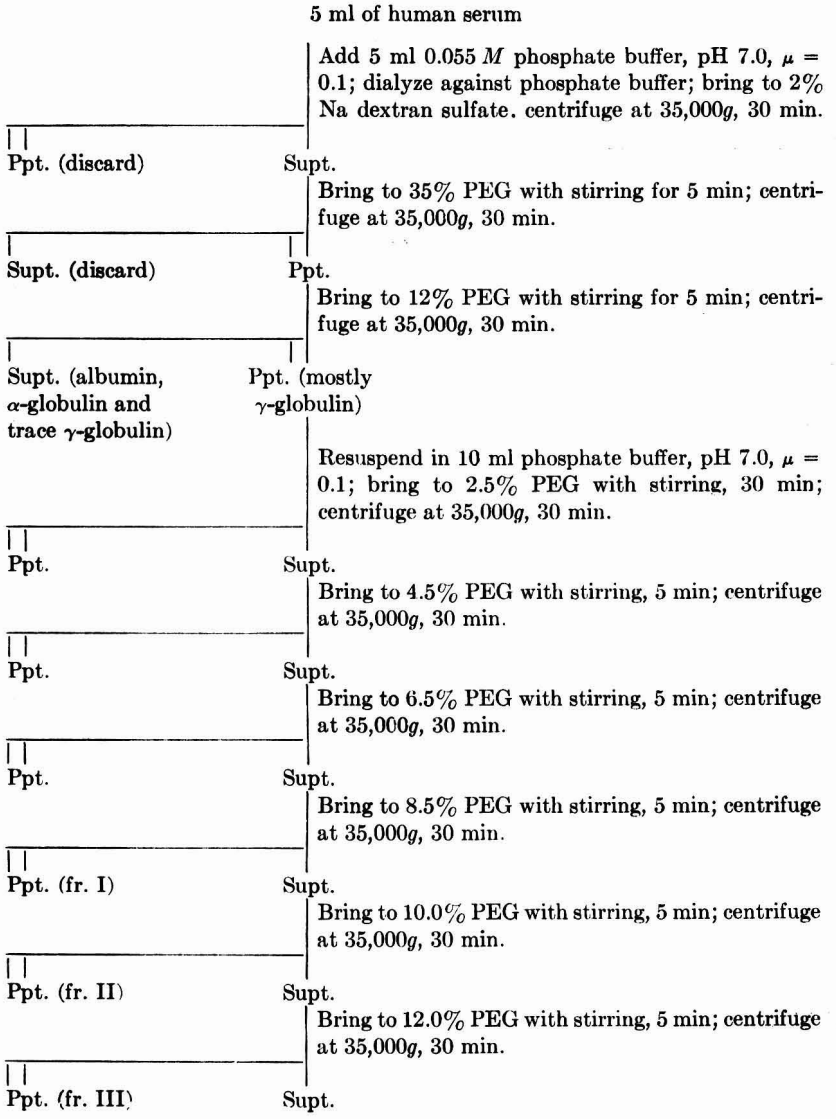

FIG. 2. Isolation of immunoglobulins from whole human serum.

buffer for 20 hr prior to PEG precipitation. Sodium dextran sulfate was added to a final concentration of 2%. The precipitate that resulted, presumably a lipoprotein complex, was removed by centrifugation at 35,000 *g* for 30 min. The supernatant was redialyzed against phosphate buffer overnight to remove residual dextran sulfate before precipitating

with PEG at 35% concentration. The precipitate was removed by centrifugation at 35,000 *g* for 30 min and dissolved in 10 ml of phosphate buffer. Further precipitation steps were carried out according to the flow chart in Figure 2. Each precipitated fraction was freed of PEG as outlined in the preceding section.

RESULTS

Precipitation of Human Serum Albumin with 35% PEG-5700. Figure 3 shows the quantity of a 1% solution of human serum albumin precipitated by 35% concentrations of PEG of different molecular weights. A complete quantitative precipitation of albumin was accomplished at this polymer concentration with PEG of molecular weight 5700. Human serum albumin served as a precipitation standard since, of the plasma proteins examined, it required the highest concentration of PEG for

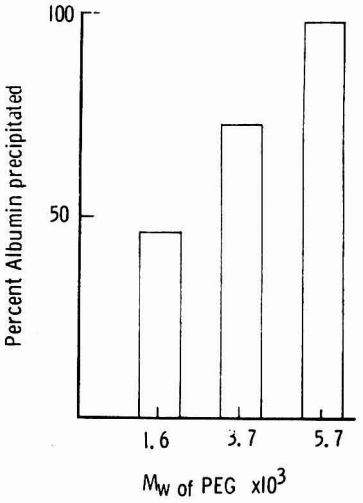

FIG. 3. Extent of precipitation of human serum albumin at 35% concentrations of PEG of different molecular weights.

complete precipitation. There appears to be a direct relationship between the extent of precipitation of albumin and the molecular weight of PEG, which was also observed at higher concentrations of PEG. Polyethyleneglycol (PEG) of higher molecular weight (e.g., 20,000) was not used in this study because of its high viscosity in water (approximately 96,000 centistokes/sec at 210°F). The immunoglobulins (Cohn fraction II) and other serum proteins examined exhibited a similar increase in the extent of precipitation with PEG of increasing molecular weight.

Isolation of Plasma Proteins with PEG-5700. A mixture of the Cohn

fractions I, II, IV, and V was prepared by dissolving 100 mg of each in 40 ml potassium phosphate buffer, pH 7.0, $\mu = 0.1$. This solution was then subjected to stepwise precipitation with PEG-5700. Figure 4 shows the precipitation patterns and the separation of individual components obtained with such a mixture by this fractionation. Fibrinogen and γ-globulins precipitated at PEG concentrations of 1–2% and 8–10%,

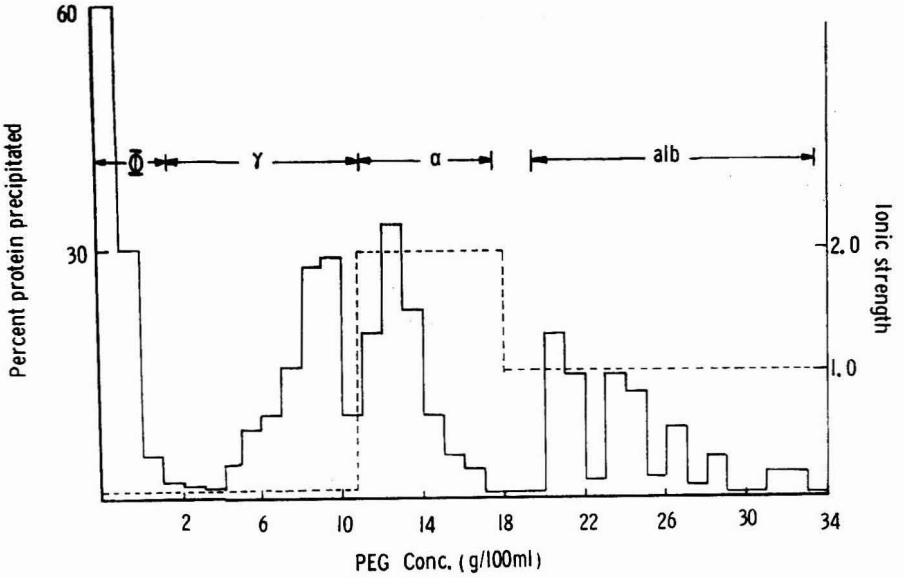

FIG. 4. Isolation of plasma proteins with PEG-5700; 100 mg each of Cohn fractions I, II, IV, and V were dissolved in 40 ml of 0.055 *M* potassium phosphate buffer, pH 7.0, $\mu = 0.1$: (—) % protein precipitated (% of each of the fractions added to the mixture); (- - -) ionic strength. The identities of the proteins in the individual fractions were established electrophoretically.

respectively, in buffers of low ionic strength. The maximum precipitation of α-globulins occurred at 12–14% PEG, provided the ionic strength of the buffer was increased to 2.0 by the addition of KCl. Albumin in a buffer of $\mu = 1.0$ precipitated at 20–35% PEG in several discrete fractions, rather than as one large peak as was the case with the globulins. Each of the discrete precipitated albumin fractions was examined ultracentrifugally and by immunoelectrophoresis. No differences were observed.

It was not feasible to isolate a clean β-globulin fraction by high-polymer precipitation; and since the commercial β-globulins (Cohn fraction III) available were insoluble in potassium phosphate buffers, even at high ionic strengths, conditions for their fractionation were not

developed. The β-globulins also appeared to interfere with the separation of other serum components, and were therefore removed by precipitation with 2% sodium dextran sulfate (16) at the beginning of the fractionation procedure. Residual sodium dextran sulfate was removed by succeeding steps.

The conditions for precipitation determined using the synthetic mixture were readily applicable to the isolation of immunoglobulins from whole serum or from commercial samples of Cohn fraction II, as is outlined in Figures 1 and 2.

Isolation and Purification of Immunoglobulins from Commercial Cohn Fraction II. Sixteen commercial samples of human immune serum globulins (fraction II), prepared by Cohn fractionation (2), were examined by immunoelectrophoresis with rabbit antiserum prepared against whole human serum. Four representative examples appear in Figure 5.

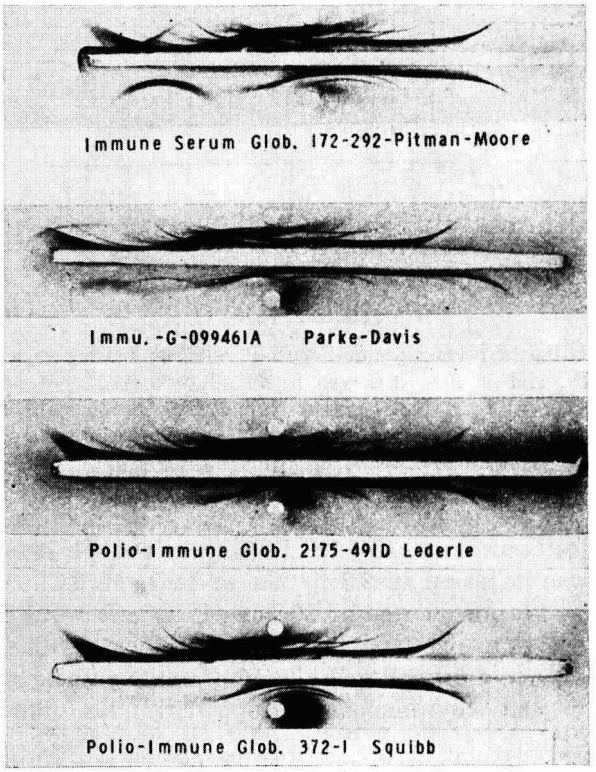

FIG. 5. Immunoelectrophoretic comparisons of four commercial immune globulins with whole human serum. Rabbit anti-human serum was the antibody. For each slide, the upper sample is whole human serum and the lower sample is the identified commercial immune globulin.

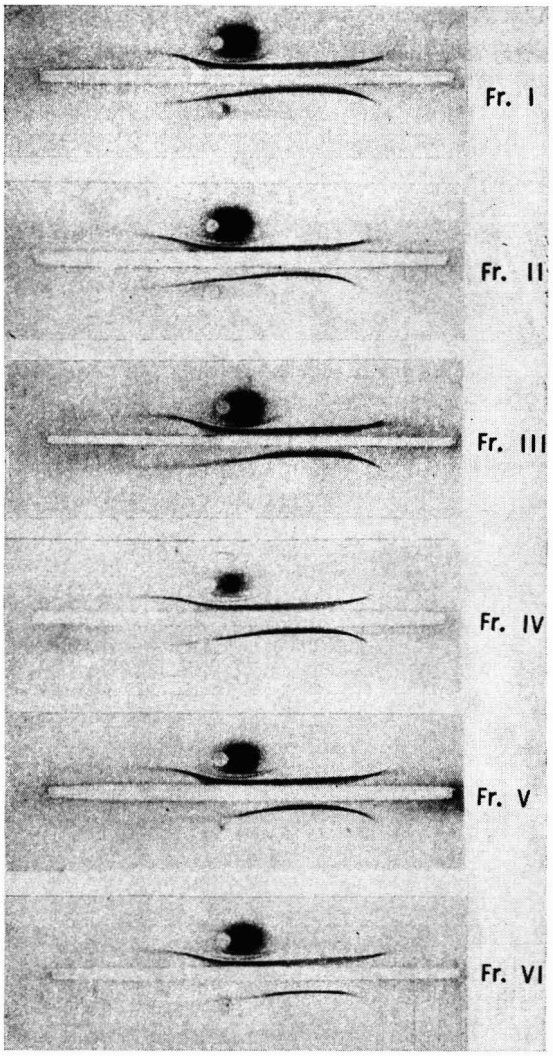

FIG. 6. Immunoelectrophoretic comparisons of Cohn fraction II (Squibb polio immune globulin), upper samples, and the fractions obtained by the high polymer precipitation outlined in Figure 1, lower samples. Antibody was rabbit anti-human serum.

It is apparent that these preparations contain contaminants in the γ region, as well as proteins migrating with the mobilities of β- and α-globulins, and even albumin.

When these preparations were treated by the procedure outlined in Figure 1 and subjected to immunoelectrophoresis in parallel with the

TABLE 1
Per Cent Recovery of Immune Globulin Fractions by High-Polymer Fractionation

Fraction precipitated	Per cent PEG concentration	Per cent recovery (dry weight basis[a])
I	2.50	4.20
II	4.50	12.36
III	6.50	12.50
IV	8.50	27.00
V	10.00	24.05
VI	14.50	9.12
VI supernatant	14.50	1.62
		$\Sigma = 90.85$

[a] Dry weight measurements were consistent with protein determinations by the procedure of Lowry *et al.* (12).

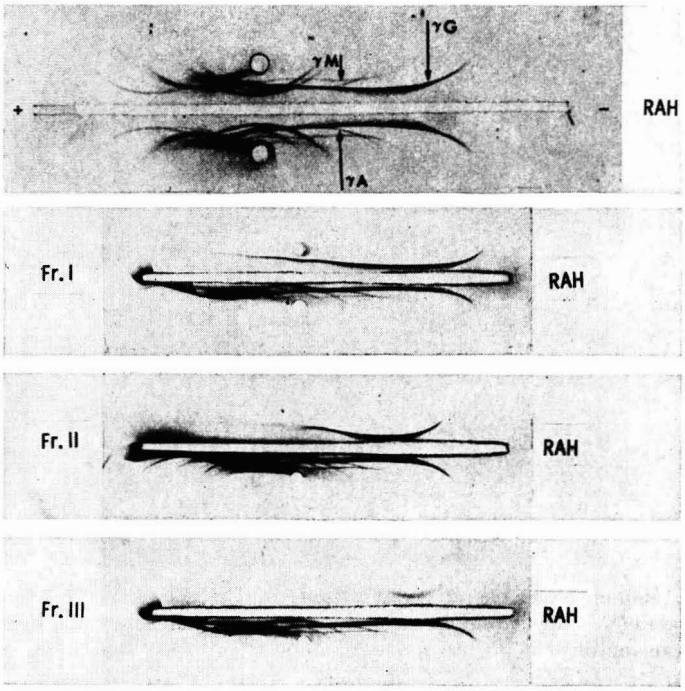

FIG. 7. Immunoelectrophoretic patterns of whole human serum (lower samples) compared with the fractions obtained by high-polymer fractionation (upper samples). The antibody was rabbit anti-human serum. The top pattern resulted when whole human serum (2× concentrated) was run against 2× concentrated antigen to delineate γ_M, γ_A, and γ_G more distinctly.

original commercial materials at similar concentrations, the patterns shown in Figure 6 resulted. Comparison of Figures 5 and 6 indicates that the γ-globulins obtained by this precipitation technique were markedly less heterogeneous than the starting materials. Similar results were obtained with other commercial preparations. The data in Table 1 show that the immunoglobulins recovered in fractions III, IV, and V

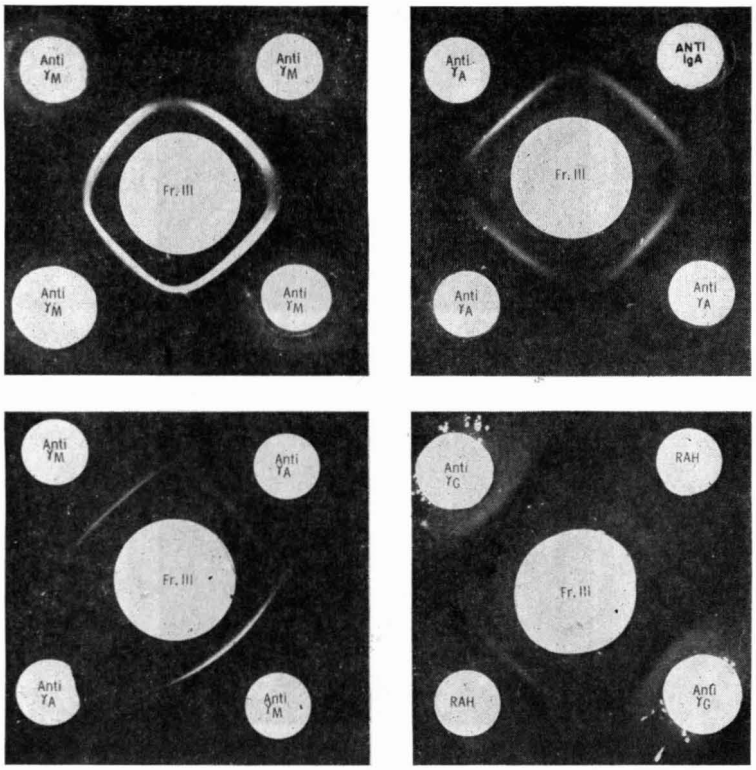

FIG. 8. Immunodiffusion patterns of immunoglobulins obtained by high-polymer fractionation. Fraction III was separated by the procedure of Figure 1.

comprise about 62% of the total proteins of the starting material, Cohn fraction II. The maximum yield of purified immunoglobulins is obtained at PEG concentrations between 8.5 and 10.5%, as indicated also in Figure 4.

Isolation of Immunoglobulins from Whole Serum. Figure 7 shows the immunoelectrophoretic patterns of purified immunoglobulins isolated from whole human serum by the procedure outlined in Figure 2. In contrast to the proteins isolated from Cohn fraction II (Fig. 6), it appears that there are three different immunoglobulins in whole human

serum that can be readily distinguished by their electrophoretic mobilities. Immunodiffusion indicated that fraction I of Figure 7 includes all three classes of immunoglobulins (γ_A, γ_M, and γ_G). Fraction III of Figure 7 appears to be an unusual γ-globulin isolated by this technique, which may be similar to the one described by Cebra and Robbins (17). The yield of immunoglobulins from 5 ml of whole serum was 40–50 mg.

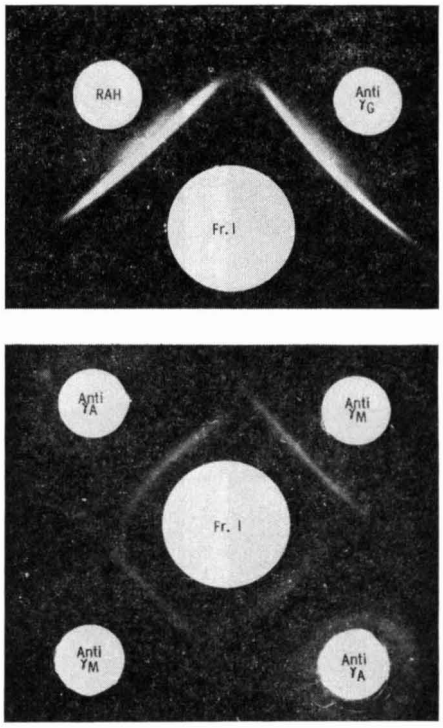

FIG. 9. Immunodiffusion patterns of fraction I (Fig. 7), an immunoglobulin isolated from whole serum by the procedure of Figure 2. The center wells were enlarged to accommodate more antigen.

Immunochemical Characterization of Isolated Immunoglobulin. Analysis of each of the "pure" immunoglobulin fractions III, IV, and V of Figure 6 by the Ouchterlony double-diffusion technique revealed a complete identity with monospecific anti-γ_G serum. However, when the fractions were run against monospecific anti-γ_M or anti-γ_A sera, trace amounts of γ_A- and γ_M-globulins were detected, as shown in Figure 8. This would indicate that all immunoglobulins, γ_A-, γ_M-, and γ_G-, are present in the fractions obtained by PEG precipitation. The same results were obtained with fraction I, isolated from whole human serum

by the procedure of Figure 2, as shown in Figure 9. The antibody specificity of the fractions was not investigated.

Sedimentation Boundary Analysis. Sedimentation boundary analysis by the procedure of Baldwin (14) provided further evidence for the

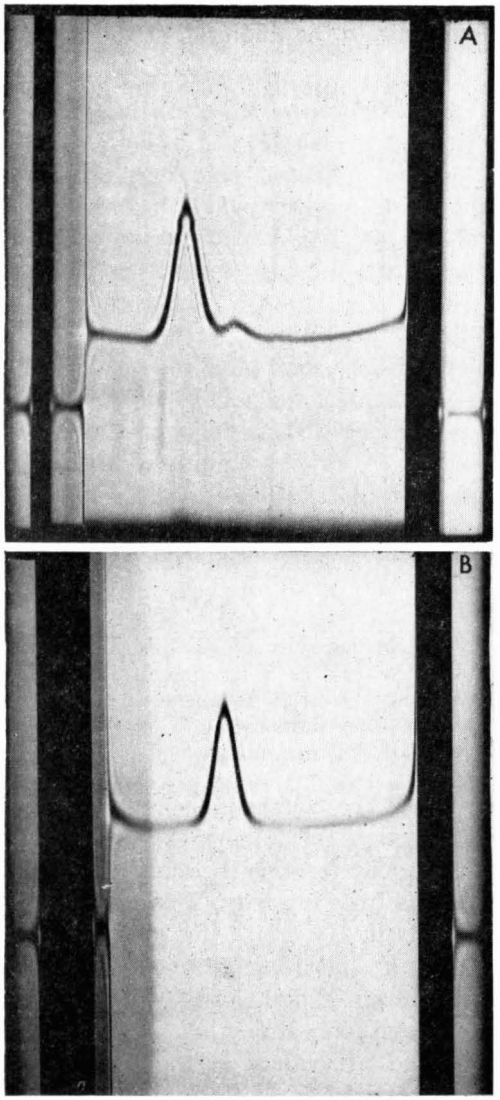

FIG. 10. Ultracentrifuge patterns of immunoglobulins: (A) 1% Cohn fraction II in 0.055 *M* potassium phosphate buffer, pH 7.0, $\mu = 0.1$, 50 min, 59,780 rpm, phase angle 65°. (B) 1% PEG purified Cohn fraction II in 0.055 *M* potassium phosphate buffer, pH 7.0, $\mu = 0.1$, 60 min, 59,780 rpm, phase angle 60°.

homogeneity of the γ-globulin obtained using this technique. Figure 10 shows the sedimentation boundaries for Cohn fraction II before and after PEG purification. A marked linearity in $g^*(s)/g^*(s)_{max} = (S - \bar{s})^2$, as shown in Figure 11, was found at low concentrations at both the trailing and leading edges for fixed values of the apparent distribution function (15), in contrast to the finding that the values of $g^*(s)/g^*(s)$-

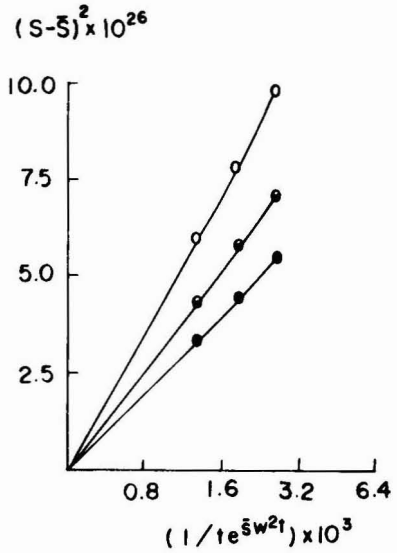

FIG. 11. Ultracentrifugal homogeneity of PEG purified immunoglobulins. Baldwin's extrapolation plot, $(S - \bar{s})^2$ as a function of $(1/te^{\bar{s}\omega^2 t})$ at fixed values of $g^*(s)/g^*(s)_{max}$ on the trailing edge of the sedimentation boundary. Data are plotted at three different protein concentrations: (○) $c = 0.86$ gm/dl; (◒) $c = 0.50$ gm/dl; (●) $c = 0.35$ gm/dl.

$_{max}$ were nonlinear in all the commercial preparations examined. However, at high concentrations of immunoglobulin, fraction III isolated according to Figure 1 and fraction I isolated according to Figure 2 showed slight deviations from linearity. This may be due to the heterogeneity of γ-globulin itself.

Effects of Varying pH and Ionic Strength. High-polymer fractionations of the immune serum globulins were also carried out at varying pH values (4.9 to 8.6) and ionic strengths ($\mu = 0.1$ to 2.0). It was found that ionic strengths and pH values in the ranges studied have little effect on the extent of precipitation of immunoglobulins. The experiments with mixtures of serum proteins and with whole serum indicate that ionic strength does apparently affect the precipitation of serum albumin and the α-globulins.

DISCUSSION

From the data presented here, it would appear that human immunoglobulins which retain many of their native properties can be isolated in high yield from both Cohn fraction II and whole serum by the simple technique of high-polymer fractionation. This technique may prove to be an effective tool for the isolation and fractionation of other complex protein mixtures permitting preservation of their structural integrity.

Since the polymers used are essentially nonionizing and since within the ranges studied variation of the pH has little effect on the precipitation of γ-globulins, the governing principle may differ from that of previous precipitation methods involving ionizing polymers (16). It is well known that the properties of polyelectrolytes in solution depend on the ionic strength. The extent to which a protein may be precipitated by such polyions is influenced by changes in the ionic environment and also by the dielectric constant of the medium. Therefore, ionic strength is important in the "salting-out" and "salting-in" of proteins. Since ionic strength does not affect the PEG precipitation of γ-globulin, it would seem that the polyglycols may function in this instance by lowering the dielectric constant of the medium.

Polson *et al.* (7) studied the effect of pH on the precipitation of plasma proteins with polyglycols and $(NH_4)_2SO_4$. Their experiments indicated that hydrogen ion concentration was a critical factor affecting the selectivity of precipitation. The results of the present work, however, suggest that it is the *dielectric constant* of the medium which influences the precipitability of protein. The experiments reported herein indicate that a low concentration of PEG is required to precipitate those proteins of high molecular weight which have a correspondingly high molecular volume, whereas higher concentrations of PEG are required to precipitate proteins of lower molecular volume. Thus, precipitation of protein by polyglycols is an inverse function of the molecular weight and molecular volume of the protein. From this observation, one might conclude that the precipitation of protein by polyglycols is a result of the removal of water from the hydration envelop of the protein molecules, causing a corresponding alteration of the dielectric constant of the surrounding medium. This would alter the steric relationships of the hydrophobic and hydrophilic groups of the protein molecules. There is no experimental evidence at present to support such a mechanism.

SUMMARY

By the use of fractional precipitation with high molecular weight nonionic polymers, immune globulins of marked homogeneity were

isolated in high yield from either whole serum or commercial samples of immunoglobulins. The isolated fractions were characterized by immunochemical and ultracentrifugal analyses. When the pH was varied from 4.9 to 8.6 and the ionic strength from 0.1 to 2.0, little effect on the precipitation of the immunoglobulins was noted. The immunochemical studies indicated that this fractionation technique is not effective in separating the γ_G, γ_M, and γ_A activities present in the γ-globulins isolated from whole serum or in commercial immunoglobulin preparations.

Preliminary experiments indicate that this technique may be used for the isolation of other serum proteins, especially albumin and the α-globulins. In these instances, however, it is important that ionic strength and pH be controlled.

The mechanism for the precipitation of serum proteins by PEG has not been established, but it is suggested that the precipitation involves a local dehydration and consequent change in the dielectric constant of the medium immediately surrounding the protein molecules.

It would appear that the high-polymer precipitation technique may be an effective and simple method for the isolation of serum proteins with preservation of much of their native properties.

ACKNOWLEDGMENTS

The authors are indebted to Drs. John Robbins and Parker Small for their stimulating advice and to Miss Victoria Kressman and Mrs. Susan Coker for excellent technical assistance.

Note added in proof: While this paper was in press, a short communication by Iverius and Laurent (18) reported the precipitation of some plasma proteins by the addition of dextran or polyethyleneglycol.

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Electronic Micrometer System for Precise Recording of Coordinates from Ultracentrifuge Plates

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The need to record position coordinates from photographs, at least in part automatically, has been met in various ways for ultracentrifugation (1–3) and similar techniques (4, 5). I wish to report a simpler digital recording system which I believe is practical for any laboratory using an analytical ultracentrifuge.

This equipment was designed with the following features in mind: (*a*) high precision, long scale, low backlash; (*b*) automatic output of digital data in printed and punched form; (*c*) light weight, low torque transducer; and (*d*) simplicity. The specification of light weight was included because of a desire to assemble a system compatible with the rather delicate stage mechanism of the Nikon microcomparator, which is quite commonly employed in ultracentrifuge laboratories. (The microcomparator used here was the Nikon Profile Projector, Model 6C, having a 1″ by 4″ cross-traveling micrometer stage, rotating stage platform, and plate holder for 2″ by 10″ photographic plates; it was supplied through Nikon Inc., 623 Steward Ave., Garden City, New York.) The transducer design adopted meets the requirements for mounting on the micrometer stage of this instrument, but could easily be placed on measuring instruments with lead screw construction.

The components of the system presently in operation include two identical photoelectric, rotational position transducers, specially designed for this application by the L & W Electronics Company (5300 Brownway, Houston, Tex.). The rotor of each transducer consists of a transparent disc which bears closely spaced opaque radial bars and is mounted coaxially on the thimble of a Starrett micrometer attached to the moving stage of the microcomparator. (The y-axis micrometer is Starrett model 468 MP, with 2″ diameter thimble, 0–25 mm scale, accuracy of 0.001 mm over full range, backlash allowance of 0.00075 mm; the x-axis micrometer is similar but has a 0–50 mm scale; manufactured by L. S. Starrett

Company, 1-165 Crescent Street, Athol, Mass.) As the rotor is turned, the radial bars interrupt two light paths between small lamps and miniature photocells housed in the stator. The resulting pulsatile output from each transducer is continuously monitored by an L & W Electronics Company five-decimal-digit reversible counter of solid state design. A discriminator circuit in each counter automatically sets the sign of counting to correspond to the direction of micrometer rotation.

On command from the operator, the net count in both x- and y-axis counters is transferred to a storage register (without erasing the count), and from there to a control unit which drives a Programatic model FL Friden Flexowriter (manufactured by Friden, Inc., 2350 Washington Ave., San Leandro, Calif.) to give counter readout in printed and punched code forms. A panel board, mounted to the right of the microcomparator, contains buttons to clear x- and y-axis counters, and to clear only the first digit on the y-axis counter. A dial-type pulsing device mounted on the panel board allows the operator to enter counts into the first, third, fourth, and fifth digits of the y-axis counter so that a code digit may be placed into the first position and a three-digit number corresponding to the distance in microns between two adjacent fringes may be added to the y-axis count. Such an addition avoids the introduction of a discontinuity into the sequence of measurements when optical clarity and accuracy require shifting from one fringe near the upper edge of a Rayleigh pattern to another closer to the center of the interference pattern. (This precaution should be unnecessary with the multislit interferometer of Billick and Bowen (6).) Mounted near the projection screen of the microcomparator is a coincidence lamp which lights when the x-axis micrometer is turned to even multiples of 100 μ, thus permitting the operator to record fringe positions at regular intervals along the x-axis without reading either micrometer. Because the system prints out the true position of the x-axis micrometer, precise setting of this micrometer is not actually required. (An elaboration of the coincidence indicating feature could facilitate taking measurements on a radius-squared, or radius-cubed, scale.) Finally, a more prominent button on the same panel actuates the mechanism for reading the net count for both axes into the Flexowriter, where it is either typed onto roll paper as two five-digit numbers, or both typed onto paper and punched into eight-channel paper punch tape. Since the micrometer counters are adjusted automatically as the comparator stage is moved through its own controls in the normal way, the operator can record all routine positions "by touch" without removing his eyes from the projection screen. (The coincidence detector, the panel board, the counters, and the control circuitry for driving the Flexowriter were supplied as two

integrated assemblies by the L & W Electronics Co. The transducer-microcomparator assembly and the panel board are shown in Figure 1. The printout unit is located a few feet away where it can be readily observed by the operator.

In principle, the range and accuracy of an electronic micrometer system of this type are limited only by the scale length and precision of the mechanical micrometer on which the transducer is mounted. In the present system, the counter capacity is 10 cm and the least count is 1 μ; least count could equally well have been set at 0.5 μ if this had seemed desirable. Because the rotor of the transducer is rigidly mounted on the

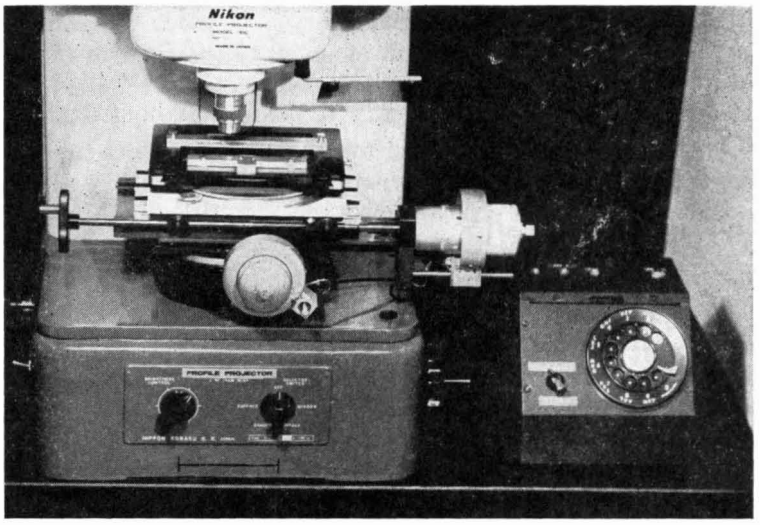

FIG. 1. Microcomparator with position transducers mounted on the x-axis and y-axis micrometers; the large projecting ring centered on each micrometer thimble is a light weight shield protecting the rotor of the transducer (not clearly shown); the small box-like appendage seen below the shield of the right-hand micrometer is the transducer stator, which houses a pair of photosensitive elements. The panel board, with its count incrementing dial and various control switches, is to the right of the microcomparator. The inscribed scale line is 10 cm long.

micrometer, it has zero backlash with respect to the micrometer. (Technically, in this design, backlash is associated with any shift in the lateral position of a ¼ in. rigid rod which orients the stator of the transducer.) The electronic count can be "zeroed" at any spot and the micrometers may be turned back and forth at any time without losing count orientation with respect to the chosen zero position. The speed of the electronic counters is considerably greater than the fastest slewing rate that can be conveniently sustained (about 1000 μ/sec). An occasional dropping

or gaining of one count apparently results from positional ambiguity in the transducer. Theoretically, this error should occur randomly with respect to sign, a maximum of once in every two reversals of the direction of motion of the micrometer. Actual performance indicates that ambiguity occurs less than once in every 100 reversals of motion.

The use of a Flexowriter or comparable alphameric encoder makes it possible to identify fully all records and to incorporate pertinent information about the experiment. The format presently employed for recording positions from ultracentrifuge interference patterns starts with a heading describing the run, including the speed and the magnification factor, and follows with a long list of x and y coordinates automatically entered as commanded from the control panel. A sample print out is illustrated in Figure 2. Special x-axis data points, such as reference positions and meniscus locations, are identified by means of a code number entered from the control panel into the first digit of the y-axis counter. Alternatively, a different editing program for computer processing might make use of code words entered from the keyboard to discriminate special data points and occasions for compensating discontinuities in y-axis readings.

The transducer units in this system are small, relatively light (about 12 oz), and have low frictional torque (slow speed torque of the micrometer-transducer assembly approximates 0.2 in. lb), and consequently offer no interference to the manual operation of the measuring stage, except for very rapid slewing. This is a distinct advantage, since stage movements can be left under the full control of an operator who can be "programmed" with great ease to align plates accurately and to judge the position of the center of either interference fringes or schlieren traces. While in principle, it would seem entirely possible to fully automate the reading of plates using a sequencing program similar to that of Bartulovich and Ward (3) and detectors operating on any of a number of principles (for instance, double-slit photometers, contour coincidence, vibrating single slit, etc. (6–8)), it was concluded that the benefits for the task at hand could not be expected to justify the very great increase in mechanical complexity and cost. This decision was supported by the fact that a human operator would still be required to place the glass plate on the stage, to align it, and probably to locate the positions of the reference edges and menisci (i.e., barring the development of an inexpensive reader having something like two orders of magnitude greater capacity than that of the remarkable "on line" scanner reported by Moore *et al.* (2), or the perfection of appropriate reading programs for the high precision model of the sophisticated film reader of Informational International, Inc. (200 Sixth Street, Cambridge, Mass.)). Although the performance of the human

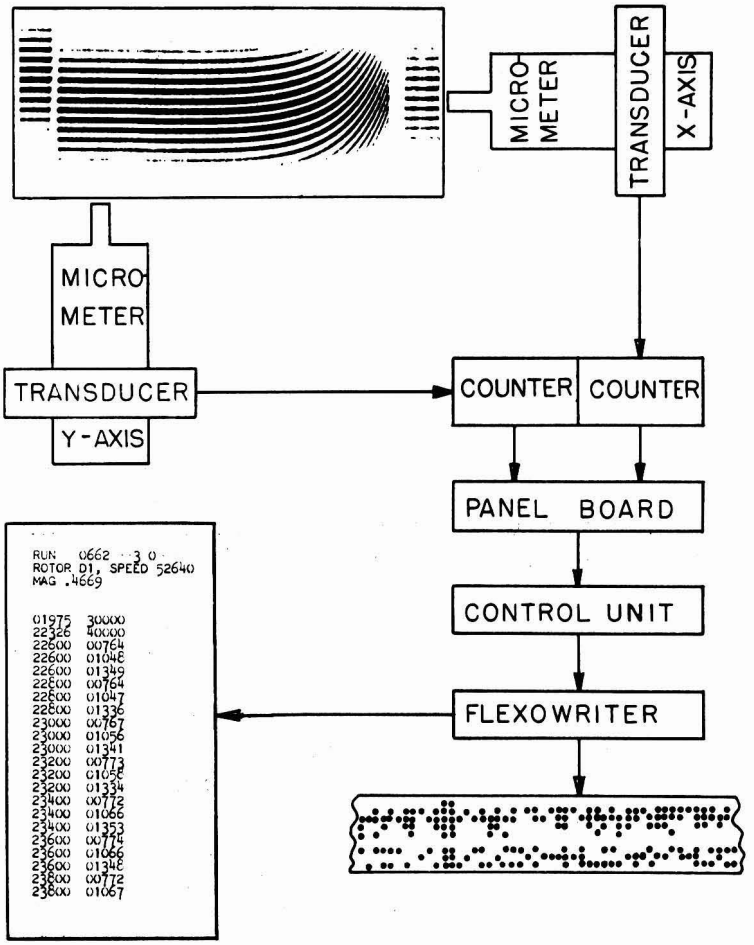

FIG. 2. Sequence of operations in recording coordinates with the electronic micrometer system. The upper left-hand corner of the diagram is a portion of a magnified image of interference fringes for an ultracentrifuge run photographed at sedimentation equilibrium, shown as it would appear on the projection screen of the microcomparator. Electrical pulses generated in the transducers as the two micrometers are turned by the operator are continuously counted by reversible electronic counters. When the operator actuates a pushbutton on the panel board, the control unit transfers the momentary net value in each counter to the Flexowriter, where it is both typed onto paper and punched into paper tape. The typed output shown has a heading describing the experiment and is followed by two columns of coordinate data pairs recorded in microns; the first digit of the right hand (y-axis) column is a code number indicating the type of measurement: "3" = position of left-hand reference; "4" = top meniscus position; "0," "1," or "2" = typical x, y pair, etc.

"eye" may become somewhat impaired by the tedium of long hours at a task and the eye strain attending frequent distance and intensity accommodations, it appears that an unfatigued operator can be quite proficient at making the kind of judgments required in plate reading. The mean deviation observed in the author's laboratory for repetitive readings of fringe position at the same spot in an interference pattern was found to be 2.5 μ (for 20 samples of 10 points each) and the square of the combined variance was 3.4 μ, when interference fringes from a Beckman-Spinco model E ultracentrifuge were viewed at a magnification of 31 $\times$ under good conditions of illumination. (This mean deviation (0.009 fringe) is roughly comparable to the mean deviation (0.005 fringe) achieved by Dew (7) with a photometric mechanism, for measurements on fringes produced in a stationary optical system, and is similar to the estimated accuracy (2% of line width) attained in determining line positions with the apparatus of Rymer and Halliday (8); it is an order of magnitude more precise than the accuracy calculated for the scanning of a plate pattern 35 mm long by a computer densitometer system (1) having a raster resolving 1000 points in its greatest dimension, and corresponds to an error about half of that claimed for the high precision model of the elaborate film reader manufactured by Informational International, Inc.) For the analysis of ultracentrifuge patterns, it therefore seems reasonable to employ an operator who is also able to pass judgment on the quality of the pattern being measured and the consequent reliability of the results, provided measuring time is significantly reduced and lighting conditions slightly improved, compared to typical conditions for manual recording.

Use of the recording micrometer system described here has shortened the time for measuring plates to approximately one-fourth of that previously required. This time saving can be quite significant for ultracentrifuge studies when the shape of moving boundaries must be analyzed or when the complete distribution of solute concentration is examined at sedimentation equilibrium. (For instance, an investigator using a three-channel ultracentrifuge centerpiece (9) for daily molecular weight experiments could be faced with the chore of reading, recording, and processing several thousand fringe positions per week, each accurate to a few microns. Unless automatic recording and data processing were possible, the required investment of human energy in time-consuming but mechanically simple tasks could seriously limit the practical application of a potentially valuable analytical technique. In addition, the laborious routine of manual data handling would supply a large opportunity for error, especially in the initial steps of recording.) The present electronic micrometer units have virtually eliminated recording errors

within the $\pm 1\ \mu$ accuracy of the system, and output is supplied in a form ready for entry into a digital computer, so that all preliminary hand manipulation of data may be by-passed and final calculations of molecular weight, sedimentation coefficient, diffusion coefficient, or interaction parameters may be carried out by available computer programs (10) as appropriate to the experiment.

SUMMARY

The reading of position coordinates from ultracentrifuge schlieren and interference plates is greatly facilitated by the use of a zero-backlash transducer that can be attached to a standard microcomparator stage. On command from the operator, an electronic system controls the operation of a Flexowriter which prints and punches paper tape records of micrometer positions to the nearest $1\ \mu$ for all points selected throughout the full range of micrometer movements. The combination of human control with automatic recording of digital data by a mechanically simple system is believed to utilize the capabilities of man and machine to best advantage in performing the measuring tasks of an ultracentrifuge laboratory.

ACKNOWLEDGMENTS

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ANALYTICAL BIOCHEMISTRY 19, 505–513 (1967)

Disc Electrophoresis of Sorghum Seed Proteins in Polyacrylamide Gels

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Zone electrophoresis in polyacrylamide gels has become a powerful tool for the analyses of protein mixtures from a number of sources. Disc electrophoresis in polyacrylamide gels as developed by Davis and Ornstein (1) and Reisfeld *et al.* (2) has several advantages over other zone electrophoretic methods. Polyacrylamide gels with different pore sizes can be prepared to take advantage of the molecular sieving effect of the gel during electrophoretic separation of proteins (1, 2). Further, by incorporating different buffer salts in gel and the electrode vessels and by introducing discontinuities in ionic strength and in the pore sizes of the gel matrix, protein zones are concentrated into thin starting discs and the zones migrate as discrete discs in an electric field (1, 2).

Electrophoretic analysis of wheat proteins by zone electrophoresis in starch gel and polyacrylamide gels has been reported by a number of workers (4–6). Starch gel electrophoresis has been used for the analyses of the protein patterns of 34 genetic varieties of wheat and related genera (4). Similar studies on the physicochemical characterization and electrophoretic analyses of the protein fractions from grain sorghum have not been available. In this laboratory, work is in progress on the fractionation and physicochemical characterization of proteins from a large number of genetic strains of grain sorghum. This report deals with the methods for the characterization of protein components of sorghum seed flour by disc electrophoresis in polyacrylamide gels. Since acrylamide can be polymerized even in the presence of protein solubilizing agents such as urea (3), the difficulties arising from the low solubility of these proteins in aqueous solutions have been circumvented by incorporating urea into the polyacrylamide gels and the electrode tray buffers.

METHODS

An Indian variety of grain sorghum (Rabi Jowar MS-35-1) obtained from the Agronomist, Agricultural Research Station, Siruguppa, Mysore

State, was used in the experiments reported here. The seeds were milled in a McGill miller to separate the seed coat from the endosperm. The endosperm was ground and the powder was sieved to pass 100 mesh.

The seed flour was defatted by shaking 100 gm of the flour with 300 ml of *n*-butanol, for 2 hr. After standing overnight at room temperature the solvent was removed by centrifugation and the powder was air-dried.

Preparation of Protein Extracts: Water extract of the flour was prepared by shaking the flour with distilled water for 1.5 hr in a wrist-action shaker. A flour to solvent ratio of 1:10 (w/v) was used. The suspension was centrifuged and the clear supernatant was lyophilized. This will be designated as the "albumin" fraction of sorghum. Salt-soluble proteins of sorghum endosperm were prepared in a similar manner by extracting the flour with 1% NaCl solution in place of water. The extract was made salt-free by dialysis against distilled water before lyophilization. This will be designated as the "globulin" fraction of the seed protein.

An alcohol extract of the flour was prepared by intermittently shaking 100 gm of the defatted flour with 1 liter of 60% aqueous ethanol at 60°C for 2 hr. After centrifugation, the supernatant was flash-evaporated at about 35° to remove the solvent. This represents the "prolamin" fraction of sorghum.

Protein nitrogen in the 10% trichloroacetic acid-precipitable portion of the extracts was determined by the conventional micro-Kjeldahl method. The amount of protein in the extracts was calculated from the nitrogen values (N $\times$ 6.25).

Preparation of Protein Samples: Water and salt extracts of sorghum flour were dissolved in freshly prepared 8 *M* urea to give a concentration of 9.6 mg protein/ml. The alcohol extract was dissolved in saturated urea solution and contained 20 mg protein/ml. The solutions were prepared just before use to minimize accumulation of ammonium cyanate in the urea solutions on storage (7). A 75 μl quantity of the protein solutions was used for each electrophoretic run.

Preparation of the Gels: Two gel systems—7.5% cross-linked, pH 9.5 standard gel (stacks at pH 8.3 and runs at pH 9.5) and 7.5% cross-linked, pH 4.3 gel (stacks at pH 5.0 and runs at pH 4.3)—were used. These will be referred to as pH 9.5 gel and pH 4.3 gel, respectively.

Cylindrical columns of gel matrices in three sections, i.e., small pore lower gel, large pore spacer gel, and the large pore sample gel, were prepared in glass tubes 6 cm long and 0.5 cm i.d. The procedure for the preparation of the pH 9.5 gel was essentially similar to that of Davis and Ornstein (1) except that urea was incorporated into the gel and, for the preparation of the pH 4.3 gel, the procedure of Reisfeld *et al.* (2) was

employed. The gel monomer solutions, buffer concentrates, and catalyst solutions were mixed in required proportions (1, 2) and stored in ice until ready for use. A weighed amount of urea was added to the "premixed" small pore and large pore gel solutions to make them 8 *M* with respect to urea, and the solutions were poured into the tubes for polymerization. The change in buffer concentration resulting from the volume change due to addition of urea was ignored.

Application of the Sample: Since it was necessary to work with dilute protein solutions, a special sample gel solution was prepared. The composition of the sample gel was the same as that of the spacer gel but the "4 parts" of water normally used (2) was not added, to offset the dilution of the sample gel solution resulting from addition of relatively large volumes of protein solution. After the lower and spacer gel solutions had polymerized, 0.15 ml of the sample gel solution was added to the tubes followed by 0.075 ml of protein solution, which was thoroughly mixed into the sample gel solution. The sample gel was overlayered with water to exclude contact with atmospheric oxygen. The tubes were placed in front of a fluorescent lamp for photopolymerization of the sample gel.

Difficulties were encountered in the polymerization of the sample gel when certain protein solutions were added to the pH 9.5 sample gel. In these cases, addition of 0.015 ml of *N,N,N′,N′*-tetramethylethylenediamine (TEMED) per milliliter of sample gel solution resulted in polymerization of the sample gel.

Electrophoretic Run: The electrode vessels consisted of round plastic trays fitted with platinum electrodes at the center of the chambers. The electrode vessels used for destaining the gels were fitted with graphite electrodes.

Composition of the four electrode tray buffers used in this work is shown in Table 1. Electrophoresis was carried out for 30 min in buffer A, or for 45 min in buffer B, or for 60 min in buffers C and D at a current strength of 6.4 mA per tube at room temperature (25–28°C).

After the electrophoretic run, the gels were removed from the tubes and placed in protein stain solutions. The gels were stained for 1 hr with amido black prepared by dissolving 0.5 gm of the dye in 100 ml of 6% (v/v) acetic acid solution. The solution was filtered and 10 mg of aluminum lactate was added to 100 ml of the dye solution. Incorporation of aluminum lactate into the dye solution was found to enhance the color intensity of the protein bands (8). The background stain from the amido black stained gels was removed by an electrical destaining procedure (1).

In addition to amido black, water-soluble "nigrosine" was used as a protein stain. Nigrosine dye solution was prepared by dissolving 0.05 gm

TABLE 1
Composition of Electrode Tray Buffers[a]

Component	pH 9.5 gel	pH 4.3 gel		
	A (ref. 1) pH 8.6 $\mu = 0.22$	B (ref. 2) pH 4.6 $\mu = 0.23$	C (ref. 3) pH 3.8 $\mu = 0.21$	D (ref. 10) pH 3.1 $\mu = 0.1$
Tris (gm)	6.0	—	—	—
Glycine (gm)	28.8	—	28.1	—
β-Alanine (gm)	—	31.2	—	—
Glacial acetic acid (ml)	—	8.0	4.0	—
Aluminum lactate (gm)	—	—	—	4.9
Lactic acid (ml)	—	—	—	5.2

[a] To be diluted to a final volume of 1 liter with distilled water. To 75 ml of the buffer solution, 60 gm of urea was added just before the run. Buffer solution was discarded after each run. Recirculation of the electrode tray buffers results in lowering of the mobility of the protein components and spreading of bands.

of the dye in 100 ml of acetic acid/water/methanol (10/50/40, v/v/v). The gels were kept in the nigrosine solution for at least 12 hr (9). The nigrosine stained gels were destained by washing the gels with several changes of 6% acetic acid. The gels were preserved in 6% acetic acid solution.

RESULTS

Only 10–12% of the total protein present in sorghum endosperm flour can be extracted with water or 1% sodium chloride under the conditions employed in these experiments, whereas nearly 45% of the total can be extracted with 60% alcohol at 60°C, as determined by nitrogen analysis

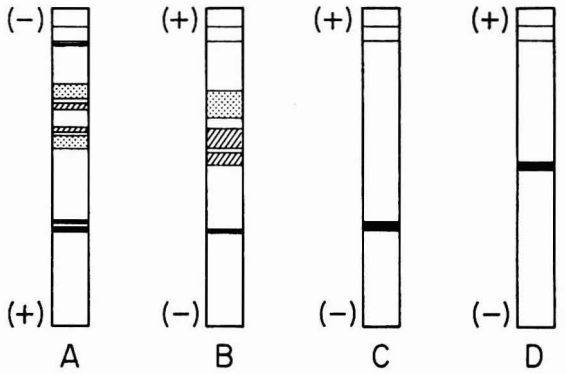

FIG. 1. Diagram of disc electrophoresis patterns of water-soluble sorghum endosperm proteins in: (A) tris/glycine buffer, pH 8.6, (B) β-alanine/acetic acid buffer, pH 4.6, (C) glycine/acetic acid buffer, pH 3.8, and (D) aluminum lactate/lactic acid buffer, pH 3.1. Migration is from top to bottom.

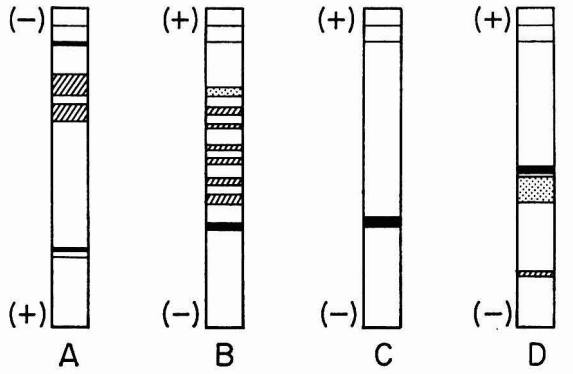

FIG. 2. Diagram of disc electrophoresis patterns of salt-soluble sorghum endosperm proteins in buffers A, B, C, and D. Migration is from top to bottom.

on the extracts. A large quantity of nonprotein matter is also present in the water and salt extracts, but the alcohol extracts contain mostly protein (11.6% nitrogen).

The protein components present in each of these extracts were analyzed in the pH 9.5 and 4.3 gel systems using buffer A (Table 1) with pH 9.5 gel and buffers B, C, and D with pH 4.3 gel. The typical electrophoretic patterns of the protein extracts obtained with each of these buffers are shown in Figures 1–3.

The water-soluble proteins of sorghum separate into four distinct bands and two faint bands when electrophoresed in buffer A (Fig. 1A). The two fast moving bands are very close to each other and form a characteristic "doublet." These bands are also seen in the electro-

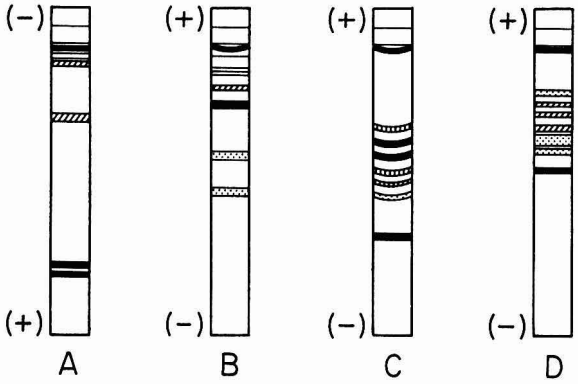

FIG. 3. Diagram showing the disc electrophoresis patterns of prolamin fraction of sorghum endosperm proteins in buffers A, B, C, and D. Migration is from top to bottom.

phoretic patterns of the salt-soluble and alcohol-soluble proteins of sorghum when run with buffer A (Figs. 2A and 3A). When the water extract is run in buffer B, the number of protein bands is reduced to four, of which the slow moving band is faint and diffuse (Fig. 1B). The albumin fraction of sorghum moves as a single component in buffers C and D (Figs. 1C and 1D). The mobility of the band is somewhat higher in buffer C than in buffer D.

The protein fraction of sorghum which is extracted with dilute sodium chloride solution shows two broad bands of low mobility in buffer A and two fast moving bands which are close together (Fig. 2). This preparation is resolved into 8 distinct components by electrophoresis in buffer B. The globulin fraction of sorghum again behaves as a homogeneous component in buffers C and D, although in buffer D two additional faint bands are seen, one of which is very diffuse. The mobilities of the globulin fraction in buffers C and D are similar to the albumin fraction in these buffers. Therefore, only buffers A and B are suitable for the analyses of the albumin and globulin fractions of sorghum.

The alcohol-soluble proteins of sorghum are poorly resolved during electrophoresis in buffer A (Fig. 3). Most of the protein sample remains at the place of application. Three protein bands of low mobility, two fast moving bands, and one band of the intermediate mobility are seen. Resolution of the prolamin fraction is somewhat better in buffer B but excellent separation is obtained in buffers C and D. Seven distinct bands are seen in both patterns. The mobility of the bands in buffer C is higher than in buffer D. An intense band appears at the interface of the large pore and small pore gels in all the buffers. This band probably consists of high molecular weight protein(s) which is unable to penetrate the small pore gel.

The pore size of 7.5% cross-linked polyacrylamide gel seems to be optimum for separation of albumin, globulin, and prolamin fractions extracted from sorghum flour. An essentially similar pattern was obtained when the prolamin fraction was subjected to electrophoresis in buffer D using 5% cross-linked gel.

In most cases, the sample gel is also stained by the protein dye, indicating that some of the protein remained in the sample gel. This may be due to the low solubility of some of the proteins at the pH of the sample gel. Denaturation of the labile proteins due to the heat produced by the current may also have occurred (6).

When urea is not included in the gel and buffer solutions, very indistinct patterns are obtained and most of the protein stays in the sample gel. It is obvious that the presence of urea increases the solubility of proteins in the buffer solutions.

Reproducibility of the electrophoretic patterns is excellent when identical samples are included in the same run or when run under identical conditions at different times. It is important that the sample solutions and buffer solutions with urea should be prepared just before the run.

The prolamins are poorly stained by the protein dyes as compared to the albumins and globulins, even though the concentration of the alcohol-soluble protein used for electrophoresis is much higher. In the alcohol extracts only the fast moving bands are intensely stained.

There are also some differences in the staining properties of sorghum seed proteins with amido black and nigrosine, as can be seen from figure 4. In some instances nigrosine stained gels show more bands than

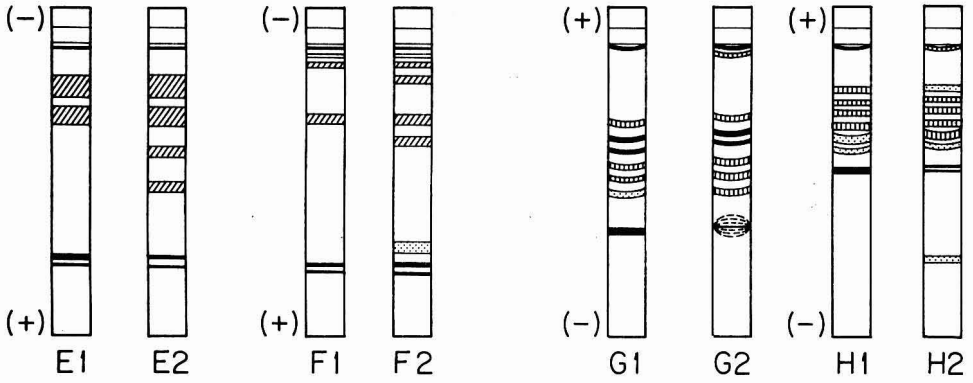

FIG. 4. Disc electrophoresis of sorghum endosperm proteins. E1 and E2 are patterns obtained by electrophoresis of salt-soluble proteins in buffer A and stained with amido black (E1) and nigrosine (E2). F1, F2, G1, G2, and H1, H2 are patterns of prolamin run in buffers A, C, and D, respectively, and stained with amido black (F1, G1, and H1) and with nigrosine (F2, G2, and H2).

those stained with amido black. Such differences are most pronounced with salt-soluble and alcohol-soluble extracts when they are subjected to electrophoresis in buffer A (Fig. 4). Although nigrosine seems to be a better protein dye for seed proteins, there are some disadvantages in using nigrosine for locating protein bands in polyacrylamide gels since the background dye cannot be removed by electrophoretic destaining procedure, and it takes a long time to leach out the background dye by mechanical washing procedures.

DISCUSSION

Solubility fractionation of the proteins of dehulled, defatted sorghum meal indicates that prolamin is the major protein fraction of this cereal and the albumin and globulin fractions constitute a small proportion of

the total protein of the flour. Although these protein fractions are not entirely group-specific and are highly complex (4), this solubility fractionation is still a standard criterion for classifying cereal proteins.

Electrophoretic analysis of the albumin, globulin, and prolamin fractions of sorghum endosperm protein in several buffer systems reveal a highly complex pattern. The albumin and globulin fractions show at least 4 to 8 distinct components after electrophoresis in two buffers. However, both the albumins and globulins of sorghum show a single protein band in glycine/acetic acid buffer (buffer C, pH 3.8) and in aluminum lactate/lactic acid buffer (buffer D, pH 3.1). This again emphasizes the need for studying the electrophoretic behavior of protein preparations at different pH values, with a number of buffer salts. The prolamin fraction gives qualitatively similar electrophoretic patterns by electrophoresis in buffers C and D and shows 7 to 8 distinct protein bands. The pH of the buffer appears to be more important in the separation of the components in a mixture rather than the kind of buffer ion present in the system.

The albumins, globulins, and prolamins seem to have some components with similar mobilities in the buffer systems used in these studies. The fast moving bands are common to all three fractions. It is not clear as yet whether the bands having closely related mobilities represent the same components or different components with similar mobilities. Elton and Ewart (11) have suggested that the fast moving components they observed in starch gel electrophoresis of wheat proteins are due to globulins, the intermediate components to albumins, and the slow moving components to gliadins. It remains to be seen whether a similar relationship exists in the mobilities of the albumin, globulin, and prolamin fractions of sorghum during acrylamide gel electrophoresis. However, the majority of the components in prolamin fraction of the sorghum endosperm protein appear to have low mobilities in the four buffers.

When working with buffers and gels which contain high concentrations of urea, precautions should be taken to prevent carbamylation of the amino and sulfhydryl groups of proteins by cyanate, which is known to accumulate in urea solutions (12, 13). Urea was added to the buffer solutions and gel solutions just before use. Since carbamylation reactions are minimized by working at pH values less than about 5.5 or above 9.0, it is likely that reaction of cyanate with functional groups of proteins is minimal in the buffer systems employed in these experiments. This assumption is further borne out by the fact that the electrophoretic patterns were highly reproducible.

These studies have shown that electrophoresis of sorghum seed proteins in polyacrylamide gels in a variety of buffers over a wide range of

pH permits separation of seed proteins which have a low solubility in aqueous buffers. With this analytical tool, varietal differences in the protein pattern of a large number of genetic strains of sorghum can be analyzed, and the effect of hybridization on the protein composition of varieties may be evaluated (4). Investigations along these lines are in progress in this laboratory.

SUMMARY

Electrophoretic analyses of sorghum flour protein by disc electrophoresis in polyacrylamide gels containing urea have been described. The albumin, globulin, and prolamin fractions of sorghum endosperm meal have been investigated, using pH 9.5 and 4.3 gel systems with four different buffers. Highly complex patterns were observed for all three protein fractions. It has been suggested that this method can provide a convenient tool for the analyses of seed proteins which are relatively insoluble in aqueous buffers.

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ANALYTICAL BIOCHEMISTRY 19, 514–528 (1967)

A Method for Sequential Quantitative Separation and Determination of Protein, RNA, DNA, Lipid, and Glycogen from a Single Rat Liver Homogenate or from a Subcellular Fraction[1]

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An accepted method for studying the metabolic activity of the cell under experimental conditions has been to measure its protein, RNA and DNA composition, and the incorporation of specific precursors into these components. This gives valuable information as to the functioning of the mechanisms involved in protein synthesis. In many cases the process has been extended to study these components in subcellular fractions. In order to evaluate the function of all the major metabolic areas, however, it is desirable to extend these studies to include other major cellular constituents such as glycogen and lipid. These measurements should be carried out simultaneously on the principal subcellular fractions of the cell. Further, if suitably labeled substances known to be involved in many metabolic pathways are administered to the animal before sacrifice, it should be possible to detect changes in the rates of metabolism as well as in the composition of these major components. Such measurements should provide clues as to the sites of action during an experimental treatment that would guide more detailed studies of the mechanisms involved in the production of observed effects.

In order to measure the incorporation of radioactivity into these specific substances, a scheme for chemical fractionation of the components of the cell is required which allows "quantitative" recovery of each component with a minimum of cross contamination of the other components. The method should be applicable to all the subcellular fractions

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and capable of simultaneous analyses of fractions derived from control and treated animals.

The major problems encountered in attempting a fractionation of this type, using the original method of Schmidt and Thannhauser (1) are (*a*) breakdown of proteins that are not acid-precipitable, during the alkaline hydrolysis of RNA (2) and (*b*) loss of RNA, DNA, and protein into lipid solvents when this step follows immediately after the first acid precipitation (3, 4). These problems have been discussed in detail by Munro and Fleck (5) and Hutchinson and Munro (6), who have appraised methods available for the extraction and determination of nucleic acids in biological materials and have suggested modifications to the method of Schmidt and Thannhauser (1).

Recently, Wannemacher *et al.* (7), using the extraction procedure suggested by Hutchinson and Munro (6), described the separation of nucleotides and protein from a single sample of liver. When this method was applied by us to rat liver homogenate and subcellular fractions for the sequential isolation of glycogen, protein, lipid, RNA, and DNA, large and variable losses of protein, RNA, and DNA occurred. The major losses occurred during the lipid extraction.

The investigation described here deals with the development of a procedure based on the suggestion of Munro and Fleck (5) that lipid extraction should be avoided before extraction of nucleic acids. This method gave reproducible results showing good recovery of these components. Also described is the simultaneous administration of glycine-C^{14}, adenine-H^3, and orthophosphate-P^{32} to rats for incorporation studies of these radioactive labels into the protein, lipid, RNA, DNA, and glycogen of the liver as a means of supplying additional information about the functioning of the various metabolic pathways.

METHODS AND MATERIALS

Animals

Male Charles River rats weighing approximately 150 gm were used. The rats were maintained on a Purina Chow diet. One hour before sacrifice the animals were injected intraperitoneally with glycine-C^{14} (μl) (4 μc in 4 μmoles/100 gm body weight), adenine-H^3 (20 μc in 0.5 μmole/100 gm body weight) and sodium orthophosphate-P^{32} (5 μc in 2.5 μmoles/100 gm body weight), all dissolved in 0.8% saline solution. The radiochemicals were obtained from New England Nuclear Corporation, Boston.

Preparation of Tissue for Analysis

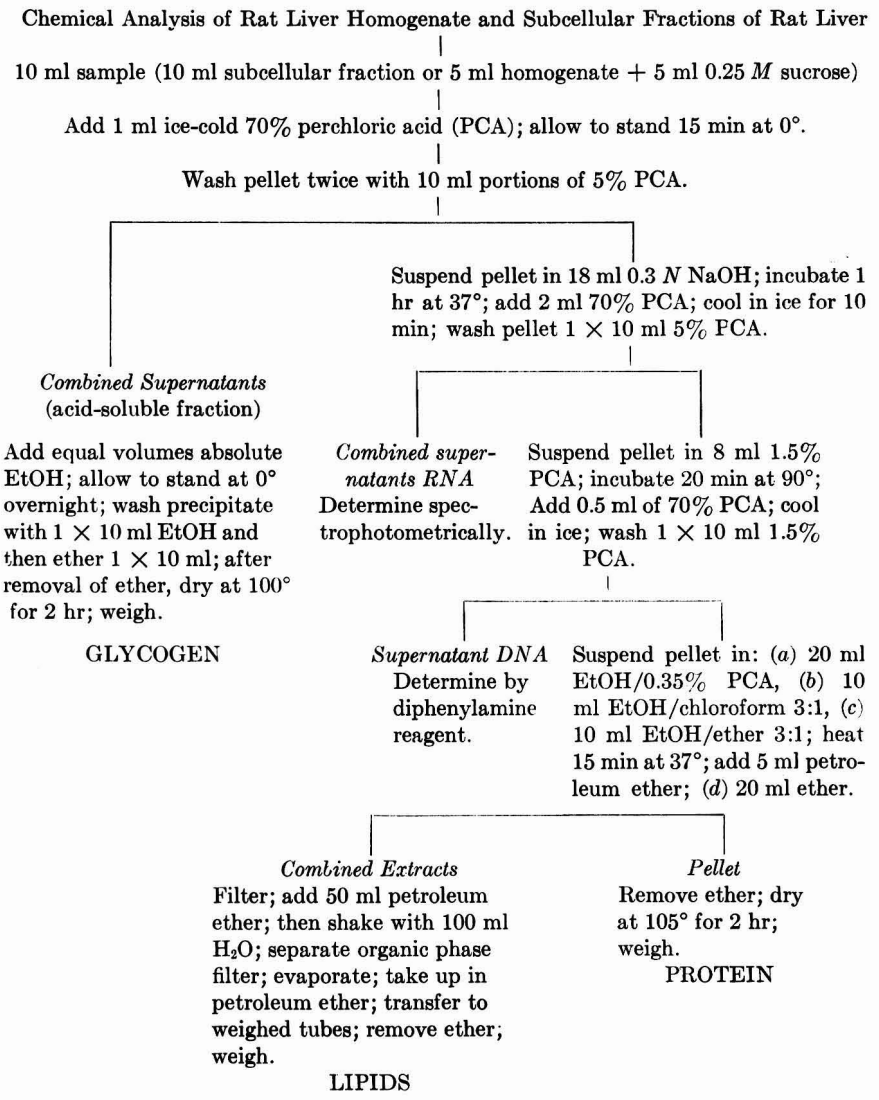
The rats were sacrificed by decapitation. The liver was rapidly removed and washed free of blood with ice-cold 0.25 *M* sucrose and then

homogenized in ice-cold sucrose 0.25 *M*/0.006 *N* NaOH (1/8 w/v), using a Potter-Elvehjem homogenizer. NaOH was added to the homogenizing medium because at this concentration (0.006 *N*) it gave an homogenate with a pH of 7. In its absence the final pH of the homogenate was 5. At pH 7 subcellular particles showed maximum stability, and the possibility of enzymic hydrolysis of the tissue constituents by acid hydrolases was minimized. Usually four livers were combined to give one large composite homogenate. This was strained through cheesecloth to remove connective tissue. A portion (5 ml) was used for determination of total RNA, DNA, lipid, protein, and glycogen; 120 ml of the remainder was fractionated into the principal subcellular fractions. Five particulate and one soluble fraction were obtained by centrifuging the homogenate successively for 10 min at 650 *g* (unbroken cells and nuclear fraction), 15 min at 4000 *g* (mitochondrial fraction), 20 min at 14,000 *g* (light mitochondrial fraction), 30 min at 20,000 *g* (heavy microsomal), and 60 min at 100,000 *g* (light microsomal). The remaining supernatant was used for assay of the soluble fraction. Each particulate fraction was suspended in 25 ml of 0.25 *M* sucrose, care being taken to remove and discard lipid that had separated out during the centrifugation procedures; 10 ml of this suspension was adequate for the analytical procedure. Because the subcellular fractions were not washed before analysis, each fraction was contaminated to some extent by the next most rapidly sedimentating fraction. An estimate of the cross contamination was obtained by measuring the enzyme content of each fraction for an enzyme that is known to be specific for that subcellular fraction. Glutamic dehydrogenase activity gives an estimate of mitochondria; acid phosphatase, of lysosomes; glucose-6-phosphatase, of microsomes; and lactic dehydrogenase, of the soluble cytoplasm. Acid phosphatase and glucose-6-phosphatase were measured by the method of Gianetto and de Duve (8), glutamic dehydrogenase by the method of Hogeboom and Schneider (9), and lactic dehydrogenase by the method of Wroblewski and La Du (10).

Methods for Sequential Separation of Glycogen, RNA, DNA, Lipid, and Protein of Tissue Homogenates and Subcellular Fractions

The first method studied was similar to that used by Wannemacher *et al.* (7) with the exception that (*a*) perchloric acid (PCA) was used in place of trichloroacetic acid because it is a more efficient protein precipitant (4) and (*b*) 0.3 *N* NaOH instead of 0.3 *N* KOH was used for hydrolysis of the RNA. NaOH was used because it does not precipitate as sodium perchlorate, which would interfere with subsequent protein determinations. Further, since sodium perchlorate is soluble in ethanol, any salt remaining after the DNA extraction would be removed during

SCHEME A



the lipid extraction and would not contribute to the final weight of the protein. Because the method resulted in large losses of tissue components, a modified scheme, which is shown as Scheme A, was developed. This differs from the first method as follows: (*a*) RNA and DNA were extracted before lipid extraction, (*b*) the pellet remaining after DNA extraction was washed with ethanol containing 0.35% PCA, instead of

with ethanol saturated with sodium acetate, and (*c*) petroleum ether was added to the pellet suspended in ethanol/ether mixture in order to precipitate dissolved protein from this solvent mixture. For a comparison of these procedures, the same amounts of homogenates or subcellular fractions and the same volumes of the reagents were used (see Scheme A). The times of incubation used for extraction of RNA and DNA are the same as those suggested by Hutchinson and Munro (6), who showed that these conditions gave maximum extraction of these components. In all cases the precipitated material was recovered by centrifugation in the PR2 International centrifuge for 10 min at 2000 rpm, using a rotor which allows 12 samples to be spun at once. The Branson Sonifier[2] was used for resuspension of the precipitates in the various reagents.

Determination of Glycogen, Protein, RNA, DNA, and Lipid

Glycogen. Glycogen extracted in the first PCA extract was precipitated by addition of absolute ethanol to a final concentration of 50%. The glycogen was recovered by centrifugation and, after washing with ethanol, was dried at 110°C for 2 hr and weighed.

Protein. Protein isolated from the homogenate or subcellular fraction was dried and weighed. This gravimetric procedure was found to give excellent agreement with values determined by the biuret method. Protein contained in the RNA and DNA extracts was determined by the method of Miller (11).

RNA. RNA was determined (*a*) by UV absorption at 260 mμ or (*b*) by the orcinol reaction (12).

DNA. DNA was determined by UV absorption at 260 mμ or by the diphenylamine reagent (13).

Lipid. Lipid was determined by direct weighing of the solvent-free, dried lipid extract.

Phospholipid. A sample of lipid (1 mg) was digested in a Pyrex tube with 0.25 ml of concentrated sulfuric acid and 0.05 ml of 30% hydrogen peroxide. The tube was heated until the solution became colorless. After cooling, the sample was diluted with water to a final volume of 8 ml and the inorganic phosphate determined by the method of Lowry (14). A factor of 22.7 was used for calculating the total phospholipid from the phosphorus content (15).

Radioisotope Counting Procedures

RNA and DNA fractions were neutralized with 6 *N* KOH. Samples containing 1 mg of RNA or about 0.2 mg DNA were used for determina-

[2] Heat Systems Co., Melville, Long Island, New York.

tion of radioactivity. Glycogen and protein were dissolved by heating with 1 *N* KOH. Then 1 ml of hydroxide of Hyamine 10-X (1 *M* in methanol) was added to 1 ml of each solution which contained about 10 mg of these fractions. The mixture was neutralized with 2 *N* HCl and made to 4 ml with water. The final pH of the glycogen solution was about 6 and for other samples, 6.5–7. These solutions or suitable portions of the other fractions were mixed with 15 ml of the dioxane scintillation system as described by Snyder (16). The H^3, C^{14}, and P^{32} radioactivity was determined simultaneously with an ANSitron liquid scintillation counter. Internal standards were used to evaluate quenching, and the corrected radioactivity values are expressed as disintegrations/min (dpm). Suitable corrections were made for the C^{14} contribution to the counts in the tritium channel.

RESULTS AND DISCUSSION

Figure 1 shows the distribution of the selected marker enzymes in each subcellular fraction. It is clear that each fraction is characterized by a concentration of the specific enzyme associated with the specific subcellular particle but that there is a considerable cross contamination, particularly between adjacent fractions. These biochemical characteristics define the subcellular fractions that are used in this study.

The protein, lipid, RNA, DNA, and glycogen isolated from a single sample of rat liver homogenate and from subcellular fractions prepared from portions of this homogenate are shown in Table 1, where the first procedure used is referred to as Method 1 and the modified method (Scheme A) as Method 2. The preparation of the acid-soluble fraction from which glycogen is derived is the same for either procedure; hence, there is no difference in the amount of glycogen isolated by Method 1 or 2. This value represents the total liver glycogen, as Roe *et al.* (17) have shown that an acid extraction is adequate to remove all the glycogen from liver, providing there is complete dispersion of the tissue in the reagent. Comparison of the amounts of protein, lipid, RNA, and DNA isolated by Method 1 or 2 shows that significantly greater yields were obtained when Method 2 was used. When Method 1 was used the greatest losses of protein and RNA and DNA occurred during the lipid extraction. These substances appeared as insoluble material at the interface of the water and lipid solvent during the lipid purification procedure. The presence of sodium acetate in the ethyl alcohol during extraction of the acid-precipitated pellet, as recommended by Munro and Downie (4), resulted in increased loss of cellular components into this solvent. Further losses occurred when the pellet was extracted with the ethanol/ether mixture. The nuclear and microsomal fractions (frac-

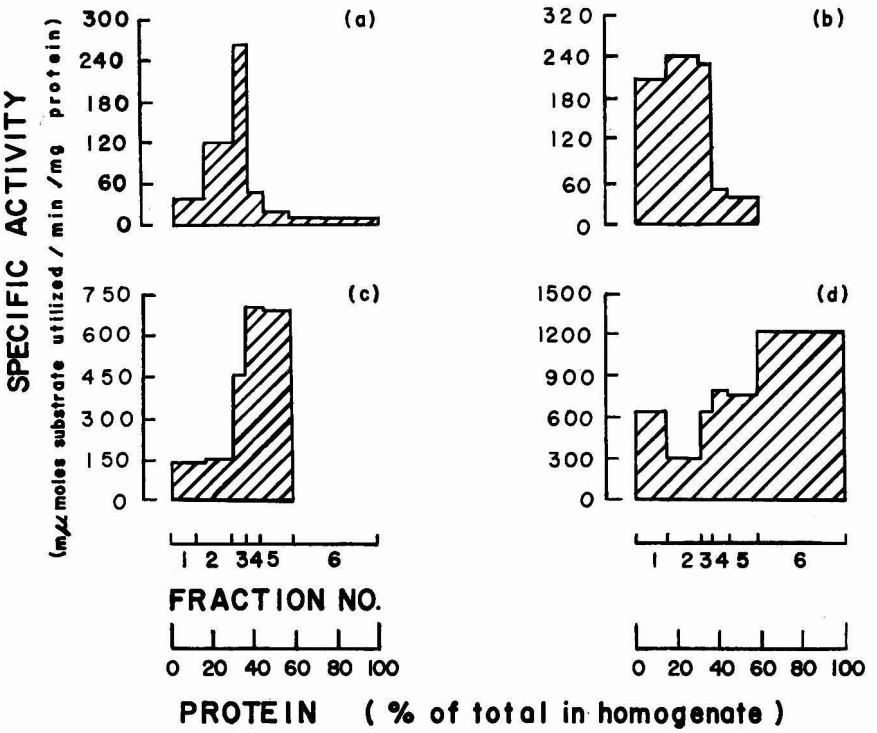


FIG. 1. Distribution pattern of enzymes in subcellular fractions of rat liver (a) acid phosphatase, (b) glutamic dehydrogenase, (c) glucose-6-phosphatase, (d) lactic dehydrogenase. Fractions are plotted in order of their isolation. Fraction 1, unbroken cells and nuclear; fraction 2, heavy mitochondrial fraction; fraction 3, light mitochondrial fraction; fraction 4, heavy microsomal fraction; fraction 5, light microsomal fraction; fraction 6, soluble fraction. Each fraction is represented on the ordinate by the specific activity of enzyme indicated. On the abscissa each fraction is represented by its percentage of the total protein in the homogenate.

tions 1 and 4) were particularly soluble in these solvents. The removal of RNA and DNA before lipid extraction as described for Method 2 made the remaining protein insoluble in ethanol containing PCA (0.35%). However, losses could still occur during extraction with the ethanol/ether mixture unless prevented by precipitation of the protein by the addition of petroleum ether to a final concentration of 33%.

Table 2 shows the relative cross contamination of the isolated DNA and RNA with protein and with each other. DNA contamination of RNA and RNA contamination of DNA was relatively small by either procedure. However, "protein" (material giving the Lowry reaction) was present in both the RNA and DNA. In the case of RNA extracted from

TABLE 1

Comparison of Chemical Composition of Rat Liver and Subcellular Fractions of Rat Liver

Results are expressed as mg/gm fresh tissue. Details of subcellular fraction and Methods 1 and 2 (modified procedure of Method 1) are given in the text. The results are mean values of 6 estimations.

Sample	Protein		Lipid		RNA		DNA		Glycogen	
	Method 1	Method 2	Method 1	Method 2	Method 1	Method 2	Method 1	Method 2	Method 1	Method 2
Homogenate	148.1 ± 26.1	194.5 ± 2.7	26.8 ± 5.5	34.4 ± 1.4	9.38 ± 0.8	11.14 ± 0.2	1.32 ± 0.8	2.06 ± 0.2	40.8 ± 0.8	41.2 ± 0
Fraction 1	26.1 ± 4.7	45.2 ± 1.7	4.1 ± 1.2	6.1 ± 0.5	1.24 ± 0.1	1.48 ± 0.2	0.83 ± 0.2	1.33 ± 0.8	—	—
Fraction 2	21.3 ± 4.4	29.8 ± .05	3.2 ± 0.7	4.7 ± 0.1	0.92 ± 0.1	1.12 ± .05	0.06	0.10	—	—
Fraction 3	13.2 ± 0.7	14.8 ± 0.5	3.0 ± 0.2	3.7 ± 0.2	1.30 ± 0.1	1.42 ± .04	0.02	0.08	—	—
Fraction 4	12.2 ± 0.7	13.3 ± .13	3.4 ± 0.3	4.0 ± 0.2	1.49 ± 0.1	1.62 ± 0.1	0.02	0.08	—	—
Fraction 5	18.8 ± 0.6	23.2 ± 0.2	6.3 ± 0.4	7.8 ± 0.3	2.31 ± .03	2.68 ± 0.4	0.04	0.14	—	—
Fraction 6	54.2 ± 5.4	62.3 ± 7.2	3.2 ± 0.2	3.6 ± 0.1	0.38 ± 0.1	1.20 ± 0.1	0.02	0.13	—	—
	103.4 ± 23.9[a]	97.4 ± 4.7[a]	86.6 ± 7.5[a]	86.9 ± 0.5[a]	81.3 ± 2.9[a]	86.3 ± 0.9[a]	78 ± 2.6[a]	90 ± 1.9[a]	—	—

[a] $\frac{\text{Sum of fractions}}{\text{Homogenate}} \times 100.$

TABLE 2

Cross Contamination of RNA and DNA with Protein and with Each Other

Data are expressed as mg contaminant/mg isolated RNA or DNA; also as amount of contaminant (in each RNA and DNA sample derived from 1 gm fresh tissue) expressed as percentage of amount isolated from 1 gm fresh tissue.

	Protein in RNA				Protein in DNA			
	mg/mg RNA		% of recovered protein		mg/mg DNA		% of recovered protein	
Sample	Method 1	Method 2	Method 1	Method 2	Method 1	Method 2	Method 1	Method 2
H	0.60	0.56	3.80	3.19	1.0	1.31	0.89	1.39
1	0.92	0.63	4.36	2.07	0.4	0.44	1.30	1.31
2	0.53	0.52	2.28	1.94	1.7	2.13	0.48	0.70
3	0.14	0.40	1.40	3.81	9.5	3.86	0.76	2.09
4	0.08	0.32	1.59	3.84	6.5	2.36	1.06	1.50
5	0.27	0.40	3.37	4.57	23.0	4.14	4.89	2.50
6	4.42	1.53	3.09	2.95	20.0	7.13	0.73	1.49
	RNA in DNA				DNA in RNA			
	mg/mg DNA		% of recovered RNA		mg/mg RNA		% of recovered DNA	
Sample	Method 1	Method 2	Method 1	Method 2	Method 1	Method 2	Method 1	Method 2
H	0.016	0.049	1.6	4.9	0.01	0.01	0.98	1.02
1	0.009	0.027	0.9	2.6	0.08	0.05	0.84	0.50
2	0.012	0.018	1.4	1.9	Nil		Nil	
3	0.008	0.012	1.1	1.2	Nil		Nil	
4	0.005	0.012	0.5	1.2	Nil		Nil	
5	0.014	0.017	1.4	1.7	Nil		Nil	
6	0.041	0.028	12.9	2.8	Nil		Nil	

the homogenate and the first two subcellular fractions the amount of protein present was similar when either procedure was used. The RNA extracted from the remaining subcellular fractions showed greater protein contamination when Method 2 was used. For DNA the amount of protein present when Method 2 was used was more than double that found when DNA was extracted according to Method 1. The increased protein in these fractions may be due to the presence of readily hydrolyzable proteins that were lost into the lipid solvent when Method 1 was used. Although the relative amounts of protein to RNA and DNA appear high, these represent only about 2 to 5% of the total protein in the fraction. The problems that may arise due to the presence of protein in these fractions are as follows: (*a*) spectrophotometric measurements of DNA and RNA have to be corrected for the absorption due to the "protein" and (*b*) the radioactivity of the protein contributes to that of the isolated RNA and DNA. Spectrophotometric measurements of RNA were essential because estimation by the orcinol reaction was found to give variable results due to the presence of interfering substances. In order to correct for the presence of protein, a factor of 0.001 OD unit/μg protein/ml solution was applied (5, 6). Similarly, spectrophotometric measurements of DNA which have been corrected with this factor gave results similar to those observed using the diphenylamine reaction.

Method 2 gave larger yields of the lipid components of the homogenate and subcellular fractions, presumably due to the breaking of lipoprotein complexes during the extraction of RNA and DNA. In both cases the total lipid isolated from the fractions was less than that from the homogenate because lipid that had separated out during the various centrifugation steps had been discarded. Therefore, the amount of lipid in the soluble fraction represents only part of the lipid originally present in the soluble cytoplasm. Determination of the phosphate content of these extracts showed that approximately 30% of the phospholipid components had been lost from lipid extracted by Method 2. This loss was reflected in the lipids of each subcellular fraction. Apparently about one-third of the lipid phosphorus was lost during the alkaline and acid extractions. The total lipid recovered was not affected, emphasizing the importance of not basing lipid determination on phosphorus content. The small amount of phosphorus involved was not detected in the RNA or DNA fractions isolated by Method 2.

C^{14}, H^3, and P^{32} derived from the injected glycine-C^{14}, adenine-H^3, and sodium hydrogen phosphate-P^{32} were present in all the substances isolated (Table 3). In the case of protein, C^{14} was the major radioactive component, with smaller amounts of H^3 and P^{32} present also. The specific

TABLE 3

Specific Activities of Chemical Components Isolated from Liver Homogenate and Subcellular Fractions by Two Procedures

Each value is expressed as DPM/mg substance and is the mean value and standard deviation of 6 determinations.

Sample	C^{14}		H^3		P^{32}	
	Method 1	Method 2	Method 1	Method 2	Method 1	Method 2
			Protein			
Homogenate	1114 ± 110	993 ± 38	100 ± 21	116 ± 16	4	5
Fraction 1	694 ± 44	560 ± 14	44 ± 28	136 ± 34	4	4
Fraction 2	624 ± 94	596 ± 38	94 ± 43	103 ± 39	3	4
Fraction 3	1485 ± 89	1401 ± 96	89 ± 70	185 ± 43	4	4
Fraction 4	1908 ± 63	1892 ± 34	163 ± 127	119 ± 32	4	4
Fraction 5	1897 ± 170	1727 ± 54	171 ± 70	331 ± 47	4	5
Fraction 6	787 ± 108	706 ± 52	108 ± 6	130 ± 15	4	4
			Lipid			
Homogenate	301 ± 7	212 ± 13	330 ± 9	271 ± 15	72 ± 13	35 ± 6
Fraction 1	290 ± 24	184 ± 16	253 ± 3	215 ± 11	55 ± 4	35 ± 6
Fraction 2	251 ± 23	164 ± 87	270 ± 16	203 ± 18	70 ± 6	40 ± 10
Fraction 3	332 ± 4	214 ± 9	379 ± 12	277 ± 22	98 ± 8	40 ± 7
Fraction 4	371 ± 17	238 ± 6	418 ± 38	305 ± 18	109 ± 8	43 ± 4
Fraction 5	369 ± 18	242 ± 8	450 ± 47	304 ± 3	122 ± 8	54 ± 3
Fraction 6	177 ± 19	162 ± 1	238 ± 16	231 ± 7	14 ± 5	3 ± 0
			RNA			
Homogenate	511 ± 49	715 ± 34	2955 ± 120	4007 ± 222	182 ± 12	320 ± 55
Fraction 1	619 ± 82	886 ± 47	7840 ± 164	8451 ± 704	212 ± 44	335 ± 32
Fraction 2	328 ± 67	558 ± 32	2278 ± 128	2924 ± 153	128 ± 25	238 ± 12
Fraction 3	406 ± 65	656 ± 18	1473 ± 234	2120 ± 200	78 ± 24	218 ± 14
Fraction 4	368 ± 195	633 ± 12	1472 ± 79	1956 ± 77	68 ± 18	210 ± 9
Fraction 5	329 ± 38	558 ± 48	2706 ± 250	2757 ± 140	78 ± 4	201 ± 7
Fraction 6	1153 ± 55	1139 ± 106	5081 ± 610	6967 ± 537	411 ± 19	362 ± 38

			RNA corrected for protein contamination[a]			
Homogenate	0	162	2895	3925	182	320
Fraction 1	0	533	7801	8351	212	335
Fraction 2	0	250	2230	2872	128	238
Fraction 3	200	100	1461	2050	78	218
Fraction 4	223	38	1467	1922	68	210
Fraction 5	0	0	2651	2625	78	201
Fraction 6	0	0	4604	6767	411	362
			DNA			
Homogenate	3779 ± 200	3120 ± 154	1626 ± 78	2581 ± 304	115 ± 14	183 ± 1
Fraction 1	489 ± 49	920 ± 174	1524 ± 129	2025 ± 176	24 ± 5	67 ± 7
Fraction 2	4134 ± 143	3175 ± 191	4149 ± 345	4412 ± 667	216 ± 54	317 ± 45
Fraction 3	—	—	—	—	—	—
Fraction 4	—	—	—	—	—	—
Fraction 5	—	—	—	—	—	—
Fraction 6	—	—	—	—	—	—
			DNA corrected for protein contamination[a]			
Homogenate	2665	1820	1526	2137	111	177
Fraction 1	200	676	1507	1973	22	67
Fraction 2	2074	1906	3996	4186	212	309
Fraction 3	—	—	—	—	—	—
Fraction 4	—	—	—	—	—	—
Fraction 5	—	—	—	—	—	—
Fraction 6	—	—	—	—	—	—
			Glycogen			
Homogenate	29 ± 2	28 ± 3	120 ± 7	110 ± 9	8	8

[a] See text for discussion of value of this correction.

activity of the protein isolated by Method 2 was somewhat lower than that isolated by Method 1.

The amount of C^{14}, H^3, and P^{32} present in lipids isolated by Method 2 was considerably less than that observed when Method 1 was used. The greatest decrease was in the P^{32} content of the lipid. This may be an additional indication of loss of phosphate of the phospholipid. However, the decreased radioactivity of these fractions may also be due to an increased recovery of less highly labeled lipids.

RNA and DNA showed considerable radioactivity in respect to H^3 with smaller amounts of C^{14} and P^{32} being present. As has been previously pointed out, because of the protein contamination of the RNA and DNA it is desirable to make a correction for this contribution to the observed radioactivity. This correction was made by subtracting the dpm (H^3, C^{14}, and P^{32}) derived from the protein associated with 1 mg of the RNA or DNA from their uncorrected specific activities. The dpm to be subtracted was calculated from the values obtained for the specific activities of the protein isolated from the respective fractions.

The differences in specific activities of the protein, nucleotides, and lipids isolated by the two methods are probably due to a more complete recovery of these components by Method 2. Further, as Munro and Downie (4) have pointed out, it may not be correct to assume that the components lost during the extraction procedure have the same specific activity as those isolated. The results obtained indicate that the protein and lipid lost by Method 1 have somewhat lower specific activity than that recovered. They also show that the nucleotides lost in Method 1 have a higher specific activity than that recovered as indicated by the decreased specific activity of protein and lipid and the increased specific activity of nucleotides isolated by Method 2. However, if this effect also applies to the protein contaminating the RNA and DNA, the corrections applied can at best be considered approximations of the contribution of the protein to the radioactivity of these fractions. Their value in experiments of this type involving the use of multiple isotopes is that it shows which of the various forms of radioactivity present in the isolated substances are probably due to the presence of contaminants. For example, in the case of RNA, application of the protein correction factor indicates that it is unlikely that C^{14} has been incorporated into RNA, whereas the correction in H^3 counts is relatively minor, showing the validity of the measurement of H^3 labeling by adenine-H^3.

With the modifications described in Method 2 it has been possible to obtain consistently good recoveries of lipid, protein, and nucleotides from rat liver homogenates and subcellular fractions of rat liver. The quantities isolated are in good agreement with figures published by Kosterlitz

(18) and considerably greater than those isolated by Wannemacher *et al.* (7). The chemical fractionation described can be carried out in a reasonable time. In our laboratory, two trained people are able to handle 24 samples at a time and complete the sequential chemical fractionation in a 6 hr period. The simultaneous use of 3 labeled precursors of these constituents of the structural and functional elements of the cell extends the scope of the use of labeled compounds in metabolic studies. It permits comparisons to be made of the numerous metabolic pathways involved in these syntheses.

SUMMARY

A procedure is described for the quantitative separation and estimation of protein, RNA, DNA, lipid, and glycogen from a single sample of rat liver or from a subcellular fraction. The method includes alkaline extraction of RNA from the perchloric acid insoluble portion of the sample, extraction of DNA by acid hydrolysis, and then recovery of lipids by solvent extraction. The procedure recommended prevents losses of protein and nucleotides into the lipid solvents and increases the recoveries of lipids.

The simultaneous measurement of incorporation of each of the radioactive labels from glycine-C^{14}, adenine-H^3, and orthophosphate-P^{32} into the chemical components studies is described. The usefulness of this procedure as an over-all screen of the functioning of the metabolic machinery is indicated.

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Analysis for Lysine, Arginine, Histidine, and Tyrosine in Biological Fluids[1]

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Despite increasing utilization of column chromatographic methods for the quantitative determination of amino acids in blood and urine, there remains the need for simple, rapid, and accurate assays for specific amino acids which do not require elaborate instrumentation. Interest of this laboratory in hyperlysinemia created a need for such an assay for lysine. The method described by Dickerman and Carter (1) appeared to satisfy the basic requirements. Modifications of the method have increased its sensitivity and greatly reduced the time required for each determination. The analysis has been successfully extended to include tyrosine, histidine, and arginine. Once the technique is established within a laboratory, 10–15 determinations in duplicate can be performed in a working day.

The assay consists essentially of decarboxylation of the amino acid by a specific enzyme, reaction of the resultant amine with dinitrofluorobenzene, extraction of the colored compound, and its quantitative determination in a spectrophotometer.

MATERIALS AND METHODS

Reagents

A. *For Decarboxylation*

1. *Lysine decarboxylase:* Just prior to use, 100 mg of lysine decarboxylase is suspended in 50 ml of 0.02 *M* potassium maleate buffer. Briefest possible application of a loose-fitting, Teflon-pestle, Potter-Elvejhem

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homogenizer aids in obtaining a homogeneous suspension. The suspension is centrifuged for 10 min at 800 *g* and the supernatant discarded. This removes the "fines" and a small amount of extraneous amines. The pellet is resuspended in 25 ml of 0.2 *M* potassium maleate buffer containing 1 mg of pyridoxal phosphate.[1]

2. *Potassium maleate buffer,* 0.2 *M,* pH 6.0: 23.2 gm of maleic acid and 0.9 gm disodium ethylenediaminetetraacetic acid are adjusted to pH 6.0 with potassium hydroxide and diluted to 1 liter. Potassium maleate buffer, 0.02 *M,* is prepared as a dilution of this solution.

3. *Hydrochloric acid,* 0.9 *M.*

B. *Dinitrophenylation of Cadaverine*

1. *Potassium borate buffer,* 1 *M,* pH 8.7: The pH of boric acid is corrected with potassium hydroxide. The solution is filtered before use.

2. 1-*Fluoro*-2,4-*dinitrobenzene* (DNFB) is prepared as a 0.4% solution in absolute ethanol. This solution is stable for at least 1 week under refrigeration. (*Caution!* Skin sensitization may be induced by the concentrated solution.)

3. *Potassium hydroxide,* 1.0 *M.*

4. *Chloroform:* reagent-grade chloroform is distilled and ethanol added to a concentration of 0.75%.

C. *For Deproteinization of Plasma*

1. *Zinc sulfate*·$7H_2O$, 90 gm/liter.

2. *Sodium hydroxide,* approximately 0.5 *M.*

Precipitation of the plasma proteins is accomplished by the method of Somogyi (6). The reagents must be titrated in order to attain optimal precipitation and this is essential for reproducible results. Five milliliters of 0.2 *M* maleate buffer is added to 20 ml of the zinc sulfate and the resulting solution is diluted to 150 ml with water. This is titrated to pH 8.1 with the 0.5 *M* sodium hydroxide. The strength of the sodium hydroxide solution is adjusted if necessary so that it takes 20 ml $\pm$ 0.1 to perform the titration. The adjusted solution must be protected to prevent CO_2 absorption.

D. *Preparation of Plasma Ultrafiltrates*

The removal of plasma proteins by ultrafiltration instead of precipitation increases the sensitivity of the method and permits its application to amino acids for which a satisfactory precipitant was not found. The technique is essentially that described by Smith (18) but uses a vacuum

[1] The activity of some preparations has been reported to be enhanced by pyridoxal phosphate.

line producing a pressure differential of 500–550 mm of mercury. A 3 to 7 in. piece of ¼″ diameter dialysis tubing (A. H. Thomas Co., Philadelphia, Pa.; tubing stored under refrigeration) is wetted, knotted on one end, and the other end slipped over a 6 in. piece of glass tubing. The junction is inserted into the rubber stopper of a side-arm test tube. Plasma is added to a level above the junction. The assembly is placed in an ice chest overnight with vacuum applied to the side arm. The yield is more than 50% of the original volume and loss by evaporation is less than 3%.

Materials

Lysine, arginine, histidine, and tyrosine, "M.A. grade," were purchased from Mann Research Laboratories, New York. Cadaverine, agmatine,[2] histamine, and tyramine were obtained from Nutritional Biochemicals Co., Cleveland, Ohio; and putrescine from Calbiochem, Los Angeles, California. The decarboxylases were purchased from Worthington Biochemical Corp., Freehold, N. J.; pyridoxal phosphate from Sigma Chemical Co., St. Louis, Mo.; 1-fluoro-2,4-dinitrobenzene from Eastman Organic Chemicals, Rochester, N. Y.; Δ^1-piperideine-2-carboxylic acid (7), and ϵ-*N*-acetyl-L-lysine (8) were prepared in this laboratory.

Lysine Assay

A. *For Solutions Containing No Protein*

1. Place an aliquot of sample in a 16 × 125 mm test tube and dilute to 0.6 ml with water. Maximal urine and plasma ultrafiltrate volumes are ordinarily 0.1 and 0.3 ml, respectively, with 0.05 and 0.3 ml being the typical volumes used. (The OD of the sample blank[3] should be less than 0.200 and the sample itself should have an OD reading of less than 0.750 or further dilution is necessary).

2. Add 0.5 ml of buffered enzyme solution and incubate at 37°C with agitation for 45 min.

3. Terminate incubation with 0.1 ml of 0.9 *N* HCl. Centrifuge at 1500 *g* for 20 min.

[2] Agmatine from two other biochemical supply houses was found to be impure by paper chromatography in *n*-butanol/acetic acid/water 12/3/5 v/v and phenol/water 4/1 w/v (18).

[3] Two aliquots of the unknown are analyzed. One is reacted with the enzyme, the other is not. The latter is a measure of the amine level, or more accurately; of the "extractable DNFB reacting substances" present in the sample. For simplicity this will be termed the "sample blank." The term "reagent blank" indicates a water sample carried through the entire analysis and compared to a tube containing chloroform, which in turn is set to zero.

4. Transfer 1 ml of the supernatant to cuvets.[4,5] Add 0.4 ml of borate buffer and 0.1 ml of 1 *N* potassium hydroxide and mix. Add 0.2 ml of 0.4% DNFB solution. Cap and mix *immediately*.

5. Incubate with agitation in the capped cuvet for 30 min at 60°C. (Thorough mixing and continuous agitation are necessary for uniform results.)

6. Cool and add 1 ml of 1 *N* potassium hydroxide and exactly 4 ml of chloroform. Shake vigorously and centrifuge.

7. Read at 400 mμ against a cuvet containing chloroform. With appropriate tubes the chloroform layer intercepts the beam of the spectrophotometer, making an additional transfer unnecessary.

Standards containing lysine concentrations ranging from 5 to 50 μg/tube and a reagent blank[3] are carried through the entire analysis.

The sample blank[3] is determined by adding 0.1 ml of 0.9 *N* HCl (ordinarily step 3) to the unknown *prior* to the enzyme solution and then continuing with the analytical procedure. If the unreacted enzyme preparation does not modify the amount of color development, a suitable sample blank may be obtained simply by omitting the enzyme and substituting an equal volume of buffer.

B. *For Plasma*

1. Place 0.5 ml of plasma and 0.5 ml of water in a 16 × 125 mm tube. Add 0.5 ml of buffered enzyme solution and incubate at 37°C for 45 min with agitation.

2. Terminate incubation with 2 ml of zinc sulfate solution.

3. Layer with 2 ml of sodium hydroxide and then mix by sudden and repeated inversion. Transfer to a clean tube[5] and centrifuge for 20 min at 1500 *g*.

4. Transfer 2 ml of the supernatant to a cuvet.[5] Add 0.4 ml of borate buffer. Mix and add 0.2 ml of DNFB solution. Cap and mix *immediately*.

5. Continue with step 5 as described under A.

A "sample blank" is made by adding $ZnSO_4$ prior to the enzyme as discussed above in section A. For complete control of the analytical procedures, standards may be prepared by adding lysine to pooled plasma at three levels, deriving a line which is extended back to zero. This would parallel the current practice in many clinical laboratories of using sera that have been reliably analyzed as standards and controls.

[4] Screw-top culture tubes with Teflon-lined caps (Kimble #45066-A, 13 × 100 mm), matched to ±0.5% at 50% transmission, are satisfactory. The light path is approximately 1 cm.

[5] A pipet equipped with a pipet filler such as a Propipette is useful for this transfer and is preferred to decanting.

RESULTS AND DISCUSSION

Two basic requirements must be satisfied for this method to be applied to the analysis of an amino acid: (*1*) a specific decarboxylase for that amino acid must be available; (*2*) the solubility characteristics of the DNFB derivative of the resultant amine must permit its selective extraction from other compounds that have reacted with DNFB.

As to the first requirement, there are at present only a few decarboxylases commercially available, though others have been described and can be prepared from suitable microorganisms. To isolate the DNFB derivative of the amine formed by decarboxylation from other DNFB reactive compounds, which are usually polar, the derivative is extracted into an organic solvent. This should be possible with most basic and neutral amino acids where enzymic decarboxylation and reaction with DNFB will produce a compound with limited solubility in alkaline solutions.

The combination of specific enzymic decarboxylation, reaction of the amine with DNFB, and subsequent extraction from other reactive compounds forms the basis of the method described by Dickerman and Carter (1). The modifications suggested here increase the sensitivity, making it more useful in biological ranges, and simplify the assay. The increased sensitivity was accomplished largely by reducing the reagent blank. Four incubations requiring 155 min have been reduced to two; for a total of 75 min. Five transfers, which increase possibilities for error, have been eliminated.

The reagent blank has been reduced from an OD of 0.350 as described by Dickerman and Carter to 0.080, partially by using a different wavelength to determine the DNFB derivative, and also by improving the conditions for forming and extracting the derivative. The reduction of the reagent blank made it possible to increase greatly the amount of DNFB used in the reaction, thereby reducing the possibility of exhausting the reagent with competing components.

Spectrophotometric Determination

In Figure 1 are presented the spectral curves of the DNFB derivative of cadaverine and of the reagent blank. The optical density peaks rather sharply at 349 mμ, where the reagent blank is also high. A better wavelength to use for the analyses is 400 mμ, where the curve for the DNFB derivative flattens, and the differential from the reagent blank is greater.

Spectrophotometric readings should be limited to a maximum OD of 0.750 when using a Bausch & Lomb Spectronic 20 or similar spectro-

photometer, though this limitation does not apply to the technically superior instruments such as the Beckman DU spectrophotometer. However, at the higher optical densities one must keep in mind the possibility of exhaustion of DNFB, particularly in biological fluids where competing reactive materials may be present. For example, in a gross amino-

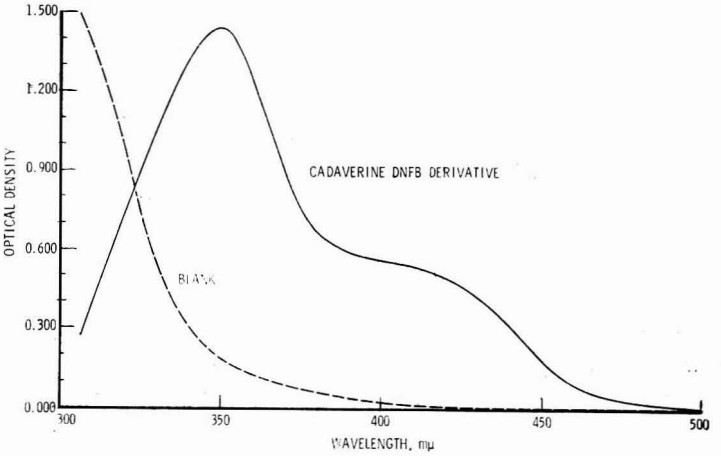


FIG. 1. Spectral curve of the DNFB derivative of cadaverine. Cadaverine readings were against the reagent blank; the reagent blank was against chloroform. Similar but lower curves were obtained with agmatine, histamine, and tyramine. The agmatine reagent blank was approximately three times that for cadaverine. Data were obtained with a Perkin-Elmer 202 ultraviolet-visible spectrophotometer using minimal slit width.

aciduria, a false low result may be obtained for lysine because of reaction of the DNFB with other amino acids. This possibility is easily excluded by repeating the analysis with a sample that is diluted with water 1:1. If the result is greater than 50% of the original determination, it would suggest exhaustion of DNFB in the first analysis.

Specificity of the Assay

The specificity of the analytical method is dependent primarily on the specificity of the decarboxylase. The extensive work of Gale and his associates (9) has demonstrated: (*1*) The enzymes are highly specific for the L-form of the free amino acid. (*2*) Substitutions on the amino or carboxyl groups prevent decarboxylation, while substitution elsewhere on the molecule either eliminates or greatly reduces the effectiveness of the enzyme.

These studies have been repeated by Dickerman and Carter for lysine decarboxylase and have been extended by our laboratory to tyrosine,

TABLE 1
Specificity of the Decarboxylase Preparations

	Substrates											
Decarboxylases	L-Lysine	L-Arginine	L-Histidine	L-Ornithine	L-Tryosine	DL- plus allo-δ-hydroxy-lysine	L-Homo-citrul-line	L-Homo-arginine	Diamino-pimelic acid	L-Phenyl-alanine	L-Dihy-droxy-phenyl-alanine	L-Asparagine
Lysine	100	3.5	<0.5	0.7	—	7	0.8	<0.5	1.9	—	—	1.1
Arginine	0.8	100	1.6	<0.5	3.7	—	<0.5	5	—	<0.5	<0.5	2.6
Histidine	<0.5	<0.5	100	<0.5	2.7	—	<0.5	<0.5	—	<0.5	<0.5	<0.5
Tyrosine	<0.5	1.0	<0.5	<0.5	100	—	<0.5	<0.5	—	1.5	9	<0.5

The enzyme preparations were incubated with 2 μmoles of each substrate. Results are presented as per cent optical density that would be obtained from equimolar amounts of the specific substrate.

Lysine decarboxylase. The following additional compounds contributed less than 0.5% of the optical density of lysine: L-Homoarginine, δ-aminovaleric acid, ε-aminocaproic acid. ε-*N*-acetyl-L-lysine, α-aminoadipic acid, DL-pipecolic acid, Δ¹-piperideine-2-carboxylic acid, L-leucine, L-proline, L-tryptophan, L-glutamine, creatinine, hippuric acid, urea.

Arginine, histidine, tyrosine decarboxylases. The following additional compounds contributed less than 0.5% of the optical density of the specific substrates: L-alanine, L-citrulline, L-cysteine, L-glutamine, L-hydroxyproline, L-leucine, L-proline, L-serine, L-tryptophan, creatinine, hippuric acid, urea.

Ammonia reacts with DNFB, raising the OD of the sample blank 1.7% of the increase caused by an equimolar amount of cadaverine in the lysine assay. Similar sample blank increases of 2.0, 4.6, and 2.2% are produced by ammonia in the arginine, histidine, and tyrosine assays, respectively, when compared to their specific amines. Ammonia does not react with the enzymes, and therefore does not influence the final calculated results.

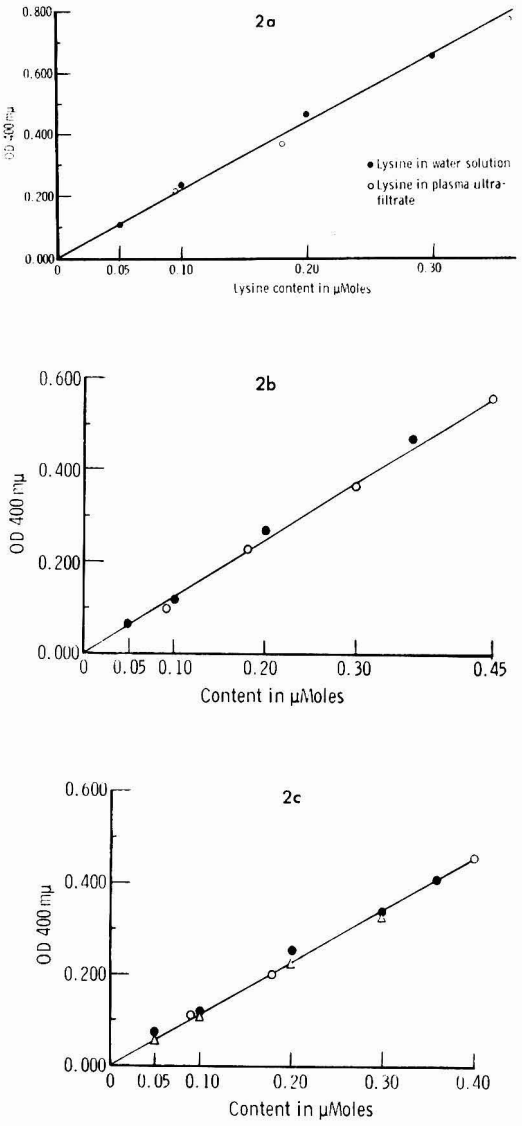
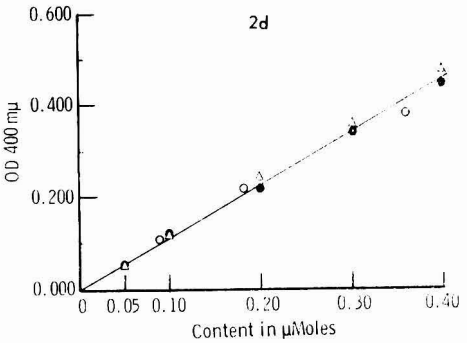


FIG. 2. Recovery of amino acids from plasma and water solutions: (a) lysine, (b) tyrosine, (c) arginine, (d) histidine. Increasing amounts of each amino acid were added to pooled normal plasma and the concentrations determined in the ultrafiltrate (○). The results are compared to water solutions of the amino acids (●) and of the analogous amines (△). In the analysis, 0.3 µmole of the amino acid is equivalent to a plasma concentration of 1 mmole/liter. All values were corrected for the concentration of amino acids present in the pooled plasma prior to supplementation.



histidine, and arginine decarboxylases and to other potentially interfering compounds, with similar results (Table 1).

The major factors interfering with the specificity of the reaction are the traces of tyrosine and lysine decarboxylases present in other decarboxylase preparations used in this study (Table 1). Barring unnaturally large excesses of certain compounds, the error from this source is likely to be small. An interesting and highly informative discussion of the subject has been published (19).

The quantitative nature of the decarboxylation reaction and the reproducibility of the analytical method are demonstrated in Figure 2. Equal amounts of lysine were added to plasma and to water. The increases in optical density are shown. Equimolar amounts of cadaverine produced identical results. Each point represents the average of two determinations. In a similar experiment, lysine was determined seven times at each concentration. The maximum deviation from the mean was ±6%, with most determinations falling within ±3%. The almost identical straight line curves for the amino acid and its amine provide evidence for the completeness of the decarboxylation reaction. Time curves have demonstrated that the reaction is complete in 20 min. Previous studies (1, 9) have shown minimal inhibition of the decarboxylase by compounds usually present in urine and plasma, although color development with DNFB is retarded by high concentrations of certain salts (1).

Application to Blood and Urine

The plasma levels of lysine can be quickly and precisely determined in normal and hyperlysinemic subjects (10, 11). Accepting 1.5 times the reagent blank as the lower limits of sensitivity of the method, plasma levels as low as 1 mg % can be determined when zinc sulfate protein precipitation is used. This somewhat reduced the amount of color de-

velopment with DNFB, but the loss is easily corrected for by treating lysine standards in similar fashion.

Other protein precipitants tested—trichloroacetic acid, metaphosphoric acid, picric acid, tungstic acid—were not satisfactory. Ultrafiltration requires more plasma and longer preparation, but does not increase work time, and gives greater sensitivity.

The plasma level in 17 normal subjects, using zinc sulfate precipitation, averaged 2.6 mg %, identical to that reported by Dickerman and Carter (1). The average in 6 normal adults, using ultrafiltration, was 3.3 mg %. This compares with averages reported in the literature, using column chromatography, of 2.72 (12), 2.22 (13), and 2.32 mg % (14), and a value of 2.95 mg % (15) found by microbial assay.

In Table 2 are presented the results of parallel determinations for lysine in plasma using column chromatography and the enzyme method.

TABLE 2
Comparison of Analyses for Lysine by Column Chromatography and by the Enzyme Method

	A		B		C	
No.	Column	Enzyme	Column	Enzyme	Column	Enzyme
1.	3.65	3.43	3.05	2.85	12.6	15.0
2.	8.09	8.42	3.39	3.41	19.8	22.5
3.	12.90	11.77	4.10	3.63		
4.	20.50	20.98	3.06	3.34		
5.	34.50	32.30				

A. Lysine was added to plasma (#1) to produce the following increments: (2) 4.38, (3) 8.76, (4) 17.53, and (5) 29.20 mg %. Ultrafiltrates were prepared and analyzed by column chromatography (16) and the enzyme method. All results are in mg %.

B. Fasting lysine plasma levels of four normal adults (16).

C. Hyperlysinemic patients (referred by Dr. Woody) (10, 11); $ZnSO_4$ precipitation was used for the enzyme determinations in this column, and amino acid chromatography was by the method of Spackman *et al.* (17).

There is good correlation of the results with each other, and with those reported in the literature for normal fasting subjects.

Plasma contains minimal amounts of substances contributing to the sample blank. However sample blanks from urine samples are often high, due in part to the large quantity of ammonia ordinarily present in urine (see Table 1).

No evidence of DNFB exhaustion has been observed in a limited series of urines examined by this laboratory when sample blanks were limited to OD 0.200. All readings were limited to the 0–0.750 portion of the scale not only to obviate the possibility of DNFB exhaustion but be-

cause of the limitations imposed by the clinical spectrophotometer (see "Spectrophotometric Determination" above).

Recoveries of lysine and cadaverine from normal and hyperlysinemic urines gave values similar to those shown in Figure 2.

APPLICATION TO OTHER AMINO ACIDS

Only slight modifications were necessary in the lysine method to determine the additional amino acids for which decarboxylases are commercially available. These changes are summarized in Table 3. The

TABLE 3
Assay Conditions

Decarboxylation		Formation of DNFB derivative			Extraction of derivative	
		Temperature, °C	Time, min	Borate buffer pH	Alkali, *N*	Organic solvent
Enzyme[a]	Buffer					
Lysine decarboxylase, *B. cadaveris* (NCTC 6578), 0.1 unit/mg	Potassium maleate, pH 6.0	60	30	8.7	1.0	Chloroform
Arginine decarboxylase, *E. coli* (NCTC 7020), 0.2 unit/mg	Potassium propionate, pH 5.2	45	60	10.0	5.0	Chloroform
Histidine decarboxylase, *Cl. welchii* (NCTC 6785), 0.07 unit/mg	Potassium acetate, pH 4.5	60	60	8.7	1.0	Chloroform
Tyrosine decarboxylase, *S. faecalis* (NCTC 6783), 0.1 unit/mg	Potassium propionate, pH 5.5	60	30	8.7	1.0	Chloroform
Ornithine decarboxylase	—	60	30	8.7	1.0	Methylene chloride

[a] The enzymes are acetone dried powders of selected bacterial strains (9). A unit of activity yields 1 μmole of CO_2/min at 37°C and a final substrate concentration of 0.083 M, as described by the supplier.

procedure is otherwise the same as for the lysine assay. If the enzyme preparation requires washing, this is done with the same buffer as is used for the decarboxylation reaction but at a concentration of 0.02 *M*.

Tyrosine

The analytical method is essentially the same as that for lysine (Table 3). Although the tyrosine decarboxylase preparation contains small amounts of phenylalanine decarboxylase, insignificant amounts of

phenylalanine are decarboxylated under the conditions of the analysis (<1.5% with equimolar amounts of substrate). When zinc sulfate is used for the precipitation of plasma proteins, the lower limit of the method is about 2 mg %. If an ultrafiltrate is used, this figure is reduced to 1 mg %.

Histidine

The only significant modification in the method is the prolongation of the reaction time with DNFB to 60 min. This raises the blank, but with an ultrafiltrate of plasma the histidine level can be satisfactorily determined as low as 1–2 mg %. Zinc sulfate precipitation is unsatisfactory.

Arginine

Although the decarboxylation with the specific enzyme introduced no problems, the reaction of agmatine with DNFB and its subsequent extraction required modifications of the basic technique. The optimal conditions for derivative formation are incubation at 45°C for 60 min at pH 10.0. Quantitative extraction into chloroform required the substitution (in step A.6) of 5 *N* potassium hydroxide for the 1 *N* solution usually used. As a result of these modifications, the reagent blank is increased, limiting the usefulness of the method at physiological plasma levels. With an ultrafiltrate of plasma, the arginine level can be determined as low as 3 mg %. Zinc sulfate precipitation is unsatisfactory.

Recoveries from urine of the agmatine-DNFB derivative varied among urine samples. An entirely quantitative determination would require an internal control such as a set of standards in aliquots of each specimen or the addition of trace amounts of radioactive arginine.

Ornithine

An ornithine decarboxylase has been described (9), but is not commercially available at present. The amine, putrescine, reacts well with DNFB but the product is nearly insoluble in chloroform. Quantitative extraction can be achieved with methylene chloride. Zinc sulfate precipitation of proteins is satisfactory, the results being similar to those obtained with cadaverine.

POSSIBLE APPLICATION TO ADDITIONAL AMINO ACIDS

To explore the possibility of applying this assay method to additional amino acids as specific amino acid decarboxylases become available, the behavior of several amines have been studied. Amine analogs of amino acids were selected that were representative of various classes of amino

acids. They are: butylamine, ethanolamine, phenethylamine and tryptamine (the analogs of norvaline, serine, phenylalanine, and tryptophan, respectively). The conditions described for cadaverine have been found satisfactory for these amines. The lower limits of usefulness for the analysis using zinc sulfate precipitation are: 4, 3, 5, and 5 mg %, respectively. Ultrafiltration extends the sensitivity of the method to 1 mg % for tryptamine and 1–2 mg % for butylamine, ethanolamine, and phenethylamine.

SUMMARY

A method for the analysis of lysine using a specific decarboxylase has been developed. The amine resulting from decarboxylation is reacted with dinitrofluorobenzene. The product is selectively extracted into chloroform and determined by spectrophotometry at 400 mμ.

The sensitivity of the method is at least one hundred times greater than manometric assay using the same enzyme. Lysine in blood plasma and urine may be easily measured at physiological levels. The method has been extended to tyrosine, arginine, and histidine, and data are presented for its extension to the amine analogs of several other amino acids.

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Operational Normality of α-Chymotrypsin Solutions by Coulometric-Amperometric Titrimetry[1]

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Enzyme preparations, even if crystalline, are never entirely pure. As a rule, furthermore, they tend to lose activity when stored for an extended period of time. It is important, therefore, to have methods available which are capable of distinguishing between active and inactive enzyme. Kinetics techniques are not adequate in that they serve only to compare a particular enzyme preparation with one of unknown but high purity. On the other hand, reagents capable of reacting specifically and relatively irreversibly with the active center of an enzyme can be utilized to titrate active enzyme, i.e., to estimate the molar concentration or normality of active enzyme in a preparation or solution.

A spectrophotometric technique for the determination of the operational normality of solutions of α-chymotrypsin has been described by Schonbaum *et al.* (1). Their technique utilizes the ability of *N-trans*-cinnamoylimidazole to react with α-chymotrypsin on a mole to mole basis to form a derivative that a relatively stable at pH 5. The extent of the acylation is determined by ultraviolet difference spectrophotometry.

More recently, a procedure was developed in this laboratory (2) using a specific chromogenic inactivator of chymotrypsin, 2-nitro-4-carboxyphenyl-*N*,*N*-diphenyl carbamate (NCDC).[2] NCDC hydrolyzes to a negligible extent at pH 7.8 in the absence of enzyme but reacts with a 1:1 molar stoichiometry with active enzyme, yielding stable diphenylcarbamyl chymotrypsin and an equimolar quantity of NHB, which, because it is yellow, can be estimated colorimetrically.

[1] This work was supported in part by a grant from the National Institutes of Health (GM-11805-09).

[2] Abbreviations used: NCDC, 2-nitro-4-carboxyphenyl-*N*,*N*-diphenyl carbamate; NHB, 3-nitro-4-hydroxybenzoic acid; DPCC, *N*,*N*-diphenylcarbamyl chloride; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone.

We have recently begun to investigate the use of coulometric techniques for the titration of active enzyme and wish to report on a method that uses DPCC (3, 4) as a titrant. DPCC is a specific inactivator of chymotrypsin, reacting on a mole to mole basis with a serine at the active site of the enzyme. For every molecule of DPCC that reacts with the enzyme, a Cl^- ion is released.

$$\text{DPCC} + \text{chymotrypsin} \rightarrow \text{DPC-chymotrypsin} + Cl^-$$

There are various accurate and sensitive methods available for the assay of Cl^- (5). We chose to utilize the technique of Cotlove *et al.* (6) in which silver ion is generated coulometrically into the solution at a constant rate. The end point (when all of the chloride ion has been precipitated) is detected by a pair of silver indicator electrodes which sense a rise in conductivity owing to the accumulation of Ag^+ ions. As little as 0.1 μmole of chymotrypsin can be titrated by this method with a precision of about $\pm 3\%$.

EXPERIMENTAL

The instrument used in these studies is manufactured by Buchler Instruments, Inc., Fort Lee, New Jersey, and is called the Buchler-Cotlove Chloridometer. In this instrument a direct current is passed between a pair of silver generator electrodes causing the release of Ag^+ at a constant rate into the solution to be titrated. At the initiation of the release of Ag^+ a time clock is automatically started. Accumulation of Ag^+, which occurs after the end point is released, is sensed by a pair of silver indicator electrodes which actuate a relay that turns off the timer. Since the release of Ag^+ occurs at a constant rate, the elapsed time is proportional to the amount of Ag^+ generated and hence to the concentration of chloride ion. For the determinations reported in this paper, the instrument was run at the "low" setting.

Titrations are run in a 0.1 *N* nitric acid/10% acetic acid solution. Prior to a series of assays it is necessary to run solution blanks as well as standard NaCl solutions. A more detailed description of the instrument and its operation can be found in the article by Cotlove *et al.* (6).

The placement of additional resistors in the generator (coulometric) circuit slows the rate of generation of Ag^+ and hence makes it possible to titrate lower concentrations of Cl^- with greater precision. The instrument as manufactured is capable of titrating 0.2 μmole of Cl^- with a precision of approximately $\pm 2\%$. The addition of 220 kohms to the generating circuit allows one to titrate 0.1 μmole of Cl^- (and hence 0.1 μmole of chymotrypsin) with a similar precision. The cooperation of Buchler Instruments in making these changes is gratefully acknowledged.

Method of Titration: The following is a representative run in which aliquots containing 0.15 μmole of chymotrypsin were assayed for chloride ions (MW 25,000). The concentrations of enzyme and inactivator in solution during the inactivation process were $3 \times 10^{-4} M$ (assuming a pure enzyme of MW 25,000) and $5 \times 10^{-4} M$ (MW of DPCC 231.7), respectively. In all runs described in this paper, the inactivator concentration was always kept at somewhat less than twice the concentration of the enzyme.

Enzyme Solution: (All solutions were in distilled water.) Forty-five milligrams of chymotrypsin was dissolved in 2 ml of 0.05 *M* Tris-nitrate buffer, pH 7.8. Since the moisture content of the enzyme changed from day to day and from lot to lot, quantities were standardized by assuming that a concentration of 0.130 mg/ml in 0.001 *N* HCl gave an optical density of 0.230 at 280 mμ in a 10 mm cuvet.

DPCC (Titrant) Solution: 17.4 mg of DPCC (Distillation Products, Inc. recrystallized 3$\times$ from methanol) was dissolved in 10 ml of methanol.

DPCC Blank: We have consistently found that this blank is negligible. Nevertheless, it should be run periodically. To a stirred solution of 2.8 ml of 0.05 *M* Tris-nitrate buffer is added 0.2 ml of DPCC solution. Stirring is continued for 20 min at room temperature (ca. 23°), after which a 0.5 ml aliquot is withdrawn and added to 2.0 ml of the acetic acid-nitric acid solution and assayed for chloride ion.

Enzyme Blank: To a stirred solution made up of 0.1 ml of methanol and 0.9 ml of 0.05 *M* Tris-nitrate buffer, pH 7.8, is added 0.5 ml of the enzyme solution. After stirring for 20 min at room temperature, a 0.5 ml aliquot is added to 2.0 ml of the acetic acid-nitric acid solution and assayed for chloride ion.

Enzyme Assay: To a stirred solution containing 1.0 ml of enzyme solution and 1.8 ml of 0.05 *M* Tris-nitrate buffer is added 0.2 ml of DPCC in methanol. The turbid solution is stirred for 20 min, after which two 0.5 ml aliquots are withdrawn and each added to 2.5 ml of 0.1 *N* nitric acid/10% acetic acid solution and assayed for chloride ion.

Standard Solutions: Standard chloride solutions are prepared by dissolving the appropriate concentrations of sodium chloride in 0.1 *N* nitric acid/10% acetic acid. The concentrations of the standard solution should be within the range of concentration of the enzyme preparation being assayed, i.e., ca. $1 \times 10^{-4} M$ to $5 \times 10^{-4} M$. A blank solution containing 2.5 ml of 0.1 *N* nitric acid/10% acetic acid was used.

Variations in the Procedure: In the preceding paragraphs, 0.5 ml aliquots were withdrawn from the inactivation solution. Each aliquot contained 0.15 μeq of enzyme and hence was taken from a solution containing

$3 \times 10^{-4} M$ chymotrypsin (assuming MW 25,000 and a purity of 100%). Chymotrypsin solutions of lower concentration can be titrated by withdrawing larger aliquots. For example, a solution containing $1.5 \times 10^{-4} M$ chymotrypsin can be titrated by assaying a 1.0 ml aliquot. Theoretically, one can use an aliquot of any size and, therefore, titrate very low concentrations of enzyme. Actually, however, the solubility product of AgCl imposes a practical limitation. We have been able to titrate solutions of chymotrypsin as low as $10^{-4} M$ routinely by withdrawing 1.5 ml of reaction mixture and adding it to 1.5 ml of a more concentrated nitric acid/acetic acid solution (0.2 N nitric acid/20% acetic acid). Appropriate changes were, of course, made in the controls and blanks. We have not had occasion to test the method at lower concentrations.

RESULTS

A study was made of the utility of the chloride assay procedure for quantities of enzyme ranging from less than 0.1 μmole to about 1 μmole. (For this purpose, the Chloridometer was standardized using the appropriate range of concentrations of NaCl solution.) The results are shown in Figure 1.

Linearity was observed down to 0.1 μmole of enzyme, each point

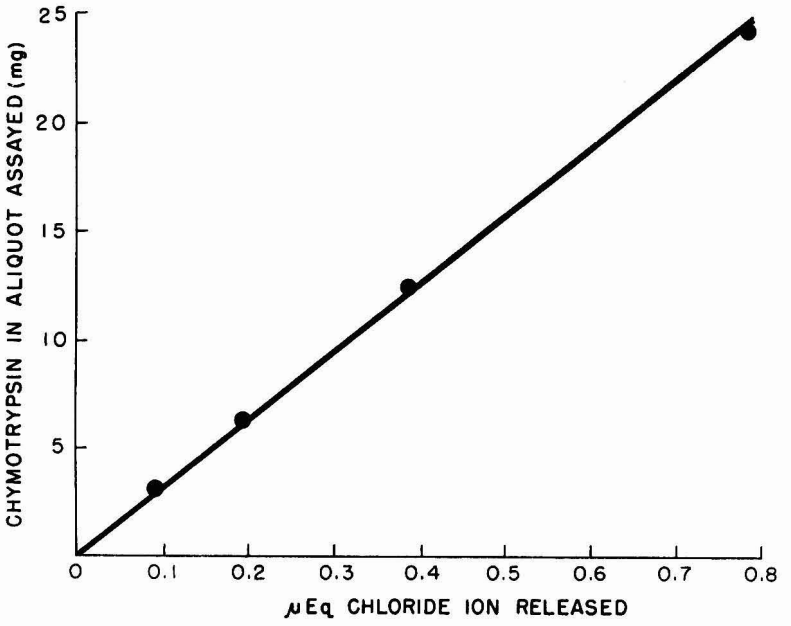

FIG. 1. Titration of range of concentrations of α-chymotrypsin. See "Experimental" for pertinent experimental conditions.

representing the mean of at least three separate runs. The precision at the lowest concentration was ±5%. In quantities of 0.2 μeq and above, a precision of ±2% could be obtained. The precision could be improved at the lower concentrations by introducing an additional 220,000 ohm resistor into the silver generating circuit (see "Experimental"). This modification served to slow down the generation of Ag^+, increasing the titration time so that it could be measured with greater precision. A precision of ±3% was then attainable for the titration of 0.1 μmole of enzyme.

The inverse of the slope of the line in Figure 1 is a measure of the purity of the lot of chymotrypsin used for the studies (Worthington 3× crystallized lot CDI 6100-1). Assuming a molecular weight of 25,000, it is 79.1% active enzyme. Assay of a portion of the same lot using the NCDC procedure (2) gave a value of 78.6% pure enzyme.

DISCUSSION

The results of the coulometric-amperometric technique described in this paper can be considered to be reliable because they are in good agreement with results obtained using NCDC, and are also in general agreement with the values obtained by Schonbaum *et al.* (1) for 3× crystallized chymotrypsin preparations using their spectrophotometric method. Its advantage over the NCDC method is its greater sensitivity. It can be used to titrate as little as 0.1 μmole of chymotrypsin at concentrations as low as 10^{-4} *M* enzyme; the NCDC technique requires about 0.3 μmole. The spectrophotometric procedure of Schonbaum *et al.* required between 0.11 and 0.22 μmole. There is very little difference in the precision of the three techniques.

The coulometric-amperometric technique should be applicable to a number of enzyme systems. All that is required is that there be a reagent capable of reacting with the enzyme with a known stoichiometry and that a halide ion result from the interaction. For example, methylphenylcarbamyl chloride, which reacts with and inactivates acetyl cholinesterase (7) might be used as a titrant for this enzyme. TLCK (8), a specific inactivator, should be a suitable reagent for the titration of trypsin. Sulfhydryl enzymes might be titrated using α-halo acids or aromatic mercuric halides. It is, thus, unnecessary to design nitrophenyl esters or similar reagents less reactive than the halides but amenable to spectrophotometric measurement.

SUMMARY

A coulometric-amperometric procedure was developed for the determination of the normality of α-chymotrypsin solutions. The procedure

utilizes the specific inactivator, *N*,*N*-diphenylcarbamyl chloride as the titrant. Its reaction with active chymotrypsin releases one equivalent of chloride ion for every equivalent of enzyme inactivated. The chloride ion concentration, which, therefore, is equivalent to the concentration of active enzyme, is measured by a coulometric-amperometric procedure. As little as 0.1 μeq of enzyme at concentrations as low as 10^{-4} *M* can be titrated with a precision of $\pm 3\%$. It is suggested that this type of analytical procedure might be applicable for the titration of other enzyme systems.

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A Simple Radioisotope Assay for Phenylalanine Hydroxylase

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Recent studies in this laboratory (1, 2) have revealed new information about the mechanism of the phenylalanine hydroxylase reaction. Briefly, these experiments show that during hydroxylation the para substituent of the substrate, phenylalanine, migrates and appears on the meta position(s) of the product, tyrosine. In addition to providing insight into the chemistry of the reaction these observations have led to the development of a simple, rapid, and sensitive method for the assay of phenylalanine hydroxylase.

The method is based on the use of *p*-tritio-L-phenylalanine as substrate. After the enzymic reaction the tyrosine which was formed is treated with *N*-iodosuccinimide to produce *m,m*-diiodotyrosine (2), and the amino acids are removed by absorption on Dowex 50. The tritium released by the combination of enzymic hydroxylation and iodination appears as tritiated water and can be used as a measure of the enzyme activity. The details of the method are described in this report.

MATERIALS

p-Tritio-L-phenylalanine (5700 μc/μmole) was purchased from Nuclear-Chicago Corporation. One micromole of carrier L-phenylalanine was added per millicurie of labeled material. The material was then chromatographed on Whatman 3 MM paper in 2-propanol/NH_3/H_2O (80/10/10). The phenylalanine was eluted from the paper and the eluate was acidified and applied to a 0.5 ml column of Dowex 50 (H^+). The column was washed with 10 ml of water and then the amino acid was eluted with 20 ml of 1 *N* HCl. The eluate was evaporated to dryness on a Rinco rotary evaporator and the residue dissolved in 1 ml of H_2O. Finally, 9 ml of alcohol was added and the isotope stored at —20°.

Commercial *N*-iodosuccinimide was recrystallized from hot dioxane with the addition of carbon tetrachloride to the cloud point. The mixture

was cooled and the crystals collected. The purified *N*-iodosuccinimide was washed with carbon tetrachloride and stored at —20°.

Pseudomonas phenylalanine hydroxylase (3) was purified 15- to 20-fold[1] and activated with Fe^{++} before use. The preparation of rat liver phenylalanine hydroxylase used was purified through the first ammonium sulfate step (4).

Many of the initial studies designed to determine the optimum conditions of iodination were carried out with *m,m*-ditritiotyrosine (New England Nuclear Corporation), which is an adequate model for *m*-tritiotyrosine, the product of the hydroxylation of *p*-tritiophenylalanine (2).

Samples were counted in a Packard 4000 series scintillation counter equipped with automatic external standardization. All counting was done with between 7 and 9% efficiency using Bray's counting solution (5). When tyrosine production was to be determined directly, the fluorometric assay of Waalkes and Udenfriend (6) was used.

METHOD

Assays were performed in a total volume of 0.25 ml containing: *p*-tritiophenylalanine (10 μl, between 130,000 and 160,000 cpm); L-phenylalanine, 1 μmole; Tris buffer, pH 7.3, 25 μmoles; DPNH, 1 μmole; 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine, 0.15 μmole (in 0.03 ml of 0.1 *M* mercaptoethanol); enzyme; and water. Usually the incubations were carried out for 10 min at 30° in air. The incubation mixtures were then heated for 1 min at 100°, immediately cooled on ice, and kept cold for the remainder of the procedure.

Sodium acetate buffer, 0.2 *M*, pH 5.5, 0.5 ml, was then added. At this point, if the precipitate was substantial, as in the assay of large aliquots of crude tissues, the sample was centrifuged in a refrigerated International centrifuge and the supernatant fraction was removed with a transfer pipet. In assays of the activity of purified enzyme this was unnecessary. To the cold supernatant solution, 0.2 ml of a freshly prepared 1% aqueous solution of *N*-iodosuccinimide was added. The mixture was allowed to remain at 0° for 5 min. Then 0.05 ml of 30% trichloroacetic acid was added to each tube.

The colored supernatant portion of each sample was placed on a Dowex 50/charcoal column. To prepare these columns, 1 ml of an 80% slurry of acid-washed Dowex 50 (H^+) was pipetted into a cotton-plugged, disposable Pasteur pipet. After the resin had settled, 0.1 ml of a 10% slurry of activated charcoal was added. The columns were then washed with 1 or 2 ml of water and were ready for use. The sample was

[1] Guroff, G., and Rhoads, C. A., in preparation.

allowed to run into the column. The tubes were each rinsed with 1 ml of water, which was then applied to the column. The combined effluent from the sample and the wash was collected in a counting vial; 10 ml of counting solution was added and the vials were capped for counting.

RESULTS AND DISCUSSION

When the activity of the purified phenylalanine hydroxylase from *Pseudomonas* spp. was measured with the isotopic method (Table 1) the results were comparable to those obtained by fluorometric assay of the tyrosine produced. The blanks amounted to about 0.1 to 0.2% of

TABLE 1
Comparison of Isotopic and Fluorometric Assays with Purified Phenylalanine Hydroxylase from *Pseudomonas* spp.

Amount of enzyme, μl	Experiment 1			Experiment 2		
	Isotopic		Fluorometric, mμmoles tyrosine	Isotopic		Fluorometric, mμmoles tyrosine
	cpm	mμmoles phenylalanine hydroxylated		cpm	mμmoles phenylalanine hydroxylated	
0	264	1.9	1.1	248	1.1	1.1
1	2,687	21.0	16.6	1,985	14.6	18.2
2	4,461	34.8	31.0	4,349	32.9	35.3
3	5,942	46.4	40.9	6,105	47.0	50.8
4	7,485	54.5	51.9	7,024	54.1	56.9
5	7,905	61.2	53.6	8,256	63.5	68.0
7	8,841	69.1	65.8	9,431	72.8	76.4
10	12,128	97.2	80.1	11,432	88.6	87.9
15	12,675	99.0	89.6	13,304	102.5	101.7
20	15,088	118.0	101.0	14,826	115.4	113.2

Incubations were carried out for 10 min at 30° in air with: L-phenylalanine, 1 μmole; *p*-tritio-L-phenylalanine, 156,000 cpm; DPNH, 1 μmole; 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine, 0.15 μmole (in 0.03 ml of 0.1 *M* mercaptoethanol); Tris buffer, pH 7.3, 25 μmoles; and water in a total volume of 0.25 ml. The enzyme preparation contained 10.5 mg of protein per milliliter and was activated with Fe^{++} before use. At the end of the incubations the samples for isotope assay were treated with *N*-iodosuccinimide as described in the text. Water (0.55 ml) and trichloroacetic acid (0.2 ml of a 30% solution) were added to the tubes to be used for fluorometric assay and the tyrosine content of the protein-free supernatant portion was measured. Each value presented in the table represents the average of duplicate tubes at each enzyme concentration.

the total tritiated phenylalanine added. Similar comparative experiments were performed using purified liver phenylalanine hydroxylase (Table 2). Again the isotopic and fluorometric assays gave comparable results. The lack of linearity at low enzyme concentration, seen with both methods, may reflect the instability of the liver enzyme at these low protein concentrations. The fact that the activity of both enzymes

TABLE 2
Comparison of Isotopic and Fluorometric Assays with Purified Phenylalanine Hydroxylase from Rat Liver

Amount of enzyme, μl	Experiment 1			Experiment 2		
	Isotopic			Isotopic		
	cpm	mμmoles phenylalanine hydroxylated	Fluorometric, mμmoles tyrosine	cpm	mμmoles phenylalanine hydroxylated	Fluorometric, mμmoles tyrosine
0	152	1.2	0	171	1.3	1.1
1	1,020	7.9	7.6	1,030	7.9	5.5
2	3,065	23.9	21.0	3,527	27.3	20.5
3	5,486	42.9	43.6	5,905	46.1	44.2
4	7,346	57.3	59.8	8,081	63.0	59.7
5	9,005	70.3	70.2	9,245	72.3	73.0
7	11,399	89.2	85.7	11,799	92.3	87.8
10	13,733	107.4	103.2	13,552	107.2	106.6
15	14,948	116.7	117.0	15,405	120.0	125.9
20	15,979	124.7	124.8	16,693	130.1	132.0

Incubations were carried out as described in Table 1 except that rat liver hydroxylase was used instead of the *Pseudomonas* enzyme and iron was omitted from the reaction mixture. The enzyme preparation contained 16.1 mg of protein per milliliter. Each value presented in the table represents the average of duplicate tubes at each enzyme concentration.

leveled off at about 10% conversion of phenylalanine to tyrosine probably indicates that the pteridine cofactor became limiting at this point.

The mμmoles of phenylalanine hydroxylated in the isotopic assay have been calculated from the cpm using the equation:

$$\text{m}\mu\text{moles} = \frac{\text{cpm of } H_2O_{\text{observed}}}{\text{sp.act. phenylalanine added}}\left(0.08 + \frac{0.92}{0.81}\right)$$

It has been found that the hydroxylation of *p*-tritiophenylalanine proceeds with the direct release of 8% of the tritium from the para position.[2] The remaining 92% is retained in the product. By iodination experiments using *m,m*-ditritiotyrosine as a model, it has been shown that 81% of the tritium of the product is released under the conditions of the assay. The over-all correction amounts to:

$$\text{m}\mu\text{moles} = \frac{\text{cpm of } H_2O_{\text{observed}}}{\text{sp.act. phenylalanine added}} \times \frac{1}{0.82}$$

Even if the retention of tritium in the product varied widely the correction factor would not change markedly. For example, for retentions of 70 and 100%, the correction factors would be 1/0.86 and 1/0.81, respectively.

[2] Guroff, G., and Daly, J., in preparation.

TABLE 3
Assay of Phenylalanine Hydroxylase in Crude Tissue Extracts

Tissue	Amount of extract, μl	Mμmoles phenylalanine hydroxylated in experiment: 1	2	3	4	5	6
Blank	—	1.2	1.2	1.0	1.0	1.0	1.1
Rat brain	5	—	1.3	—	—	—	—
	10	—	1.3	—	—	—	—
	30	—	1.0	—	—	—	—
	40	—	—	1.0	—	—	—
	80	—	1.0	1.2	—	—	—
	120	—	1.1	—	—	—	—
	140	—	—	0.9	—	—	—
Rat liver	1	7.8	4.4	—	6.4	—	4.4
	2	14.9	8.3	14.7	13.8	11.7	9.8
	4	29.8	13.6	26.6	27.5	21.3	19.9
	8	44.1	27.3	56.8	54.1	39.8	—
	16	—	49.1	68.4	75.3	61.0	58.2
	32	—	—	—	85.6	—	78.9
	64	—	—	—	84.7	—	98.3
Guinea-pig brain	40	—	—	1.1	—	—	—
	80	—	—	1.0	—	—	—
	140	—	—	1.0	—	—	—
Guinea-pig liver	8	—	—	3.7	4.6	—	—
	16	—	—	6.1	5.8	—	—
	32	—	—	11.0	9.4	—	—
	64	—	—	17.4	16.0	—	—

Tissue extracts were prepared by homogenizing the tissue in 2 vol of cold 0.01 *M* acetic acid and centrifuging at 15,000 × g for 10 min in a Lourdes refrigerated centrifuge. The supernatant portions were then assayed for 10 min at 30° in the presence of dimethyltetrahydropteridine, DPNH, Tris buffer, and L-phenylalanine as described in Table 1. Fe^{++} was not added nor was the enzyme activated. Each experiment represents assays of tissue extracts from different animals done on different days.

Application of the isotopic method to crude tissue extracts (Table 3) showed that extremely small amounts of tissues, such as rat liver, could be assayed. The data indicate that livers from both rats and guinea pigs had substantial phenylalanine hydroxylase activity but that the brains of these species had none. It should be noted, however, that these experiments were with undialyzed homogenates of whole brain at pH 7.3. Under these conditions the phenylalanine hydroxylase activity of brain tyrosine hydroxylase (7) would not have been observed.

The repeated assay of a sample of lysed *Pseudomonas* spp. cells (Table 4) illustrated the reproducibility of the method. The standard deviation of the data obtained is ±5.4%.

Based on information now available about the mechanism of the

TABLE 4
Assay of Phenylalanine Hydroxylase in Lysed *Pseudomonas* spp.

Amount of lysate, μl	Mμmoles phenylalanine hydroxylated in experiment:			
	1	2	3	4
0	2.2	2.4	2.1	2.1
5	7.6	10.0	7.9	10.0
10	13.5	15.4	14.4	14.7
20	21.3	22.2	20.3	20.7
30	24.5	25.1	23.5	21.9
40	28.7	27.1	26.6	29.2
60	32.7	33.7	33.1	32.0
80	31.3	33.3	34.3	34.9

Pseudomonas cells were washed three times with dilute mercaptoethanol and taken up in 4 vol of 0.05 *M* Tris, pH 7.3. The cell suspension was broken in a French press and centrifuged at 15,000 × g for 45 min in a refrigerated centrifuge. The supernatant portion was used for these assays. The lysate was incubated with Fe^{++} and then assayed as described in Table 1.

phenylalanine hydroxylase reaction (1, 2) the assay method can be depicted as shown in Figure 1. The product of iodination under these conditions has been shown to be exclusively *m,m*-diiodotyrosine (2). Preliminary data indicate that the phenylalanine hydroxylase reaction exhibits a slight isotope effect. The effect, however, if real, is small and is observed only as a slight increase in the specific activity of the substrate after most of it is used up. In any case, the consistency of the isotopic data with the fluorometric assay under these conditions of low substrate conversion proves that isotope effects are negligible.

Assays which depend upon the direct replacement of carbon-bound tritium at the position of hydroxylation have been used in the study of tyrosine hydroxylase (8), proline hydroxylase (9, 10), and tyrosinase (11). The mechanism of the phenylalanine hydroxylase reaction, which involves migration of the tritium rather than its direct replacement, makes the iodination step necessary in this assay. Other experiments have shown that this method can be applied to measure the phenylalanine hydroxylase activities of partially purified mast cell tryptophan hydroxylase and of adrenal tyrosine hydroxylase. Indeed, assays of this type may be of wide value in the study of hydroxylases since other such enzymes have now been shown to have mechanisms of action similar to that of the phenylalanine hydroxylase reaction, specifically, the tryptophan-5-hydroxylase from mast cells (12) and the aryl hydroxylase of rat liver microsomes (13).

The assay method described here is a somewhat more sensitive measure of phenylalanine hydroxylase activity than the nitrosonaphthol

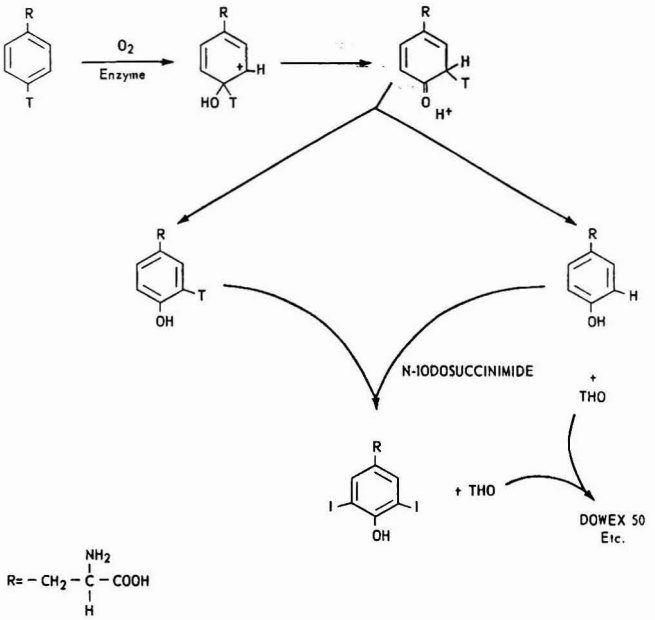

FIG. 1. Reactions leading to the production of tritiated water in the assay of phenylalanine hydroxylase.

method for tyrosine. The hydroxylation of 2 mμmoles of phenylalanine should be easily observable with the isotope method whereas the fluorometric method has a practical limit of about 5 mμmoles. Among the other advantages of the method are its speed and simplicity. A most important point is that the assay is eminently applicable to small amounts of crude tissues where endogenous tyrosine limits the sensitivity of the nitrosonaphthol method or where further metabolism may remove part of the tyrosine formed. Also, use of this method permits a study of the effects of tyrosine itself and related compounds on the activity of the hydroxylases.

The combined advantages of sensitivity and lack of interference by endogenous materials should make this method useful for the examination of small samples of crude tissue such as those obtained from biopsy procedures. An important application of the method, then, would be in the study of clinical or experimental phenylketonuria.

SUMMARY

An isotopic assay for phenylalanine hydroxylase activity has been devised. The assay involves incubation of the enzyme with *p*-tritio-L-phenylalanine followed by the addition of *N*-iodosuccinimide to iodinate

the tyrosine produced. The tritium released by this combination appears as tritiated water and is proportional to the amount of phenylalanine hydroxylated. Among the advantages of this method are its speed, simplicity, applicability to crude preparations, and utility in studies of the effects of *p*-phenols on hydroxylating systems. It should also prove valuable in the study of clinical or experimental phenylketonuria.

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Preparative Separations of Oligonucleotides Containing Ara-Cytidine by Continuous Electrophoresis

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During the synthesis of a series of dinucleoside phosphates, nucleotides, and simple esters of these nucleotides (1), product separations were carried out by ion-exchange chromatography on Dowex 1X2 when $2' \rightarrow 5'$ and $3' \rightarrow 5'$ isomers had to be resolved.

Since column chromatography was time consuming and could lead to rearrangements and degradation (2), a new method for isolating the products of a reaction was investigated, viz., the "carrier-free" continuous-flow electrophoresis systems of Barrolier, Watzke, and Gibian (3) and Hannig (4).

METHODS AND RESULTS

In order that the molecules of the compound desired would have a charge differing from those of other possible constituents in the reaction mixture, the pH's were chosen using the following information (Table 1): (*a*) the number and kind of base forming the molecule, (b) the pK_a values of the base(s), and (*c*) number of phosphate groups and their pK_a's.

TABLE 1
Approximate pK_a Values of Different Functional Groups (6)

Functional group	pK_{a_1}	pK_{a_2}	pK_{a_3}
Cytosine	4.3–4.7	12.1	
Adenine	<0	3.5–4.2	9.8
Uracil	0.5	9.2–9.5	>13
Phosphate	0.7–1.6	5.9–6.6	

The compounds shown in Table 2 have been isolated by continuous electrophoresis from their respective reaction mixtures except for compounds 8–11, where the $2' \rightarrow 5'$ and $3' \rightarrow 5'$ isomers were initially resolved on Dowex 1X2 by the method of Taylor and Hall (7). The conditions and buffers used are shown in Table 3.

TABLE 2
Products of the Condensation Reactions (1)

No.	Compound desired	Abbreviation	Starting materials	
			Protected nucleotide	Protected nucleoside
1	*ara*-Cytidylyl-[5′ → 5′]-*ara*-cytidine	pCaCa	O ‖ $\phi OPCl_2$	N^4,2′,3′-*O*-Tribenzoyl-*ara*-cytidine
2	*ara*-Cytidine 5′-*O*-phenylphosphate	ϕpCa	O ‖ $\phi OPCl_2$	N^4,2′,3′-*O*-Tribenzoyl-*ara*-cytidine
3	*ara*-Cytidylyl-[5′ → 5′]-deoxyuridine	pCadU	3′-*O*-Acetyldeoxyuridine 5′-phosphate	N^4,2′,3′-*O*-Tribenzoyl-*ara*-cytidine
4	Deoxyuridylyl-[3′ → 5′]-*ara*-cytidine	dUpCa	5′-*O*-Trityldeoxyuridine 3′-phosphate	N^4,2′,3′-*O*-Tribenzoyl-*ara*-cytidine
5	*ara*-Cytidylyl-[5′ → 5′]-adenosine	pCaA	N^4,2′,3′-*O*-Triacetyladenosine 5′-phosphate	N^4,2′,3′-*O*-Tribenzoyl *ara*-cytidine
6	*ara*-Cytidylyl-[5′ → 5′]-deoxycytidine	pCadC	N^4,3′-*O*-Diacetyldeoxycytidine 5′-phosphate	N^4,2′,3′-*O*-Tribenzoyl-*ara*-cytidine
7	*ara*-Cytidylyl-[5′ → 5′]-cytidine	pCaC	N^4,2′,3′-*O*-Triacetylcytidine 5′-phosphate	N^4,2′,3′-*O*-Tribenzoyl-*ara*-cytidine
8	Adenylyl-[2′ → 5′]-*ara*-cytidine	A^pCa	N^4-Benzoyl-2′,3′-*O*-diacetyl-*ara*-cytidine 5′-phosphate	5′-*O*-Trityl-N^4-benzoyl-adenosine
9	Adenylyl-[3′ → 5′]-*ara*-cytidine	ApCa	N^4-Benzoyl-2′,3′-*O*-diacetyl-*ara*-cytidine 5′-phosphate	5′-*O*-Trityl-N^4-benzoyl-adenosine
10	*ara*-Cytidylyl-[3′ → 5′]-deoxyadenosine	CapdA	N^4,3′-*O*-Diacetyldeoxyadenosine 5′-phosphate	5′-*O*-Trityl-N^4-benzoyl-*ara*-cytidine
11	*ara*-Cytidylyl-[2′ → 5′]-deoxyadenosine	Ca^pdA	N^4,3′-*O*-Diacetyldeoxyadenosine 5′-phosphate	5′-*O*-Trityl-N^4-benzoyl-*ara*-cytidine

TABLE 3
Continuous-flow Electrophoresis Conditions

Compound	Approx. charge at operating pH	Electrode Buffer	Separation cell: Buffer	Separation cell: pH	Separation cell: Flow rate, ml/hr	Electrode voltage	Current, mA	Sample inlet position (Tube No.)	Polarity[a]	Desired product fractions
pCaCa	+1	1 *N* HOAc	0.5 *N* HOAc	2.3	74	1800	75	34	(−, +)	10–18
ϕpCa	0									32–37
pCadU	−½	0.029 *N* HOAc, 0.024 M Et_3N	Electrode buffer	5.0	74	1900	110	41	(+, −)	16–23
dUpCa	−½	0.029 N HOAc, 0.024 M Et_3N	Electrode buffer	5.0	74	1750	80	34	(+, −)	17–20
pCaA	+1	1 *N* HOAc	0.5 *N* HOAc	2.3	74	1800	70	34	(−, +)	24–31
pCadC	+1	1 *N* HOAc	0.5 *N* HOAc	2.3	82	2140	90	38	(−, +)	15–25
pCaC	+1	1 *N* HOAc	0.5 *N* HOAc	2.3	89	2050	90	7	(+, −)	21–30
A[p]Ca[b]	+1	1 *N* HOAc	0.5 *N* HOAc	2.3	74	1750	75	34	(−, +)	15–22
ApCa[b]	+1	1 *N* HOAc	0.5 *N* HOAc	2.3	74	1750	75	34	(−, +)	18–25
CapdA[b]	+1	1 *N* HOAc	0.5 *N* HOAc	2.3	74	1800	70	7	(+, −)	24–30
Ca[p]dA[b]	+1	1 *N* HOAc	0.5 *N* HOAc	2.3	78	1800	70	7	(+, −)	22–28

[a] Polarity: (+, −) a cation will migrate from the inlet toward the collection tubes with the larger numbers (34 → 48); (−, +) a cation will migrate toward the collection tubes with the smaller numbers (34 → 1).

[b] The mixtures of the 2′ → 5′ and 3′ → 5′ isomers were initially resolved on Dowex 1*X*2 by the method of Taylor and Hall (7).

Continuous electrophoresis was carried out at 10°C in the Brinkmann continuous-flow electrophoresis apparatus (after Hannig (4, 5)) using volatile buffers (Table 3) for the separation cell and electrode compartment. The sample was dissolved in separation cell buffer and the conductivity adjusted by dilution to approximate that of the buffer in the separation cell and fed in at a suitable point near the top of the plates at a rate of 1.2 to 2.0 ml/hr; 0.5 to 2.0 gm of reaction mixture could be processed per 24 hr day. To reduce losses of sample due to migration into the electrode compartments, ion-exchange membranes (Nepton

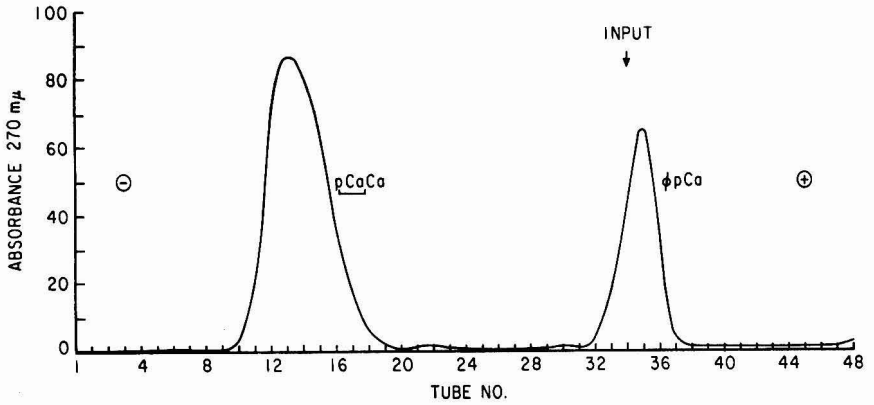

FIG. 1. Isolation of *ara*-cytidylyl-[5′ → 5′]-*ara*-cytidine (pCaCa) and *ara*-cytidine 5′-*O*-phenylphosphate (φpCa) by continuous electrophoresis.

CR61 cation membrane at the anode and Nepton AR111 anion membrane at the cathode) were employed. During the separations, the current was held constant and, in the course of the run, the voltage increased a few percent. At the end of each run, the characteristic ultraviolet absorbance of an aliquot of each fraction was measured. The fractions corresponding to the main absorbance peaks (cf. Fig. 1) were pooled and lyophilized. Since the apparatus employs no stationary phase, the products can be isolated in volatile buffer free of any contamination from support media. The materials in the peaks were characterized by paper chromatography, high-voltage paper electrophoresis, thin-layer chromatography, and, for the desired compounds, also by elemental analysis and nuclear magnetic resonance spectroscopy (1).

As might be expected, some of the apparently pure fractions contained more than one component when examined by paper chromatography or thin-layer chromatography, e.g., the fractions indicated (Table 3) for compounds 6 and 7 contained colored material which was adsorbed on a Dowex 1 formate column (0.1 *N* formic acid elution). For the con-

ditions shown, however, the fractions indicated for compounds 1–5 and 8–11 contained only the desired isomerically pure (i.e., one intranucleotide linkage type) product.

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Quantitative Micro-Determination of Lipid Carbon in Microorganisms

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In microbiological work it is often desired to determine changes in the total cellular content of protein, carbohydrate, and lipid in relation to changes in light intensity, temperature, or mineral element deficiencies. The methods most generally used for estimating total lipid are (*a*) gravimetric determination of extracted lipid, (*b*) calculations involving the caloric content of cells (*R* value) as estimated by carbon, hydrogen, nitrogen elementary analyses (1), and (*c*) subtraction of the weight of protein and carbohydrate from the total dry weight. These procedures all require milligram quantities of cellular material and hence are not useful in experiments in which the number of samples limits the dry weight of cells per sample to less than one milligram. Gas chromatography and specific colorimetric analyses (2) enable one to determine specific lipid components, e.g., fatty acids, with high sensitivity, but these are not necessarily suitable for rapid and simple determinations of the total amount of lipid in a large number of samples.

This paper describes a method for the quantitative determination of lipid in microorganisms in samples containing as little as 20 μg of lipid. The procedure is based upon oxidation of the solvent-extracted lipids to carbon dioxide and measurement of the liberated carbon dioxide by infrared analysis. The results obtained by this method for various microorganisms and spinach chloroplasts are compared with those obtained by conventional gravimetric and colorimetric methods.

METHODS

Procedure for Determination of Lipid Carbon

Extraction of Lipids. Samples of suspended microorganisms were pipetted into 5 or 10 ml tapered centrifuge tubes and centrifuged, and the

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supernatant was discarded. Sample volumes were chosen so as to contain between 20 and 600 μg lipid. The centrifuged cell pack was extracted with at least 10 times its volume, but not less than 1 ml, of chloroform/methanol (2/1 by vol) at 35–40°C for 4 hr, during which time the tubes were held on a vortex mixer at least five times. After centrifuging at 1500 *g* for 5 min, the chloroform/methanol layer was transferred to a clean test tube with a thin Pasteur pipet equipped with a rubber bulb. This extraction procedure was then repeated two more times. The extracts were combined and filtered through a 25 mm silver filter (Selas Flotronics, Spring House, Pa.) with a pore size of 0.2 μ to remove any particulate material which had been transferred with the pipet. The filtrate was transferred to a 10 ml ampoule (Kimble No. 12011-L) which had been rinsed with distilled water and heated at 450°C for 4 hr to eliminate all organic carbon. A glass paper filter (Whatman GF/C, 24 mm), which had previously been heated at 450°C for 2–3 hr to remove organic carbon, was pushed down to the bottom of the ampoule to provide more surface area during removal of the organic solvent. Blank determinations consisting of 4 ml of the chloroform/methanol solvent were included in all steps of the procedure.

Removal of Organic Solvent. The chloroform/methanol solvent was removed by heating in a forced air oven at 35–40°C for 12–24 hr, after which the ampoules were placed in a vacuum desiccator for 1–2 hr at 5–50 μ Hg pressure.

Oxidation of Lipid to Carbon Dioxide. The following additions were made to each ampoule: (*a*) 5.0 ml of triple-distilled water (the third distillation was in an all-Pyrex still with refluxing over 1% $K_2S_2O_8$ and 0.2% H_3PO_4) (3); (*b*) 400 mg of $K_2S_2O_8$ (recrystallized by heating a saturated solution containing 1% H_3PO_4 at 60°C for 3 hr, cooling at room temperature, drying at 50°, and pulverizing the crystals); (*c*) 0.25 ml of 3% H_3PO_4. Inorganic carbon (CO_2) was removed from the acidified sample as follows. A short piece of silicone rubber tubing (o.d. 10 mm, i.d. 5 mm) was placed over the neck of the ampoule and a small plug of silicone stopcock grease pushed into the open end of the silicone tubing. A stainless-steel cannula (diameter 1.0 mm) with a tapered point was inserted through the side of the silicone tubing so that the end was at the bottom of the ampoule. Nitrogen gas (purity 99.997% minimum) freed of its organic carbon and carbon dioxide by passage through activated carbon, molecular sieve, and Ascarite was then flushed through the ampoule for 6 min to remove carbon dioxide. The cannula was removed and the plug of grease pushed down into the tubing. The neck of the ampoule was then sealed with a gas-air torch and the ampoule were

autoclaved for 40 min at 130° (24 psi) to convert organic carbon to carbon dioxide.

Measurement of Carbon Dioxide. The carbon dioxide resulting from the oxidation was measured on a Beckman model No. 15A infrared CO_2 gas analyzer. The procedure was similar to that described by Menzel and Vaccaro (3) with the following modifications: (*a*) the stream of nitrogen was not stopped during breaking of the ampoule neck and insertion of the cannula into the ampoule; (*b*) the nitrogen was passed through activated

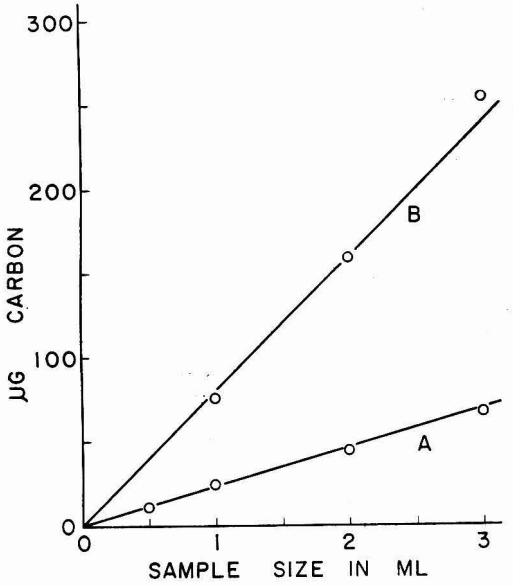

FIG. 1. Recovery of CO_2 after oxidation of lipid carbon of *Navicula pelliculosa* as a function of sample volume: (A) samples from an exponential culture; (B) samples from an old culture which had been silicon deficient and kept in dim light for five weeks. Cell densities in the young and old cultures were 6.1×10^6 and 6.3×10^6 cells per milliliter, respectively. Each point represents the average of two determinations.

charcoal, molecular sieve, and Ascarite before passage into the ampoule; (*c*) instead of recording peak height the total area under the curve was obtained by means of a disc integrator; (*d*) the CO_2 analyzer was equipped with a 1 in. detection cell.

Sensitivity and Precision of Method. As shown in Figure 1 the range over which lipid carbon can be reliably determined by this procedure is 10–300 μg lipid carbon. Eight replicate samples (2 ml) of a silicon-deficient culture of *Navicula pelliculosa* gave a mean of 131 μg carbon, with a standard deviation of 2.8 μg carbon.

Cultures of Microorganisms and Preparation of Spinach Chloroplasts

Bacteria-free cultures of *Dunaliella tertiolecta* (marine green flagellate), *Navicula pelliculosa* (freshwater diatom), and *Chlorella ellipsoidea* were grown at 18–20°C in defined media with continuous illumination of about 4000 lux from fluorescent lamps. The nutrient media used for these organisms are given in references (4), (5), and (6), respectively. A sample of bakers' yeast (from a cake of Fleischmann's compressed yeast) was suspended in 0.1 *M* phosphate buffer at pH 7.0 by stirring with a magnetic bar. Spinach chloroplasts were obtained by disruption of the leaves in isotonic sucrose solution with a Waring Blendor, followed by differential centrifugation. The pellet of chloroplasts obtained at 650 *g* was resuspended in isotonic sucrose (0.8 *M* sucrose, 0.02 *M* Tricine, 0.01 *M* NaCl) at pH 7.9.

Determination of Lipid Content by Three Different Methods

(*a*) *Carbon Dioxide Analysis.* Samples of suspensions of the microorganisms were centrifuged at 1500 *g* for 5 min in tapered 5 or 10 ml centrifuge tubes and the supernatant removed with a Pasteur pipet connected to a vacuum line. Sample volumes were chosen so as to contain about 100 μg lipid carbon (e.g., 1–4 ml of a culture of *N. pelliculosa* containing 6×10^6 cells/ml). The cell pellet was then extracted and the lipid estimated as described above. It was not feasible to determine lipid carbon of the chloroplasts in this manner due to the small volume of chloroplast suspension available and to the concentration of sucrose in the solution. Chloroplast lipid was therefore determined by difference between the total carbon content (see below) and the carbon content after extraction of lipids. Samples of the chloroplast suspension were filtered on glass filters, which were then extracted for 10 min with 2–3 ml of the chloroform/methanol solvent. The lipid organic phase was removed by suction and the extraction repeated 3–4 times. The filter was then put in an ampoule and the carbon content determined.

To determine total organic carbon, aliquots of the suspensions of microorganisms were filtered at 25 cm (Hg) vacuum through glass filters which had been freed of organic carbon by heating at 450°C for 2–3 hr. The filter was rinsed with 5 ml of sterile medium free of organic carbon and then transferred to an ampoule. The organic carbon content was determined as described above. The procedure with chloroplasts was similar except that the filter was rinsed with 5 ml of 0.6 *M* ammonium formate, which was then removed by heating at 50° for 2 hr (7).

The lipid content was calculated in two ways: (*1*) the lipid carbon was expressed as a percentage of the total cell organic carbon; (*2*) after

multiplying the lipid carbon and total carbon values by 1.7 and 2.0, respectively, the weight of the lipid was expressed as a percentage of the total cellular dry weight. These factors of 1.7 and 2.0 were used on the basis of data available in the literature (1, 7–12).

(*b*) *Gravimetric Determination.* One to two liters of the cell suspensions was centrifuged at 1500 *g* to give 1–2 ml of packed cells, which were then extracted repeatedly with chloroform/methanol as described above. The lipid fraction and the nonlipid fractions were dried to constant weight in a forced-air oven at 40°C in tared weighing bottles and the dry weight was determined on a Mettler balance.

(*c*) *Fatty Acid Determination.* The estimation of total lipid by determination of the fatty acid content was done by the pinacyanol reaction as described by Strickland and Parsons (13). This procedure estimates the weight of "stearic acid equivalents" and does not measure other lipid components.

RESULTS AND DISCUSSION

The results of the lipid analyses on the algae, yeast, and chloroplasts are shown in Table 1. The first column of percentages is derived directly from the carbon values obtained by CO_2 analysis. To convert these values

TABLE 1

Lipid Content of Microorganisms and of Chloroplasts Determined Gravimetrically, by Combustion and Measurement of CO_2, and by Fatty Acid Determination (all figures are averages of at least three separate determinations)

Cellular material	Lipid carbon as percent of total cellular carbon	Lipid as percent of total dry wt		
		Method of determination		
		Gravimetric	CO_2	Fatty acids
Navicula pelliculosa	36	34	31	21
Chlorella ellipsoidea	16	20	13	—
Dunaliella tertiolecta	31	29	27	—
Bakers' yeast	6.7	—	5.6	5.5
Spinach chloroplasts	46	—	38	—

to a total dry weight basis, the carbon values were multiplied by the factors 1.7 and 2.0 to give the percentages shown in the column headed "CO_2."

The data in Table 1 demonstrate that the determination of lipid by the CO_2 method agreed with the value obtained by gravimetric means for *N. pelliculosa* and for *D. tertiolecta.* For both these organisms, the values showing the lipid content as determined gravimetrically were higher than the values calculated from the CO_2 data, but were lower than the value

showing lipid carbon as a percent of the total cellular carbon. Such variation can be expected, as the extrapolation of organic carbon values to a weight basis depends upon the factors 2.0 and 1.7. These are average values, and any one culture may show significant differences. Spoehr and Milner (1) have shown that the carbon content of *Chlorella pyrenoidosa,* when expressed as percent of ash-free dry weight, can vary from 49.5 to 70.1%. When one is following the change of lipid with time in kinetic experiments it is not necessary to use these factors, as it is sufficient to consider the lipid carbon as a function of the total cellular carbon. This is not a serious problem, therefore, and is encountered only when one compares results with those obtained by gravimetric means.

The value obtained for lipid content of *C. ellipsoidea* was about 50% higher when determined gravimetrically than when determined by CO_2 analysis. This may have been caused by incomplete extraction of the lipids from the cells. Many strains of *Chlorella* are difficult to extract completely for chlorophyll and other lipid components (14). The cell pack used for gravimetric determination of lipid was exhaustively extracted for several days, but still remained light green at the termination of the extraction procedure. The small volumes extracted for lipid analysis by combustion to CO_2 were not extracted for as long a period as the sample for gravimetric determination. To obtain good lipid analyses of cells such as *Chlorella,* it may be necessary to break the cells to facilitate extraction of the lipid molecules.

There was not enough chloroplast material to permit any gravimetric determination of lipid content. The value obtained by the CO_2 method was 38% lipid by weight, which is in excellent agreement with the value of 37% found by Menke (15).

The lipid content of yeast as determined by fatty acid analysis (5.5%) was very close to the value obtained by measurement of lipid carbon (5.6%). With *N. pelliculosa,* however, the lipid as determined by fatty acid complement was only 60–70% of that obtained gravimetrically or by CO_2 analysis. According to Strickland (personal communication), values obtained for lipid by the fatty acid procedure commonly are much lower than the values obtained gravimetrically after extraction of lipids by conventional procedures. This method of lipid determination by fatty acid analysis has about the same sensitivity as the method of measurement of lipid carbon after combustion. There are, however, two disadvantages of the pinacyanol reaction for lipid analysis. First, the method does not measure total lipids, but only fatty acids containing 12 or more carbon atoms. The final value obtained is in terms of "stearic acid equivalents" of lipid. This can lead to signifi-

cant errors in terms of "total lipid," as Milner (14) has shown that the fatty acids of *Chlorella* can vary from 28 to 87% of the total lipid fraction. Second, the color complex formed between pinacyanol and fatty acids is not stable; timing of all steps throughout the procedure is very important and makes it impractical to do more than 10 samples at once.

The fraction which is termed "total lipid" is the sum of the compounds which were extracted by chloroform/methanol. This organic phase contains a small percentage of low molecular weight compounds which are removed by a saline water wash (16, 17). This is not significant in the results described in this paper, since we used identical methods for extraction of the lipid fractions for gravimetric analysis and for CO_2 analysis.

Determination of carbon content by infrared analysis is based upon the assumption that all the carbon of organic compounds is quantitatively oxidized to CO_2. Since the figures in Table 1 are all percentages, the similarities between the values obtained for the content of lipid by gravimetric means as compared to CO_2 analysis might be misleading if the carbon method were only oxidizing a small fraction of the total cellular material. In our experiments the total weight of cellular material per unit volume suspension determined gravimetrically was compared to the weight obtained by multiplying the total carbon content by a factor of 2.0. There was agreement in all comparisons. The conclusion that this method oxidizes all the cellular carbon to CO_2 is supported by the data of Menzel and Vaccaro (3), which show close to 100% recovery in CO_2 for a variety of nonlipid organic materials. The quantitative recovery in CO_2 of carbon from fatty acids has been verified in controls run with stearic and palmitic acids. To test for completeness of recovery from cellular lipids, the lipids from 2.0 ml packed cells of *N. pelliculosa* were extracted with 100 ml chloroform/methanol and the total organic carbon determined on four samples (two of 0.1 ml and two of 0.2 ml). At the same time the total dry weight of lipid was also determined by drying the remaining 99.4 ml. The total dry weight of lipid was 101 mg, while the four carbon samples indicated 57, 59, 60, and 60 mg carbon in the original 100 ml of extract. The ratio of total dry weight of lipid divided by the organic carbon content was thus 1.71, which agrees with the calculated value based on chemical composition of cellular lipids.

The analysis of lipid carbon by combustion to CO_2 has been used in our laboratories to follow the cellular concentration of lipid in diatoms which have been synchronized by a silicon-starvation period. These studies, which also include data on protein, carbohydrate, cell carbon, DNA, RNA, and pigments, will be reported elsewhere.

SUMMARY

A method is described for the quantitative determination of the carbon content of lipids extracted from microorganisms. The procedure depends upon conventional solvent extraction of the lipids followed by combustion of all lipid carbon to CO_2. The CO_2 is then measured by infrared gas analysis. The technique is extremely reliable and applicable for samples containing between 10–300 μg carbon. Results comparing the lipid content of various algae determined by this combustion technique show good agreement with the lipid content as determined by conventional gravimetric methods.

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D-Alanine Interference in Microbiological Assays of Vitamin B_6 in Human Blood

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Microbiological assays for three naturally occurring forms of vitamin B_6—pyridoxine, pyridoxal, and pyridoxamine—have been reported to give incongruous results when applied to blood (1). The total vitamin B_6 content of whole blood, hydrolyzed in acid to free the vitamin from protein and phosphate, appears to be many times lower than the sum of its parts. The differential assay uses three test organisms, all of which respond to one or more forms of the free vitamin (2). *Saccharomyces carlsbergensis* measures total vitamin B_6; *Lactobacillus casei,* pyridoxal; *Streptococcus faecium* or *Streptococcus faecalis,* pyridoxal and pyridoxamine. Storvick (1) has demonstrated that the apparent vitamin B_6 content of blood hydrolyzates can be increased for *L. casei* and *S. faecium* but not for *S. carlsbergensis* by prolonging acid hydrolysis from the usual 5 hr to 10 to 20 hr.

Since D-alanine (3–8) and undefined mixtures of peptides (4, 9) are known to stimulate growth of lactic acid bacteria but not yeast, the possibility that these substances might be formed during acid hydrolysis of blood was investigated. Evidence is presented here that the growth response of *L. casei* and *S. faecium* but not of *S. carlsbergensis* reflects the increase in D-alanine and peptide content of blood samples with increasing hydrolysis time.

METHODS

Pooled samples of clotted human blood were homogenized with 20 vol of 0.055 *N* HCl (10); aliquots were hydrolyzed at 121°C for zero to 20 hr. The hydrolyzates were cooled, adjusted to about pH 5.5 with KOH, diluted to a convenient volume, and heated 5 min at 121°. The precipitate which formed during heating was sedimented by centrifuging to yield a clear, yellowish brown solution suitable for the analytical work described below. Microbiological assays were carried out according to the procedures described by Storvick (11) with *S. faecium* ϕ 51

(NIRD Culture No. 1229), *L. casei* ATCC 7469, and *S. carlsbergensis* 4228 (ATCC 9080) as test organisms.

Evidence of the presence of D-alanine in hydrolyzates was obtained by three methods: (*1*) the spectrophotometric method of Srinivasan, Corrigan, and Meister (12), (*2*) chromatography of keto acid 2,4-dinitrophenylhydrazones formed after incubating the sample with D-amino acid oxidase and treating it with 2,4-dinitrophenylhydrazine reagent, and (*3*) chromatography of the amino acid formed by catalytic hydrogenation of the keto acid 2,4-dinitrophenylhydrazone (13). To convert any D-alanine present in samples to the keto acid 2,4-dinitrophenylhydrazone, 1 ml aliquots of clarified hydrolyzate were incubated with 8 mg D-amino acid oxidase and 120 μmoles sodium pyrophosphate buffer, pH 8.3, in a final volume of 3 ml for 30 min at 37°C. The reaction was stopped by adding 2 ml 0.3% 2,4-dinitrophenylhydrazine in 2 *N* HCl. After 30 min at room temperature, the phenylhydrazones were extracted with 5 ml diethyl ether. A 3 ml aliquot of the ether extract was evaporated to 0.5 ml under nitrogen; 10 μl aliquots of the concentrated extract were spotted on a thin-layer plate coated with a 0.25 mm layer of silica gel H. The chromatogram was developed for 30 min in benzene/glacial acetic acid (80/20, v/v). Standards for chromatography were prepared by converting 5 mg D-alanine or sodium pyruvate to pyruvic acid 2,4-dinitrophenylhydrazone by the procedures described above. A suitable aliquot of standard for chromatography was 2 to 5 μl of the unconcentrated (5 ml) ether extract containing about 5 to 15 μg pyruvic acid 2,4-dinitrophenylhydrazone.

To isolate 2,4-dinitrophenylhydrazones from blood hydrolyzates for catalytic hydrogenation, the chromatography system described above was used on a preparative scale. Concentrated ether extracts of 2,4-dinitrophenylhydrazones were streaked on a thin-layer plate coated with a 0.25 mm layer of silica gel H; the chromatogram was developed as described above. Silica gel containing material corresponding in R_f value to pyruvic acid 2,4-dinitrophenylhydrazone standard was scraped with a razor blade from the thin-layer plate into a centrifuge tube. The 2,4-dinitrophenylhydrazone was eluted from silica gel by adding 5 ml benzene/acetic acid (80/20, v/v), mixing briefly, and centrifuging to sediment the silica gel. The benzene/acetic acid extract was decanted into a 12 ml centrifuge tube, immediately evaporated to dryness at room temperature under nitrogen, and hydrogenated in 1 ml water with 2 mg platinum oxide catalyst for 16 hr at 40 lb pressure (13). The products of hydrogenation were chromatographed on thin-layer plates coated with 0.25 mm layers of silica gel G in the solvent systems described in Table 1. Amino acids were detected by spraying the chromatograms with

TABLE 1
Evidence of Alanine in Hydrogenated 2,4-Dinitrophenylhydrazones Isolated from Hydrolyzed Blood

Solvent system	R_f values		
	Serine	Alanine	Unknown from blood hydrolyzates
1. Chloroform/methanol/14% ammonium hydroxide (20/20/10, v/v)	0.54	0.62	0.62
2. *N*-Propanol/28% ammonium hydroxide (67/33, v/v)	0.33	0.38	0.39
3. 96% aqueous ethanol/28% ammonium hydroxide (77/23, v/v)	0.38	0.48	0.48
4. *n*-Butanol/acetic acid/water (60/20/20, v/v)	0.15	0.21	0.20

Whole blood hydrolyzed 20 hr was incubated with D-amino acid oxidase to convert any D-alanine present to pyruvic acid. The keto acid was isolated as the 2,4-dinitrophenylhydrazone and hydrogenated to regenerate the amino acid. Pyruvic acid 2,4-dinitrophenylhydrazone standard yields alanine after catalytic hydrogenation. This table shows that the material isolated from blood hydrolyzates contains ninhydrin-positive spots corresponding in R_f value to that of alanine standard in four solvent systems. R_f values are for thin-layer chromatography on plates coated with 0.25 mm layers of silica gel G. The detecting reagent was ninhydrin (15).

ninhydrin reagent and heating the chromatogram 30 min at 70°C to develop color. The ninhydrin reagent contained 200 mg ninhydrin, 95 ml methanol, and 5 ml 2,4,6-trimethylpyridine (15).

Quantitative determinations of D-alanine were carried out with minor modifications of the procedure of Hoeprich (16). Aliquots of blood hydrolyzate or 0.1 to 0.5 μmole D-alanine standard were incubated with 8 mg D-amino acid oxidase and 60 μmoles sodium pyrophosphate buffer, pH 8.3, in a final volume of 1.5 ml at 37°C. The reaction was stopped at zero time or after 30 min by adding 1.5 ml 15% (w/v) trichloroacetic acid. (Zero time blanks for each sample were used to correct for the possible presence of keto acids in hydrolyzates prior to treatment with D-amino acid oxidase). After sedimentation of proteins by centrifuging, a 2 ml aliquot of the supernatant was neutralized with sodium hydroxide and diluted to 3 ml with water; 1 ml aliquots of the neutralized extract were assayed spectrophotometrically for keto acids by the 2,4-dinitrophenylhydrazone method described by Hoeprich (16).

The hydrolysis of blood proteins was followed by the quantitative ninhydrin procedure of Rosen (17). The protein content of whole blood was determined by a modified biuret procedure (18).

Catalase and partially purified D-amino acid oxidase were purchased from Worthington Biochemical Corp., Freehold, N. J., crystalline lactic

dehydrogenase and chromatographically homogeneous D-alanine from Calbiochem, Los Angeles, Calif., and silica gels from Brinkmann Instruments, Inc., Westbury, N. Y.

RESULTS

Figure 1 shows the striking discrepancies in the vitamin B_6 content of the same sample of pooled human blood as reflected by the growth of the three test organisms. The total vitamin B_6 content of the sample as measured by growth of *S. carlsbergensis* appears to be lower than that of the pyridoxal or pyridoxal plus pyridoxamine content as measured by growth of the lactic acid bacteria.

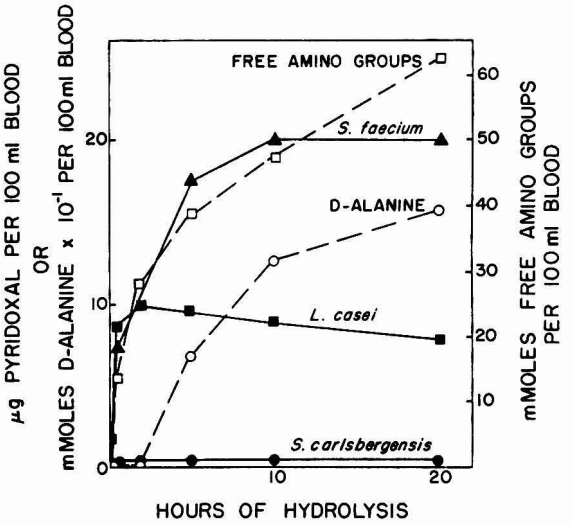

FIG. 1. Effect of hydrolysis time on the apparent vitamin B_6 content of whole blood. Prolonged hydrolysis in 0.055 *N* HCl at 121°C increases the apparent vitamin B_6 content of human whole blood for *S. faecium* ϕ 51 and *L. casei* 7469 but not for *S. carlsbergensis* 4228. Since *L. casei* responds only to pyridoxal and *S. faecium* only to pyridoxal and pyridoxamine, these microorganisms might be expected to show less growth than *S. carlsbergensis*, which utilizes all three forms of the vitamin. Growth of the lactic acid bacteria, *S. faecium* and *L. casei*, but not of the yeast reflects the increase in D-alanine and free amino content of blood samples with hydrolysis time.

To find an explanation for these curious results, blood samples were tested for D-amino acids by incubating them in an assay system containing D-amino acid oxidase, lactic dehydrogenase, and NADH. Meister (12) has demonstrated that only D-alanine, D-serine, and D-α-aminobutyric acid have activity in this system as indicated by the disappearance of NADH absorption at 340 mμ. NADH consumption was observed

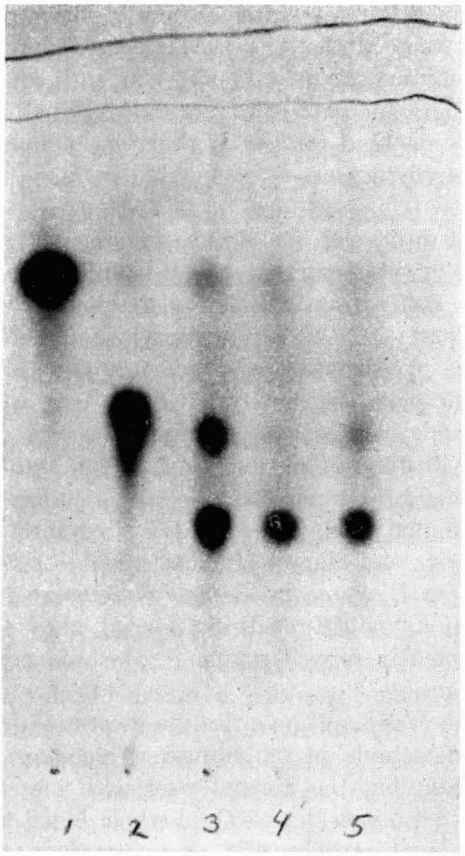

FIG. 2. Results of quantitative thin-layer chromatography on silica gel H in benzene/acetic acid (80/20, v/v) of 2,4-dinitrophenylhydrazones isolated from hydrolyzates of human whole blood. Spots corresponding in R_f value to the 2,4-dinitrophenylhydrazone of pyruvic acid standard can be detected in samples hydrolyzed 20 hr but not in aliquots of the same sample hydrolyzed zero hours. *Identification:* (1) water blank, (2) 2,4-dinitrophenylhydrazone prepared from sodium pyruvate standard, (3) 2,4-dinitrophenylhydrazone prepared by treating D-alanine with D-amino acid oxidase and isolating the keto acid as the 2,4-dinitrophenylhydrazone, (4) 2,4-dinitrophenylhydrazone isolated from blood hydrolyzed zero hours, (5) 2,4-dinitrophenylhydrazone from blood hydrolyzed 20 hr. For details of procedure, see text.

in blood samples hydrolyzed 20 hr but not in blanks containing samples hydrolyzed zero hours.

To determine whether the compound active in the spectrophotometric assay was D-alanine, it was oxidized to the corresponding keto acid with D-amino acid oxidase, converted to the 2,4-dinitrophenylhydrazone, and

chromatographed quantitatively with the results shown in Figure 2. (For procedural details, see "Methods.") D-Alanine standard yields pyruvic acid 2,4-dinitrophenylhydrazone and two unidentified spots. Figure 2 shows the chromatographic pattern of material isolated from 20 hr blood hydrolyzates to be identical to that of D-alanine standard. No pyruvic acid 2,4-dinitrophenylhydrazone is detectable in blood hydrolyzed zero hours.

The solvent used to develop the chromatogram in Figure 2 (benzene/acetic acid, 80/20, v/v) separated the 2,4-dinitrophenylhydrazone of pyruvic acid (R_f 0.48) from that of α-ketobutyric acid (R_f 0.57) but not from that of β-hydroxypyruvic acid (R_f 0.49). To confirm that the phenylhydrazone from 20 hr blood hydrozates was that of pyruvic acid, the material with R_f 0.48 was isolated from thin-layer plates and hydrogenated to regenerate the amino acid. The 2,4-dinitrophenylhydrazone of pyruvic acid yields alanine, and that of β-hydroxypyruvic acid, approximately equal quantities of alanine and serine (13). The amino acid from blood hydrolyzates was chromatographed against serine and alanine standards. Table 1 shows that a spot which corresponds in R_f value to alanine is detectable in four solvent systems. A trace of ninhydrin-reacting material whose R_f corresponded to serine was detected in solvent system 1. It could not be detected in other solvent systems.

Figure 1 shows the results of quantitative determinations of D-alanine. D-Alanine is not detectable in unhydrolyzed blood or in samples hydrolyzed 2 hr or less, but the amount rises with increasing hydrolysis time to a value of 1.6 mmoles per 100 ml whole blood at 20 hr. This is about 40 times the concentration of L-alanine reported to occur free in serum (19) and suggests the major source of D-alanine is racemized L-alanine from hydrolyzed blood proteins.

The hydrolysis of blood proteins was followed by measuring the increase in free amino groups. Figure 1 shows there was a steady increase with hydrolysis time from 1.0 mmole per 100 ml blood in samples hydrolyzed zero hours to 62.4 mmoles per 100 ml blood in samples hydrolyzed 20 hr.

DISCUSSION

The growth-stimulating effects of peptides (4, 9, 20–28) and D-alanine (3–8) for lactic acid bacteria have been studied extensively. Peptides are, in themselves, potent growth stimulants because they bypass the competition for absorption and utilization which depresses growth in a medium imperfectly balanced in amino acids (9). When D-alanine is added to a medium that contains each of the L-amino acids and is

enriched with a crude source of peptides such as enzymic casein hydrolyzate, certain lactic acid bacteria including *L. casei* (9), the test organism for pyridoxal, can grow without vitamin B_6. A key role of pyridoxal phosphate in the metabolism of lactic acid bateria under such conditions is as a cofactor in the enzymic racemization of L-alanine. Consequently, a preformed supply of D-alanine and other metabolites whose biosynthesis requires pyridoxal phosphate permits growth in absence of the vitamin. The pyridoxal-sparing effect of D-alanine for *L. casei* is not evident when the microorganism is grown in a medium containing only free amino acids as the nitrogen source; no growth stimulation occurs in the presence of 100 μg D-alanine/ml medium. When the medium is supplemented with enzymic casein hydrolyzate, 5 μg D-alanine/ml permits half-maximum growth (4). In our assays, 5 hr blood hydrolyzates supplied 5 μg D-alanine/ml medium; 10 hr hydrolyzates, 9.4 μg/ml; and 20 hr hydrolyzates, 11.7 μg/ml. In the presence of partially hydrolyzed blood proteins, these amounts of D-alanine might be expected to give false high values for the pyridoxal content of blood.

D-Alanine has been reported to stimulate growth of *S. faecium,* the test organism for pyridoxal and pyridoxamine, in the presence of the vitamin but not in its absence (7, 8). To eliminate error from this source, D-alanine is added to the basal assay medium. Gregory (7) and Møller (8) have concluded that *S. faecium* cannot substitute D-alanine for vitamin B_6 from experiments with media containing synthetic amino acids or acid-hydrolyzed casein as a nitrogen source. Neither tested the growth response of *S. faecium* to D-alanine on a medium supplemented with peptides. Figure 1 shows that the growth of *S. faecium* on blood hydrolyzates reflects the increase in D-alanine and peptide content with hydrolysis time. It is thus probably clear that peptides enhance the growth-stimulating effects of D-alanine for *S. faecium* as they do for *L. casei.* However, our data do not exclude the possibility that unknown substances contribute to the increased growth of *S. faecium* on samples subjected to prolonged hydrolysis.

Although Snell (5) and others (7, 8) have pointed out that D-alanine is a potential source of error in the microbiological assay of vitamin B_6, it is not surprising that there has been no previous report of D-alanine interference. D-Alanine is rare in nature. Some is formed from L-alanine in microbiological media heated 20 min at 121°C, but the extent of racemization is less than 1% (29). Only samples with an usually high ratio of protein to vitamin B_6 might be expected to yield after hydrolysis the relatively large amounts of D-alanine needed to replace the vitamin. Snell estimated that about 10,000 to 25,000 μg of D-alanine is needed to replace 1 μg of pyridoxal for *L. casei* in a peptide-enriched medium

(5). Møller (8) predicted that interference from D-alanine might occur in samples with a protein to vitamin B_6 ratio of 10^4 to 1. In the human blood we analyzed, the ratio of protein to vitamin was about 10^6 to 1. It is interesting that Storvick (1) found the exaggerated growth response of *L. casei* does not occur in blood samples deproteinized before hydrolysis with trichloroacetic acid.

Toepfer and Lehman (30) have described a method for separating the three forms of vitamin B_6 chromatographically and using a single microorganism, *S. carlsbergensis,* to measure each form. This procedure should be explored as a way of measuring the three forms of vitamin B_6 in blood without interference from D-alanine and peptides.

SUMMARY

Prolonged acid hydrolysis of whole blood prior to microbiological assay increases its apparent vitamin B_6 content for the lactic acid bacteria, *L. casei* and *S. faecium,* but not for the yeast, *S. carlsbergensis.* Since yeast growth reflects all three forms of vitamin B_6 while the lactic acid bacteria respond either to pyridoxal or to pyridoxamine and pyridoxal, the differential assay for vitamin B_6 gives incongruous results when applied to blood. We find the growth of *S. faecium* and *L. casei* but not that of *S. carlsbergensis* reflects the increase in D-alanine and peptide content of the samples with increased hydrolysis time. These substances, known to stimulate growth of the lactic acid bacteria but not yeast, suggest a possible explanation for the curious finding that the total vitamin B_6 content of whole blood estimated microbiologically appears to be many times lower than the sum of its parts.

ACKNOWLEDGMENTS

Dr. Esmond E. Snell suggested that D-alanine and peptides might interfere with the microbiological assay of vitamin B_6 in blood in unpublished discussions at the International Symposium on Vitamin B_6, New York, N. Y., 1964. We are indebted to him for helpful discussions during the progress of this work, to Dr. William E. Thiessen and Mr. William D. Kray for assistance with catalytic hydrogenation, and to Miss Sandra Beiderbecke, who carried out preliminary experiments.

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Isotope Effects in the Decarboxylation of 1-^{14}C-Dicarboxylic Acids Studied by Means of the Schmidt Reaction

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The presence of measurable kinetic isotope effects in some biochemical reactions (1, 2) has been of great help in the elucidation of reaction mechanisms. Knowledge of the chemical reactions involved permits pinpointing of the rate-determining step. When "carbon by carbon" degradation of complex molecules is conducted to determine the location and extent of a radioactive label, a knowledge of potential isotope effects may forestall misinterpretation of data.

For instance, in studies of fatty acid elongation using u.l. ^{14}C-acetate, the ratio of the specific activity of the ^{14}C of the carboxyl group to that of the alkyl group is indicative of the extent of elongation (3). The Schmidt reaction (4, 5) is the technique of choice for the determination of the carboxyl carbon of fatty acids. The technique of Van Slyke (6) is used for the determination of all the carbons of organic compounds, i.e., alkylamines (the remainder of the carbon chain of a fatty acid after the Schmidt reaction). A ratio of 1 obtained from the specific activity of the CO_2 from the carboxyl carbon vs. the specific activity of the CO_2 from the alkyl carbons would indicate total *de novo* synthesis without elongation of pre-existing medium-chain fatty acids (the specific activity here defined as disintegrations-per-minute/millimole CO_2, or dpm/mmole). Thus, a value larger than 1 would indicate chain elongation. Knowledge that the quantitative yield (80/90%) for the Schmidt reaction (4, 5) for various fatty acids (7, 8) and known values for ^{14}C/^{12}C isotope effects (ca. 15%) from other reactions suggest a value greater than 0.7–0.75 as the more realistic indication of change from *de novo* biosynthesis to elongation.

The purpose of this investigation was to determine if isotope effects

exist in the decarboxylation of dicarboxylic acids and to evaluate the influence of chain length on the ^{14}C-isotope effect between two carboxylic functional groups, one of which was ^{14}C-labeled. For this purpose the following series of 1-^{14}C-dicarboxylic acids was investigated: malonic, succinic, glutaric, adipic, pimelic, suberic, azaleic, and sebacic. The ratio of % yield of $^{14}CO_2$ to $^{12}CO_2$ trapped radioassayed and measured manometrically was employed as a method of assay if there was a preferential decarboxylation of $^{12}COOH$ over $^{14}COOH$ and the extent of this effect. The isotope effect data would be expected to indicate a preferential breaking of the ^{12}C—^{12}C bond in comparison to the ^{12}C—^{14}C bond. The results would also be expected to shed light on the rate-determining step of the Schmidt reaction.

MATERIALS AND METHODS

The glass apparatus and techniques employed have been described (8, 9). The radioactive dicarboxylic acids were purchased from: New England Nuclear Corporation—malonic 1-3 ^{14}C acid, succinic 1-4 ^{14}C acid, adipic 1-5 ^{14}C acid; Nuclear Science and Engineering Corporation—glutaric 1-5 ^{14}C acid, pimelic 1-7 ^{14}C acid, suberic 1-8 ^{14}C acid, sebacic 1-10 ^{14}C acid; Nuclear Research Chemicals—azelaic 1-9 ^{14}C acid. Each of these acids was recrystallized and considered pure only when by paper chromatography (ethanol/ammonia/water, (80/4/16, v/v/v) one radioactive spot was obtained (which was the same as the one marked by the indicator dye). Each of the acids had been prepared by the reaction of $^{14}CN^-$ and ^{12}C α-ω-haloalkyl compounds or ω-halo acids followed by hydrolysis. Thus, the only ^{14}C-labeled portion of the molecule would be the carboxyl group. The sodium azide and fuming sulfuric acid used were from the Mallinckrodt Co. Because bacterial growth has been observed in stock solutions of some of these acids, sterile techniques were utilized in all preparations and manipulations. The high specific activity ^{14}C-labeled acids or their sodium salts (1 mCi/mmole) were diluted with ^{12}C acid or the sodium salt in approximately a 1:40 ratio, which was calculated to yield a specific activity suitable for reliable radioactive assay (2–6000 dpm/mmole CO_2). The solutions of dicarboxylic acids were kept frozen when not in use. For each experimental run, the stock solution was thawed and an aliquot (containing 0.5 to 1.3 mmole) transferred to the reaction flask by sterile technique, lyophilized, and used immediately. During the experimental runs, great care was taken to exclude any CO_2 from the atmosphere. CO_2-free nitrogen was used throughout the work.

In previous publications (10–12) CO_2 was trapped as $BaCO_3$ in a $Ba(OH)_2$ solution layered with toluene and gravimetrically assayed.

TABLE 1
Observed Isotope Effects $k^{14}C/k^{12}C$ in the Decarboxylation of 1-^{14}C-Decarboxylic Acids

	$HOO^{12}C-(CH_2)_n-{}^{14}COOH$, where n =							
Run No.	(1) Malonic	(2) Succinic	(3) Glutaric	(4) Adipic	(5) Pimelic	(6) Suberic	(7) Azaleic	(8) Sebacic
1	0.90	0.91	0.86	0.91	0.87	0.93	0.88	0.93
2	0.83	0.93	0.87	0.88	0.85	0.93	0.89	0.99
3	0.87	0.94	0.84	0.87	0.88	0.95	0.89	1.00
4	0.85	0.90	0.84	0.85	0.86	0.95	0.89	0.97
5	0.82	0.89	—	—	—	0.89	0.92	0.98
sum	4.27	4.57	3.41	3.51	3.46	4.65	4.47	4.87
$\bar{x}$	0.854	0.914	0.8525	0.8775	0.865	0.930	0.894	0.974
S.D.	0.0321	0.0207	0.0151	0.0250	0.0129	0.0245	0.0152	0.027
S.E.	0.01435	0.00925	0.00755	0.01250	0.00645	0.01095	0.00679	0.01207

Within group SD = 0.0228

Analysis of variance:

(a) Among all acids $P < 0.01$

(b) Short-chain vs. long-chain acids $P < 0.01$

(c) Odd-numbered vs. even-numbered acids $P < 0.01$

TABLE 2
Decarboxylation of 0.778 Mmole 1-^{14}C-Azaleic Acid[a]

Time, min	Total mmole CO_2 collected	Total dpm collected	Calculated specific activity	% decarboxylation		Ratio of % yields $^{14}C/^{12}C$
				Calc. from $^{12}CO_2$ data	Calc. from $^{14}CO_2$ data	
1	0.0117	52	4444	1.50	1.32	0.88
4	0.0237	105	4430	3.08	2.66	0.86
7	0.0554	246	4440	7.12	6.23	0.87
11	0.0694	311	4481	8.92	7.87	0.88
15	0.0818	366	4474	10.51	9.27	0.88
18	0.1169	522	4465	15.03	13.22	0.88
21	0.1479	675	4567	19.14	17.09	0.89
25	0.1792	850	4743	23.03	21.52	0.93
30	0.2088	1090	5233	26.77	27.60	1.03
35	0.2716	1462	5383	34.91	37.01	1.06
40	0.3038	1651	5434	39.05	41.80	1.07
45	0.3399	1829	5381	43.69	46.31	1.06

[a] Five different samples of 1-^{14}C-azaleic acid were previously used for the determination of the specific activity of the carboxyl carbon. The average SA value of 0.778 mmole obtained was 5076 dpm/mmole CO_2 (carboxyl). Thus, for the sample used in this Schmidt reaction the total radioactivity count for the carboxyl carbon was 3949.4 dpm and the total CO_2 was 0.778 mmole (all calculations based on one carboxyl group).

For this study CO_2 was trapped in a 5% ethanolamine solution (13). The CO_2 content was determined on an aliquot of the ethanolamine-carbonate solution by use of the Natelson microgasometric (14) modification of the Van Slyke technique (15). The radioactivity was assayed in another aliquot of the ethanolamine-carbonate solution by liquid scintillation technique to ±0.1% S.D. (16).

In a separate experiment, repeated five times, the values for (*a*) total mmoles CO_2 and (*b*) total dpm $^{14}CO_2$ from each acid were determined by complete combustion (15). The values of both mmoles of $^{12}CO_2$ and dpm of $^{14}CO_2$ obtained for each time interval were expressed as % yield from the substrate added. The CO_2 from the reaction flask was continuously collected using a new container after each short-time interval. The results for each individual container were added. A $^{14}C/^{12}C$ ratio of yields, for a time interval, was obtained by dividing % yield from the $^{14}CO_2$ data by % yield from the $^{12}CO_2$ data. This ratio of $^{14}C/^{12}C$ yields can easily be related to the isotope effect $k^{14}C/k^{12}C$ for the early stages of the reaction (18, 19). After 15–20% of reaction, because the specific activity of the substrate is sufficiently different from the original, more difficult calculations are necessary to determine the isotope effect.

Five independent experimental Schmidt reactions were carried out for the evaluation of $^{14}C/^{12}C$ isotope effects for each of the 1-^{14}C-dicarboxylic acids used. The results were averaged and the standard deviation determined. The equation of Melander (19) was used to calculate the isotope effect. Data employed for this calculation were those obtained during the first 20% yield of the reaction.

RESULTS

The results of the individual experiments and the statistical evaluation of the data are presented in Table 1. In Table 2 are the data and calculations for a typical experiment.

DISCUSSION

The results indicate a definite kinetic preferential decarboxylation of ^{12}COOH over ^{14}COOH. Primary kinetic isotope effects for $^{14}C/^{12}C$ have been calculated (18) and reported (10–12) in the 10–18% range. The results (3–15%) fall within these values and thus suggest a "primary" kinetic isotope effect. The mechanistic steps for the Schmidt reaction (4, 5) are:

$$R—CH_2—COOH \rightarrow R—CH_2—CONHOH \rightarrow R—CH_2—NCO \rightarrow R—CH_2—NH_2 + CO_2$$

The rate-determining step, compatible with the observed data, is the breaking of the C—C bond between the methylene carbon and the

adjacent CO group during the Lossen rearrangement ($R—CH_2—CONHOH \rightarrow R—CH_2—NCO$). Absence of a ^{14}C-kinetic isotope effect would have suggested a step other than the Lossen rearrangement as rate determining.

Statistical evaluation of the data, obtained by grouping the short-chain acids (first 4) and the longer chain acids (last 4), suggests that increased chain length only slightly decreases the size of the isotope effect. This difference is too small to suggest steric hindrance due to chain lengthening. Differences are obtained by grouping of isotope effect data of odd and even chain acids. These are small, although statistically significant.

SUMMARY

The isotope effect of 1-^{14}C-dicarboxylic acids during decarboxylation by the use of the Schmidt reaction was determined for malonic, succinic, glutaric, adipic, pimelic, suberic, azelaic, and sebacic acids. Results indicated a kinetic predilection for the loss of the ^{12}C-carboxyl group over the ^{14}C one. The observed isotope effects were in the range of 3–15%. The rate-determining step in the Schmidt reaction appears to be a break of the C—C bond between the CO and adjacent methylene carbon during the Lossen rearrangement. Results suggest small but statistically significant isotope effect differences due to increased chain length and in grouping of odd versus even-chain dicarboxylic acids.

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ANALYTICAL BIOCHEMISTRY 19, 584–586 (1967)

An Improved Sample Applicator for Column Chromatography

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A method and device have been devised which substantially reduce the time normally spent in applying a sample to be separated by descending column chromatography and, especially, gel filtration. In addition, the method greatly reduces the possibility for disruption of the bed and provides a means for application of the sample in a minimum volume to improve the final resolution of the separation. The apparatus used is very economical and simple to construct and use.

DESCRIPTION

The apparatus (Fig. 1) is constructed of: (*1*) capillary glass or plastic tubing of the desired length inserted into a rubber stopper so that the rod is firmly held by friction; (*2*) two short lengths of 22-gage stainless-steel hypodermic tubing (Small Parts Inc., Miami, Fla.) with one bent at right angles into an L shape by heating with a high intensity flame; (*3*) sufficient small-bore polyethylene tubing (medical grade polyethylene tubing, PX022, Becton-Dickenson and Company, Rutherford, N. J.); and (*4*) a conventional syringe of convenient size for sample application or, preferably, a motor-driven syringe for better uniformity of application.

PROCEDURE

To the sample is added a low molecular weight substance that will not interfere with subsequent analysis of the sample. Sucrose, 4–6% w/v, for example, has been commonly used for routine separation of serum proteins and protein subunits (1). The column is packed and equilibrated in the usual manner. When equilibration of the column is completed, the buffer reservoir is disconnected. The tip of the applicator is carefully inserted beneath the buffer to within 2 to 4 mm of the column bed and stabilized by the stopper at the top of the column. The sample is gently expelled from the syringe which forms a thin band because of the high density of the sample. When the addition of the sample is completed, the

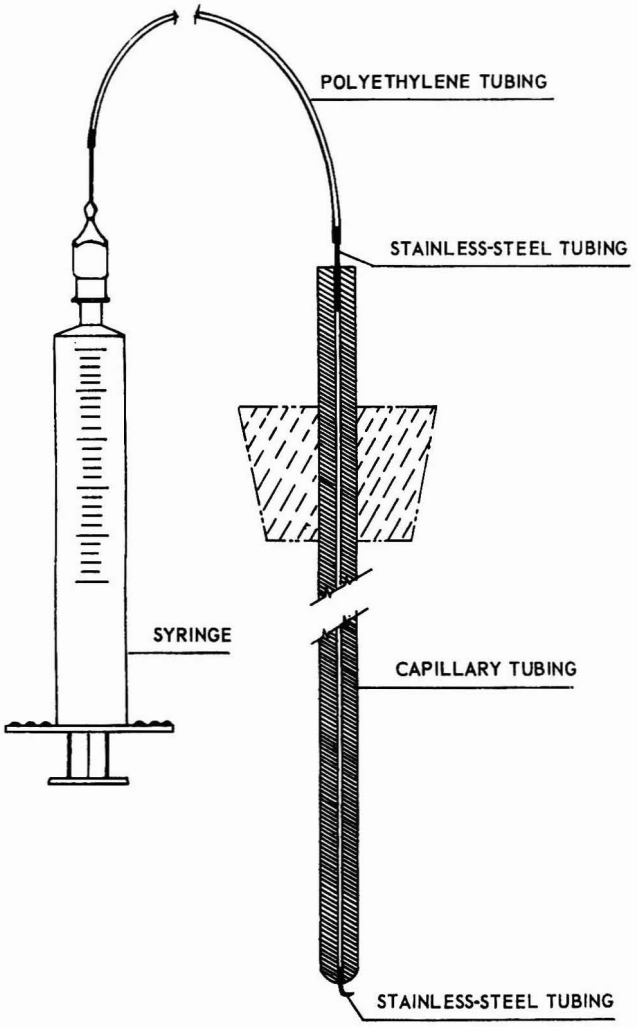

FIG. 1. Chromatography sample applicator.

applicator is gently removed. The buffer reservoir is reconnected and collection of effluent is initiated, all without the necessity of adjusting the level of the buffer over the column bed. The uniformity of entrance of sample into the bed may be observed clearly by the sharp refraction at the buffer/sample interface or even more readily if a colored component, e.g., hemoglobin, or a high polymer dextran dye (Blue Dextran 2000, Pharmacia Fine Chemicals, Piscataway, N. J.) is incorporated into the sample.

DISCUSSION

Pipets or simple syringes used previously (2) for sample addition may easily allow disruption of the bed during application. The apparatus and method described have been used successfully in our laboratory for some time and have proved to be practical and convenient. This technique has been particularly useful for use with column beds of low density, especially Sephadex, and limits the possibility of mechanically disrupting the bed during sample application.

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SHORT COMMUNICATIONS

Stabilization of Serum Cholinesterase in Dried Starch Gel

Starch gel immobilized cholinesterase supported on a pad of open cell urethan foam has proved useful in the polarographic detection of cholinesterase inhibitors in air and water (1). The stability of this immobilized enzyme under various storage and use conditions, including those in which no refrigeration is available, is a matter of concern in perfecting the analytical procedures and establishing the reliability of the method under various climatic conditions.

Previous enzyme stability studies were primarily concerned with maintenance of enzyme activity during isolation procedures or in reactions with various substrates. In these studies it was shown that most of the activity of aqueous solutions of cholinesterase and similar enzymes was retained if the temperature was kept low, if the pH was kept near the isoelectric point of the enzyme, and if additives such as the following were employed: serum albumin, gum arabic, gum acacia, insulin, glycogen, boiled starch, castor oil, amino acids, and gum mastic (2–8). It is generally recognized, however, that enzymes are more stable in the dry state than when in solution even though there are little or no published data regarding the effect of elevated temperatures on the stability of dry cholinesterase with or without additives.

The original starch gel immobilized enzyme pads for use in the polarographic detection apparatus contained glycerin to promote rapid rewetting of the dried enzyme pads (1). Although these pads kept well at refrigerator temperatures, their use after storage at room temperature and above gave erratic analytical results, indicating the destruction of the enzyme. It was suspected that the glycerin was either reacting with the enzyme or was attracting water that was interfering with the stability of the enzyme. The present study was undertaken to determine whether glycerin or water was responsible for the destruction of the enzyme at temperatures of 37–60°C and to find substances which would provide the

necessary rewetting properties to the pads without enzyme destruction. It will be noted that in the accelerated aging tests conducted at 60°C a desiccant was employed to minimize the effect of moisture.

Methods. The uniform distribution of the starch gel-enzyme mixture in the urethan foam sheet is essential to preparing enzyme pads with a uniform enzyme content. The following procedure was found to be satisfactory. Sheets of ¼ in. open-cell urethan foam were cut with a pattern so that they were exactly 4 × 6 ins. The warm (47°C) 8% starch mixtures containing blue dye (FDC Blue #1), enzyme, and/or additives were taken up into a 10 ml syringe (no needle) and applied to the center of the urethan foam sheet all at once. The sheet was placed on a warm glass plate larger than the urethan sheet; a plastic household rolling pin was used to distribute the starch slurry as quickly as possible, in such a way that (*a*) the blue color was evenly distributed and (*b*) only a minimum amount of the starch remained on the rolling pin or on the glass plate. The sheets were immediately suspended in a refrigerator to gel, and were later dried in a vacuum desiccator under reduced pressure overnight (finally at 0.05 mm of Hg).

The horse-serum cholinesterase used in these studies was prepared by Armour & Co. It had a specific activity of about 3 units/mg based on the hydrolysis of acetylcholine.

The candidate glycerin substitutes were evaluated on the basis of their ability to promote the wetting of dried starch, and also their ability to keep the dried starch film on urethan foam when it is flexed. The materials considered were: carboxymethylcellulose (Hercules Powder Co.), Carbowax-400 (Carbide Chemical Co.), Kraystay K (Kraft Foods Co.), sodium chloride, sorbitol, sucrose, Triton X-100 (Rohm and Haas Co.), and dimethyl sulfoxide. Dimethyl sulfoxide was eliminated from further consideration because it softened the urethan foam support. Sorbitol, Triton X-100, and Kraystay K were found to be the most promising, and were chosen for the storage-stability tests with enzyme.

The quantity of each of the additives used is shown in Table 1, column 2. The round enzyme pads for use in the whole experiment were cut on day zero with a ⅜ in. cork borer, and immediately placed in screw-cap bottles containing a layer of magnesium perchlorate as desiccant. The bottles were then placed in the refrigerator or the 60°C oven for the storage-stability test. At the appropriate time intervals, pads were withdrawn from the bottles and assayed for their enzyme activity.

These pads were assayed for their cholinesterase activity by a colorimetric procedure based on the method of Ellman, Courtney, Andres, and Featherstone (2) in which the color developed and measured is due to the reaction of thiocholine with 5,5′-dithiobis(2-nitrobenzoate) to give

TABLE 1
Storage-Stability Tests of Experimental Enzyme Pads

Additive	Quantity[b]	Storage temp.,	Enzyme activity per pad[a]				
			Day 0[c]	Day 7[d]	Day 14[d]	Day 28[d]	Day 56
None	—	4	0.429	0.390	0.563	0.443	0.473
		60	0.429	0.248	0.228	0.075	0.001
Glycerin	5	4	0.428	0.321	0.338	0.242	0.214
		60	0.428	<0.001	0	0	0
Kraystay K[e]	1	4	0.735	0.948	0.978	0.798*	0.767
		60	0.735	0.357	0.509	0.256*	0.074
Triton X-100[f]	1	4	0.794	0.731	0.830	0.547	0.668
		60	0.794	0.315	0.267	0.111	0
Sorbitol	5	4	0.516	0.462	0.544	0.527	0.375
		60	0.516	0.045	0.038	0.051	<0.001

[a] Activity is expressed as micromoles of butyrylthiocholine iodide hydrolyzed per minute per pad. Pads were cut from ¼″ urethan foam with a ⅜″ cork borer.

[b] The amount of additive in grams per 100 grams of 8% starch slurry in tris buffer. The starch slurry contained 800 mg horse serum cholinesterase per 100 ml.

[c] Each figure represents the average of five pads.

[d] Each figure represents the average of three pads except at * which indicates the average of six pads.

[e] Kraystay K—neutral mixed calcium, sodium, and potassium salts of a sulfonated polysaccharide (carrageenan) produced by Kraft Foods.

[f] Triton X-100—water-soluble nonionic alkyl aryl polyether alcohol produced by Rohm and Haas.

the yellow anion of 5-mercapto-2-nitrobenzoic acid. In our procedure the butyryl ester of thiocholine was substituted for the acetylthiocholine substrate and therefore units of enzyme activity are defined in terms of micromoles of butyrylthiocholine hydrolyzed per minute.

In our enzyme assay procedure, one of the ⅜ in. circular enzyme pads is gently macerated in 5 ml of pH 7.4 tris buffer (0.1 *M*) containing about 10 units of α-amylase (from hog pancreas, Worthington Biochemicals) at room temperature for 30–60 min. (In a separate experiment it was shown that the α-amylase did not hydrolyze the butyrylthiocholine iodide substrate.) At the end of this incubation period an aliquot (usually 0.5 ml) of the supernatant was added to a cuvet containing 2.5 ml of a substrate indicator mixture (SIM):

Dithiobis(nitrobenzoate) solution (396 mg/100 ml), 2.0 ml
Butyrylthiocholine iodide (21.6 mg/ml, freshly prepared), 0.4 ml
Tris(hydroxymethyl)aminomethane buffer, 0.1 *M*, pH 7.4, 47.6 ml

The rate of change of absorbancy at 412 mμ was determined with a Beckman DK-1 recording spectrophotometer. The activity is expressed as

micromoles of butyrylthiocholine iodide hydrolyzed by the enzyme in one pad in one minute:

$$\text{activity} = \frac{\Delta A \times 10^3 \times V}{13{,}600 \times F}$$

where ΔA is change in absorbancy units per minute, V is volume of liquid in the cuvet in milliliters, 13,600 is the extinction coefficient, and F is the fraction of one enzyme pad represented by the size of the aliquot added to the cuvette.

Results. The storage stability test of a series of experimental enzyme pads are presented in Table 1. The design of this experiment was based on some preliminary stability studies with glycerin-containing enzyme pads stored at 4, 30, 47, and 60°C. The high variability in enzyme activity observed in the preliminary experiment suggested either (*a*) that the pads had not received uniform quantities of enzyme at the start of the experiment or (*b*) that glycerin and/or water reacts with cholinesterase at temperatures of 30° and above. In order to eliminate the possibility that the pads containing glycerin were not completely dry and that water rather than glycerin was responsible for the destruction of enzyme activity, we conducted the storage tests in the presence of a desiccant; magnesium perchlorate was chosen for this purpose. For the storage tests the enzyme pads were separated from the desiccant by a screen wire support. Despite the close proximity of the pads and the desiccant possible contamination of the enzyme pads with the desiccant was not a problem, since experimentation showed that magnesium perchlorate is not an inhibitor of serum cholinesterase.

To ensure the uniform application of the starch-enzyme mixture to the urethan foam sheets, a standardized application procedure was worked out. Analysis of a number of enzyme pads cut from a series of sheets and statistical treatment of the data showed that the deviation of weight of dried starch enzyme mixture on the individual pads was ±5%.

Table 1 shows the stability of various starch enzyme products at 4 and 60°C. An examination of the activities of the starch enzyme products at time zero indicated that there is an immediate variation of enzyme activity, which must be attributed to the presence or absence of the specific additives. The increase in enzymatic activity observed for the starch-enzyme pads containing either a water-soluble gum (Kraystay K) or a wetting agent (Triton X-100) is difficult to explain. The stabilization of cholinesterase by gum arabic, gum acacia, and boiled starch has been explained on the basis of one phenomenon, namely, the physical adsorption of the enzyme to the surface of the stabilizer (8). It is there-

fore surprising that the protective and potentiating effects of Kraystay K and Triton X-100 are greater than the effects of starch alone. Calcium and certain other divalent ions are known to activate cholinesterase (10) after it has been inhibited by removal of the divalent ions by dialysis. The calcium component of Kraystay K was suspected to be a factor in the increased enzyme activity; however, we found that the addition of calcium chloride to the enzyme does not produce a similar increase in activity.

At 60°C the pads containing dry glycerin lose their activity rapidly so that there is no more than a trace of activity at the seventh day. On the other hand, the starch gel alone and the additives other than glycerin gave enzyme pads which retained their activity for prolonged periods at 60°. It is concluded that the glycerin undergoes a chemical reaction with the enzyme under these test conditions.

Summary. Starch gel immobilized serum cholinesterase pads prepared for use in the polarographic analysis of air and water streams for the presence of cholinesterase inhibitors have now been shown to be unstable at temperatures of 30°C and above when glycerin is present. It is postulated that the glycerin itself rather than water is responsible for the inactivation observed since the storage of these pads in the presence of magnesium perchlorate does not prevent the glycerin-induced destruction of the enzyme. Three enzyme additives were selected and compared for their ability to preserve the activity of starch gel immobilized cholinesterase when it is stored at 60° for periods up to 56 days. Both Kraystay K and Triton X-100 are significantly better additives than glycerin when judged solely on the basis of preservation of enzyme activity. Starch gel alone is also a good protector of this enzyme and pads with no additives other than the starch retained 50% of their activity after two weeks at 60°.

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FDNB Staining of Proteins Separated by Disc Electrophoresis

Disc electrophoresis in polyacrylamide-gels has become a very valuable tool for the study of proteins. Methods exist for staining and thus visualizing separated bands. However, separated bands might contain more than one protein or one protein with multiple peptide chains or both. Conventional staining methods provide no evidence on the identity of chain termini. Slicing of gel electrophoresis columns for subsequent *N*-terminal amino acid analysis is usually avoided because of the uncertainty that exists of the exact location of the bands, especially those that are close together. This difficulty can be overcome by staining the disc electrophoresis column directly with FDNB (1),[1] slicing the colored band, hydrolyzing the DNP-protein, and identifying the *N*-terminal DNP-amino acids by chromatographic procedures.

Polyacrylamide-gel columns (7%) were prepared as described by Davis (2). Electrophoresis usually was performed for 30 min in tris-glycine buffer (ionic strength 0.01, pH 8.3) with a current of 5 mA per gel column. Conventional detection of the separated protein components was achieved by staining the columns for 1 hr with amido schwartz dye followed by electrical destaining, as described by Davis (2).

[1] Abbreviations: FDNB, 2,4-dinitrofluorobenzene; DNP, dinitrophenyl.

Aliquots (5 λ) of 5% aqueous solution of crystalline bovine serum albumin purchased from Armour Laboratories were used for these experiments. After electrophoresis the gel columns were placed in the storage tubes, which were filled with 2.5% (v/v) solution of FDNB in ethanol (3). The tubes were stoppered and placed in the dark for 16 hr. Since the interior of the gel column is alkaline the reaction of the protein with FDNB proceeds by diffusion of the reagent into the gel. Shorter incubation times resulted in incomplete staining of the bands. During the incubation time the gels became opaque and their size was reduced. No bands were seen at this time. Then the columns were removed from the tubes and washed several times with ether to remove excess reagent. Subsequently, the gel columns were transferred in an Erlenmeyer flask and were covered with 100 ml of ether containing 5 ml of 2 *N* HCl. The mixture was shaken gently occasionally and was replaced several times until no yellow color was eluted from the gel columns. This procedure took several hours to be completed. At the end of the extraction, the gel columns became clear again, their original size was recovered, and the protein bands appeared dark yellow against a pale yellow background. The number of bands observed by FDNB staining of a column of bovine serum albumin was the same as with amido schwartz staining. Thus, three major fractions (monomer, dimer, and trimer) were obtained, followed by several minor components. However, only the major band (monomer) was analyzed for *N*-terminal amino acids. FDNB staining of the bovine serum albumin bands appears to be as sensitive as amido schwartz staining although the difference in color of the two stains favors the latter.

The major bands from several FDNB-stained bovine serum albumin gel columns were then cut and combined in small test tubes. Usually 20 to 30 gel columns was sufficient for each chromatographic analysis. Hydrolysis of the DNP-protein was performed by the general procedure of heating the sample in a sealed tube with constant-boiling HCl under nitrogen at 105°C for 16 hr (3). To check for artifact formation, portions of the gel column which did not contain protein bands were subjected to hydrolysis and this hydrolyzate was analyzed similarly to the protein sample. After hydrolysis the polyacrylamide-gel was precipitated by cooling. The hydrolyzate was filtered and diluted with water, and the *N*-terminal DNP-amino acids were extracted with ether. Chromatographic analysis of the DNP-amino acids was performed in the "toluene" /1.5 *M* phosphate two-dimensional system (4). Additional identification of the DNP-amino acids was made by hydrolyzing them in concentrated NH_4OH, then identifying the resulting free amino acids by paper chromatography (5). Aspartic acid was found to be the only *N*-terminal amino acid present.

In order to test the application of the method to proteins with multiple chain terminals, human γ-globulin (Fraction II) purchased from Mann Research Laboratories was subjected to similar analysis, as described above. Aliquots (10 λ) of a 5% solution of the γ-globulin in saline were used for these experiments. The same number of bands was found with FDNB and amido schwartz staining. Glutamic and aspartic acids were identified as the *N*-terminal amino acids present in transverse slices of the major γ-globulin band.

The method described above might be useful in further characterizing protein components separated by disc electrophoresis. The advantage of the method is realized in the identification of one or multiple peptide chains located in the same protein band provided the chain termini do not end in identical amino acids. In the latter case, quantitation will be necessary to establish the multiplicity of the chains. The quantitative aspects of the above procedure are currently under investigation in our laboratory.

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Authors' Notes

Since publication of the article, "Determination of Free Magnesium," *Analytical Biochemistry,* **14,** 135 (1966), it has come to the attention of the author that a method for such determination, also dependent on the magnesium-eriochrome complex, was previously described by Dr. Mackenzie Walser, *Analytical Chemistry,* **32,** 711 (1960). I should like to direct the readers' attention to this prior publication.

R. D. BERLIN

Vol. **18,** No. 2 (1967), in the article, "An Improved Vertical Polyacrylamide Gel Electrophoresis Apparatus. Application to Typing and Subtyping of Haptoglobins," by Robert C. Woodworth and Leslie G. Clark, pp. 295–304:

Page 296: in Footnote 4 an offer was made to supply detailed scale drawings of the apparatus. Owing to legal technicalities, the offer must be withdrawn.

R. C. WOODWORTH
L. G. CLARK

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