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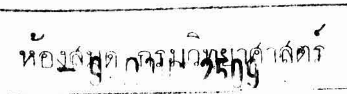

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An Ion-Exchange Column Chromatographic Method for the Separation and Quantitative Analysis of Neutral Monosaccharides[1]

EARL F. WALBORG, JR.,[2] LENA CHRISTENSSON,

AND SVEN GARDELL

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Received February 16, 1965

Methods for the column chromatographic analysis of neutral monosaccharides are based on three principles of separation: adsorption, partition between a water and organic phase, and ion-exchange. The chromatographic separations utilizing carbon columns and other adsorbents have been reviewed by Binkley (1). Partition column chromatography on a cellulose support has been reported by Hough, Jones, and Wadman (2), and a method utilizing starch columns has been described by Gardell (3). Samuelson (4–7) has reported several partition chromatographic methods employing ion-exchange resins.

Most methods for ion-exchange chromatography of the neutral monosaccharides are based on the fact that certain polyhydroxy compounds react with the borate ion to give anionic complexes (8). Khym and Zill (9, 10) were the first to develop an ion-exchange column chromatographic method for the separation of the neutral monosaccharides. Nakamura and Mori (11) have reported the ion-exchange chromatographic separation of sugars in 50% ethanol. Hallén (12) has further developed this principle of separation.

Attempts to improve the system of Hallén (12) have resulted in the development of a column chromatographic system which allows the separation of the borate complexes of neutral sugars to be performed at neutral pH and elevated temperature.

[1] This research was supported by a grant from the Swedish Medical Research Council (No. F-139-13-A).

[2] Supported by a U. S. Public Health Service Postdoctoral Fellowship (No. 5-F2-GM-12,732-02) from the Division of Research Grants. Present address: Department of Biochemistry, The University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, Texas, 77025.

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MATERIALS

Columns. Jacketed glass columns, having an internal diameter of 6 mm and a length of 155 cm, were used. The external diameter of the water jacket was 25 mm. The water jacket extended to within 5 mm of the end of the column, so that the temperature of the entire column of resin could be controlled. A piece of small plastic tubing (internal diameter of approximately 1 mm) was inserted through the center of a small rubber stopper, and the stopper then inserted into the lower end of the column. The columns were constructed in this manner in order to minimize any mixing of the eluate after passage through the resin.

Resin. Dowex 2-X8 (200/400 mesh, medium porosity, total capacity 3.0 ± 0.3 meq per dry gm)[3] was obtained from Fluka AG, Buchs SG, Switzerland.

Sugars. Commercial sugar preparations of the highest quality were used. L-Arabinose, D-fructose, L-fucose, D-galactose, D-lyxose, D-mannose, L-rhamnose, D-ribose, and D-xylose were analyzed reagents obtained from Mann Research Laboratories, 136 Liberty St., New York 6, N. Y. Analytical-grade D-glucose was obtained from J. T. Baker Chemical Co., Philipsburg, N. J.

Proteins. Crystallized bovine plasma albumin was a product of The Armour Laboratories, Chicago, Ill. Ovine luteinizing hormone was kindly supplied by Dr. Darrell N. Ward, of the Biochemistry Department, The University of Texas M. D. Anderson Hospital and Tumor Institute, Texas Medical Center, Houston, Texas.

Buffers. Buffer A: 0.4 *M* boric acid, 1.0 *M* glycerol, 0.5 ml toluene per liter, adjusted to pH 6.80 ± 0.05 at 50°C with NaOH (approximately 70 meq per liter required for adjustment). *Buffer B:* 0.4 *M* boric acid, 1.0 *M* glycerol, 0.050 *M* NaCl, 0.5 ml toluene per liter, adjusted to pH 6.80 ± 0.05 at 50°C with NaOH. The buffers were filtered prior to use.

The chemicals used in preparing the buffers were analytical reagents obtained from E. Merck AG, Darmstadt, Germany.

[3] In order to obtain more uniform particle size, the resin was fractionated by settling. To 500 gm of moist resin in a 4-liter beaker was added 3.5 liters of water; the resin was stirred and allowed to settle for 30 min. The median settling distance was 16–18 cm. After settling the fine particles were decanted. This procedure was continued until most of the fine particles had been removed. The fine particles decanted after the 30-min settling were designated Fraction I. The remaining resin was suspended, stirred, and allowed to settle for 7 min, and the particles remaining in suspension were decanted and saved. This was repeated until the supernatant liquid was clear. The particles of intermediate size, Fraction II, were saved and used in the preparation of the columns. The yield of Fraction II was 25–50% of the starting material, depending upon resin batch. The remaining resin, which settled in 7 min, was designated Fraction III.

METHOD

Preparation of Resin. The resin (150 ml of settled Fraction II, which is sufficient for packing two columns) was treated in the following manner:

1. Transferred to a sintered glass filter and converted to the hydroxide form by washing with 1 liter of 2 *N* NaOH in four portions.
2. Washed with 2 liters of distilled water to remove excess NaOH.
3. Washed with 2 liters of 0.4 *M* boric acid, 1.0 *M* glycerol, 0.050 *M* NaCl, 0.5 ml toluene per liter (the solution used in preparing Buffer B) in four portions. The final pH of the above slurry should be 6.50 or less.
4. Suspended in 250 ml of the above solution, warmed to 50°C, and the pH adjusted to 6.80 ± 0.05 at 50°C.
5. Equilibrated at 50°C with three 250-ml changes of Buffer B.
6. Equilibrated finally by adding 350 ml of Buffer B, warming to 50°C, and deaerating under reduced pressure.
7. After allowing the resin to settle the total volume was adjusted to 450 ml, giving a slurry consisting of 1 part settled resin to 2 parts buffer. This slurry was kept at 50°C while packing the columns.

Packing and Equilibration of Columns. The columns, maintained at 50°C, were packed in 4–5 sections under gravity flow essentially according to the manner described by Moore, Spackman, and Stein (13). A thin pad of fine glass wool was placed at the bottom of the column to prevent resin particles from obstructing the outlet tubing. The initial height of the columns was 150–153 cm. A small plug of fine glass wool was inserted in the column approximately 3 cm above the top of the resin to aid in deaerating the buffer before entering the resin. The column was equilibrated at a flow rate of 3.0 ± 0.2 ml/hr at 50°C with buffer deaerated under reduced pressure prior to use. During equilibration further packing of the resin occurred, so that the final column height was 145–150 cm. Insufficient preliminary equilibration of the resin caused temporarily higher retention volumes for each sugar.

Following preliminary equilibration, the void volume of such columns was conveniently determined by adding a 1-ml sample of water. The first thirty 1-ml fractions were analyzed using the aniline/acetic, orthophosphoric acid reagent (14) and the depression of the column blank employed as an indication of the void volume. The void volume was 19 ± 2 ml.

Preparation of Hydrolyzates for Chromatographic Analysis. Hydrolysis in 1–2 *N* sulfuric acid has been used for the liberation of neutral sugars from glycoproteins (15–18). Procedures for neutralization and removal of salt from the hydrolyzates have employed barium hydroxide (17), barium carbonate (15), and a strong anion-exchange resin in the

carbonate form (16). The use of resin in the neutralization procedure has been employed in the analyses reported here because it permitted the quantitative recovery of monosaccharides from hydrolyzates and required a minimum of manipulation. During the application of this chromatographic method to the analysis of the neutral monosaccharides of several glycoproteins, the following procedure for hydrolysis and subsequent neutralization was adopted:

1. To each 5–7 mg of glycoprotein was added 1 ml 1 *N* H_2SO_4 and hydrolysis performed at 100°C for 8–12 hr.

2. After cooling, the hydrolyzate was neutralized with Dowex 2-X8, Fraction III, in the bicarbonate form (1 gm of resin per meq of H_2SO_4). As discussed later, an internal standard can be added after hydrolysis and prior to Step 2.

3. The resin-hydrolyzate was slurried and filtered. The hydrolysis tube was rinsed with four 1-ml aliquots of distilled water, and these rinses in turn used to wash the resin remaining on the filter.

4. The combined filtrate and washes were frozen and lyophilized in an evacuated desiccator over NaOH pellets.

Sample Application and Operating Conditions. Standard sugar mixtures or hydrolyzates were dissolved in Buffer A. Samples (200 μl or 1.0 ml) were applied under gravity flow. A 200-μl sample was followed by a wash of 200 μl each of Buffers A and B, respectively, whereas a 1-ml sample was followed by a 0.5-ml wash each of Buffers A and B, respectively. Elution with Buffer B was performed at 50°C at a flow rate of 3.0 ± 0.2 ml/hr, maintained by the use of a constant-volume pump. One-milliliter fractions were collected. Between samples the column was allowed to equilibrate with approximately 100 ml of Buffer B.

Analysis of Samples. The fractions were analyzed for sugar using the aniline/acetic, orthophosphoric acid method described in another communication (14). Three milliliters of reagent was added to each 1-ml fraction, the samples were heated for 2 hr at 100°C, and the absorbance was read at 370 and 360 mμ (14).

RESULTS

Resolution. The separation of a standard sugar mixture is shown in Fig. 1. The retention volumes of the various sugars relative to glucose are tabulated in Table 1. These were calculated by subtracting the void volume of the column from the elution volume of each sugar and calculating the ratio relative to glucose (equal to 100). The relative retention volumes shown in Table 1 were calculated from standard sugar mixtures applied as 1-ml samples and using columns prepared from the same resin batch. The size of the sample can cause slight variations (approximately

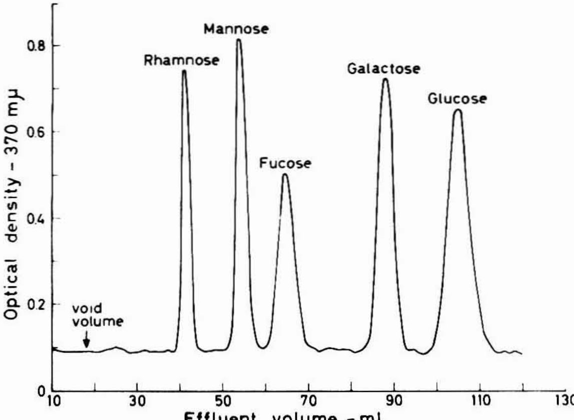

FIG. 1. Column chromatographic separations of a standard neutral monosaccharide mixture containing 0.40 μmole rhamnose, 0.75 μmole mannose, 0.50 μmole fucose, 1.00 μmole galactose, and 1.25 μmoles glucose.

TABLE 1
CONSTANTS FOR THE CHROMATOGRAPHIC SYSTEM

Monosaccharide	Recovery[a]	Retention volume relative to glucose
Rhamnose	101.0 ± 2.5 (21)	27
Mannose	93.8 ± 2.5 (12)	42
Lyxose	96.7 ± 2.5 (8)	52
Fucose	100.7 ± 1.9 (6)	54
Ribose	97.1 ± 1.7 (6)	75
Arabinose	96.9 ± 3.5 (5)	77
Galactose	91.0 ± 0.8 (5)	80
Glucose	96.5 ± 1.8 (5)	100
Xylose	100.8 ± 3.6 (7)	113
Fructose	92.9 ± 2.9 (6)	116

[a] Recovery expressed as per cent ± standard deviation. Numbers within parentheses indicate the number of determinations.

±3%) in the relative retention volumes. The relative retention volumes of fructose and ribose showed slight but significant variations with resin batch, but only in one case was the order of elution altered, i.e., fructose being eluted before xylose. Monosaccharide pairs whose relative retention volumes differ by 12 or more units (relative to glucose equal to 100) can be separated. When analyzing hydrolyzates of glycoproteins, it is more convenient to express the retention volumes relative to rhamnose.

These can be readily calculated from the retention volumes relative to glucose given in Table 1.

Recoveries. In Table 1 are compiled the recoveries of the various neutral sugars. These were calculated from sugar mixtures applied as 200-μl and 1-ml samples. No significant differences in recoveries could be attributed to the sample volume.

Analysis of Monosaccharide Components of Glycoproteins. In order to determine the effect of amino acid and peptide components on the chromatographic system and also to check the quantitation of neutral monosaccharides obtained from hydrolyzates neutralized according to the previously described method, quadruplicate 12-mg samples of crystalline bovine serum albumin were submitted to hydrolysis in 2 ml of 1 N H_2SO_4 for 8 hr at 100°C. After hydrolysis, an aliquot of monosaccharide standard solution containing rhamnose, mannose, fucose, galactose, and glucose was added. These simulated glycoprotein hydrolyzates were then neutralized with resin in the bicarbonate form according to the procedure mentioned before. The dried hydrolyzates were dissolved in Buffer A and submitted to column chromatographic analysis. The recoveries of monosaccharides were as follows: rhamnose, 99.9% ± 3.6 S.D. (standard deviation); mannose, 91.8% ± 3.4 S.D.; fucose, 96.6% ± 4.0 S.D.; galactose, 88.9% ± 1.4 S.D.; and glucose, 95.0% ± 0.7 S.D. No monosaccharides could be detected in bovine serum albumin samples analyzed directly. By comparing these values with the recovery data in Table 1, it can be seen that over 95% of the monosaccharides were recovered. No

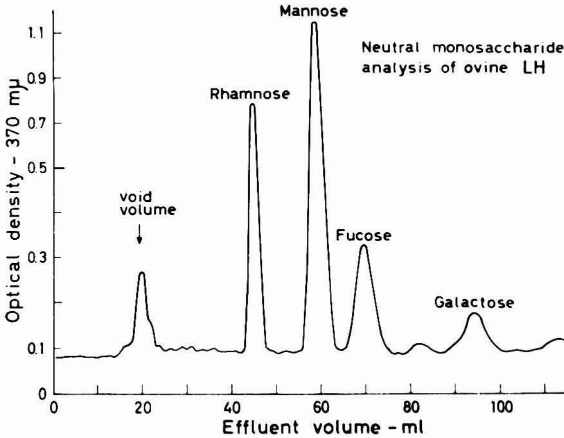

FIG. 2. Column chromatographic analysis of the neutral monosaccharide components of ovine luteinizing hormone. Rhamnose was added as an internal standard to check the quantitation of the neutralization procedure.

significant losses of any individual sugar occurred; therefore the small losses incurred were probably due to the manipulations involved in the neutralization. Since glycoproteins ordinarily contain no rhamnose, it is convenient to correct for small losses incurred in the neutralization procedure by adding rhamnose as an internal standard immediately following hydrolysis and prior to neutralization.

The column chromatographic analysis of the neutral sugars of several glycoproteins has been performed. The chromatographic profile of a hydrolyzate of ovine luteinizing hormone (LH) is shown in Fig. 2. The retention volumes relative to rhamnose indicate the presence of mannose, fucose, and galactose. Rhamnose, which was used as an internal standard, was recovered quantitatively. The quantitative analysis, which indicated 0.272 μmole mannose, 0.063 μmole fucose, and 0.038 μmole galactose per mg of protein, is essentially in agreement with previous analyses (19) using the method of Hallén. The nature of the peak which emerges at the void volume is unknown.

DISCUSSION

A wide variety of column chromatographic methods have been applied to the analytical problem of monosaccharide separation and quantitation. Since earlier work employing adsorption chromatography proved useful only for preparative scale separations, methods utilizing partition and ion-exchange principles were applied to this problem. Three methods possess considerable resolving power: the partition chromatographic methods of Arwidi and Samuelson (7) and Gardell (3) and the ion-exchange chromatographic method of Hallén (12). Since the method of Arwidi and Samuelson requires rather large effluent volumes, milligram quantities of monosaccharide are necessary for analysis. The quantitative aspects of this method have not been reported. The method of Gardell possesses considerable resolving power and sensitivity, as well as good quantitation, but it is difficult to apply to hydrolyzates because of the effect of salt on the organic solvent system. The ion-exchange chromatographic system employing sugar-borate complexes described by Hallén was a notable advance toward a method possessing sensitivity, good quantitation, and applicability to hydrolyzates.

The ion-exchange column chromatographic method described herein is the result of attempts to increase the resolution of Hallén's system. Initial attempts utilizing increased column temperatures were not encouraging. Operation of Hallén's system at 30°–50°C showed that considerable destruction (10–20%) of the sugars occurred. This was not particularly surprising since the alkaline conditions of chromatography are favorable for the alteration of the sugars by the Lobry de Bruyn-Van

Ekenstein transformations (20). Indeed, the recoveries from Hallén's system (approximately 23°C) are 90–95% (19), indicating some alteration of the sugars even at this temperature. Since sugar-borate complexes occur at lower pH, even under acid conditions (8), the possibility of performing the elution at lower pH was investigated employing boric acid in phosphate buffer (pH 7–8). As the pH was lowered, buffer of less ionic strength, and consequently lower buffering capacity, was required for elution. In order to increase the amount of ionizable borate at low pH and at the same time obtain a higher capacity buffer, a system containing glycerol and boric acid was employed. The use of a boric acid/glycerol buffer has enabled the chromatographic separation of the neutral sugars to be performed at neutral pH.

For the quantitative column chromatographic analysis of hydrolyzates it is important that the hydrolyzates be prepared for chromatographic analysis in a manner in which no major losses of neutral monosaccharides occur. The procedure utilizing neutralization with strong anion-exchange resin in the bicarbonate form has been adopted and the quantitative recovery of sugars after neutralization demonstrated. Rhamnose has been used as an internal standard to correct for small mechanical losses incurred during neutralization of glycoprotein hydrolyzates. The analytical method has been applied to several glycoproteins and tissues. The chromatogram showing the analysis of ovine LH (Fig. 2) allows a comparison with previous analyses using the method of Hallén. It can be seen that the present method gives sharper separation and a more acceptable column baseline.

The primary improvement offered by the chromatographic system described herein is the ability to perform the chromatography at neutral pH. This has removed one of the limiting factors present in previous ion-exchange systems and should provide the basis for the development of systems possessing higher resolution and faster flow rates.

SUMMARY

A method for the separation and quantitation of micromole quantities of many of the naturally occurring neutral monosaccharides has been described. This method is based on the ion-exchange chromatography of the sugar-borate complexes on a strong anion-exchange resin. The utilization of a boric acid/glycerol buffer has allowed the chromatographic separation to be performed at pH 6.8 and at an elevated temperature. This system possesses a high degree of resolution and permits neutral monosaccharides to be quantitated with a precision of ±5% or better in the case of some monosaccharides.

REFERENCES

1. Binkley, W. W., *Advan. Carbohydrate Chem.* **10,** 55 (1955).
2. Hough, L., Jones, J. K. N., and Wadman, W. H., *Nature* **196,** 448 (1948).
3. Gardell, S., *Acta Chem. Scand.* **7,** 201 (1953).
4. Samuelson, O., and Swenson, B., *Acta Chem. Scand.* **16,** 2056 (1962).
5. Dahlberg, J., and Samuelson, O., *Svensk Kem. Tidskr.* **75,** 178 (1963).
6. Samuelson, O., and Swenson, B., *Anal. Chim. Acta* **28,** 426 (1963).
7. Arwidi, B., and Samuelson, O., *Anal. Chim. Acta* **31,** 462 (1964).
8. Böeseken, J., *Advan. Carbohydrate Chem.* **4,** 189 (1949).
9. Khym, J. X., and Zill, L. P., *J. Am. Chem. Soc.* **73,** 2399 (1951).
10. Khym, J. X., and Zill, L. P., *J. Am. Chem. Soc.* **74,** 2090 (1952).
11. Nakamura, M., and Mori, K., *Biochim. Biophys. Acta* **34,** 546 (1959).
12. Hallén, A., *Acta Chem. Scand.* **14,** 2249 (1960).
13. Moore, S., Spackman, D. H., and Stein, W. H., *Anal. Chem.* **30,** 297 (1958).
14. Walborg, E. F., and Christensson, L., *Anal. Biochem.* **13,** 186 (1965).
15. Bragg, P. D., and Hough, L., *Biochem. J.* **78,** 11 (1961).
16. Eylar, E. H., and Jeanloz, R. W., *J. Biol. Chem.* **237,** 622 (1962).
17. Spiro, R., *J. Biol. Chem.* **235,** 2860 (1960).
18. Walborg, E. F., and Ward, D. N., *Texas Rept. Biol. Med.* **21,** 601 (1963).
19. Walborg, E. F., and Ward, D. N., *Biochim. Biophys. Acta* **78,** 304 (1963).
20. Speck, J. C., *Advan. Carbohydrate Chem.* **13,** 63 (1958).

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A Colorimetric Method for the Quantitative Determination of Monosaccharides[1]

EARL F. WALBORG, JR.[2] AND LENA CHRISTENSSON

From the Department of Physiological Chemistry, University of Lund, Lund, Sweden

Received February 16, 1965

In conjunction with the development of a column chromatographic method for the separation of monosaccharides (1), it was necessary to use a method for the detection of sugars which was applicable to the routine analysis of column effluents containing high concentrations of glycerol. In addition to this criterion, a method possessing accuracy of quantitation, high sensitivity (detection of 5–50 μg/ml), and applicability to a wide range of monosaccharides was desired.

In general the conventional methods using strong mineral acids cannot be employed in the presence of organic solvents because of side reactions of strong acid on the organic solvents. In the case of some of these reactions glycerol itself forms chromogens, e.g., the anthrone reagent has been employed for the determination of the sugar alcohols, including glycerol (2). The phenol-sulfuric acid method of Dubois *et al.* (3), which possesses all the criteria of quantitation, sensitivity, and applicability to a wide range of sugars, cannot be used because of interference due to glycerol.

Another group of reagents employs the reaction between sugars and the aromatic amine salts of organic and inorganic acids. Partridge (4) utilized aniline phthalate and oxalate as qualitative reagents for the identification of sugars on paper chromatograms. Hough, Jones, and Wadman (5) have investigated spray reagents which utilized salts of a number of aromatic amines. The aniline phthalate reagent has proved useful in detecting a wide variety of sugars, e.g., aldopentoses, aldohexoses, and hexuronic acids as well as their methylated derivatives.

[1] This research was supported by a grant from the Swedish Medical Research Council (No. F-139-13-A).

[2] Supported by a U. S. Public Health Service Postdoctoral Fellowship (No. 5-F2-GM-12,732-02) from the Division of Research Grants. Present address: Department of Biochemistry, The University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, Texas.

The ketohexoses do not react with this reagent. Bryson (6) and Mukherjee and Srivastava (7) have also described spray reagents consisting of aniline phosphate and *p*-anisidine phosphate, respectively. The spectrum of monosaccharides detectable with these reagents is broadened to include ketohexose.

Quantitative reagents using aniline trichloroacetate and aniline acetate have been described by Gardell (8) and Hallén (9), respectively. As noted by Gardell, this type of reagent is much more compatible with organic solvents than reagents containing mineral acids.

The quantitative method of analysis described herein utilizes aniline dissolved in a mixture of acetic and orthophosphoric acids. This reagent provides a sensitive, quantitative method for the determination of a broad spectrum of monosaccharides and is readily applicable to the analysis of column effluents containing glycerol.

MATERIALS

Chemicals. Commercial sugar preparations of the highest quality were used. L-Arabinose, 2-deoxy-D-ribose, D-fructose, L-fucose, D-galactose, D-lyxose, D-mannose, L-rhamnose, D-ribose, and D-xylose were obtained from Mann Research Laboratories, 136 Liberty St., New York 6, N. Y. D-Glucose was obtained from J. T. Baker Chemical Co., Philipsburg, N. J. D-Glucuronic acid was obtained from Fluka AG, Buchs SG, Switzerland. Boric acid and glycerol were analytical-grade reagents obtained from E. Merck AG, Darmstadt, Germany. Crystallized bovine serum albumin was a product of The Armour Laboratories, Chicago, Ill.

The Aniline/Acetic, Orthophosphoric Acid Reagent. The reagent was prepared by adding 200 ml of glacial acetic acid to 6 ml of redistilled aniline. To this was added 100 ml of 85% orthophosphoric acid. Glacial acetic acid and 85% orthophosphoric acid were analytical-grade chemicals obtained from E. Merck AG. Aniline for use in the reagent was prepared by twice distilling analytical-grade aniline in the presence of sodium hydroxide and zinc dust. The distillation was performed under nitrogen. The colorless aniline was stored in a glass-stoppered amber bottle at 4°C. Aniline prepared and stored in this manner remained suitable for use for at least two months.

METHOD

To 1 ml of sugar solution (5–50 μg sugar) in 17–18 $\times$ 160 mm test tubes was added 3 ml of the aniline/acetic, orthophosphoric acid reagent. The samples were mixed and placed in a water bath at 100°C for 2 hr. Since a rather long heating time is required for maximal color development, a bath which allowed minimal heating of the upper part of the

tube was utilized in order to avoid evaporation. Evaporation was further minimized by the use of glass marbles to cover the tubes during heating. Less than 2% evaporation was experienced under the conditions at which the samples were heated. After heating, the samples were allowed to cool for at least 20 min in a water bath at 30°. Cooling at lower temperatures produced a more viscous solution and thus made measurement of absorbance in cuvettes more difficult. Measurement of absorbance was performed in 1-cm cuvettes in a Beckman spectrophotometer (model B). Routinely all samples and blanks were read against water as a blank. Absorbance measurements were routinely performed within 4 hr after completion of heating.

RESULTS

Determination of Optimal Heating Time. To determine the length of heating required for maximal color development, samples of galactose, fructose, fucose, and arabinose were heated at 100°C for varying lengths of time up to 3 hr. These samples were dissolved in Buffer A (1) of the following composition: 0.4 M boric acid, 1.0 M glycerol, 0.5 ml toluene per liter, adjusted to pH 6.80 ± 0.05 at 50° with NaOH. The effect of the duration of heating is shown in Fig. 1. In view of these data, 2 hr

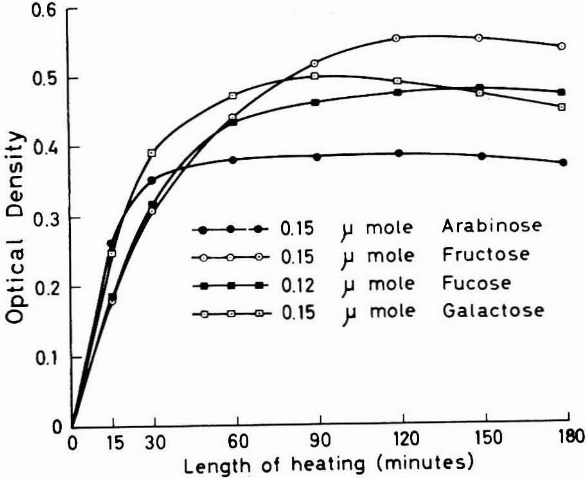

FIG. 1. Effect of duration of heating on color development. Absorbances were measured at the absorption maximum for each monosaccharide.

was chosen as optimal length of heating. Samples of sugar dissolved in water exhibited no differences in rate of color development on heating. Glucuronic acid showed a much slower rate of color development and no plateau of color development was reached even after 3 hr of heating.

Determination of Absorption Spectra of the Monosaccharides. The absorption spectra (335–500 mμ) for mannose, arabinose, fructose, and fucose dissolved in Buffer A (1) are shown in Fig. 2. The absorption

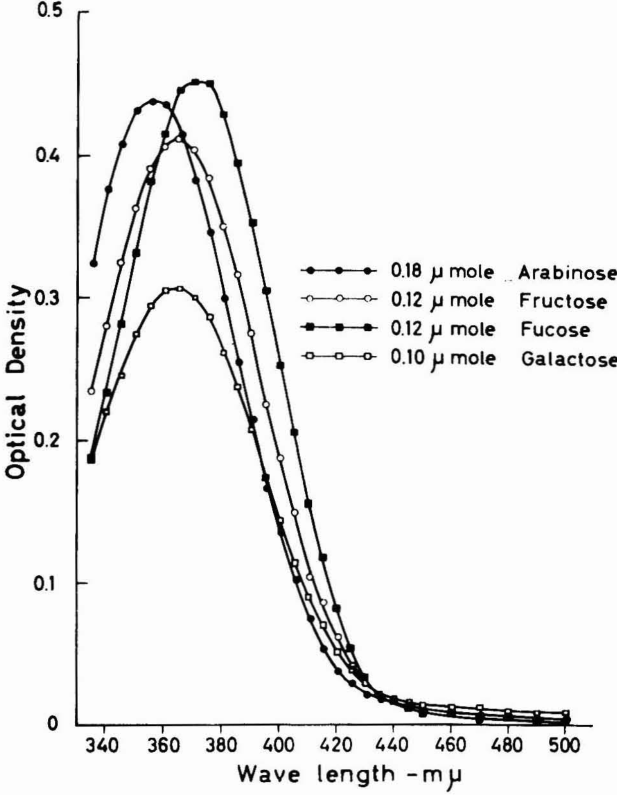

Fig. 2. Absorption spectra for monosaccharides.

maxima for all monosaccharides investigated are listed in Table 1. The aldopentoses exhibited an absorption maxima at 355 mμ, the aldohexoses and fructose at 365 mμ, and the 6-deoxyaldohexoses at 370 mμ. Monosaccharides dissolved in water exhibited the same characteristic absorption spectra as that obtained in Buffer A. The chromogen formed with glucuronic acid after heating for 2 hr exhibited an absorption spectrum differing from the other sugars. A broad absorption plateau was exhibited in the region 330–380 mμ rather than a distinct peak, as in the case of the other monosaccharides. Deoxyribose could not be detected using this method.

Correlation between Sugar Concentration and Intensity of Color.

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TABLE 1
ABSORPTION MAXIMA AND MOLECULAR EXTINCTION COEFFICIENTS OF MONOSACCHARIDES USING THE ANILINE/ACETIC, ORTHOPHOSPHORIC ACID REAGENT

Monosaccharide	Absorption maxima ± 3 mμ	Molecular extinction coefficient[a]	
		in Buffer A	in water
Arabinose	355	9,780 ± 140	8,550 ± 280
Lyxose	355	10,000 ± 300	8,870 ± 300
Ribose	355	10,400 ± 300	8,800 ± 270
Xylose	355	10,100 ± 300	8,550 ± 240
Galactose	365	12,500 ± 300	11,800 ± 300
Glucose	365	11,900 ± 400	11,300 ± 300
Mannose	365	11,900 ± 200	11,500 ± 300
Fructose	365	14,600 ± 400	13,900 ± 200
Fucose	370	16,100 ± 200	13,200 ± 200
Rhamnose	370	14,600 ± 400	13,400 ± 400

[a] Molecular extinction coefficient ± standard deviation. Each was calculated from a total of 15 values obtained from 3 standard curves containing 5 different concentrations.

Representative standard curves for several of the monosaccharides are shown in Fig. 3. The molecular extinction coefficients of the various monosaccharides, determined both in water and Buffer A (1), are presented in Table 1. The optical density of the blanks read at 355–370 mμ in 1-cm cuvettes was 0.075 ± 0.007 for Buffer A and 0.015 ± 0.007 for water. The molecular extinction coefficient for glucuronic acid determined after 2 hr of heating and at a wavelength of 365 mμ was 4200 ± 650 standard deviation (6 determinations).

Stability of the Chromogen. In order to determine the stability of the chromogen formed, the absorbance of replicate samples of galactose, fucose, fructose, and arabinose was measured at 1, 6, 24, and 96 hr after heating was completed. The variation of the absorbance was ±5% or less. Since the slight variations which occurred were random, it can be stated that the chromogen is stable for at least 96 hr. After 96 hr the blank increased approximately 15%.

Stability of the Reagent. The reagent is stable at room temperature for at least 2 months. Identical standard curves were obtained for glucose and arabinose after 21 days and for fructose and fucose after 2 months. The maximum increase in blank values was approximately 25%.

Effect of Alteration of Ratio of Acetic Acid to Orthophosphoric Acid in the Reagent. Two reagents, containing altered ratios of acetic to orthophosphoric acid, were prepared: Reagent A contained 220 ml acetic acid and 80 ml orthophosphoric acid, whereas Reagent B contained 180 ml acetic acid and 120 ml orthophosphoric acid. Both reagents contained 6 ml redistilled aniline. The molecular extinction coefficients of arabinose,

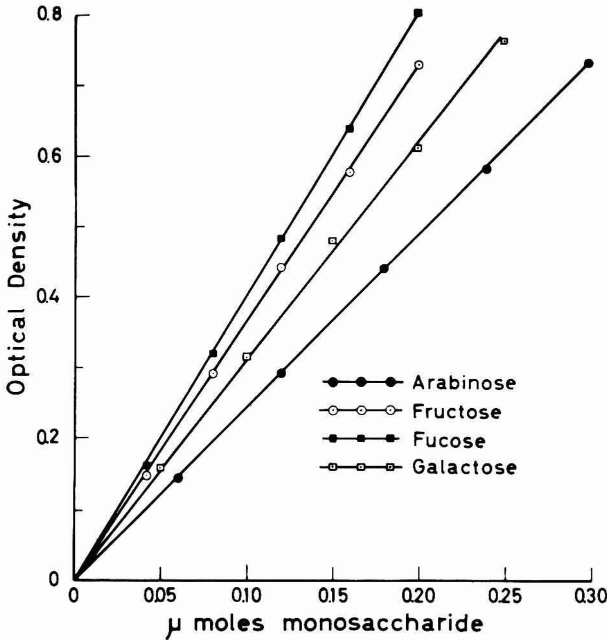

FIG. 3. Standard curves for monosaccharides. Absorbances were measured at the absorption maximum for each monosaccharide.

galactose, fructose, and fucose dissolved in Buffer A (1) were determined. All other conditions of analysis were the same as previously described (see "Method"). The molecular extinction coefficients using Reagent A were as follows: arabinose 8,600; galactose 11,700; fructose 12,400; and fucose 15,000. Corresponding values for Reagent B were: arabinose 10,800; galactose 12,800; fructose 15,200; and fucose 16,700. Thus higher molecular extinction coefficients were obtained with increasing orthophosphoric acid concentration of the reagent. As the concentration of orthophosphoric acid is increased, a more viscous reagent is obtained. The reagent chosen possessed high sensitivity and a viscosity which permitted unhindered routine use.

Effect of Sample Volume on Color Development. Samples containing 0.10 μmole glucose dissolved in 0.80, 1.00, and 1.20 ml Buffer A (1) were submitted to analysis by the described procedure. The resulting absorbances, corrected to the same final volume, indicated that the decreased sample volume caused a 3–4% increase in the absorbance, whereas the increased sample volume caused a 6–7% decrease in the absorbance. It is thus necessary that the volume of the sample be adequately controlled to obtain accurate analyses.

Effect of Salt on Color Development. Galactose or arabinose, dissolved

in water or Buffer A (1), containing added NaCl to a concentration of 0.10 *M*, exhibited no significant alterations in their molecular extinction coefficients. However, if the concentration of NaCl was increased to 0.25 *M*, a 6–9% increase of the molecular extinction coefficients was experienced.

Effect of Peptides and Amino Acids on Color Development. Galactose standards, dissolved in water or in Buffer A (1) and containing increasing amounts of bovine serum albumin (BSA), were analyzed. The presence of BSA and/or its products of hydrolysis had no effect upon color development at least up to a concentration of 150 μg BSA/ml.

Dilution of Samples. If the absorbance of a sample is too high (above an absorbance of 0.8) for accurate reading, the sample may be diluted with a mixture consisting of 2 parts glacial acetic acid and 1 part 85% orthophosphoric acid. Fourfold dilutions can be performed in this manner without altering the linearity of the standard curves or the molecular extinction coefficients. Dilution of samples with 96% ethanol or blank solution produced lower molecular extinction coefficients. Addition of water to samples greatly reduced the absorbance; thus it is important that samples be protected from accidental contamination with water both during heating and after color development.

DISCUSSION

The colorimetric method described here provides a simple method for the sensitive quantitative determination of a wide variety of neutral monosaccharides. With regard to its sensitivity and/or the number of sugars detected it is superior to similar previous quantitative reagents described by Gardell (8) and Hallén (9). The present method is approximately twice as sensitive as that of Gardell, but similar in sensitivity to that described by Hallén. The range of neutral monosaccharides detected by this method has been broadened to include ketohexose. Preliminary experiments indicate that this reagent will also be useful in the sensitive determination of neutral oligosaccharides. This work will be reported in another communication. Another distinct advantage of this method is the stability of the reagent.

Using the aniline/acetic, orthophosphoric acid reagent, different classes of neutral monosaccharides exhibit different absorption maxima: the aldopentoses, 355 mμ; the aldo- and ketohexoses, 365 mμ; and the 6-deoxyaldohexoses, 370 mμ. The existence of different absorption maxima is in contrast to the methods of Gardell and of Hallén in which the same absorption maximum is exhibited for all the classes of monosaccharides mentioned above. When analyzing column effluents containing any of the above classes of sugars, accurate quantitation of all of

these classes can be made by reading the absorbance at two wavelengths: 360 mμ, which lies near both the aldopentose and the aldo- and ketohexose absorption maxima; and 370 mμ, the absorption maximum for the 6-deoxyaldohexoses. By reading at these two wavelengths a different ratio is obtained for each class of neutral monosaccharide. During column chromatographic analysis this ratio is a useful qualitative tool in the identification of peaks or in the detection of mixtures of different classes of sugars within the same peak.

The properties of the reagent with regard to its simplicity, sensitivity, range of sugar detection, and stability make it suitable as a reagent for the automatic determination of sugars in column effluents.

Although this colorimetric method has been developed for application to a particular column chromatographic method, its special properties of quantitation, sensitivity, and range of detection make it applicable to other problems of sugar determination as well.

SUMMARY

A sensitive colorimetric method for the quantitative analysis of neutral monosaccharides utilizing an aniline/acetic, orthophosphoric acid reagent has been described. The method permits quantitation of a variety of monosaccharides (aldopentoses, aldo- and ketohexoses, and 6-deoxyaldohexoses) in amounts of 5–180 μg with a precision of ±5%. Since different absorption maxima are exhibited for the aldopentoses, aldo- and ketohexoses, and 6-deoxyaldohexoses, qualitative distinction between these classes of sugars is possible. Application of this method to the analysis of effluents from column chromatograms is discussed.

ACKNOWLEDGMENT

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REFERENCES

1. WALBORG, E. F., JR., CHRISTENSSON, L., AND GARDELL, S., *Anal. Biochem.* **13,** 177 (1965).
2. GRAHAM, H. D., *J. Food Sci.* **28,** 440 (1963).
3. DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A., AND SMITH, F., *Anal. Chem.* **28,** 350 (1956).
4. PARTRIDGE, S. M., *Nature* **164,** 443 (1949).
5. HOUGH, L., JONES, J. K. N., AND WADMAN, W. H., *J. Chem. Soc.* **1950,** 1702.
6. BRYSON, J. L., *Nature* **167,** 864 (1951).
7. MUKHERJEE, S., AND SRIVASTAVA, H. C., *Nature* **169,** 330 (1952).
8. GARDELL, S., *Acta Chem. Scand.* **5,** 1011 (1951).
9. HALLÉN, A., *Acta Chem. Scand.* **14,** 2249 (1960).

ANALYTICAL BIOCHEMISTRY 13, 194–198 (1965)

Specific Purification of Avidin by Column Chromatography on Biotin-Cellulose[1]

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Avidin, a protein of low molecular weight isolated mainly from egg white, binds natural D-biotin to form a relatively stable complex with an estimated dissociation constant of 10^{-15} M (1). The complexing of biotin by avidin appears to require the ureido ring and aliphatic chain portions of the vitamin, but does not depend on a free carboxyl function as evidenced by the similar binding of biocytin (2) and oxybiotin methyl ester (3).

Chromatography on bentonite (4) and carboxymethylcellulose (5) have been used to obtain essentially pure avidin, but more specific techniques were desirable. Chromatographic methods which utilize the specific complexing of certain biological materials have found occasional use in their purification. Moreover, the relatively small size and good stability of the avidin molecule together with its ability to complex tightly with that portion of biotin not involving the carboxyl group suggested the possibility of esterifying biotin to cellulose for formation of an avidin-specific absorbent.

The present study describes the synthesis and chromatographic use of biotin-cellulose for the specific purification of avidin.

MATERIALS AND METHODS

Commercial avidin and crystalline D-biotin were purchased from Nutritional Biochemicals Corp. The avidin contained 1 to 1.25 units/mg where 1 unit corresponds to 1 μg of biotin bound under specified conditions (5). C^{14}-Biotin was obtained as carbonyl labeled from Nuclear of Chicago. Thionyl chloride and pyridine were from Eastman Organic Chemicals. The cellulose powder used was Whatman ashless CF 11. Ammonium carbonate (30% NH_3) was reagent grade from Mallinckrodt.

[1] This investigation was supported in part by Research Grant AM-08721 from the National Institutes of Health and by funds from the State University of New York.

Synthesis of Biotin-Cellulose. One gm of biotin with 5 μc of radioactivity was dried over P_2O_5 *in vacuo*. This C^{14}-biotin was dissolved in 15 ml of thionyl chloride which was freshly purified under anhydrous conditions by distillations from quinoline and linseed oil (6). The C^{14}-biotin acid chloride crystallized during removal of excess thionyl chloride by evaporation under reduced pressure (7). This acid chloride was taken up in 100 ml of anhydrous pyridine and stirred into a suspension of 400 ml of pyridine containing 10 gm of cellulose powder that had been dried at 50° over P_2O_5 *in vacuo*. The mixture was stoppered and allowed to shake at 30° overnight. The C^{14}-biotin-cellulose was filtered off and thoroughly washed in liter amounts of solution by resuspending and filtering ten times from water, twice from ethanol, and once from diethyl ether. The material was dried *in vacuo* and appropriate samples suspended with thixotropic gel in Bray's solution (8) for counting of radioactivity in a Packard Tri-Carb Liquid Scintillation Spectrophotometer. Approximately 10% of original C^{14}-biotin was esterified to the cellulose for a biotin-cellulose compound containing 0.1% biotin by weight.

Column Chromatography of Avidin. Commercial avidin of approximately 10% purity was dissolved in small volumes of 0.2 to 0.225 *M* ammonium carbonate and poured over columns of cellulose or biotin-cellulose which had been previously washed with the ammonium carbonate. Elution of protein by various aqueous solutions was followed in aliquots of the fractions collected by measuring the absorbancy at 280 mμ in a Beckman DU Spectrophotometer. C^{14}-Biotin was added to other aliquots of the fractions, which were then dialyzed in seamless cellophane tubing for 24 hr against cold 0.2 to 0.225 *M* ammonium carbonate. Aliquots (1 ml) of the dialyzed preparations were dissolved in 10 ml of Bray's solution for counting of radioactivity. As reported previously (9), the amounts of avidin activity in the fractions were calculated from the corrected counts per minute retained in the dialyzed preparations above that found in a simultaneously dialyzed water control.

RESULTS AND DISCUSSION

Appropriate means for the selective retention and elution of avidin from biotin-cellulose were investigated by absorption of avidin with conditions reported optimal for binding biotin under the slightly alkaline medium (pH 8.9) and moderate ionic strength (0.6 μ) afforded by 0.2 *M* ammonium carbonate (10). As seen from the data in Table 1, most protein material from impure commercial avidin was washed through both the cellulose control and biotin-cellulose by the ammonium carbonate eluant. Further rinsing with water removed most of the small

TABLE 1

RELATIVE CONDITIONS FOR RETENTION AND ELUTION OF AVIDIN FROM CELLULOSE AND BIOTIN-CELLULOSE

Commercial avidin (20 mg with 20 to 25 units of activity) in 5 ml of 0.2 *M* ammonium carbonate was poured over a 1 × 10 cm column of cellulose or biotin-cellulose previously washed with 0.2 *M* ammonium carbonate. C^{14}-Biotin (3.6 μg with 0.5 μc of radioactivity) was added to 10-ml aliquots of each fraction collected and all the solutions dialyzed for 24 hr against 4 liters of cold 0.2 *M* ammonium carbonate.

Column, 2 gm	Eluant, 50 ml	Protein, total mg	Specific activity, counts/min/mg
Cellulose control:			
	0.2 *M* $(NH_4)_2CO_3$	20.3	97,780
	Water	0.6	7,750
	0.2 *M* $(NH_4)_2CO_3$ + 10^{-4} *M* biotin	0.1	0
Biotin-cellulose:			
	0.2 *M* $(NH_4)_2CO_3$	18.9	108,170
	Water	0.2	206,810
	0.2 *M* $(NH_4)_2CO_3$ + 10^{-4} *M* biotin	0.7	6,063

amount of protein remaining on cellulose, but a final rinse with ammonium carbonate plus biotin was needed to accomplish this with biotin-cellulose. Most of the avidin activity was washed through both cellulose and biotin-cellulose by the initial ammonium carbonate, but significant amounts of avidin with a 2-fold greater specific activity was subsequently eluted from biotin-cellulose by water. Although the avidin-retaining capacity of the small amount of biotin-cellulose used was considerably exceeded by the relatively large amount of material put through the column, these findings demonstrate that a significant quantity of avidin was bound to the biotin residues on cellulose in the presence of ammonium carbonate and released when the ionic strength of the medium was markedly dropped with water as eluant.

Essentially complete purification of avidin could be accomplished by column chromatography on amounts of biotin-cellulose which were sufficient to retain most avidin while allowing extraneous material to wash through during gradual decreases in ionic strength of the eluant. As illustrated in Fig. 1, most protein was not retained on columns even when the ratio of cellulose or biotin-cellulose to material chromatographed was relatively large and the concentration of ammonium carbonate decreased linearly. However, though avidin was not significantly enriched on the cellulose, the retention on biotin-cellulose was sufficient to allow approximately 10-fold enrichment of activity. The fractions from biotin-cellulose that had maximal avidin activity contained over 12 units/mg, which may be compared with pure avidin with a reported specific activity of 13.8 units/mg (1).

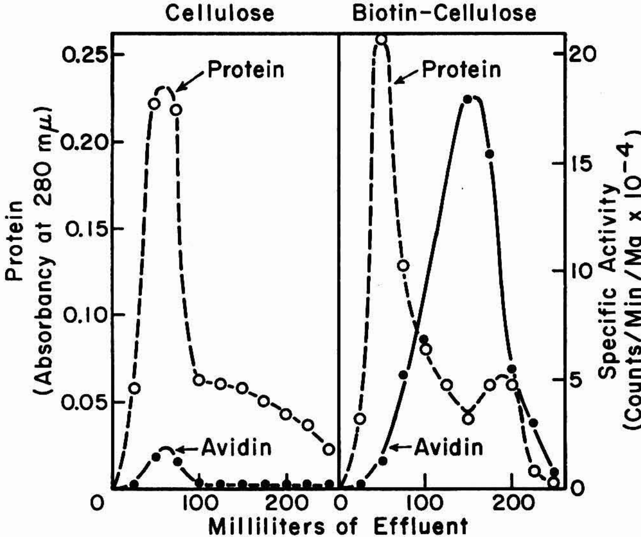

FIG. 1. Column chromatography of avidin on cellulose and biotin-cellulose. Commercial avidin (10 mg with 10 to 12.5 units of activity) in 5 ml of 0.225 *M* ammonium carbonate was poured over a 1.5 × 25 cm column (10 gm) of cellulose or biotin-cellulose previously washed with 0.225 *M* ammonium carbonate. Ten 25-ml fractions were collected from each column by eluting with a reversed linear gradient from 0.225 *M* ammonium carbonate to water. C^{14}-Biotin (3.6 μg with 0.2 μc of radioactivity) was added to 10-ml aliquots of each fraction and the solutions dialyzed against 4 liters of cold 0.225 *M* ammonium carbonate. Conventional specific activity expressed as units/mg is 0.7 times the values shown on the ordinate scale to the right. Thus, the fraction with maximum specific activity eluted from the biotin-cellulose had a conventional specific activity of 18 × 0.7 = 12.6 units/mg. The milligrams of avidin present in each 25 ml fraction is calculated then as 25 times the absorbancy at 280 mμ times the ratio of conventional specific activity to that of pure avidin. Thus, the fraction with maximum specific activity eluted from the biotin cellulose contained 25 × 0.04 × 12.6/13.8 = 0.91 mg.

Previous methods for the final purification of avidin involved nonspecific and somewhat more laborious chromatography on such materials as bentonite (4) and carboxymethylcellulose (5). The present method using biotin-cellulose has the obvious advantage of greater specificity and is quite facile. Furthermore, the biotin-cellulose is again made ready for use after a run by simply rewashing with the starting ammonium carbonate solution. The reasonable stability of the esterified biotin under conditions employed herein allows repeated use of this material.

The use of biochemically specific absorbents to selectively purify biological materials which are not readily amenable to other fractionation procedures is becoming of considerably importance. The general principle

of attachment of specific reagents or natural substances to cellulose or cellulose derivatives has recently been successfully applied for purifications of enzymes (11, 12), antibodies (13), and nucleic acids (14). The relative ease of synthesis of many such specific absorbents, the small number of reactive sites generally needed, and the simplicity of the over-all method are real advantages which should lead to further development of the general technique.

SUMMARY

1. Biotin-cellulose has been synthesized by converting biotin with thionyl chloride to biotin acid chloride and esterifying the latter in pyridine to cellulose.

2. Avidin has been specifically purified by chromatography over biotin-cellulose using a linear gradient from high to low concentration of ammonium carbonate.

REFERENCES

1. GREEN, N. M., *Biochem. J.* **89,** 585 (1963).
2. WRIGHT, L. D., VALENTIK, K. A., NEPPLE, H. M., CRESSON, E. L., AND SKEGGS, H. R., *Proc. Soc. Exptl. Biol. Med.* **74,** 273 (1950).
3. WINNICK, T., HOFMANN, K., PILGRIM, F. J., AND AXELROD, A. E., *J. Biol. Chem.* **161,** 405 (1945).
4. FRAENKEL-CONRAT, H., SNELL, E. E., AND DUCAY, E. D., *Arch. Biochem. Biophys.* **92,** 80 (1952).
5. MELAMED, M. D., AND GREEN, N. M., *Biochem. J.* **89,** 591 (1963).
6. FIESER, L. F., *Experiments in Organic Chemistry,* 2nd ed., p. 381. Heath, Boston, 1941.
7. WOLF, D. E., VALIANT, J., AND FOLKERS, K., *J. Am. Chem. Soc.* **73,** 4142 (1951).
8. BRAY, G. A., *Anal Biochem.* **1,** 279 (1960).
9. LAUNER, H. F., AND FRAENKEL-CONRAT, H., *J. Biol. Chem.* **193,** 125 (1951).
10. WEI, R. D., AND WRIGHT, L. D., *Proc. Soc. Exptl. Biol. Med.* **117,** 341 (1964).
11. ARSENIS, C., AND MCCORMICK, D. B., *J. Biol. Chem.* **239,** 3093 (1964).
12. ARSENIS, C., AND MCCORMICK, D. B., *J. Biol. Chem.* in press (1965).
13. TUPPY, H., AND KÜCHLER, E., *Biochim. Biophys. Acta* **80,** 669 (1964).
14. ERHAN, S., NORTHRUP, G. L., AND LEACH, F. R., *Proc. Natl. Acad. Sci. U. S.* **53,** 646 (1965).

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Rapid Separation and Continuous Monitoring of Enzyme Activity in Chromatographic Column Effluents

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Many enzymes have been shown to exist in multiple molecular forms called isoenzymes (1). Isoenzymes have been separated by electrophoresis (2), DEAE-cellulose chromatography (3), and ion-exchange chromatography (4). Methods for the estimation of isoenzyme activities in mixtures have been based on differences in heat stability (5), kinetic properties (6), and affinity for ion-exchange gels in batch procedures (7).

Because it is relatively nondestructive, column chromatography is desirable as a preparative procedure. Column procedures are usually time consuming, requiring the collection and assay of many fractions. Even with the use of instrumentation for the complete automation of enzyme assays of the fractions (8), much time and many manipulations would be required for a single experiment involving a large number of fractions.

Continuous analysis of enzyme activity in column effluents can greatly reduce the time required for separation experiments, increase the analytical information about the resolution of separations, and yield quantitative data immediately at the end of each separation procedure. One method for continuous monitoring of enzyme activity in column effluents has been described (9). This paper describes the use of a continuous enzyme analyzer (10, 11) with an automated column programer (12) for the continuous monitoring and rapid separation of isoenzymes on DEAE-Sephadex ion-exchange gels.

METHODS

Apparatus: The enzyme analyzer (10, 11) and column programer (12) have been described in detail elsewhere. A diagram of the experimental setup is shown in Fig. 1. The column programer automatically adds buffer solutions to the DEAE column in sequence, allowing each buffer to drop to the level of the gel bed before the addition of the next. Up to ten buffer solutions can be used in a program. The shape of the

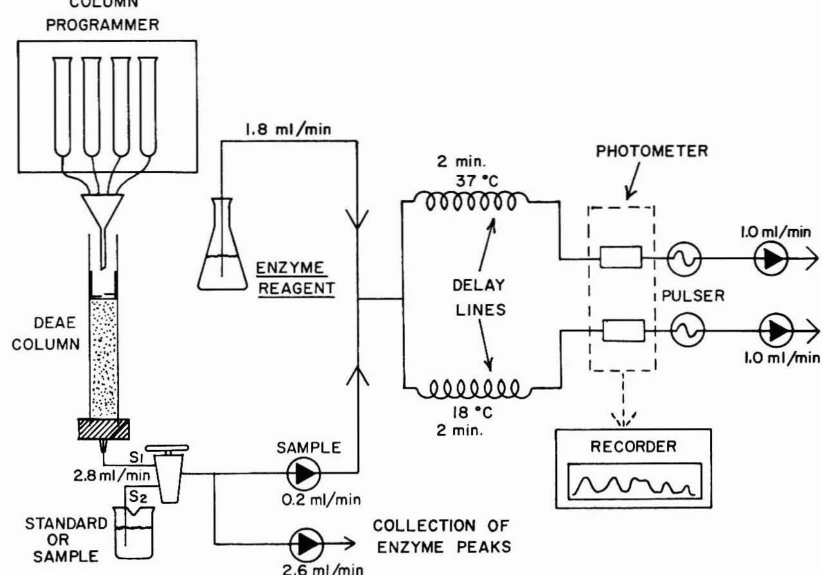

FIG. 1. Diagram of DEAE column and continuous enzyme analyzer.

gradient is determined by the amount of solution put into each buffer reservoir. After the last buffer has been used, the programer automatically shuts off the enzyme analyzer and stops the column flow. The flow rate of the column is constant at 2.8 ml/min, being the resulting sum of the metered flow rates of the sample stream, 0.2 ml/min, and the main effluent stream for the collection of enzyme peaks, 2.6 ml/min. The flow rate of the sample stream into the enzyme analyzer can be varied to decrease or increase the sensitivity of the analyzer, depending on the amount of enzyme activity originally applied to the column. Flow rates as low as 0.05 ml/min have been used. A flow rate of 0.2 ml/min was used for all the studies described in this particular paper.

A three-way stopcock permits the selection of either the column effluent stream, S_1, or a second stream, S_2, which can be used to introduce buffer and standards to set the baseline or calibrate the analyzer before a run.

In the enzyme analyzer, the sample stream is mixed with a stream of enzyme reagent to form a reaction stream which is split into two delay lines at different temperatures. After a fixed time delay, the two reaction streams flow through two photometer cells. The absorbance difference between the cells is monitored continuously by a photometer (10) and is a continuous measure of the enzyme activity. The response of the chart recorder is linear with enzyme activity (11).

Dehydrogenase Reagent: Dehydrogenase activity was determined as previously described (11) by use of reactions (1) and (2):

$$\text{substrate} + \text{NAD} \overset{\text{DH}}{\rightleftharpoons} \text{product} + \text{NADH}_2 \quad (1)$$

$$\text{NADH}_2 + \underset{\text{(blue)}}{\text{dye-ox.}} \overset{\text{PMS}}{\rightleftharpoons} \text{NAD} + \underset{\text{(colorless)}}{\text{dye-red.}} \quad (2)$$

The production of reduced nicotinamide adenine dinucleotide ($NADH_2$) by the dehydrogenase enzyme reaction is coupled to a second reaction which bleaches a blue dye in the presence of a catalyst, phenazine methosulfate (PMS). Different dehydrogenases can be measured by changing the substrate in the reaction system. For example, when the substrate is lactate, lactic dehydrogenase (LDH) is measured and, when it is malate, malic dehydrogenase (MDH) is measured. Several dehydrogenases have been detected in this manner.

The dehydrogenase reagent consisted of 200 mg NAD, 10 mg PMS, and 20 ml blue dye (34 mg 2,6-dichlorophenolindophenol in 100 ml water) diluted to 200 ml with 0.1 M phosphate buffer, pH 7.4. For LDH determination, 4 ml lactic acid, 60% syrup (Sigma Chemical Co., St. Louis, Missouri), was added. For MDH, 4 ml 2 M d,l-malic acid, pH 7.4, was added. A detailed study of the dehydrogenase method used here can be found elsewhere (11).

Transaminase Reagent: The determination of transaminase activity was based on a system previously described (13) which uses reactions (3), (4), and (5):

$$\text{amino acid} + \alpha\text{KG} \overset{\text{GXT}}{\rightleftharpoons} \text{keto acid} + \text{glutamate} \quad (3)$$

$$\text{glutamate} + \text{NAD} \overset{\text{GDH}}{\rightleftharpoons} \text{NADH}_2 + \alpha\text{KG} + \text{NH}_4^+ \quad (4)$$

$$\text{NADH}_2 + \underset{\text{(blue)}}{\text{dye-ox.}} \overset{\text{PMS}}{\rightleftharpoons} \text{NAD} + \underset{\text{(colorless)}}{\text{dye-red.}} \quad (5)$$

In reaction (3), the transfer of an amino group from an amino acid to α-ketoglutarate (αKG) is catalyzed by the transaminase enzyme. In reactions (4) and (5), the production of glutamate by the transaminase reaction is coupled to a glutamic dehydrogenase (GDH) reaction using essentially the same system described above for dehydrogenase measurements. The transaminase measured is determined by the amino acid added to the system. For example, addition of aspartic acid gives the reaction for glutamic oxalacetic transaminase (GOT), and of alanine, for glutamic pyruvic transaminase (GPT).

The enzyme reagent for transaminase determinations was metered in two streams. The first stream, metered at 1.0 ml/min, contained a solution of 300 mg NAD, 10 mg PMS, 7 ml 0.1 M αKG, and 33 ml blue dye

solution diluted up to 200 ml with 0.1 *M* phosphate buffer, pH 7.4. A second stream, metered at 0.8 ml/min, contained 50 ml prepared GDH solution (13) and either 20 ml 0.4 *M* aspartic acid or 50 ml 0.05 *M* cysteinesulfinic acid (Calbiochem, Los Angeles, California), diluted up to 100 ml with 0.1 *M* phosphate buffer. The reagent was metered in two streams to prevent any transaminase activity in the GDH preparation from affecting reagent stability (13). Complete details of the transaminase assay system and reagent studies can be found elsewhere (13).

Preparation of Tissue Homogenates: Human tissue was collected at autopsy and frozen immediately; 50 gm of tissue was thawed, cut into 3- to 5-gm pieces, soaked in physiological saline for about 5 min, and rinsed in 0.1 *M* phosphate buffer, pH 7.4; 100 ml phosphate buffer was added to the rinsed tissue and the tissue was homogenized in a Waring Blendor for about 2 min. The homogenate was strained through a cheesecloth and centrifuged for 30 min at 24,000 $\times g$ at 5°C in a Sorvall RC-2 centrifuge. The supernatant was divided into 5-ml aliquots and frozen. Frozen aliquots were discarded after 2 weeks.

For use, a frozen 5-ml aliquot was thawed and processed by gel filtration on a column previously described (11) to remove low molecular weight substances. About a 5-ml fraction of peak enzyme activity was collected from the gel column instead of quantitatively collecting all of the enzyme. The 5 ml of processed homogenate was used for separation studies.

For quantitative studies of recovery and distribution, the processed homogenate was diluted into an activity range where all peaks from the DEAE column would be in the analytical range of the enzyme analyzer; 3 ml of the diluted homogenate was applied to the column.

Preparation of Columns: 60 gm Sephadex DEAE A-50, coarse, dry particles was placed in a dry chromatographic column previously described (12). An air stream was passed up through the column from the bottom until about 20% of the dry material was "distilled" off. The 80% of dry material remaining in the column was suspended in 4 liters of distilled water and allowed to equilibrate overnight.

Fines were removed by repeated washings with distilled water and decantation. Next, the Sephadex was soaked in 0.5 *M* NaCl for at least 1 hr and, finally, equilibrated with 5 suspensions in 500 ml 0.05 *M* tris buffer, pH 7.5, followed by decantation. The remaining Sephadex is adequate to pack one 160-ml column.

While the yield using air fractionation of the dry material and repeated washings was low, it was necessary to obtain columns with sufficiently high flow rates for rapid separations. Sephadex could be used over several times by repeating the washings and equilibrating steps to

regenerate the gel. Sephadex used up to 10 times still gave good reproducible results.

Each column was packed to about 160 ml with a 1:1 Sephadex A-50 buffer suspension. After packing, several milliliters of tris buffer was passed through the column to ensure packing and the sample applicator (12) was placed on top of the gel bed, preparing the column for use.

Buffer Solutions: 0.05 *M* tris buffer was made by adding 6.06 gm Sigma 7-9 (Sigma Chemical Co.) to 500 ml distilled water, adjusting to pH 7.5 with 1 *N* HCl, and diluting to 1 liter with distilled water. NaCl buffer solutions were prepared by adding NaCl to make 0.05, 0.10, 0.125, 0.150, 0.175, 0.200, 0.225, 0.250, 0.275, and 0.300 *M* NaCl-tris solutions. Solutions were stored at room temperature.

Procedure: Before adding any solutions, the column programer is advanced manually so that it will start at solution reservoir number 1 (12). The enzyme analyzer is plugged into the programer if it is to be shut off automatically after the run. Finally, the NaCl-tris solutions are poured into the programer solution reservoirs (12). With enzyme reagent in the reagent stream and buffer being metered into the sample stream through S_2 in Fig. 1, a baseline is established and set at 0.2 on the chart with the zero control (11). To calibrate the instrument for recovery on quantitative column runs, a dilution of homogenate or a known standard solution is metered in stream S_2. When the recorder pen reaches steady state, the pen response is set to the desired position with the sensitivity control (11).

After calibration is complete and with the instrument running at baseline with buffer in S_2, column follower electrodes are inserted into the sample applicator as previously described (12). The stopcock is switched to stream S_1, and any buffer in the column is allowed to drop to the column bed. The stream is switched back to S_2 while 3 ml of diluted homogenate is added to the column with a 5-cc syringe which has about 2 ft of polyethylene tubing attached. The stream is switched back to S_1 and the homogenate is allowed to flow into the column. As the homogenate drops to the level of the gel bed, 2 to 3 ml of tris buffer is applied to the column with a wash bottle to wash the last traces of homogenate into the column. As the last buffer flows into the column, the automatic start button on the programer is pushed. The first NaCl-tris solution is automatically added to the column, starting the column program. The selector switch on the programer is set for the number of solutions to be used in the procedure and the auto-shutoff switch is turned on. After the last buffer is used, the programer stops the column flow and turns off the enzyme analyzer.

Agar Gel Electrophoresis: LDH isoenzymes from the column were

identified by an agar gel electrophoresis similar to that already described (14). After electrophoresis, agar slides were stained for lactic dehydrogenase (LDH) activity by incubation in a solution containing 20 mg nitroblue tetrazolium (NBT), 2.5 mg PMS, 5 ml lactic acid, 60% syrup, and 25 mg NAD diluted to 50 ml with phosphate buffer, pH 7.4 (all from Sigma Chemical Co.). To stain for MDH, the lactate was replaced with 5 ml 2 *M* malic acid, pH 7.5. Incubation for 30 min at 45°C was usually adequate to develop the bands.

RESULTS

Separation of LDH Isoenzymes: Figure 2 shows tracings of recordings for the separation of LDH isoenzymes with 30, 50, 70, and 90 ml of NaCl-tris solutions in each reservoir. Five peaks of activity were eluted

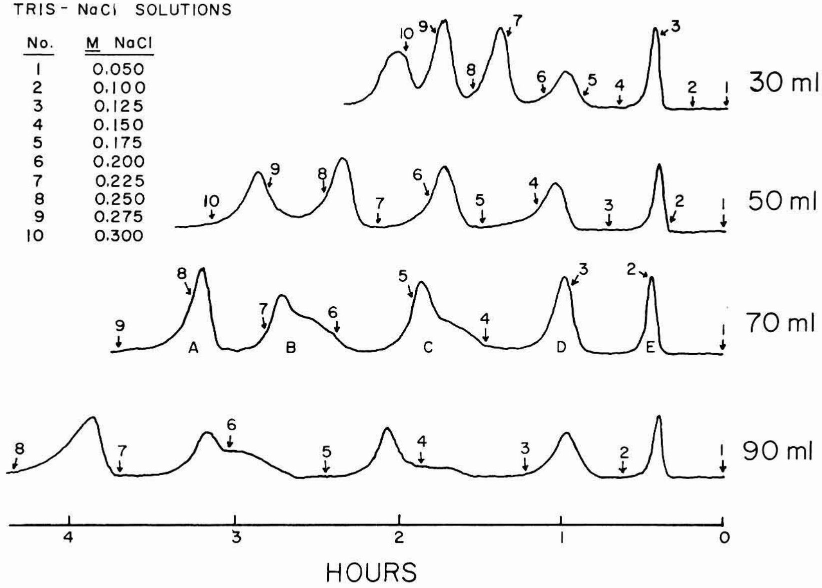

FIG. 2. Recorder tracings of the separation of LDH isoenzymes with different elution programs: The separation of LDH isoenzymes was repeated with 30, 50, 70, and 90 ml of NaCl-tris solution in each reservoir. The numbers indicate at what point on the recording, from right to left, each solution was added to the column.

from the column, from right to left. The arrows show at what point each solution was added to the column. Each recording is with a mixture of heart, liver, and spleen homogenates to give about an equal distribution of the 5 isoenzymes peaks.

With the 30-ml program the procedure was complete in about 2.5 hr, while about 4.5 hr was required for a 90-ml program. The activity did not return to baseline between every peak for the 30- or 50-ml programs as it did for the 70- and 90-ml programs. The separation of the first two peaks eluted, D and E, was not significantly affected by the programs while the last two bands, A and B, were most affected. A 70-ml program was selected for routine use since the activity returned to baseline between each peak with a minimum of time required. With a

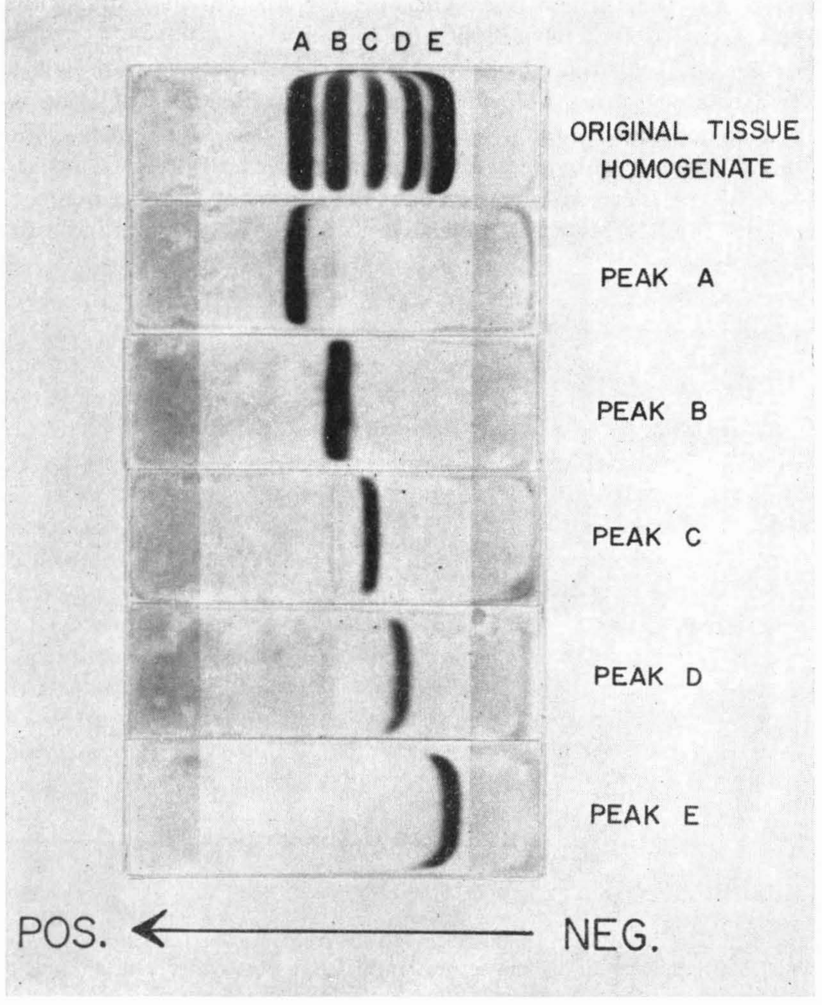

FIG. 3. Agar gel electrophoresis of LDH isoenzymes in a tissue homogenate before and after separation on a DEAE ion-exchange column.

70-ml program, artifacts on peaks B and C begin to appear. This might be, as will be shown later, due to the shape of the stepwise gradient used on the column.

The LDH isoenzymes were identified by collecting the activity under each peak, A through E in the 70-ml program in Fig. 2, and separating each fraction along with the original homogenate by agar gel electrophoresis. The results are shown in Fig. 3, where peak A, which is eluted from the column last, corresponds to the fastest moving electrophoretic LDH band and peak E corresponds to the slowest moving electrophoretic fraction. For LDH, the 5 isoenzymes are eluted from the column in the reverse order of their electrophoretic mobility.

Further to study the column procedure, 5-ml fractions were collected from a column during a 70-ml program separation. The chloride concentration in each fraction was determined with an Aminco-Cotlove automatic titrator (American Instrument Co., Silver Spring, Maryland). A plot of the chloride concentration was superimposed on a recorder tracing of the LDH activity, as shown in Fig. 4. The arrows indicate at

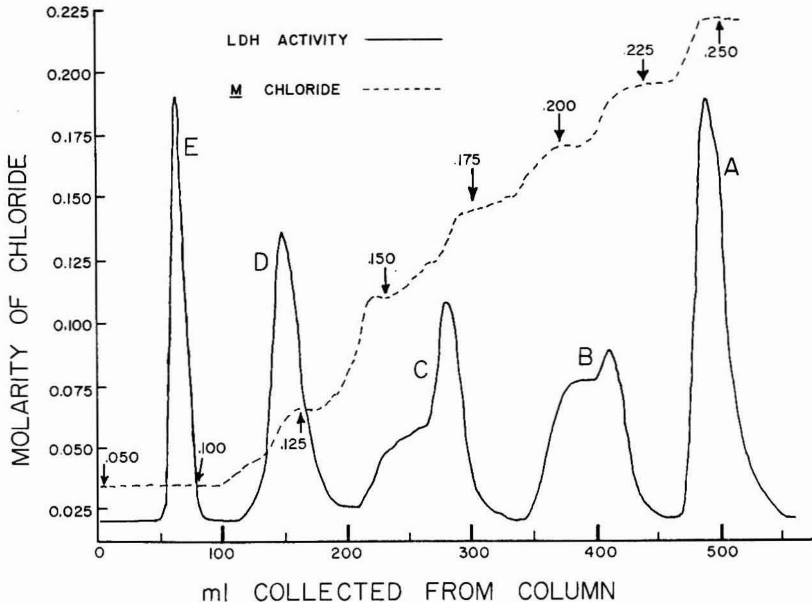

FIG. 4. Study of chloride gradient for 70-ml program: 5-ml fractions were collected from a column during the separation of LDH isoenzymes with a 70-ml program. A plot of chloride concentration was superimposed on the LDH activity recording. Arrows indicate at what point during the procedure each solution was added to the column.

what point each tris-NaCl solution was added to the column. From the data in Fig. 4, it appears that inflections in the chloride concentration might account for the sudden increase in activity during the elution of peaks B and C. Attempts to vary the gradient in nonlinear steps by varying the amounts of tris-NaCl solution in each reservoir did not improve the separation of the LDH isoenzymes. A linear stepwise increase in salt concentration always gave the best results. Because of complications due to gel bed shrinkage (12) during the elution procedure, the stepwise elution of columns, allowing each solution to drain to the gel bed, gave more reproducible separations than initial experiments using a continuous gradient.

Recovery and Distribution of LDH Isoenzymes: Several experiments were performed with different human tissue homogenates to demonstrate the use of the column and enzyme analyzer as a quantitative tool. The total recovery of LDH activity from the column and distribution of the 5 isoenzyme activities were determined for heart, liver, skeletal muscle, spleen, and kidney homogenates.

For quantitative studies, it was necessary to dilute the processed tissue homogenate so that the isoenzyme peaks would not exceed full scale of the enzyme analyzer during the experiment, enabling peak areas to be measured. Before starting a column run dilutions of the processed tissue homogenate were metered in stream S_2 to steady state to determine the activity response of the homogenate. Occasionally, diluted samples of crystalline LDH of known activity were run to calibrate the recorder or to determine the activity of the homogenate in LDH units (11). It was not necessary, as will be shown later, to know the actual activity of a homogenate to make quantitative measurements of recovery and distribution. Generally, 3 ml of homogenate which was about 100 times as active as the acceptable dilution metered in S_2 was applied to the DEAE A-50 column. Since the response of the enzyme analyzer is linear with activity (11), if the sensitivity setting of the enzyme analyzer is left unchanged after calibrating with the diluted homogenate in stream S_2, an area of chart paper which represents the total activity applied to the column can be calculated from the following formula:

$$\text{total area} = CU \times R \times ML \times (S/F)$$

where CU is the chart response obtained at steady state with the diluted homogenate, R is the ratio of the activity of the homogenate applied to the column to the activity of the diluted homogenate used to obtain CU, ML is milliliters of homogenate applied to the column, S is the chart speed in in./min, and F is the flow rate of the column in ml/min. This formula assumes that the column flow rate is constant, which is true

because the column flow is metered by peristaltic pumps. The calculated area is given in units of chart response-inches, chart response being the vertical axis and inches the horizontal.

For each run, the area representing the total activity applied to the column as calculated from the formula above and the areas under each peak were cut out of the recording paper and weighted on an analytical balance. The sum of the weights of the peaks was compared to the total area weight to calculate total per cent recovery. The distribution of activity was calculated from the weight of each peak using the sum of the peaks as 100% of the activity.

Recorder tracings for several homogenates are in Fig. 5. As shown,

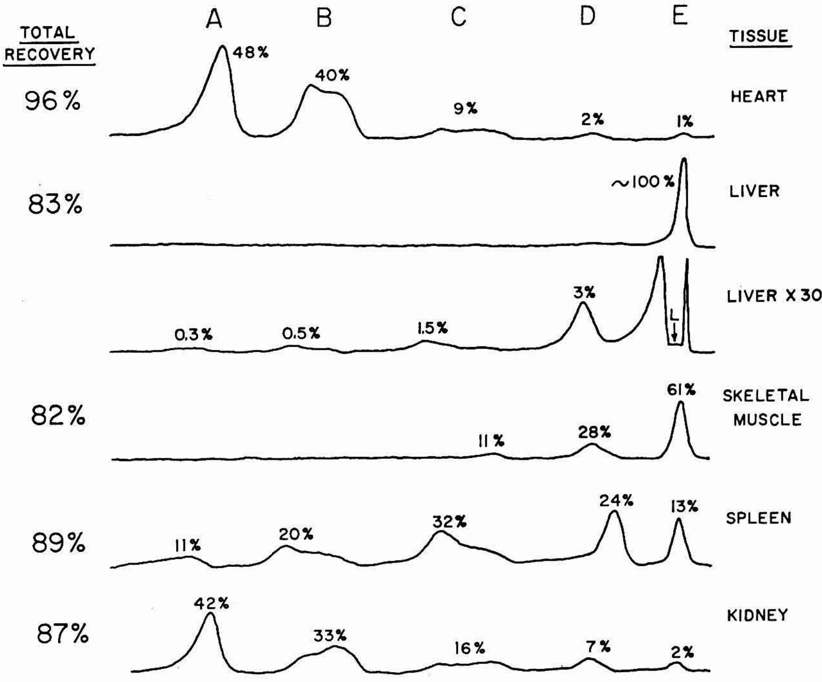

FIG. 5. Study of distribution and total recovery of LDH isoenzymes in tissue homogenates.

heart and kidney are richest in the electrophoretically faster moving fractions, A and B, while skeletal muscle and liver are predominantly the slow-moving fractions. When liver diluted into the analytical range was used, essentially all the activity was in the slowest moving fraction. When a liver homogenate 30 times as active was used, other peaks were detected, as may be seen in the Liver ×30 recording in Fig. 5. The major

peak, E, was so active that all reagents in the enzyme analyzer were depleted, resulting in inversion of the peak response (arrow at L). The figures given on each tracing, with the exception of the Liver ×30 recording, represent the average of at least two separate experiments. Distribution of the major peaks of activity was generally reproducible to about ±1% while total recovery varied by about ±10%. A total recovery significantly greater than 100% was not obtained for any tissue. The highest recoveries were generally obtained with tissues richest in the fastest moving fractions, such as heart and kidney, while lowest recoveries were obtained with tissues rich in the slower, more labile fractions, such as in liver and skeletal muscle.

Separation of Other Isoenzymes: The isoenzymes of malic dehydrogenase (MDH) and transaminase activity, using aspartic acid (GOT activity) and cysteinesulfinic acid (CSA activity) for substrates, were separated. Except for the reagents used in the enzyme analyzer, all conditions were identical as for the separation of LDH isoenzymes with a 70-ml program. Results are shown in Fig. 6. An LDH recording is shown first for reference. The major isoenzyme fractions obtained for GOT (15) and MDH (16) are consistent with previous studies. The

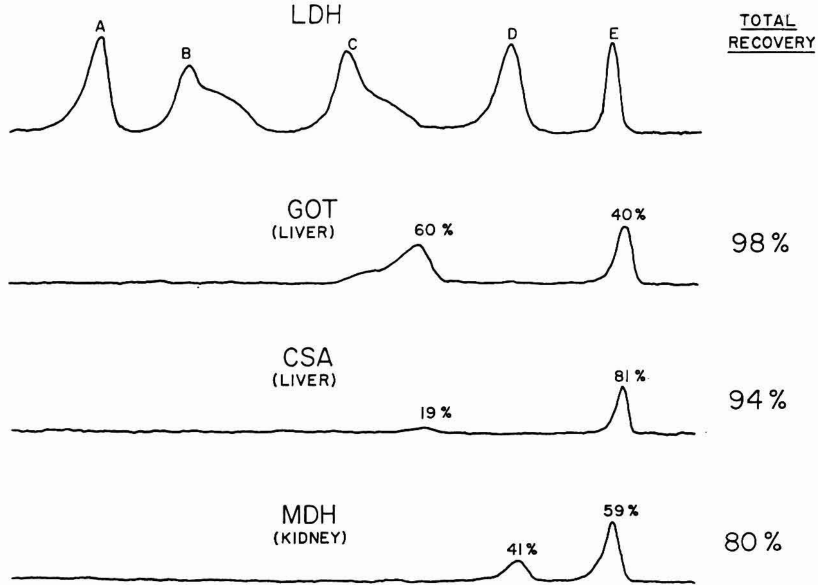

FIG. 6. Separation of other dehydrogenase and transaminase isoenzyme activities: The conditions for the separation of MDH, GOT, and CSA were identical to those for the separation of LDH with the 70-ml program. Only the reagents in the enzyme analyzer were changed.

separation of CSA into two peaks which elute in the same positions as the GOT peaks is consistent with the idea that CSA and aspartic are substrates for the same enzyme, GOT (17). The difference of the distribution of transaminase activity with aspartic and CSA is of interest. There have been some reports of as many as four or five isoenzymes for GOT (18).

Electrophoresis of the MDH peaks showed that the fastest moving fraction was eluted first, an order of elution opposite that for LDH.

DISCUSSION

The use of an enzyme analyzer with an automatic column programer to continuously monitor and quantitate the enzyme activity eluted has been demonstrated. The separations were relatively rapid compared to other column procedures. The method was demonstrated with both dehydrogenase and transaminase enzyme systems.

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REFERENCES

1. Wroblewski, F., ed., *Ann. N. Y. Acad. Sci.* **94,** 655–1030 (1961).
2. Van der Helm, H. J., Zondag, H. A., Hartog, H. A. P. H., and Van der Kooi, M. W., *Clin. Chim. Acta* **7,** 540 (1962).
3. Gelderman, A. H., Gelboin, H. V. and Peacock, A. C., *J. Lab. Clin. Med.* **65,** 132 (1965).
4. Dioguardi, N., Agostoni, A., Fiorelli, G., and Mannucci, P. M., *Enzymol. Biol. Clin.* **4,** 31 (1964).
5. Stranjord, P. E., Clayson, K. J., and Frier, E. R., *J. Am. Med. Assoc.* **182,** 1099 (1962).
6. Dawson, D. M., Goodfriend, T. L., and Kaplan, N. O., *Science* **143,** 929 (1964).
7. Richterich, R., Schofroth, P., and Aebi, H., *Clin. Chim. Acta* **8,** 178 (1963).
8. Pitot, H. C., and Pries, N., *Anal. Biochem.* **9,** 454 (1964).
9. Hoober, J. K., and Bernstein, I. A., *Anal. Biochem.* **9,** 467 (1964).
10. Blaedel, W. J., and Hicks, G. P., *Anal. Chem.* **34,** 388 (1962).
11. Hicks, G. P., and Updike, S. J., *Anal. Biochem.* **10,** 290 (1965).
12. Hicks, G. P., and Nalevac, G. N., *Anal. Biochem.* **12,** 603 (1965).
13. Hicks, G. P., and Blaedel, W. J., *Anal. Chem.* **37,** 354 (1965).
14. Yakulis, V. J., Gibson, C. W., and Heller, P., *Am. J. Clin. Path.* **38,** 378 (1962).
15. Romel, W. C., and La Mancusa, S., J., *Clin. Chem.* **11,** 131 (1965).
16. Englard, S. L., Siegel, L., and Breiger, H. H., *Biochem. Biophys. Res. Commun.* **3,** 323 (1960).
17. Singer, T. P., and Kearney, E. B., *Biochem. Biophys. Acta* **14,** 570 (1954).
18. Decker, L. E., and Rau, E. M., *Proc. Soc. Exptl. Biol. Med.* **112,** 144 (1963).

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Ion-Exchange Thin-Layer Chromatography

XIII. Resolution of Complex Nucleoside Triphosphate Mixtures[1]

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For the purpose of pool size determinations of ribo- and deoxyribonucleoside triphosphates, we have developed a new method having the following characteristics:

1. High resolving power, enabling a complete resolution of all common triphosphate precursors of nucleic acids to be carried out within a short time.

2. Great sensitivity, making possible analyses of small amounts of biological material.

3. Suitability for qualitative and quantitative routine analyses.

4. Reproducibility.

Of all techniques available at the present time, the new method of anion-exchange thin-layer chromatography on PEI-cellulose[2] (2–5) appeared to be most promising. The qualitative (3, 4) and quantitative (5) aspects of this technique have been discussed in detail. A separation of CTP, UTP, ATP, GTP, and ITP from each other and from other mononucleotides can be obtained on PEI-cellulose layers (4). However, a mixture containing also deoxyribonucleoside triphosphates cannot be resolved by this procedure. By the procedures described in the present paper, nine common ribo- and deoxyribonucleoside triphosphates are

[1] This work was supported by a travel grant from the University of Copenhagen to one of us (J. N.) and by grants-in-aid from the U. S. Atomic Energy Commission (AT(30-1)-2643), the U. S. Public Health Service (CA 5018-08), the National Science Foundation (22138), and the Wellcome Trust. This is publication No. 1225 of the Cancer Commission of Harvard University.

[2] A cellulose anion-exchange material obtained by impregnating unmodified or modified cellulose with poly(ethyleneimine) (molecular weight 30,000–40,000) (1). For abbreviations of nucleotides, see "Abbreviated Instructions to Authors" as published in the issues of *J. Biol. Chem.*

completely separated on PEI-cellulose layers from each other and from other mononucleotides. Part of this work has been reported in a preliminary form (6).

EXPERIMENTAL

Materials

All nucleotides were obtained commercially as sodium salts from Sigma Chemical Company, St. Louis, Missouri, and from California Corporation for Biochemical Research, Los Angeles, California. Solvents were prepared from analytical reagent-grade materials. Kodirex X-ray film[3] was used for autoradiography.

Preparation of PEI-Cellulose Plates[4]

Glass plates (3, 4) or plastic sheets (7) were coated using a suspension of cellulose powder MN 300[5] in a poly(ethyleneimine) hydrochloride solution. All plates were given a preliminary ascending wash with NaCl solution followed by water (4). If they were not used within a few days they were stored in darkness in the cold (0° to 4°) (3) or, preferably, at —10° to —20°.

Chromatography

All nucleotide solutions were approximately 0.002 M in water (pH about 6). Samples were applied as described elsewhere (3, 4). Ascending chromatography was carried out in closed rectangular tanks containing solvent to a height of 0.7–1.2 cm. It was not necessary to saturate the tank atmosphere with solvent vapors. After chromatography, the plates were dried in a stream of warm air and then examined and photographed in incident short-wave ultraviolet light (3) in a dark room.

Solvents and Chromatographic Procedures

Two two-dimensional procedures (I and II), which give the desired separation of nucleoside triphosphates, are described here in detail.

Procedure I. The following solvents were used: *Solvent I,1:* 1.0 M LiCl was saturated with boric acid at room temperature (22°) and brought to pH 7.0 by the addition of ammonia (specific gravity 0.90).

[3] Kodak Ltd., London (Great Britain), supplied by Kodak A/S, Copenhagen (Denmark).

[4] Plates prepared from commercial PEI-cellulose powders and commercial PEI-cellulose plates may give separations that are different from those described in this paper.

[5] Macherey and Nagel, Düren (Germany). Obtained from Brinkmann Instruments, Westbury, New York.

Solvent I,2: 2.0 *N* HCOOH/1.6 *M* LiCl (1/1, v/v). *Solvent I,3a:* 0.50 *M* $(NH_4)_2SO_4$. *Solvent I,3b:* 0.70 *M* $(NH_4)_2SO_4$.

After applying the nucleotide solution at the starting spot X (see Fig. 1), the chromatogram was developed with solvent *I,1*. The plate was dried in a stream of warm (50°) air after the solvent front had reached a line about 12 cm above the start. To remove LiCl and borate, the plate was laid for 15 min in a flat dish (25 × 25 cm) containing 800–1000 ml anhydrous methanol. Solution was accelerated by occasional agitating.

After drying, the chromatogram was developed in the same direction with solvent *I,2*. This development was carried out in the cold (0°–5°) and again terminated when the solvent front reached a line 12 cm above the origin. Subsequently, the plate was dried for 4–5 min in a stream of cold air, then for about 3 min in a stream of warm (50°) air and laid in a flat dish containing a solution of 600 mg tris(hydroxymethyl)aminomethane (free base) in 500 ml anhydrous methanol. After 5 min, the plate was dried in a stream of cold air and treated for 10 min with 500 ml anhydrous methanol. The removal of HCOOH and LiCl was again accelerated by agitating.

After drying, parallel lines were scratched into the bottom part of the layer (3), and all parts of the layer not needed for chromatography in the second dimension were scraped off with a sharp spatula (4). Compounds close to the solvent front of the first dimension, e.g., all nucleoside monophosphates, nucleoside diphosphate sugars, CDP, and ADP, are excluded from further chromatography in this way. Development in the second direction was carried out using a stepwise elution procedure (3) with solvents *I,3a* and *3b* at room temperature or at 0°–5° (see Results). Solvent *I,3a* was allowed to ascend to 4 cm above the starting line of the second dimension, and the plate was then transferred without intermediate drying to a tank containing solvent *I,3b*. Chromatography was terminated when the solvent front reached a distance of 12–16 cm from the starting line. After drying, the plate was examined under short-wave ultraviolet light and photographed. The whole procedure takes 4.5 to 5.5 hr.

If only a separation of ribonucleoside polyphosphates was intended, procedure I was simplified as follows: Solvent *I,1* was omitted; solvent *I,2* was run up to 10–12 cm at room temperature or in the cold. Tris/methanol treatment and development in the second dimension were carried out as described above. The procedure takes 2.5 to 3 hr.

Procedure II. The following solvents were used: *Solvent II,1a:* 2.0 *N* acetic acid/2.0 *M* LiCl (1/1, v/v). *Solvent II,1b:* 4.0 *N* acetic acid/2.5 *M* LiCl (1/1, v/v). *Solvent II,2a:* 2.5 *M* ammonium acetate contain-

ing 3% boric acid; the solution was adjusted to pH 7.0 by addition of ammonia (specific gravity 0.90). Solvent *II,2b:* 3.5 *M* ammonium acetate containing 4% boric acid, pH 7.0 (ammonia).

The plate was developed at room temperature in the first dimension using a stepwise elution procedure with solvents *II,1a* and *1b*. The plate was transferred from solvent *II,1a* to solvent *II,1b* when the solvent front reached a line 4 cm above the starting line. When the front was 15 cm above the start, the plate was dried thoroughly—20 min in warm air (40°)—to remove as much of the acetic acid as possible. Subsequently, it was treated for 20 min with 1000 ml anhydrous methanol (as described in Procedure I), dried in a stream of cold air, and "scratched" (see above). Development in the second dimension was carried out at room temperature using solvent *II,2a* up to 4 cm above the starting line followed by solvent *II,2b* up to 14 cm. This procedure takes about 4 hr.

Chromatography of Extracts from Biological Material

The methods described in the present paper can be applied directly to alcohol, trichloroacetic acid, or perchloric acid cell extracts—see also (8). The plates may be given a preliminary methanol treatment (3) and/or a preliminary ascending development with methanol/water (1/4, v/v) up to 10–15 cm in order to remove salts and other interfering substances. Chromatography in the first dimension should be carried out perpendicularly to the methanol/water development.

A separation of labeled nucleoside triphosphates from *E. coli* B grown in the presence of deoxyadenosine (9) was obtained in the following way. The bacteria were grown for two generations anaerobically in low-phosphate broth (0.33 m*M* orthophosphate) containing ^{32}P-labeled orthophosphate (specific activity in the medium: 3 μc/μmole orthophosphate). Glucose (0.2%) was used as the carbon source. At zero time, deoxyadenosine was added to the medium (final concentration 2.5 m*M*) and growth continued. Forty minutes later, a 5-ml aliquot of the culture (corresponding to about 0.6 mg bacterial dry weight) was filtered through a Millipore filter. The filter was immediately immersed in 4.5 ml 5% trichloroacetic acid (0°) and shaken vigorously. After centrifugation for 5 min at 10,000 rpm, the supernatant solution was extracted several times with ether to remove trichloroacetic acid and the water phase was lyophilized. The residue was dissolved in 100 μl water and a 10-μl aliquot was applied to each starting spot of two plates, followed by addition to the same spot of 5 μl of a marker solution containing 5 mμmoles of ATP, GTP, UTP, CTP, dATP, dGTP, dTTP, and dCTP. The plates were treated for 5 min with 500 ml anhydrous methanol (3) and developed in two dimensions according to Procedures I and II, respectively.

Subsequently, autoradiography was carried out (exposure time 24 hr in either case).

Quantitative Analyses

Quantitative spectrophotometric assays of model compounds were carried out using PEI-cellulose on plastic sheets (7). After chromatography (Procedure I), the compounds were transferred from the layer to paper wicks (5) using 15 μl water, followed by 30 μl of 0.7 *M* $MgCl_2$ as eluants. Adjacent blank areas were treated in the same way. One ml of 0.7 *M* $MgCl_2$/2 *M* tris hydrochloride, pH 7.4 (100/1, v/v), was used for elution from the paper wicks. After centrifugation, extinctions at 260 mμ were measured against blank eluates.

^{32}P-Labeled compounds were assayed as follows. After part of the layer surrounding the spot had been scraped off, the spot was moistened with 15–25 μl water, immediately transferred to planchets using a thin metal spatula, and counted in a Frieseke and Hoepfner end-window counter (8).[6]

RESULTS

As seen in Fig. 1, a complete separation of CTP, dCTP, UTP, dTTP, ATP, dATP, GTP, dGTP, and ITP from each other and from diphosphates is obtained by Procedure I. There is also no overlapping with orthophosphate, nucleoside monophosphates, sugar nucleotides, DPN, and TPN (not shown in Fig. 1).

The borate system (solvent *I,1*) separates deoxyribonucleoside triphosphates from their ribonucleoside analogs (and also corresponding ribonucleoside and deoxyribonucleoside diphosphates). The rate of migration decreases in the order: deoxyribonucleoside diphosphates > ribonucleoside diphosphates > deoxyribonucleoside triphosphates > ribonucleoside triphosphates containing the same base. With regard to the base the following sequence is observed: uridine (thymidine) compounds > cytidine compounds > adenosine compounds > guanosine compounds. Because ribonucleoside diphosphates are very close to corresponding deoxyribonucleoside triphosphates, there is some overlapping between diphosphates and triphosphates.

The main effect of the subsequent development with the formic acid system (solvent *I,2*) is a clear class separation of diphosphates from triphosphates (3). After chromatography with this solvent, the slowest diphosphate (GDP) is clearly ahead of all triphosphates. The migration rate is, to a lesser extent, also influenced by the base moiety of the nucleotides (3).

[6] Frieseke and Hoepfner, Erlangen (Germany).

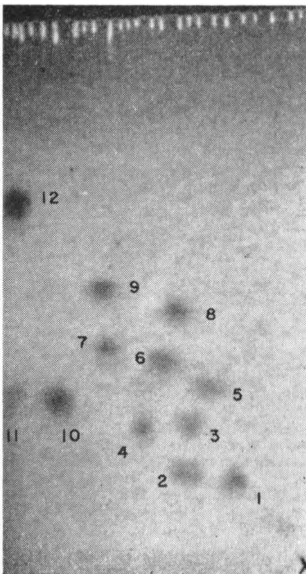

FIG. 1. Two-dimensional separation of ribo- and deoxyribonucleoside polyphosphates on a 0.5-mm PEI-cellulose layer. Applied to the starting spot X: 5–10 mμmoles of each compound. Development: Procedure I (see text). First dimension from right to left, 12 cm. Second dimension from bottom to top, 13 cm, at +2°. Compounds: 1 = GTP; 2 = dGTP; 3 = ATP; 4 = dATP; 5 = ITP; 6 = CTP; 7 = dCTP; 8 = UTP; 9 = dTTP; 10 = GDP; 11 = dGDP (impurity in the commercial dGTP used); 12 = UDP. Photographed by short-wave ultraviolet light.

Subsequent development with the ammonium sulfate system (solvents *I,3a* and *3b*) in the second dimension separates chiefly according to the base moiety; migration rates decrease in the order: uridine (thymidine) compounds > cytidine compounds > adenosine compounds > guanosine compounds. Diphosphates precede corresponding triphosphates. The pentose moiety is only of minor importance. In addition, the migration rates of some triphosphates are temperature dependent. By carrying out the chromatography at 0°–5° instead of at room temperature, the separation of adenosine triphosphates from cytidine triphosphates can be improved. Increasing the $(NH_4)_2SO_4$ concentration (0.8 *M*, no stepwise elution) also increases the distance between ATP and CTP. These phenomena can be interpreted as "salting out" effects (10). In the case of biological extracts one can take advantage of these effects if a quantitative determination of CTP in the presence of a large excess of ATP is intended (compare Figs. 3 and 4).

Figure 2 demonstrates a separation of ribonucleoside di- and triphosphates using the simplified Procedure I, which does not separate

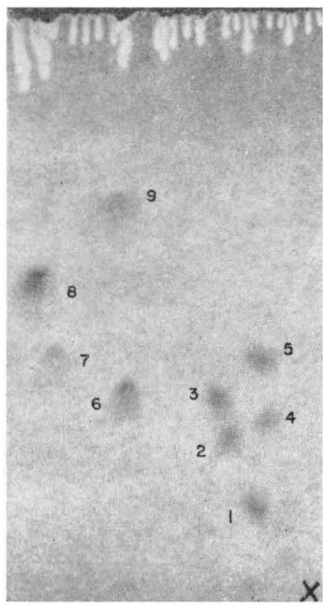

FIG. 2. Two-dimensional separation of ribonucleoside polyphosphates on a 0.5-mm PEI-cellulose layer. Applied to the starting spot X: 5–15 mμmoles of each compound. Development: simplified Procedure I (see text). First dimension from right to left, 10 cm. Second dimension from bottom to top, 13 cm, at +22°. Compounds: 1 = GTP; 2 = ATP; 3 = CTP; 4 = ITP; 5 = UTP; 6 = GDP; 7 = ADP; 8 = CDP; 9 = UDP. Photographed by short-wave ultraviolet light.

deoxyribonucleotides from ribonucleotides due to the omission of borate. The development in the second dimension was carried out at room temperature; a comparison between Figs. 1 and 2 shows how the migration rates of ATP and CTP are influenced by the different temperatures.

A comparison of Figs. 1 and 3 with Fig. 4 (see below) shows that Procedure II results in a spot pattern completely different from the spot pattern obtained with Procedure I. This is partly due to the fact that separation of deoxyribonucleotides from corresponding ribonucleotides is being achieved in the first dimension in Procedure I and in the second dimension in Procedure II. Although the over-all resolution obtained by Procedure I is more distinct, a combined use of both procedures might facilitate the identification of unknown compounds.

Figures 3 and 4 show autoradiograms of chromatograms of a ^{32}P-labeled extract from *E. coli* B. The chromatograms, each corresponding to about 0.06 mg bacterial dry weight, were developed according to Procedures I and II, respectively. It can be seen that all common ribonucleoside and deoxyribonucleoside triphosphates, except dGTP, show

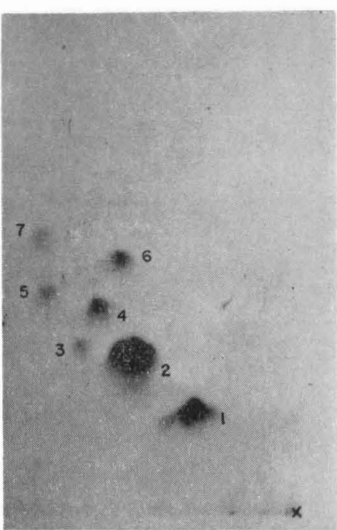

FIG. 3. Autoradiogram of a two-dimensional separation of ^{32}P-labeled ribo- and deoxyribonucleoside triphosphates on a 0.5-mm PEI-cellulose layer. Applied to the starting spot X: 10 μl of a trichloroacetic acid extract of *E. coli* B (see text for details of growth and extraction). Development: Procedure I. First dimension from right to left, 12 cm. Second dimension from bottom to top, 13 cm, at +2°. Exposure time: 24 hr on Kodirex X-ray film. Compounds: 1 = GTP; 2 = ATP; 3 = dATP; 4 = CTP; 5 = dCTP; 6 = UTP; 7 = dTTP.

up on the autoradiograms after an exposure time of 24 hr. The radioactivity of ^{32}P-labeled ATP and dTTP on the chromatograms (autoradiograms, Figs. 3 and 4) was determined as described above. The amounts calculated from the radioactivity of the spots and the known specific activity of ^{32}P-labeled orthophosphate in the medium were as follows: the ATP spot corresponded to 0.2 mμmole and the dTTP spot to 0.016 mμmole.

Using the procedure for quantitative spectrophotometric assay (5), recoveries of model compounds chromatographed according to Procedure I were generally found to be 90% or more. The small loss of nucleotide material might arise from the methanol treatments. Amounts determined were in the range of 7–10 mμmoles.

The limit of detection under short-wave ultraviolet light is, in the case of Procedure I, about 0.5–2 mμmoles of each triphosphate depending upon the specific extinction coefficients of the nucleotides.

Separations on PEI-cellulose layers are highly reproducible; plates prepared from seven different batches of poly(ethyleneimine) and from more than ten batches of cellulose powder gave practically identical

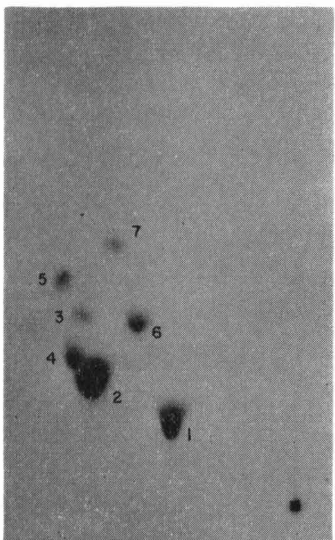

FIG. 4. Autoradiogram of a two-dimensional separation of ^{32}P-labeled ribo- and deoxyribonucleoside triphosphates on a 0.5-mm PEI-cellulose layer. Applied to the starting spot X: 10 μl of a trichloroacetic acid extract of *E. coli* B (see text for details of growth and extraction). Development: Procedure II. First dimension from right to left, 15 cm. Second dimension from bottom to top, 13 cm. Exposure time: 24 hr on Kodirex X-ray film. Compounds: 1 = GTP; 2 = ATP; 3 = dATP; 4 = CTP; 5 = dCTP; 6 = UTP; 7 = dTTP.

results. In contrast, we found that DEAE- and ECTEOLA-cellulose preparations for thin-layer chromatography obtained from different companies showed large variations from batch to batch, and even different lots obtained from the same company were found to differ in chromatographic performance—see also (11).

DISCUSSION

In order to compare the novel method described in the present paper with column chromatography, paper chromatography, and paper electrophoresis of nucleoside triphosphates, it seems appropriate to outline briefly the results that can be obtained by these conventional methods—see also (12).

Column Chromatography of Nucleoside Triphosphates (13–22)

No separation of all eight common ribo- and deoxyribonucleoside triphosphates from each other and from other mononucleotide classes on a single column has been reported. Using polystyrene-type (13–20) or cellulose-type (21, 22) anion-exchange columns, separations of three

or four triphosphates from each other and from the diphosphate group have been described, but there was no separation of corresponding deoxyribo and ribo compounds. Cohn and Bollum (16) separated some deoxyribo- and ribonucleoside monophosphates from each other on an anion-exchange column using a borate system.

Paper Chromatography of Nucleoside Triphosphates (23–29)

For resolution of complex nucleotide mixtures, time-consuming development in two dimensions is required. Although paper chromatography can be used for analysis of some ribonucleoside triphosphates in biological extracts, a separation of all common ribo- and deoxyribonucleoside triphosphates from each other and from other mononucleotide classes is not possible by present techniques of paper chromatography.

Paper Electrophoresis of Nucleoside Triphosphates (30)

Paper electrophoresis, although capable of separating a number of acid-soluble tissue nucleotides, seems to offer no advantage when compared with paper chromatography of nucleoside triphosphates. No satisfactory separations of these compounds by paper electrophoresis have been reported in the literature.

Combination of Different Conventional Techniques

For determinations of nucleoside triphosphates, a combination of column chromatography, paper chromatography, and spectrophotometry (8, 31, 32) or of column chromatography, colorimetry, and spectrophotometry (29) has been used. These combined techniques are, however, not well suited for routine determinations of nucleoside triphosphates in a great number of samples, because each analysis of an individual compound requires from several days to two weeks.

It is clear from the preceding paragraphs that the separation of all common ribo- and deoxyribonucleoside triphosphates cannot be accomplished by one conventional procedure alone.

The results presented demonstrate that anion-exchange thin-layer chromatography on PEI-cellulose complies with our requirements. Complex mixtures of mμmole quantities of ribonucleoside and deoxyribonucleoside triphosphates can be separated in a few hours. The method is thus much faster and more sensitive than conventional separation techniques for these compounds. The procedures described can be modified to resolve similarly complex mixtures of nucleoside monophosphates and diphosphates.[7] The method can be applied directly to tissue ex-

[7] Unpublished experiments.

tracts and is highly reproducible. In combination with quantitative assay procedures, it should be useful for triphosphate determinations.

SUMMARY

CTP, dCTP, UTP, dTTP, ATP, dATP, GTP, dGTP, and ITP can be completely separated from each other and from other mononucleotides by two-dimensional anion-exchange thin-layer chromatography on PEI-cellulose. The chromatograms can be evaluated quantitatively and recoveries exceed 90%. Extracts from biological material can be chromatographed directly.

ACKNOWLEDGMENTS

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REFERENCES

1. Randerath, K., *Angew. Chem.* **74,** 780 (1962), *Intern. Ed.* **1,** 553 (1962).
2. Randerath, K., *Biochim. Biophys. Acta* **61,** 852 (1962).
3. Randerath, K., and Randerath, E., *J. Chromatog.* **16,** 111 (1964).
4. Randerath, E., and Randerath, K., *J. Chromatog.* **16,** 126 (1964).
5. Randerath, E., and Randerath, K., *Anal. Biochem.* **12,** 83 (1965).
6. Randerath, K., and Neuhard, J., *Federation Proc.* **24,** 669 (1965).
7. Randerath, K., and Randerath, E., *in* "Nucleic Acids," a volume of "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.). Academic Press, New York, in preparation.
8. Neuhard, J., and Munch-Petersen, A., *Biochim. Biophys. Acta,* in press.
9. Munch-Petersen, A., and Neuhard, J., *Acta Chem. Scand.* **17,** 891 (1963).
10. Randerath, K., *Experientia* **20,** 406 (1964).
11. Fahn, S., Albers, R. W., and Koval, G. J., *Anal. Biochem.* **10,** 468 (1965).
12. Saukkonen, J. J., *Chromatog. Rev.* **6,** 53 (1964).
13. Cohn, W. E., *J. Am. Chem. Soc.* **72,** 1471 (1950).
14. Cohn, W. E., *in* "The Nucleic Acids" (E. Chargaff and J. N. Davidson, eds.), Vol. 1, p. 211. Academic Press, New York, 1955.
15. Cohn, W. E., *in* "Chromatography" (E. Heftmann, ed.), p. 554. Reinhold, New York, 1961.
16. Cohn, W. E., and Bollum, F. J., *Biochim. Biophys. Acta* **48,** 588 (1961).
17. Hurlbert, R. B., Schmitz, H., Brumm, A. F., and Potter, V. R., *J. Biol. Chem.* **209,** 23 (1954).
18. Plaisted, P. H., and Reggio, R. B., *Contrib. Boyce Thompson Inst.* **22,** 71 (1963).
19. Anderson, N. G., Green, J. G., Barber, M. L., and Ladd, F. C., Sr., *Anal. Biochem.* **6,** 153 (1963).
20. Schnitger, H., Papenberg, K., Ganse, E., Czok, R., Bücher, T., and Adams, H., *Biochem. Z.* **332,** 167 (1959).
21. Staehelin, M., *Biochim. Biophys. Acta* **49,** 11 (1961).
22. Davey, C. L., *Biochim. Biophys. Acta* **61,** 538 (1962).

23. THOMSON, R. Y., *in* "Chromatographic and Electrophoretic Techniques" (I. Smith, ed.), Vol. 1, p. 231. Interscience, New York, 1960.
24. BERGQUIST, R., AND DEUTSCH, A., *Acta Chem. Scand.* **9,** 1398 (1955).
25. TSUBOI, K. K., AND PRICE, T. D., *Arch. Biochem. Biophys.* **81,** 223 (1959).
26. TSUBOI, K. K., *Arch. Biochem. Biophys.* **83,** 445 (1959).
27. MOSCARELLO, M. A., LANE, B. G., AND HANES, C. S., *Canad. J. Biochem. Physiol.* **39,** 1755 (1961).
28. PLESNER, P., *Acta Chem. Scand.* **9,** 197 (1955).
29. MUNCH-PETERSEN, A., AND NEUHARD, J., *Biochim. Biophys. Acta* **80,** 542 (1964).
30. KLOUWEN, H. M., *J. Chromatog.* **7,** 216 (1962).
31. GOLDSTEIN, D. B., BROWN, B. J., AND GOLDSTEIN, A., *Biochim. Biophys. Acta* **43,** 55 (1960).
32. FRANZEN, J. S., AND BINKLEY, S. D., *J. Biol. Chem.* **236,** 515 (1961).

ANALYTICAL BIOCHEMISTRY 13, 223–228 (1965)

Analysis of DNA Preparations by a Variation of the Cysteine-Sulfuric Acid Test

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Characteristic absorption spectra are given by carbohydrates and carbohydrate-containing substances on reaction with cysteine and sulfuric acid (1–8). Various methods using these reagents have been employed for the estimation of deoxyribonucleic acid (DNA) (1–3, 7–9); they differ principally in the final concentration of sulfuric acid, which affects the position and intensity of the absorption maximum. This report describes a variation used during an investigation of the nucleic acids of *Bordetella pertussis* bacteria (10). The reaction conditions were similar to those used in the "CyRI" method of Dische and his co-workers (4, 5), but the results for DNA were different, owing to the presence of chloride ions in the test solution. As a result, a strong absorption at 472 mμ was obtained with DNA; cysteine was not necessary for the reaction. The test also gave an approximate measure of the extent of contamination by ribonucleic acid (RNA) and aldohexose, and allowance for their slight contribution to the DNA absorption could be made.

METHOD

The material to be tested was dissolved in an aqueous solution, 0.1 M in sodium chloride and 0.01 M in trisodium citrate, which had been adjusted previously to neutrality with acetic acid. A solution of water in concentrated sulfuric acid (1:6, v/v; 2.25 ml) was mixed in small portions with the test solution (0.47 ml), with cooling in an ice-water bath. Together with a "blank" prepared just beforehand, the test mixture was held at 0° for a further 3 min. Both solutions were then left standing at room temperature for 3.5 min, were heated in a boiling water bath for exactly 3 min, and were cooled rapidly under running water. After allowing a few minutes for dispersal of air bubbles, the spectrum was read on a Unicam SP 500 spectrophotometer, using 5 mm cells.

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The contents of the cells were then drained back into the liquid remaining in the test tubes, and the cells were cleaned and dried. A solution of L-cysteine hydrochloride (3% w/v in water, 0.05 ml) was added quickly to the blank and mixed in rapidly by shaking. The sample solution was immediately treated in the same way. After a short pause to allow air bubbles to disperse, both solutions were transferred to the cells and the spectrum was again measured.

RESULTS

The results of the first stage of the reaction, that is before addition of cysteine hydrochloride, will be considered first. The spectra obtained with DNA, RNA, and the galactose-containing polysaccharide, agar, are shown in Fig. 1. The strong absorption given by DNA at 472 mμ is

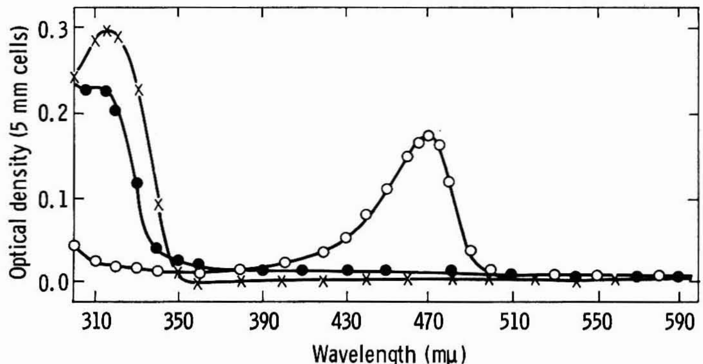

FIG. 1. Spectra given by DNA (○—○), RNA (●—●) and agar (×—×), before addition of cysteine. Concentrations per ml of test solution, prior to adding sulfuric acid solution, were 93 μg Na-DNA, 134 μg Na-RNA, and 43 μg agar. The DNA, from an avirulent strain of *B. pertussis* (10), was undenatured, was contaminated with about 0.5% of RNA and 2% of protein, and was free from aldohexose.

interesting. In the presence of cysteine hydrochloride, a weaker absorption has been obtained at this wavelength at a higher final concentration of sulfuric acid (6), and becomes more intense as the acid concentration is further increased (7, 8). An absorption at 375 mμ can also build up slowly (1, 3). At lower acid concentrations, the absorption maximum is at 490 mμ (1, 2). Chloride ions were probably present only in small amounts in these variations of the method, whereas in the present work the test solution was 0.1 *M* with respect to sodium chloride. Table 1 shows that the absorption at 472 mμ is greatly increased by chloride ions. Citrate, sulfate, and sodium ions are not effective at the concentrations tested. Chloride ions are often present in DNA preparations, and

TABLE 1

EFFECT OF ADDED SALTS ON ABSORPTION GIVEN BY DNA AT 472 mμ, BEFORE ADDING CYSTEINE

Various salts were added to aliquots of a solution of salt-free DNA in water (93 μg Na-DNA/ml), to give the molarities shown. The solutions were then treated with sulfuric acid-water (6:1 v/v) as described in the text. SSC = 0.15 *M* in sodium chloride and 0.015 *M* in trisodium citrate.

Salt and molarity	Optical density at λ_{max} (approx. 472 mμ in all cases)
No salt added	0.019
0.17 × SSC	0.134
0.50 × SSC	0.163
0.67 × SSC	0.169
1.0 × SSC	0.168
2.0 × SSC	0.178
0.15 *M* sodium sulfate	0.022
0.15 *M* trisodium citrate	0.019
0.15 *M* sodium chloride	0.176
0.15 *M* L-cysteine hydrochloride	0.100

it is clear that their concentration must be controlled in this, and perhaps in other variations of the cysteine-sulfuric acid test for DNA.

The absorption maximum at 472 mμ was obtained in the absence of added cysteine, whereas it appears generally to be assumed that cysteine is necessary for this reaction. The 2% of protein in the DNA would not contribute any appreciable amount of this amino acid, and it may be concluded that the presence of cysteine is not necessary. This is in agreement with observations made by Bakay *et al.* (8) and by Staron and co-workers (11). Cysteine may in fact partially inhibit the increase in absorption due to chloride ions (Table 1).

The variation of absorption at 472 mμ with concentration of DNA is shown in Fig. 2. A linear relationship is obtained between 20 and 480 μg of DNA per ml. The base compositions of the DNA from virulent and avirulent strains of *B. pertussis* (10) and from herring sperm differ considerably, but do not have any marked effect on the extinction coefficients.

The absorptions given by RNA and a hexose polysaccharide at 472 mμ are not entirely negligible (Fig. 1). Thus contamination of a DNA sample by an equal weight of either of these substances would raise the absorption at 472 mμ by about 5%. The extent of contamination by RNA and aldohexose can be estimated by comparing the spectra obtained before and after adding cysteine hydrochloride. The DNA spectrum remains virtually unaltered on addition of cysteine, whereas with RNA and agar the absorptions at around 315 mμ are replaced by peaks at 390 and 410 mμ, respectively. Difference spectra illustrating these points

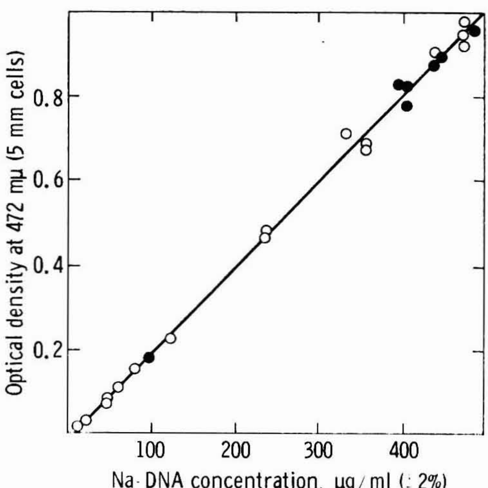

FIG. 2. Correlation of DNA concentration and optical density at 472 mμ: (○) herring sperm DNA, (●) DNA from virulent or avirulent strains of *B. pertussis*. The concentrations are those in the test solution (0.1 *M* in NaCl and 0.01 *M* in Na citrate) before adding sulfuric acid solution. Optical densities were measured in 5-mm cells 15 min after heating with sulfuric acid, and cysteine was absent. Any absorption due to RNA or hexose in the samples was deducted from the readings.

are shown in Fig. 3. The presence of pentose and aldohexose in a DNA preparation will be revealed, therefore, in a difference spectrum of this type by a negative absorption in the 315 mμ region and a positive absorption at around 400 mμ. The exact positions of the peaks will depend on the relative amounts of pentose and hexose; their heights will be a measure of the extent of contamination. The difference between the optical density differences at 390 and 422 mμ ($\Delta OD_{390} - \Delta OD_{422}$), is very small for agar and DNA, whereas it is highly positive for RNA (Fig. 3). It can therefore be used to calculate the RNA concentration, although with an accuracy limited by slight deviations from zero of the value of ($\Delta OD_{390} - \Delta OD_{422}$) for hexoses (3, 6). The RNA contribution can then be deducted from the optical density difference at 410 mμ, to allow an estimate of the hexose concentration. This type of calculation was used previously by Dische (1, 3) and Brody (6). The accuracy of the results will be further limited by variation in the extinction coefficients for different hexoses (4); pentose may be present in polysaccharides as well as in RNA.

The reaction products absorbing at 390 and 410 mμ are unstable, and the timing of readings is therefore important. The peak at 390 mμ given by RNA decays much more rapidly than the 410 mμ hexose peak, and

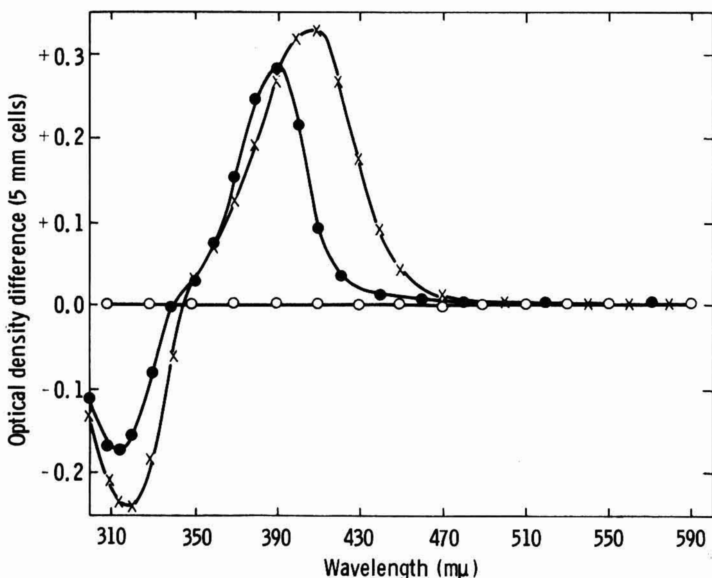

FIG. 3. Differences between spectra obtained in presence and absence of cysteine, for DNA (○—○), RNA (●—●), and agar (×—×). Results shown in Fig. 1 were deducted from those obtained for the same solutions after adding cysteine hydrochloride. The latter spectra were read between approximately 4 and 20 min after addition of cysteine.

this makes possible an alternative, but rather complicated, method of calculating the amounts of these substances (10). In the case of DNA, an absorption peak at 375 mμ builds up very slowly after addition of cysteine. The ratio of water to sulfuric acid in the final mixture must be controlled, because this affects the intensities and stabilities of the various absorption maxima (3, 7, 8). The slightly lower ratio of sulfuric acid to water might explain the lack of reactivity of pentoses in the "CyRI" method of Dische *et al.* (4).

DISCUSSION

The experiments show that chloride ions increase the intensity of the absorption at 472 mμ obtained with DNA. The amount of absorption is also dependent on the final sulfuric acid concentration (7, 8), and a higher acid concentration with chloride present might have given even more favorable results. It is likely that chloride ions are not *essential* for the reaction with DNA, since some absorption at 472 mμ was obtained even in their apparent absence (Table 1). They were probably also absent in a method described by Staron *et al.* (11), who used a higher concentration of sulfuric acid and no cysteine.

The approximate extent of contamination by RNA (pentose) and aldohexose is measured by comparing spectra before and after adding cysteine. Other classes of carbohydrate could probably also be characterized in this way, and earlier reports (1, 3–5) give some indication of the results to be expected for them. More information is required, however, on the effect of chloride, and perhaps other ions, on this and other variations of the cysteine-sulfuric acid test. Dische and co-workers have shown that chloride ions can affect the reaction with hexoses (4); preliminary tests with ribose suggest that they have little effect on the pentose reaction.

SUMMARY

DNA can be estimated by reaction with sulfuric acid in the presence of chloride ions. The intensity of absorption, at 472 mμ, is dependent on chloride ion concentration; cysteine is not necessary for the reaction. The extent of contamination by RNA and aldohexose can be approximately estimated by comparing the spectra obtained before and after adding cysteine.

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REFERENCES

1. Dische, Z., *in* "The Nucleic Acids" (E. Chargaff and J. N. Davidson, eds.), Vol. 1, p. 285. Academic Press, New York, 1955.
2. Stumpf, P. K., *J. Biol. Chem.* **169,** 367 (1947).
3. Dische, Z., *J. Biol. Chem.* **181,** 379 (1949).
4. Dische, Z., Shettles, L. B., and Osnos, M., *Arch. Biochem.* **22,** 169 (1949).
5. Dische, Z., *J. Biol. Chem.* **204,** 983 (1953).
6. Brody, S., *Acta Chem. Scand.* **7,** 495 (1953).
7. Brody, S., *Acta Chem. Scand.* **7,** 502 (1953).
8. Bakay, B., Kirschner, L., and Toennies, G., *Arch. Biochem. Biophys.* **65,** 500 (1956).
9. Keir, H. M., and Smellie, R. M. S., *Biochim. Biophys. Acta* **35,** 405 (1959).
10. Bacon, M. F., Ph.D. Thesis, University of London, 1963.
11. Staron, T., Allard, C., and Chambre, M. M., *Compt. Rend.* **254,** 765 (1962).

ANALYTICAL BIOCHEMISTRY 13, 229–233 (1965)

On the Activation Spectrum for DNA Phosphorescence

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Deoxyribonucleic acid (DNA) has a relatively low phosphorescent yield. In studying the ultraviolet-activated emission of DNA, or indeed of any substance with a low quantum yield, one is faced with the following question. Is the emission really due to the DNA or is it perhaps due to an impurity with a high quantum yield? A partial answer to this question may be obtained by showing that the activation spectrum for emission is identical to the absorption spectrum. The reason that this is but a partial answer is that the energy, after absorption by DNA, may be transferred to other molecules that may be present (1).

This paper describes a relatively simple procedure for showing that the activation spectrum of DNA phosphorescence is identical to its optical absorption spectrum. The method should be generally applicable to phosphorescence studies of other substances.

MATERIAL AND METHODS

Commercial salmon sperm was purified by methods described by Marmur (2). The DNA (A-Grade, Calbiochem.) was dissolved at 4°C in 0.0015 M citrate buffer, pH 7, containing 0.015 M NaCl. The solution was clarified by centrifugation at $12{,}000 \times g$ and the supernatant retained. NaCl was added to give a final concentration of 1 M. The DNA was precipitated by adding an equal volume of 95% ethanol. It was redissolved in the above-mentioned citrate buffer. Sodium acetate and EDTA at pH 7 were added to give a final concentration of 0.3 M sodium acetate and 10^{-4} M EDTA. The DNA was precipitated by adding 0.6 vol of isopropyl alcohol and collected in the manner described by Marmur (2). It was redissolved in the citrate buffer and dialyzed at 4° against 10 vol of 10^{-2} M NaCl for four days with at least four changes of the external bath. The DNA was concentrated about fourfold in a Buchler flash evaporator at 36°. Aliquots were stored at —25° until used.

To test for residual amino acids, aliquots containing 72 mg of DNA were analyzed on an amino acid analyzer. No detectable amounts of

tyrosine or phenylalanine were found although trace amounts of other amino acids at the level of 0.01 to 0.02% were observed.

Residual ribonucleic acid (RNA) was determined by incubating samples in alkali according to the method of Schmidt and Thannhauser (3) and fractionating on a Sephadex G-25 column. By this method the residual RNA content was estimated to be 1.5%.

All phosphorescent measurements were made using an Aminco-Keirs Spectrophosphorimeter with a photomultiplier photometer manufactured by Hruska Radio Company, Lutherville, Maryland.

Spectra were taken at 77°K in a glass containing equal volumes of glycerol and water. The DNA concentration was 0.5 mg/ml. The size and shape of the sample tubes are important and will be discussed below.

Discussion of Instrumental Response

The response, S, of the photomultiplier may be written in the form:

$$S = KqI_0\phi \tag{1}$$

where I_0 = exciting light intensity in quanta per unit time per unit area cross section, ϕ = fraction of the incident energy absorbed, and q = quantum yield for phosphorescence. K is essentially a geometrical factor depending on the relationship of the emitted light to the detecting monochromator slits. Our considerations are independent of the absolute value of K.

I_0 and ϕ are both functions of λ, the wavelength of the exciting light. It is important for the analysis presented here that K not be a function of λ. In using a phosphorimeter in which the exciting and detecting monochromators are perpendicular to each other, K will become a function of λ if sample tubes of too large a diameter are used. To see this, consider a concentrated sample. If the sample is excited at the wavelength of maximum light absorption, a large fraction of the phosphorescence will occur near the surface of the sample closest to the light. At longer wavelengths where the absorbance is lower, the exciting light will penetrate farther into the sample. For samples positioned symmetrically with respect to the exit slits, K will be larger in this instance than in the former case. Thus, if sample tubes with too large a diameter are used, K will be a function of λ. In practice, we have found that sample tubes of 0.6 mm diameter or less are adequate for use with DNA samples of 0.5 mg/ml concentration. With such samples the activation maximum occurs at 275 mμ. The activation maximum of samples in tubes of a larger diameter is shifted to longer wavelengths. As an example, we have found that in sample tubes of 3 mm diameter the maximum is at 290 mμ.

Consider a coordinate axis with z as the symmetry axis of a cylindri-

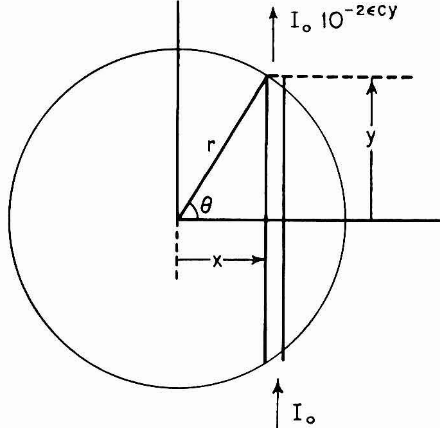

FIG. 1. Cross section of sample tube. Sample is irradiated by light of intensity I_0. In any interval, dx, the intensity after traversing the sample is $I_0\ 10^{-2\epsilon cy}$.

cal sample tube of radius r and height h (Fig. 1). If E = quanta absorbed per unit time:

$$E = 2hrI_0 - 2hI_0 \int_0^r 10^{-2\epsilon cy} dx \tag{2}$$

where ϵ is the molar extinction coefficient and c is the concentration of the sample. Let:

$$\mu = 2 \times 2.3\epsilon c$$

Then:

$$E = 2hrI_0[1 - g(\mu r)] \tag{3}$$

where

$$g(\mu r) = \int_0^{\pi/2} e^{-\mu r \sin\theta} \sin\theta\, d\theta \tag{4}$$

Expanding the exponential and integrating:

$$g(\mu r) = \sum_{n=0}^{\infty} (-1)^n \frac{2n}{n!(n+1)!} \left[\Gamma\left(\frac{n+2}{2}\right)\right]^2 (\mu r)^n \tag{5}$$

For values of μr of interest, the series converges rapidly and numerical values of $g(\mu r)$ are easily obtained. $g(\mu r)$ is plotted in Fig. 2.

S is then given by:

$$S = 2hrKqI_0[1 - g(\mu r)] \tag{6}$$

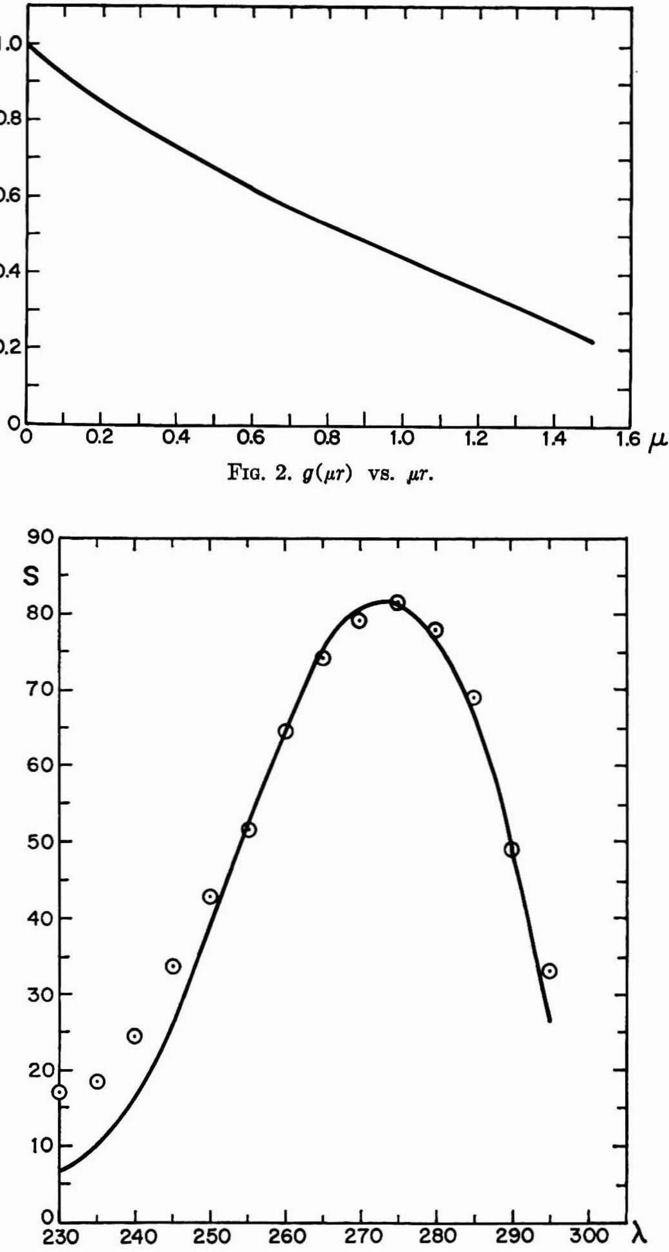

FIG. 2. $g(\mu r)$ vs. μr.

FIG. 3. Correlation of absorbance of DNA and activation of DNA phosphorescence. The curve is the activation spectrum predicted by equation (6). The points are experimentally observed values normalized at 275 mμ. The emission monochromator was set at 450 mμ.

EXPERIMENTAL RESULTS

Relative values of I_0 were obtained by using quinine bisulfate and rhodamine B as quantum counters (4). μ was obtained from the optical absorbance as measured on diluted DNA samples in a Cary spectrophotometer. The wavelength dependence of S predicted from equation (6) is compared with the observed activation in Fig. 3. It may therefore be concluded that the activation spectrum for phosphorescence is the same as the absorption spectrum of DNA.

ACKNOWLEDGMENT

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REFERENCES

1. Isenberg, I., Leslie, R. B., Baird, S. L., Jr., Rosenbluth, R., and Bersohn, R., *Proc. Natl. Acad. Sci. U. S.* **52**, 379 (1964).
2. Marmur, J., *J. Mol. Biol.* **3**, 208 (1961).
3. "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.) Vol. III, p. 678. Academic Press, New York, 1957.
4. Melhuish, W. H., *J. Opt. Soc. Am.* **52**, 1256 (1962).

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Radiometric Assay of Tyrosinase and Theoretical Considerations of Melanin Formation[1,2]

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One of the most fascinating problems in the biochemical aspects of pigmentation is the study of tyrosinase activity. The more commonly used tyrosinase assay procedures include colorimetric, histochemical, manometric, and radiometric techniques. Among these methods, the manometric determination of oxygen consumption is most popular, presumably due to its simplicity and rapidity. However, as oxygen consumption is a common feature of many enzymic and nonenzymic reactions, it does not provide a sufficiently specific basis for the assay of tyrosinase in a mixed enzyme system, e.g., tissue homogenate. Critical evaluation of other reported assay procedures also indicates the lack of tyrosinase specificity. Theoretically, however, with the fulfillment of certain criteria, the radiometric method would be the most specific and sensitive.

In any tyrosinase assay procedure, the results may be affected by many factors. In a homogenate, among the less obvious variables are substrate autoxidation (1–4), enzyme inhibitor(s) and/or activator(s) (3–26), stability of enzyme preparation (4), naturally occurring oxidizing and reducing agents, amino acids (27), other enzyme systems and their endogenous substrates, and the multimetabolic utilization of the specific substrate added. To meet the critical requirements of a completely extracted enzyme preparation of known stability and the establishment of a valid control incorporating all nonspecific reactions occurring during the course of the given assay conditions, a highly sensitive, specific, and reproducible radiometric tyrosinase assay has been developed. The findings utilizing this method have yielded some insight into the mechanism of melanin formation.

A basic precept of the method is that the tyrosinase activity is represented by the difference between the gross and control radioactivity of material insoluble in trichloroacetic acid (TCA). Such differences

[1] This investigation was supported by White Laboratories, Inc.

[2] Contribution number 132, Department of Biology, Wayne State University.

are considerable with the use of purified mushroom tyrosinase (4). However, the use of goldfish skin with relatively low tyrosinase activity (33) provided a means of critical evaluation of this and other factors during the course of assay development. That TCA-insoluble material represents melanin formed during the course of the assay is based upon the facts that: (*a*) sodium diethyl dithiocarbamate completely inhibits incorporation of labeled substrate, (*b*) oxygen restriction reduces the tyrosinase activity, and (*c*) 6 *N* HCl hydrolysis of the TCA-insoluble material does not reduce net radioactivity. Thus, although it is not possible to confirm directly that newly formed melanin is the end product, the above studies (4) provided strong but indirect evidence that tyrosinase is the enzyme measured. Further evidence is provided in the present report. In this study, tyrosinase activity refers to the combined activity of monophenolase and diphenolase, as there is little doubt that the two activities are derived from a single enzyme (26, 28–30).

MATERIALS AND METHODS

A total of 146 goldfish (*Carassius auratus* L.) weighing 5.52 ± 0.18 gm were utilized in this study. The color variety upon which most of this work is based is the black moor. The fish were maintained under constant conditions of temperature (25°C), photoperiod (12 hr), and diet. All animals were acclimated to laboratory conditions for at least three weeks prior to use.

The fish were individually captured and immediately decapitated, and the body skin with scales and fins was removed without delay. Care was taken to avoid contamination of the samples with muscle which is tightly bound to the skin of fish. The average skinning time per fish did not ordinarily exceed 2 min. The skin was immediately placed in a tared chilled beaker, weighed, and frozen (—27°) for at least 5 hr.

The homogenate was prepared in an ice-cooled micro-Waring Blendor. The frozen skin was ground with 0.1 *M* sodium phosphate buffer (31), pH 6.8, for 2 min (40 ml phosphate buffer, per 1 gm fresh skin weight). The debris was permitted to settle for several minutes and the homogenate was decanted and immediately refrigerated (0° to 4°). Approximately 9 ml of the homogenate was fractionated into particulate and soluble fractions by ultracentrifugation ($144{,}000 \times g$, 40 min, 0° to 4°). The particulate fraction was resuspended by grinding in a volume of the above buffer equal to that of the ultracentrifugate. The soluble fraction was utilized without further manipulation. A sample (0.2 ml) of the preparation equal to 5 mg of fresh skin was used for the assay of tyrosinase activity. Forty-nine samples (20 homogenates, 15 particulate, and 14 soluble fractions) were analyzed for protein nitrogen by the Folin-

Ciocalteau method (32). The samples derived from 5 mg fresh skin contained 48.2 ± 2.4, 31.9 ± 2.2, and 18.8 ± 1.0 μg protein nitrogen in the homogenate, particulate, and soluble fractions, respectively.

The tyrosinase assay was performed in rimless culture tubes (16×100 mm) containing a constant total incubation volume of 1 ml. The substrate for the enzyme was C-14-tyrosine. DL-Tyrosine-2-C-14 or uniformly labeled L-tyrosine-C-14 were diluted with L-tyrosine carrier in the above buffer to the final specific activity of 0.329 mc/mmole. As only the L form was considered pertinent, the final specific activity of L form available with DL-tyrosine-2-C-14 was 0.173 mc/mmole. The incubation mixture contained 221 mμmoles L-tyrosine when uniformly labeled L-tyrosine was used or 210 mμmoles L-tyrosine when DL-tyrosine-2-C-14 was used, 10 mμmoles L-dopa, 110 μg chloramphenicol, and 200 units penicillin G in 1 ml of the above buffer containing 100 μmoles phosphate. The dopa and antibiotic solutions were prepared immediately prior to use. The tubes were loosely covered with Saran Wrap and incubated at 30° for 16 hr. A portion of each enzyme preparation was heat inactivated (15 min in boiling water) and incubated as described above as the corresponding control. The reaction was terminated with 1 ml 10% TCA containing 200 μg L-tyrosine carrier. As autoxidation of dopa (or tyrosine) and its metabolites to melanin may only be partially inhibited by TCA at 30° and freezing the incubated mixture aids in the removal of radioimpurities (Table 8), the TCA-treated samples were stored for at least 4 hr at —27° until prepared for counting. All assays and controls were run in duplicate.

Four ml of 5% TCA was added to the thawed sample and the mixture was filtered through a 23-mm diameter membrane filter (pore size 0.45 μ). The incubation tube was thoroughly washed four times with 4 ml 5% TCA each time and each washing filtered consecutively. The inside wall of the Pyrex chimney funnel and the sample on the membrane filter were then washed in the following sequence: 2 ml 5% TCA, eight times (total 16 ml); 2 ml distilled water, eight times (total 16 ml); 2 ml 0.1 N HCl, four times (total 8 ml); 2 ml distilled water, four times (total 8 ml). The funnel was then removed and its inner base was rinsed with 2 ml distilled water over the sample on the membrane filter. The edge of the membrane filter then was washed dropwise with 16 ml distilled water, and the sample was air dried, placed in a 1-in. nickel-plated steel planchet and counted to 10,000 counts in a thin window gas-flow counter using GM gas.

With the extremely small size (Table 1) the samples could be assumed to be infinitely thin. The net enzymic activity in cpm per assay was obtained by taking the difference between the gross value obtained from

TABLE 1
DRY WEIGHT OF INCUBATES ON MEMBRANE FILTER

Category	Number of samples	Weight range, mg	Weight, mg $\bar{x} \pm$ S.E.
Homogenate	22	0.24–0.65	0.43 ± 0.02
Particulate	6	0.14–0.36	0.25 ± 0.04
Soluble	6	0.13–0.29	0.21 ± 0.03

enzyme preparation and the control value obtained from heat-inactivated enzyme preparation.

In order to determine whether loss of particulate activity occurred with the use of 0.45-μ pore size filters, the filtrates were collected from 8 samples treated and washed with TCA as described above. Each filtrate was then refiltered through a 10-mμ pore size filter and washed and fixed as described above, and the radioactivity determined. As the mean activity in these filtrates (28 ± 1 cpm) was less than twice background (15 cpm), the TCA precipitated melanin-protein mixture is effectively collected by the 0.45-μ pore size filter. The slight degree of radioactivity present in the 10-mμ filters may result from the tyrosine-holding capacity of the membrane filters.

Various aspects of the methodology employed were studied in order to evaluate their influence upon the enzyme. The tyrosinase activity of the skin homogenate before and after low-speed centrifugation at $600 \times g$ or $800 \times g$ was examined. The effects of storage at low temperature upon the enzyme in the skin, homogenate, and fractions were determined and related to time and degree of dispersion. The effect of regrinding the freshly prepared and frozen (—27°) enzyme preparations upon activity was evaluated. The effects of variation in incubation time, temperature, and shaking were studied. In addition, substrate saturation experiments determined the total quantity of amino acid necessary. The linear relationship between the quantity of enzyme preparation and assayed activity was demonstrated. Further, 17 C-14 labeled amino acids other than tyrosine were utilized to determine the specificity of the reaction. An alternate procedure utilizing labeled dopa as the substrate for comparison with radiotyrosine was also tested. Moreover, the effect of dopa on the control value and tyrosinase activity, the amounts of dopa utilized in the determination of tyrosinase activity, and the nature of the control value including autoxidation received attention.

RESULTS

Centrifugation. Ordinarily, in enzymic preparative procedures, cell debris is removed by low-speed centrifugation, $800 \times g$ (33, 34). How-

ever, the tyrosine activity of the 7 homogenates assayed before and after centrifugation ($600 \times g$ or $800 \times g$, 10 min, 0° to 4°C) showed a mean *decrease* in activity of 31.8% (range: 13.4–48.8%) after centrifugation. Thus, low-speed centrifugation was deliberately omitted from the assay procedure. In 2 homogenates, each precipitate and supernate ($800 \times g$) was assayed separately and after recombination. The mean tyrosinase activity occurring in the precipitate was 38.5%, in the supernate 61.4%, and in precipitate-supernate combination 97.7% of the activity of the noncentrifuged homogenate.

Stability. The enzyme preparations from the skins of goldfish, especially those of black fish, are unstable. It is possible to both prepare and fractionate the homogenates in less than 2 hr. The mean tyrosinase activity of 12 different homogenates immediately after preparation and after 3 hr (0° to 4°C) was 362 ± 65 cpm and 366 ± 67 cpm, respectively. Each pair of corresponding values of the 12 homogenates used is not significantly different. Thus, the cold enzyme preparations show no loss of activity if assayed within 3 hr posthomogenization.

The stability of the enzyme preparations stored at 0° to 4° for periods up to 12 hr is indicated in Table 2. When fresh, the enzymic activity

TABLE 2
STABILITY OF TYROSINASE WITH TIME IN HOMOGENATES AT 0° TO 4°C
(mean activity expressed in cpm)

Preparation	0 hr	1.5 hr	3 hr	4 hr	5 hr	12 hr
1	1002	997	—	1015	—	907
2	150	155	152	109	107	84
3	165	—	160	—	—	110

of preparation 1 was more than 6 times that of preparation 2. After 12 hr at 0°, preparation 1 lost 9.5% of its original activity. Preparation 2, on the other hand, showed a 27.3% decrease in tyrosinase activity after 4 hr and a 44.0% loss at 12 hr. Preparation 3 showed a 33.3% loss of enzymic activity after 12 hr. Thus, storage in the cold does not prevent the loss of enzymic activity.

Storage of enzyme preparations at —27° does not reduce their instability. Enzyme preparations from 17 different homogenates, 7 different particulate fractions, and 6 soluble fractions frozen from 1–12 days at —27° showed an average loss of enzymic activity of 40.2, 55.0, and 32.8%, respectively. In addition, the individual variance in tyrosinase is as great as the 24-hr loss of tyrosinase activity (range: 10.2–52.4%). The data from 5 preparations (Table 3) indicate both

TABLE 3
STABILITY OF TYROSINASE WITH TIME IN HOMOGENATES AND FRACTIONS AT −27°C

Preparation	Time frozen	% activity lost[a]		
		H	P	S
1	20 hr	39.7	57.1	38.1
2	40 hr	39.9	52.9	33.6
3	8 days	40.2	69.5	39.7
4	11 days	12.7	46.4	24.2
5	11 days	34.9	42.4	34.9

[a] Compared with activity of fresh preparation. H, homogenate; P, particulate fraction; S, soluble fraction.

the variation and loss of activity. Generally, the particulate fraction was more unstable than the soluble fraction or homogenate.

Freezing. Tyrosinase activity is increased from a mean of 158 ± 31 cpm of 5 fresh unfrozen skin samples to a mean of 390 ± 39 cpm of 39 skin samples continuously frozen at —27°C from 5 hr to 27 days. However, skin continuously frozen (—27°) for 6 months lost almost all tyrosinase activity. As individual variation in skin tyrosinase level is large, it is difficult to determine the precise length of time necessary to completely liberate tyrosinase. However, tyrosinase is present in equivalent amounts in skin of the right and left sides of black goldfish (35). Thus, immediate assay of fresh skin of one side of the animal and the other side after a period of freezing would indicate the change. In 7 such paired assays, the skin frozen (—27°) for 1–3 days showed a mean increase of 75.6% (range: 20.0–160.7%), in 6 cases. In the one remaining case, freezing did not increase or decrease tyrosinase yield. A similar study utilizing grey goldfish skin also produced similar results. In 5 of 6 paired assays, the skin frozen (—27°) for 1 day showed a mean increase of 18.3% (range: 7.9–28.6%). Again, in 1 case the yield remained unchanged after freezing. The increased tyrosinase yield after freezing, thawing, and homogenization may be produced by membrane and/or cell particle rupture with more effective enzyme release.

Grinding. Inactivation of tyrosinase at —27°C may result from covering the active center by the aggregation of particulate or other material in the preparation. Enzyme preparations (homogenates or fractions) assayed fresh were frozen at —27°C for different periods of time and assayed with and without grinding in a chilled tissue grinder (Table 4). The decrease in tyrosinase activity after storage at —27° was partially recovered by regrinding. Even the decreased activity of the soluble fraction could also be recovered in part despite the initial lack of particulate material.

TABLE 4
TYROSINASE RECOVERED BY REGRINDING IN A CHILLED TISSUE GRINDER

Preparation[a]	Time frozen at −27°C	Activity lost, %	Activity recovered, %
H	40 hr	52.4	14.6
H	3 days	68.8	23.0
H	20 hr	39.7	24.1
P	20 hr	57.1	33.3
S	20 hr	38.1	21.0

[a] See footnote, Table 3.

The effect of regrinding upon freshly prepared homogenates was also considered. The mean tyrosinase activity of 6 fresh preparations before and after regrinding was 313 ± 111 cpm and 311 ± 108 cpm, respectively. Thus, Blendor preparations contain the minimal particle size necessary for the mechanical release of tyrosinase activity.

Substrate Saturation. Tyrosinase activity was constant when the total amount of L-tyrosine per assay was greater than 28.5 μg (157 mμmoles) (Fig. 1). Since individual variance in tyrosinase is large, the activity is expressed as % of maximal activity. The high value obtained with 95 μg L-tyrosine per tube may be the result of excess radiotyrosine,

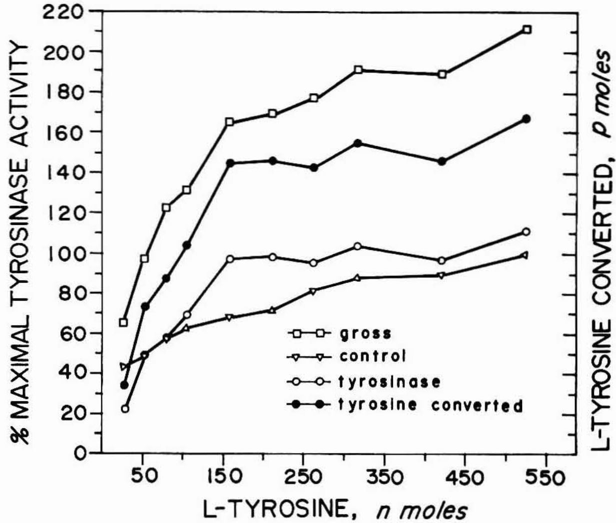

FIG. 1. Effect of substrate level on tyrosinase activity at 30°C: maximal tyrosinase activity (%) calculated from mean activity (cpm) in substrate range 157–524 nmoles. Substrate DL-tyrosine-2-C-14; specific activity L form, 0.173 mc/mmole. (Both ordinates are numerically identical.)

which is difficult to remove completely. As the homogenate is a mixture of many enzyme systems, some molecules of tyrosine may be more or less attracted by other materials present in the homogenate. Consequently, more tyrosine may be needed than simply to achieve saturation for tyrosinase. Therefore, the substrate saturation value here reported is probably greater than necessary for pure tyrosinase.

Incubation Time. As may be expected, the length of incubation has a profound effect upon the result of the assay. With increased time an increased conversion of substrate occurs (Fig. 2). However, a concurrent

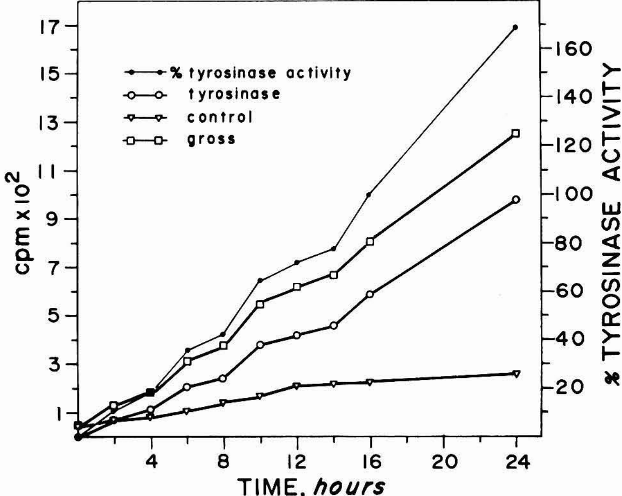

FIG. 2. Effect of length of incubation at 30°C on tyrosinase activity. Substrate uniformly labeled L-tyrosine; specific activity, 0.329 mc/mmole.

effect is an increase in the control values. Thus, it is apparent that the utilization of a zero time control may introduce considerable error. In order to avoid the possibility of this and other errors (such as bacterial contamination) which may obscure the assay, an incubation time of 16 hr was utilized. It is entirely possible that lengthening the incubation time will increase the sensitivity of the assay, providing adequate controls are utilized, bacterial contamination does not occur, and time is not a critical factor.

Temperature. The effect of temperature on tyrosinase activity was determined in a series of temperature studies on each of 3 homogenates using the standard incubation time of 16 hr. Tyrosinase activity was detectable even at —27°C (Fig. 3). The inactivation temperature for tyrosinase in the homogenate was approximately 50°. Despite the maxi-

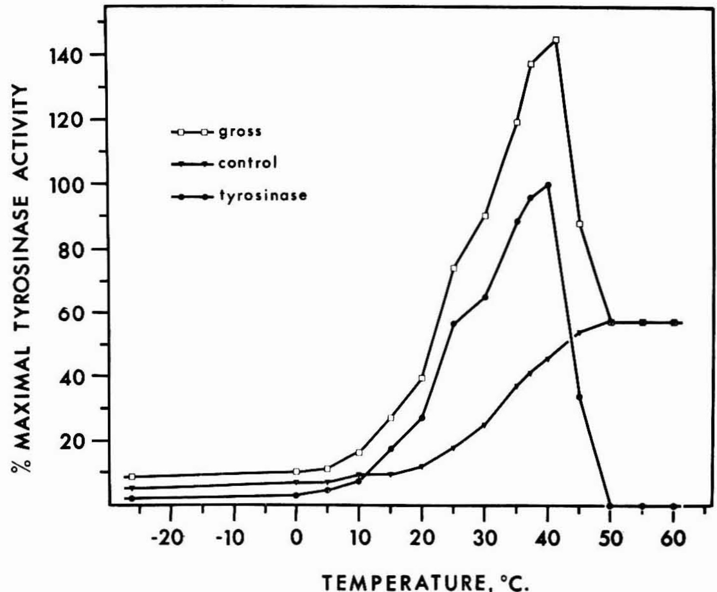

FIG. 3. Effect of temperature on tyrosinase activity. Substrate DL-tyrosine-2-C-14; specific activity L form, 0.366 mc/mmole.

mal activity at 40°, the incubation temperature of 30° is preferable. As the temperature is increased above 30° the viscosity of the homogenate is increased, thus introducing considerable difficulties in both filtration and subsequent removal of radioimpurities.

Shaking. Shaking the incubation mixture improves contact between enzyme, substrate, and cofactor(s) as well as facilitating the entrance of oxygen. This should enhance the tyrosinase activity in the assay procedure. However, comparing enzymic activity in an 1-day old homogenate (E_2) incubated with and without shaking (200 rpm) at 37°C revealed decreased activity (18.5%) after shaking (Table 5). A more pronounced decrease (36.6%) occurred with shaking a 2-day old homogenate (E_3). Since the decrease appeared proportional to the age of the enzyme preparation, a freshly prepared homogenate (E_1) was utilized. In this case, an increase of 23.3% in tyrosinase activity occurred. Shaking at 150 rpm at 37° also increased tyrosinase activity of fresh preparations (E_4, 7.7%; E_5, 8.3%) and decreased the activity of aged enzyme preparation (1 day: E_4, —16.3%; E_5, —3.4%. 2 days: E_4, —25.1%; E_5, —44.5%).

It is possible that the particles in the enzyme preparation stored at —27° (or even at 0°) are aggregated, and shaking accelerates this aggre-

TABLE 5

COMPARISON OF TYROSINASE ACTIVITY IN FRESH AND AGED HOMOGENATES WITH AND WITHOUT SHAKING DURING INCUBATION (mean activity expressed in cpm)

			No shaking						Shaking		
			30°C			37°C			37°C		
Homogenate	Age	Substrate[a]	Gross	Control	Tyrosinase	Gross	Control	Tyrosinase	Gross	Control	Tyrosinase
E_1	Fresh	DLT-2-C-14	255	72	183	387	117	270	477	144	333
E_2	1 day	DLT-2-C-14	519	83	436	675	130	545	612	168	444
E_3	2 days	DLT-2-C-14	253	91	162	335	141	194	298	175	123
E_4	Fresh	ULT-C-14	612	136	476	749	174	575	800	181	619
E_4	1 day	ULT-C-14	—	—	—	642	181	461	585	199	386
E_4	2 days	ULT-C-14	—	—	—	497	190	307	422	192	230
E_5	Fresh	ULT-C-14	279	133	146	430	166	264	467	181	286
E_5	1 day	ULT-C-14	—	—	—	410	174	236	400	172	228
E_5	2 days	ULT-C-14	—	—	—	346	191	155	302	216	86

[a] DLT-2-C-14, DL-tyrosine-2-C-14. ULT-C-14, uniformly labeled L-tyrosine-C-14.

gation so that the active centers of the enzyme are partially covered. In newly prepared enzyme preparations, shaking does not aggregate the finely separated particles and thus increases the chance of contact between reactants and oxygen. The control values were generally increased with shaking irrespective of enzyme preparation age.

Controls. Three series of experiments were designed to clarify the nature of the control value in the assay. In the first series, the enzyme preparation was incubated in the presence or absence of dopa (Table 6).

TABLE 6
EFFECT OF DOPA ON CONTROL AND TYROSINASE VALUES AT 30°C
(mean activity expressed in cpm)

		No dopa			DL-Dopa, 4 μg		
Homogenate	Substrate[a]	Gross	Control	Tyrosinase	Gross	Control	Tyrosinase
1	ULT-C-14	452	328	124	704	356	348
2	ULT-C-14	110	94	16	265	126	139
3	ULT-C-14	138	109	29	372	130	242
4	DLT-2-C-14	135	86	49	1052	101	951

[a] See footnote *a*, Table 5.

The control and gross assay values were lower in the absence of exogenous dopa. The tyrosinase activity decreased 64.4, 88.4, 88.0, and 94.9% in homogenates 1 through 4, respectively, below the corresponding dopa containing incubates. Therefore, it appears that exogenous dopa not only sparks tyrosinase but also increases the control values.

TABLE 7
EFFECT OF L-DOPA LEVEL ON CONTROL AND TYROSINASE VALUES IN ONE HOMOGENATE, UTILIZING UNIFORMLY LABELED L-TYROSINE AS SUBSTRATE
(mean activity expressed in cpm)

L-Dopa, μg	Gross	Control	Tyrosinase
0	250	242	8
0.25	289	251	38
0.50	325	259	66
1.00	355	271	84
1.50	377	280	97
2.00	377	272	105
3.00	392	288	104
4.00	385	282	103
6.00	381	307	74
8.00	359	303	56
16.00	352	302	50

In the second series, the homogenate was incubated with varying amounts of dopa (Table 7). The control values increased with increasing dopa levels. However, the enzymic conversion of tyrosine reached a maximum at 2 μg L-dopa per incubate and declined after 4 μg per incubate despite increased control values (Fig. 4).

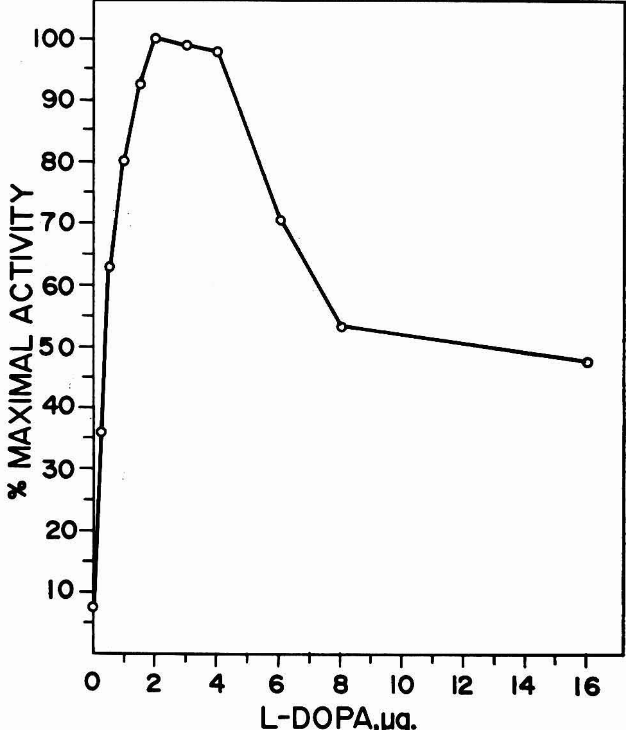

FIG. 4. Effect of L-dopa level on tyrosinase activity. Substrate uniformly labeled L-tyrosine, specific activity, 0.329 mc/mmole.

In the third series, the substrate and other components without the enzyme were incubated in the presence or absence of acid (Table 8). Enzyme was not added at the end of incubation. Little difference was observed between addition of TCA or HCl at the end of incubation. Further, a 100% increase in volume did not greatly affect the control value. TCA or HCl in the incubation mixture partially inhibited the incorporation of labeled tyrosine. Freezing (—27°C) the incubated mixture reduced the control value 28.7%. Omission of the freezing step increased the control value 40.2%.

Quantity of Enzyme Preparation. With enzyme preparations contain-

TABLE 8
EFFECTS OF HCl (0.1 *N*) OR TCA (5%) IN INCUBATION MIXTURE LACKING THE ENZYME PREPARATION AND UTILIZING UNIFORMLY LABELED L-TYROSINE

Category	Incubation mixture	Postincubation addition HCl	Postincubation addition TCA	−27°C[a]	Mean cpm	% change[b]
I	1 ml	0	1 ml	+	378	−28.7
II	1 ml	0	1 ml	−	530	+40.2
III	1 ml	1 ml	0	+	382	+ 1.1
IV	1 ml + 1 ml deionized H_2O	0	2 ml	+	361	− 4.5
V	1 ml + 1 ml TCA	0	0	+	213	−41.0
VI	1 ml + 1 ml HCl	0	0	+	238	−34.1

[a] Four hours minimum postincubation.
[b] Calculations of II, III, and IV based on I; calculations of V and VI based on IV; calculation of I based on II.

ing 1 to 20 mg of fresh skin per incubate, the tyrosinase activity was proportional to the amount of tissue used in the assay (Fig. 5).

Dopa. Labeled dopa and labeled tyrosine were utilized to estimate the rate of dopa oxidation into the end product (Table 9). Little difference

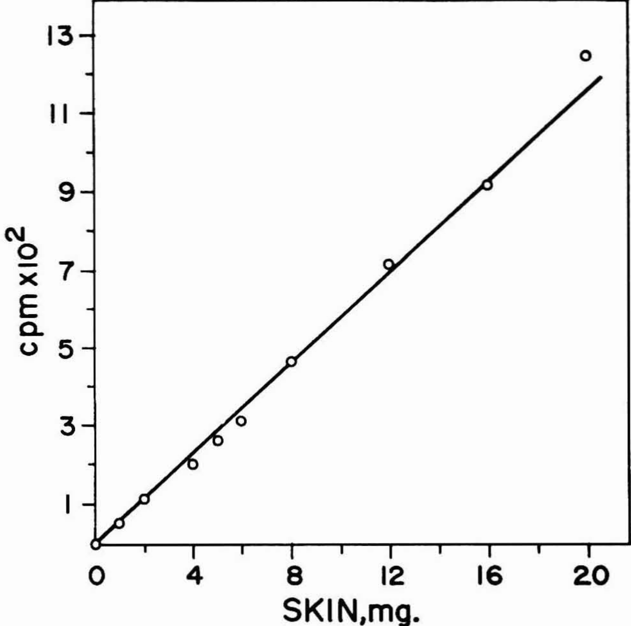

FIG. 5. Effect of quantity of enzyme preparation on tyrosinase activity. Substrate uniformly labeled L-tyrosine, specific activity, 0.329 mc/mmole.

TABLE 9
COMPARISON OF LABELED DOPA AND TYROSINE WITH CONSTANT SPECIFIC ACTIVITIES AS SUBSTRATES FOR TYROSINASE
(mean activity expressed as cpm)

Group	DL-Dopa, μg	DL-Dopa-2-C-14 (0.329 mc/mmole), μg	L-Tyrosine, μg	Uniformly labeled L-tyrosine-C-14 (0.329 mc/mmole), μg	Gross	Control	Tyrosinase
1	0	4	0	0	464	285	179
2	0	40	0	0	1362	1174	188
3	0	4	0	40	871	522	349
4	0	4	40	0	443	279	164
5	4	0	0	40	434	271	163

in tyrosinase activity between groups 1 and 2 with the same substrate specific activity was demonstrable, despite a tenfold difference in dopa concentration. However, the control and gross values increased with the increased dopa concentration. Comparing groups 1 and 4, the presence of unlabeled tyrosine had little inhibitive effect on dopa conversion. The tyrosinase activity in group 3 using both labeled dopa and tyrosine approximated the sum of groups 4 and 5. The tyrosinase activity obtained as the difference between groups 3 and 5, or taken alone from group 4, is actually twice that of group 5, since only L-dopa is converted into melanin.

Specificity. Utilization of 18 different labeled amino acids (Tables 10 and 11) as substrates for the enzyme indicates that the assay is specific for tyrosinase, although some incorporation of aromatic amino acids may occur. Comparison of serine with tyrosine (Table 10) demonstrated the dopa requirement for tyrosine in the assay and the lack of effect with serine. The appearance of small amounts of label in groups 3 and 7 demonstrated that tyrosinase was not involved, as dopa was added to group 3 but not group 7. When compared with groups 2 and 6, low activity of groups 3 and 7 may be due to the absence of serine carrier.

Incubation of homogenates with various labeled amino acid substrates in molar equivalent amounts, revealed no significant incorporation of the aliphatic amino acids (Table 11). Protein synthesis is not involved as the presence of several amino acids is required (36) and TCA precipitates protein. Since no difference occurred between DL-serine-3-C-14, specific activity 0.256 mc/mmole (Table 10) (group 4), and uniformly labelled L-serine-C-14, specific activity 0.329 mc/mmole (Table 11), the specific activity of the substrate is not important if incorporation does not occur.

The large degree of incorporation of the labeled substrate, L-tyrosine,

TABLE 10
COMPARISON, AT COMPARABLE MOLARITY, OF TYROSINE AND SERINE CONVERSION BY SKIN HOMOGENATES
(mean activity expressed in cpm)

Group	DL-Dopa, μg	DL-Serine-3-C-14 (0.256 mc/mmole), μg	DL-Serine-3-C-14 (2.56 mc/mmole), μg	Uniformly labeled L-Tyrosine-C-14 (0.329 mc/mmole), μg	L-Tyrosine, μg	Gross	Control	Tyrosinase
1	4	0	0	40	0	704	356	348
2	4	23.35	0	0	0	27	26	1
3	4	0	2.335	0	40	36	22	14
4	4	23.35	0	0	40	26	24	2
5	0	0	0	40	0	452	328	124
6	0	23.35	0	0	0	27	22	5
7	0	0	2.335	0	40	35	23	12
8	0	23.35	0	0	40	23	22	1

demonstrated that the reaction was catalyzed by tyrosinase. The presence of enzyme systems utilizing other aromatic amino acids (L-histidine, L-phenylalanine, L-tryptophan) may have resulted in the conversion of these amino acids. However, with the small amounts of enzyme preparation utilized, the incorporation of label will be specific if exogenous radioactive tyrosine is the substrate.

TABLE 11

COMPARISON OF 18 LABELED AMINO ACIDS, AT EQUIVALENT MOLARITY, AS SUBSTRATES FOR TYROSINASE

(mean activity expressed in cpm)

Substrate[a]	Substrate, μg/incubate	Gross	Control	Net
L-Alanine	19.67	19	19	0
L-Arginine	38.46	98	93	5
L-Asparagine	29.17	27	26	1
L-Aspartic acid	29.38	22	20	2
L-Glutamic acid	32.48	21	20	1
L-Glycine	16.57	23	22	1
L-Isoleucine	28.96	28	27	1
L-Leucine	28.96	36	35	1
L-Lysine	32.27	75	67	8
DL-Methionine	32.94	57	56	1
L-Proline	25.42	27	22	5
L-Serine	23.20	29	28	1
L-Threonine	26.30	27	25	2
L-Valine	25.86	26	22	4
L-Phenylalanine	36.47	194	164	30
L-Histidine	34.25	187	135	52
L-Tryptophan	45.08	705	598	107
L-tyrosine	40.00	505	258	247

[a] All UL-C-14, except DL-methionine (2-C-14) and L-tryptophan (ring-C-14). All substrates were utilized in the constant specific activity of L form, 0.329 mc/mmole.

Reproducibility and Precision. The reproducibility and precision of this radiometric assay were determined by running 30 assays with 30 controls from 1 homogenate. The mean control value was used as the control for each assay. Individual reproducibility was defined as % mean tyrosinase activity. The mean tyrosinase activity in cpm per 5 mg fresh skin was 873 ± 5 and the average reproducibility was 100.0 ± 0.8. The index of precision (37) for tyrosinase activity in cpm and reproducibility in % mean tyrosinase activity was 0.027 and 0.153, respectively. Thus, the assay method meets the necessary statistical criteria for reproducibility and precision.

DISCUSSION

The value of the control incubate varies with substrate level, length of incubation, and temperature. It increases as the dopa concentration or temperature is raised and remains constant when the substrate reaches a certain level or the temperature exceeds 40°C. However, in an oversaturated[3] substrate system, a linear relationship between tyrosinase activity and duration of incubation is present during the 24-hr interval studied. Thus, tyrosinase assayed at a given substrate level, temperature, and time requires a specific control. A zero time sample, under the same conditions, is not an adequate control. High substrate levels, high temperatures, and long incubation periods are not desirable as these make removal of impurities difficult, increase viscosity and thus retard filtration, and favor bacterial contamination, respectively. Further, high control values may mask low tyrosinase levels. Therefore, the combination of assay procedures is critical.

High levels of control radioactivity may result from: (*1*) tyrosine-holding capacity of membrane filter paper, (*2*) tyrosine-holding capacity of enzyme preparation, (*3*) radioactivity obtained by an unknown mechanism(s) during incubation. The tyrosine-holding capacity of the membrane filter and enzyme preparation are low (*4*). The contribution of the third factor is high when compared to the sum of the former factors. It is variable with time and incubation temperature, amount of dopa, amount of labeled tyrosine (or dopa) and its specific activity, and the unknown nature of enzyme preparation. Thus, the radioactivity incurred by the third factor during incubation is the difference between the control value (inactivated enzyme) and the zero time sample with this enzyme preparation.

The uncertain part of the radioactivity of the control value may be contributed by the autoxidation of tyrosine in the presence of dopa. Foster (2, 3) has suggested that autoxidation of tyrosine may occur under certain conditions. This may involve the presence of both cupric ions and a threshold concentration of dopa. The dopaquinone formed from dopa in the presence of Cu^{++} oxidizes tyrosine to dopa and dopaquinone itself is reduced to dopa. However, this diverges from the conventional concept that the tyrosinase is specific for the conversion of L-tyrosine into dopa. Furthermore, the dopa-dependent incorporation of radiotyrosine into melanin from the incubation mixture takes place in the absence of heat-inactivated enzyme preparation which is assumed to contain copper from tyrosinase (4). This indicates that autoxidation

[3] The amount of substrate present is much greater than that needed to saturate the active sites of the enzyme present.

of tyrosine may occur by the catalytic action of dopa without the participation of Cu^{++}.

Either the enzymic or autoxidative ready conversion of dopa into melanin or the dopa-dependent conversion of tyrosine into melanin may be explained by the different degree of attraction between the melanogenic intermediates. This suggestion is based upon the consideration of structural similarities and polarities of the intermediates and may be independent of the tyrosinase-specific dopa formation from tyrosine. It appears that tyrosine-like or dopa-like compounds are attracted by the intermediate(s) between dopa and melanin for polymerization and copolymerization, and that dopa and its intermediates are more easily attracted than tyrosine. In the classic Raper-Mason scheme (38), melanin is believed to be formed by repeated oxidative condensation or polymerization of indole-5,6-quinone, and that an intermediate of tyrosine or dopa must be cyclized and decarboxylated before indole-5,6-quinone is formed. Polymerization involves the anionoid center of the pyrrole ring of one molecule and the cationoid center of the benzenoid part of another (39, 40). However, recent studies (41–46) have shown some degree of incorporation of the carboxyl group of tyrosine or dopa into the polymer during melanogenesis. Tyrosine or dopa and their intermediates, both before and after cyclization, with and without decarboxylation, may be copolymerized, possibly with indole-5,6-quinone or its unit of melanin (39, 40). Consideration of the basic principle of the indole-quinone reaction (39, 40) indicates several possible types of oxidative condensations in melanin formation. With structural similarity and polarity, polymerization of indole-5,6-quinone must be the major pathway of melanogenesis. Also, some degree of heterogeneity of melanin may be produced by cross linkage resulting from the attraction and polymerization at different sites of the same monomer. Also, due to the effects of structural similarity and polarity, copolymerization of indole-5,6-quinone-2-carboxylic acid with indole-5,6-quinone or its unit of melanin may occur (43, 44, 46) to a small but significant degree in the end product. For the same reason, copolymerization of tyrosine, dopa, and its intermediates with indole-5,6-quinone or its unit of melanin, in a similar manner but to a smaller degree, is also possible (39, 40). Dopa and its intermediates appear to copolymerize more readily than tyrosine (41). Copolymerization in melanogenesis is strongly supported by the different degree of the incorporation of tyrosine and dopa carboxyl groups into melanin (41). Therefore, the attraction hypothesis permits the explanation and consolidation of the experimental findings dealing with the roles of the tyrosinase reaction and the autoxidation in melanogenesis. It is clear then that the uncertain part of the radioactivity of

the assay control value may be contributed by (*a*) the copolymerization of tyrosine with indole-5,6-quinone or its unit of melanin formed from dopa, (*b*) the autoxidative radiotyrosine-dopa conversion with the subsequent autoxidation of radiodopa to melanin by both polymerization and copolymerization, and (*c*) the combination of (*a*) and (*b*).

Not only does dopa act as a catalyst in the oxidation of tyrosine, but it is also an intermediate in the oxidative pathway (5, 13, 26, 28–30, 47). As a catalyst to spark the reaction, either enzymic or nonenzymic, the amount of dopa present will be proportional to the amount of tyrosine converted in the end product. As an intermediate in the oxidative pathway, the amount of dopa present will be inversely proportional to the amount of tyrosine converted in the end product. Further, as an intermediate, the more dopa present, the more melanin will be formed from dopa. The melanin and its precursor(s) then may attract labeled tyrosine or its intermediate(s) to contribute activity in the end product. Therefore, the incorporation of the radiotyrosine into the end product is the net result of all the positive and negative effects produced from dopa. Since the autoxidation of dopa may occur more readily than the enzymic conversion of tyrosine and as the tyrosinase stimulated dopa-melanin conversion and tyrosine-melanin conversion are essentially competitive, the utilization of dopa (both autoxidatively and enzymically) dominates the enzymic utilization of tyrosine at high L-dopa levels (above 4 μg). Within a dopa level limit (2 μg), the dopa-sparked enzymic utilization of tyrosine appears to be much greater than the other effects since the activity in the end product increases as the dopa level increases. From these considerations, the selection of the correct quantity of dopa as a catalyst is critical in the accurate measurement of tyrosinase activity.

As autoxidation of tyrosine, dopa, and intermediates is not completely inhibited by TCA or HCl at the incubation temperature of 30°C, autoxidation may continue at room temperature after the enzymic reaction is stopped with TCA. However, such autoxidation is limited if the sample is kept at −27°. Moreover, if autoxidation occurs to a similar degree in both enzyme and control assay tubes after addition of TCA, the net tyrosinase activity will be constant. In the previously described radiometric tyrosinase assay (33, 34), the TCA precipitate was collected by centrifugation. Under such conditions, the continued autoxidation of the intermediates to melanin would seriously affect the results.

The control value in the absence of the enzyme preparation may be much higher than that in the presence of the heat-inactivated enzyme preparation. This may result from the possible presence of a heat-stable inhibitor of dopa or tyrosine autoxidation (4). Fish skin tyrosinase is completely inactivated in boiling water for 15 min (4). Heat inactivation

of tyrosinase or phenolase has been reported (1, 3, 15, 28, 48–53). The use of a heat-inactivated enzyme preparation as the valid control can be ascertained experimentally in two ways. The sum of the radioactivity (net tyrosinase activity) of the particulate and soluble fractions is closely equal to the radioactivity of the homogenate. The gross value of the enzyme preparation and the control value of the heat-inactivated preparation are similar if tyrosinase activity is not present or completely inactivated during long storage at —27° in the enzyme preparation (4). The radiometric assay here described measures tyrosinase activity since a precise control eliminates the errors caused by the autoxidation of dopa and, perhaps, tyrosine as well as other known and unknown factors.

Using dopa as substrate, the estimated rate of tyrosinase reaction is approximately twice that of tyrosine (Table 9). Comparing the amounts of tyrosine and dopa used, the substrate saturation level of each is quite different, 10 mμmoles L-dopa or 157 mμmoles L-tyrosine. No difference in tyrosinase activity is detectable when 4 μg and 40 μg DL-dopa is used. It is possible that tyrosine is metabolized in several pathways but dopa in fewer, so that less dopa will be needed to saturate tyrosinase in a mixed enzyme preparation. However, the autoxidation of dopa, when used as a substrate in the assay, may obscure low levels of tyrosinase activity. Thus, the use of dopa as a substrate is not recommended.

The linear relationship between the amount of an enzyme preparation and its activity (Fig. 5) indicates that tyrosinase inhibiting factors are not involved in the range studied. The dilute enzyme preparations utilized may have resulted in the ineffective levels of inhibitors or interfering materials, as well as other enzyme systems, and thus will not influence the radiometric tyrosinase assay. Moreover, the minute amount of enzyme preparation requires less substrate (tyrosine) and in turn, the induction period produced by high tyrosine concentrations (26, 28) is lacking or reduced to an insignificant interval during the 16-hr incubation.

With antibiotics, bacterial contamination is avoided during the 16-hr period. If tyrosinase is assayed in the absence of antibiotics or in the presence of insufficient antibiotics, the results may not truly demonstrate tyrosinase activity even with a similar control. The utilization of tyrosine or dopa by bacteria will mask the lower tyrosinase activity (4). In the presence of antibiotics, the long incubation period increases the accuracy of the assay.

In addition to terminating the tyrosinase reaction, TCA also precipitates endogenous protein in the incubation mixture. The radiomelanin formed in the course of the assay appears to be aggregated and carried down with the protein precipitate. This facilitates the collection of the

end product. However, losses in collection of the end product cannot be avoided with centrifugation as described originally (33, 34).

The freezing and thawing technique has been shown to liberate enzymic activity in other systems (54–58). In the present investigation, freezing and thawing of intact fish skin enhances, but the freezing and thawing of fish skin homogenate or particulate and soluble fractions lowers, the tyrosinase activity. Thus, fish skin tyrosinase is not stable to such treatment. The cellular organization of the skin may provide some type of protection.

The loss of tyrosinase activity by low-speed centrifugation of the homogenate from mouse melanoma has been demonstrated by Seiji *et al.* (59). Measuring oxygen consumption, the nuclear fraction obtained at $700 \times g$ contained 28% of the total activity of the homogenate. The activity of this fraction was reduced to a negligible amount when purified to 70% pure nucleus. In the present investigation, the fish skin homogenate lost considerable tyrosinase activity after low-speed centrifugation ($600 \times g$ or $800 \times g$). Therefore, centrifugation, to obtain a clear homogenate for tyrosinase assay, is not a practical approach.

The utilization of labeled aromatic amino acids other than tyrosine with the radiometric assay needs further investigation. Incorporation of L-phenylalanine into the TCA precipitate may indicate tyrosine-melanin formation as phenylalanine-tyrosine conversion has been shown in vertebrates, invertebrates, and microorganisms (60–68). Incorporation of L-tryptophan may indicate ommochrome formation as found in insects (69–73). Also, incorporation of histidine may indicate histidine- or histamine-melanin formation since the existence of natural histamine-melanin has been suggested (74, 75). If the melanins truly are formed enzymically from histidine or tryptophan in the fish skin, it would be of interest to determine the enzymes responsible for such conversion.

The assay under the conditions studied is specific for tyrosinase as only tyrosine and not the aliphatic amino acids tested were incorporated into the end product. Further, the assay requires dopa. Although protein synthesis is inhibited by the antibiotics utilized, it is not possible to determine whether protein synthesis is required for the formation of melanin itself. The quantity of protein needed for melanin synthesis is probably present in the enzyme preparation, if required.

In the assay, the tyrosinase activity obtained by using radiotyrosine in the presence of unlabeled dopa represents the conversion of radiotyrosine but not unlabeled dopa into melanin. Similarly, the tyrosinase activity obtained with radiodopa in the presence of unlabeled tyrosine represents only the conversion of radiodopa but not that of unlabeled tyrosine into melanin. Considering that the enzymic melanin formation

from dopa is twice as that from tyrosine (black moor goldfish skin), the actual melanin formation under the present assay system will be three times the amount of radioactive tyrosine converted. Therefore, the assay should be performed by using labeled tyrosine as substrate and labeled dopa as both catalyst and substrate. Further, since the relative rates of tyrosine-dopa conversion and dopa-melanin conversion may be species-specific, the total melanin formation will be more meaningful if the assay is performed by labeling both tyrosine and dopa. However, for simplicity, labeled tyrosine alone may be used, but a factor must be determined for a given species to obtain the total tyrosinase activity of both tyrosine-melanin and dopa-melanin conversion.

In the manometric assay, the tyrosinase unit is described in terms of μl of oxygen uptake. As oxygen consumption is not specific for tyrosinase in a mixed enzyme preparation, a definitive unit is necessary. Although the tyrosinase activity has been represented previously in cpm, tyrosinase activity may be critically defined in terms of moles of L-tyrosine converted into melanin. Thus, for convenience in comparison of tyrosinase levels, the following tyrosinase unit is proposed: 1 unit of tyrosinase activity is defined as the amount of tyrosinase activity required to convert 1 picomole of L-tyrosine to melanin under the conditions of the described assay during a 16-hr incubation period at 30°C. Specific activity is defined as the number of tyrosinase units per μg protein nitrogen.

SUMMARY AND CONCLUSIONS

A radiometric assay of tyrosinase with high sensitivity, specificity, and reproducibility has been developed. Using enzyme preparations obtained from goldfish skin and tyrosine-C-14 (or dopa-C-14) as substrate, this method meets the major critical requirements of (*a*) a completely extracted enzyme preparation of known stability which may be specifically assayed under given conditions and (*b*) the establishment of a valid control incorporating all nonspecific reactions occurring during the course of the given assay conditions.

A meaningful and useful tyrosinase unit is proposed. One unit of tyrosinase activity is defined as the amount of tyrosinase activity required to convert 1 picomole of tyrosine to melanin under the conditions of the described assay during a 16-hr incubation period at 30°C. The specific activity of tyrosinase is defined as the number of tyrosinase units per μg protein nitrogen.

The results have led to the formulation of an attraction hypothesis concerning both tyrosinase activity and autoxidation of tyrosine and dopa in melanogenesis. Both polymerization of indole-5,6-quinone into

melanin according to the classical Raper-Mason scheme and copolymerization of the intermediates in melanogenesis discovered in recent years may be explained as primarily due to the different degree of attraction between the intermediates (including dopa and tyrosine) resulting from their structural similarity and polarity.

REFERENCES

1. RILEY, V., HOBBY, G., AND BURK, D., *in* "Pigment Cell Growth" (M. Gordon, ed.), pp. 231-266. Academic Press, New York, 1953.
2. FOSTER, M., *Proc. Natl. Acad. Sci. U. S.* **36,** 606-611 (1950).
3. FOSTER, M., *J. Exptl. Zool.* **117,** 211-246 (1951).
4. CHEN, Y. M., unpublished data.
5. HIRSCH, H. M., AND ZELICKSON, A. S., *Cancer Res.* **24,** 1137-1153 (1964).
6. MAKOWER, R. U., *Plant Physiol.* **39,** 520-522 (1964).
7. GRUPPER, C., LEGRAND, J. C., AND GONNARD, P., *Arch. Klin. Exptl. Dermatol.* **219,** 784-789 (1964).
8. MARSDEN, C. D., *Quart. J. Microscop. Sci.* **102,** 469-474 (1961).
9. DEGAWA, T., *Nippon Hifukergakkai Zasshi* **71,** 651-669 (1961).
10. HARADA, M., AND KOTO, M., *Annotationes Zool. Japon.* **33,**(3), 159-166 (1960).
11. HARADA, M., *Kagaku (Tokyo)* **30,** 259 (1960).
12. BOUCHILLOUX, S., AND MAYRARQUE-KODJA, A., *Bull. Soc. Chim. Biol.* **42,** 1045-1064 (1960); *Compt. Rend.* **251,** 1920-1922 (1960).
13. KARKHANIS, Y., AND FRIEDEN, E., *J. Biol. Chem.* **236,** PC1-2 (1961).
14. BOYLEN, J. B., AND QUASTEL, J. H., *Nature* **193,** 376-377 (1962).
15. QUEVEDO, W. C., JR., AND ISHERWOOD, J. E., *J. Invest. Dermatol.* **34,** 309-316 (1960).
16. ENCKE, W., *Z. Vererbungslehre* **89,** 697-706 (1958).
17. ZUSSMAN, R. A., AND VICKER, E. E., AND LYON, I., *Mycopathol. Mycol. Appl.* **14,** 205-214 (1961).
18. CHAKSABORTY, D. P., DEB, C., AND MUKKERJEE, M., *Sci. Cult. (Calcutta)* **25,** 386-387 (1960).
19. OHNISHI, E., *Japan. J. Zool.* **11,** 69-74 (1953); *Annotations Zool. Japon.* **27,** 188-193 (1954); *Japan. J. Zool.* **12,** 179-188 (1958); *J. Insect Physiol.* **3,** 219-229 (1959).
20. HOROWITZ, W. H., AND FING, M., *in* "Amino Acid Metabolism" (W. D. McElroy and B. Glass, eds.), pp. 207-218. Johns Hopkins Press, Baltimore, 1955.
21. KARLSON, P., AND SCHWEIGER, A., *Z. Physiol. Chem.* **323,** 199-210 (1961).
22. CLEAVER, U., AND KARLSON, P., *Exptl. Cell Res.* **20,** 623-626 (1960).
23. BODINE, J. H., ALLAN, T. H., AND BOELL, E. J., *Proc. Soc. Exptl. Biol. Med.* **37,** 450-453 (1937).
24. ISAKA, S., *Nature* **169,** 74-75 (1952).
25. OSHIMA, C., SEKI, T., AND ISHIZAKI, H., *Genetics* **41,** 4-20 (1956).
26. LERNER, A. B., AND FITZPATRICK, T. B., *Physiol. Rev.* **30,** 91-126 (1950).
27. HEIMANN, W., AND ANDLER, ST., *Z. Lebensm.-Untersuch. Forsch.* **117,** 121-129 (1962).
28. LERNER, A. B., AND FITZPATRICK, T. B., *J. Biol. Chem.* **178,** 185-195 (1949).
29. FITZPATRICK, T. B., AND KUKITA, A., *in* "Pigment Cell Biology" (M. Gordon, ed.), pp. 489-524. Academic Press, New York, 1959.
30. MASON, H. S., *Nature* **177,** 79-81 (1956).

31. GOMORI, G., *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. 1, p. 143. Academic Press, New York, 1955.
32. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDAL, R. J., *J. Biol. Chem.* **193**, 265–275 (1951).
33. KIM, K. H., AND TCHEN, T. T., *Biochem. Biophys. Acta* **59**, 569–576 (1962).
34. CHAVIN, W., KIM, K. H., AND TCHEN, T. T., *Ann. N. Y. Acad. Sci.* **100**, 678–685 (1963).
35. CHEN, Y. M., AND CHAVIN, W., in preparation (1965).
36. CANTAROW, A., AND SCHEPARTZ, B., *Biochemistry* pp. 534, 537. Saunders, Phila. (1962).
37. YOUNG, H. D., "Statistical Treatment of Experimental Data," p. 71. McGraw-Hill, New York, 1962.
38. THOMSON, R. H., *in* "Comparative Biochemistry, Vol. 3" (M. Florkin and H. S. Mason, eds.), pp. 727–753. Academic Press, New York, 1962.
39. BÚLOCK, J. D., *J. Chem. Soc.* **1961,** 52–58.
40. BÚLOCK, J. D., AND HARLEY-MASON, J., *J. Chem. Soc.* **1951,** 703–712.
41. CHEN, Y. M., AND CHAVIN, W., in preparation (1965).
42. PIATTELLI, M., AND NICOLAUS, R. A., *Tetrahedron* **15,** 66–75 (1961).
43. PIATTELLI, M., FATTORUSSO, E., MAGNO, S., AND NICOLAUS, R. A., *Tetrahedron* **18,** 941–949 (1962); **19,** 2061–2072 (1962).
44. NICOLAUS, R. A., PIATTELLI, M., AND FATTORUSSO, E., *Tetrahedron* **20,** 1163–1172 (1964).
45. CLEMO, G. G., DUXBURY, F. K., AND SWAN, G. A., *J. Chem. Soc.* **1952,** 3464–3468.
46. SWAN, G. A., *Ann. N. Y. Acad. Sci.* **100,** 1005–1019 (1963).
47. LERNER, A. B., *Adv. Enzymol.* **14,** 73–128 (1953).
48. GREGORY, K. F., AND HUANG, J. C. C., *J. Bact.* **87,** 1281-1286 (1964).
49. MATSUYAMA, S., *Nippon Nogeikagaku Kaishi* **35,** 405–408 (1961).
50. GUOTH, S., AND JURASEK, L., *Drevas. Lyskum* **6,** 165–174 (1961).
51. JANKOV, S., *Fruchtsaft-Ind.* **7,** 13–32 (1962).
52. YANKOV, S. I., *Biokhimiya* **27,** 235–239 (1962); *Konserv. i Ovoshchesuohil. Prom.* **17**(2), 32–36 (1962).
53. DIEMAIR, W., KOCH, J., AND HESS, D., *Z. Lebensm.-Untersuch. Forsch.* **113,** 381-387 (1960).
54. BEAUFAY, H., AND DEDUVE, C., *Biochem. J.* **73,** 604-609 (1959).
55. BENDALL, D. S., AND DEDUVE, C., *Biochem. J.* **74,** 444-450 (1960).
56. JAGANNATHAN, V., AND SCHWEET, R. S., *J. Biol. Chem.* **196,** 551-562 (1952).
57. SANADI, D. R., LITTLEFIELD, J. W., AND BOCK, R. M., *J. Biol. Chem.* **197,** 851–862 (1952).
58. KOEPSELL, H. J., AND JOHNSON, M. J., *J. Biol. Chem.* **145,** 379-386 (1942).
59. SEIJI, M., SCHIMAO, K., BIRBECK, M. S. C., AND FITZPATRICK, T. B., *Ann. N. Y. Acad. Sci.* **100,** 497-533 (1963).
60. MOSS, A. R., AND SCHÖENHEIMER, R., *J. Biol. Chem.* **135,** 415-429 (1940).
61. UDENFRIEND, S., AND BESSMAN, S. P., *J. Biol. Chem.* **203,** 961-966 (1953).
62. FUKUDA, T., *J. Biochem. (Tokyo)* **43,** 137-142 (1956).
63. UDENFRIEND, S., AND COOPER, J. R., *J. Biol. Chem.* **194,** 503-511 (1952).
64. KAUFMAN, S., *J. Biol. Chem.* **226,** 511-524 (1957).
65. UDENFRIEND, S., *et al.*, *J. Biol. Chem.* **208,** 731-739, 741-750 (1954).
66. DAGLEY, S., *et al.*, *J. Gen. Microbiol.* **8,** 1–7 (1953).
67. BARRATT, R. W., *et al.*, *J. Bact.* **71,** 108–114 (1956).

68. Bricteux-Grégoire, S., Verly, W. G., and Florkin, M., *Nature* **177,** 1237–1238 (1956).
69. Frezeau-Braesch, S., *Bull. Biol. France Belq.* **94,** 525–627 (1960).
70. Linzen, B., *Z. Physiol. Chem.* **333,** 145–148 (1963).
71. Frezeau-Braesch, S., *Nature* (*Paris*), No. **3336,** 172–177 (1963).
72. Butenandt, *et al., Ann. Chem.* **590,** 75–90 (1954); *Z. Physiol. Chem.* **301,** 109–114, 115–117 (1955); **305,** 284–289 (1956).
73. Kirkkana, H., *Adv. Genetics* **5,** 107–140 (1953). Kühn, A., *Naturwissenschaften* **43,** 25–28 (1956).
74. Robert, P., and Zürcker, H., *Dermatologica* **100,** 217–241 (1950); **104,** 276–294 (1952).
75. Robert, P., and Schmidli, B., *Dermatologica* **106,** 219–230 (1953); **108,** 343–351 (1954).

ANALYTICAL BIOCHEMISTRY 13, 259–264 (1965)

A Spectrophotometric Method for the Determination of Nucleoside Triphosphates (Pyrophosphorolysis of Nucleoside Diphosphate Sugars)

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In earlier papers (1–3) we reported the enzymic synthesis and pyrophosphorolysis of ADP-Glc, ADP-Man, GDP-Glc, GDP-Man, IDP-Glc, and IDP-Man[1] by extracts of mammalian tissues. In order to establish the specificity of the different enzymes under consideration, rapid and reliable assay methods were needed to measure nucleoside diphosphate sugar pyrophosphorolysis when the sugar was not glucose. When the sugar is glucose, the reactions may be followed spectrophotometrically by coupling the pyrophosphorolysis with pyridine nucleotide reduction (4) in a system containing phosphoglucomutase (EC 2.7.5.1), glucose-6-P dehydrogenase (EC 1.1.1.49), and NADP. The formation of 1 mole of NADPH then corresponds to the pyrophosphorolysis of 1 mole of nucleoside diphosphate sugar, and the reactions are followed by recording the change in optical density at 340 mμ.

Several methods were tested in order to find a similarly rapid method for measuring the reaction rates with nucleoside diphosphate hexoses containing sugars other than glucose. Measurement of (*1*) PPi, (*2*) reducing sugar in the presence of phosphomonoesterase, (*3*) nucleoside triphosphates in the presence of ADP, nucleoside diphosphokinase (EC 2.7.4.6), hexokinase (EC 2.7.1.1), glucose, and glucose-6-P dehydrogenase (EC 1.1.1.49), and (*4*) chromatography of nucleoside diphosphate sugars was not sufficiently rapid or specific. A chromatographic method based on ion-exchange paper chromatography of the nucleoside diphosphate sugars (5) proved useful for the qualitative detection of reaction products and for examining the specificity of the different pyrophosphorylases, but such methods are less rapid and quantitative than spectrophotometric procedures.

[1] Abbreviations are those set forth by the International Union of Biochemistry (1964).

The interesting observation by Adam (6) and its confirmation by Heldt (7), that 3-P-glycerate kinase (ATP:3-phospho-D-glycerate-1-phosphotransferase EC 2.7.2.3) has a broad specificity with respect to nucleoside triphosphates, offered the possibility of a good method for quantitating the formation of nucleoside triphosphates such as those appearing in nucleoside diphosphate sugar pyrophosphorolysis. Direct coupling of the nucleotidyltransferase (pyrophosphorylase) reaction to 3-P-glycerate kinase (EC 2.7.2.3) and glyceraldehyde-3-P dehydrogenase (D-glyceraldehyde-3-phosphate:NAD oxidoreductase EC 1.2.1.12) in the presence of NADH and hydrazine was investigated (reactions 1–4).

$$\text{nucleoside diphosphate sugar} + \text{PPi} \xleftrightarrow{\text{EC 2.7.7—}} \text{sugar-1-P} + \text{nucleoside triphosphate} \quad (1)$$

$$\text{nucleoside triphosphate} + \text{3-P-glycerate} \xleftrightarrow{\text{EC 2.7.2.3}} \text{1,3-diphosphoglycerate} + \text{nucleoside diphosphate} \quad (2)$$

$$\text{1,3-diphosphoglycerate} + \text{NADH} \xleftrightarrow{\text{EC 1.2.1.12}} \text{glyceraldehyde-3-P} + \text{NAD} + \text{Pi} \quad (3)$$

$$\text{glyceraldehyde-3-P} + \text{hydrazine} \rightarrow \text{hydrazone} \quad (4)$$

Oxidation of NADH was followed by the change in optical density at 340 mμ. The measurement of nucleoside triphosphate:sugar-1-phosphate nucleotidyltransferases with this system is now reported, and the advantages and limitations of the method are discussed.

MATERIALS AND METHODS

The 3-P-glyceric acid (sodium salt), 3-P-glycerate kinase (EC 2.7.2.3), and glyceraldehyde-3-P dehydrogenase (EC 1.2.1.12) were obtained from Boehringer or Sigma, and NADH from Sigma. Nucleoside diphosphate sugars except UDP-Glc were synthesized by the method of Michelson (8, 9). The solutions of 3-P-glycerate kinase (EC 2.7.2.3) and glyceraldehyde-3-P dehydrogenase (EC 1.2.1.12) were prepared essentially as described by Adam (6), except that phosphate buffers were used instead of pyrophosphate. The mammalian nucleotidyltransferases were partially purified as described (3) unless stated otherwise. Oxidation of NADH and reduction of NADP were followed at 340 mμ using a Beckman DU spectrophotometer equipped with an automatic cuvet changer, optical density converter, and a recorder (10).

All reaction mixtures were made up in cuvets with a 1-cm light path containing in μmoles: Mg^{++} 1.0, PPi 1.0, neutralized hydrazine sulfate (6) 0.25, 3-P-glycerate 0.6, NADH 0.125, nucleoside diphosphate sugar 0.2, excess 3-P-glycerate kinase (EC 2.7.2.3) and glyceraldehyde-3-P

dehydrogenase (EC 1.2.1.12), and enzyme extract to a total volume of 0.5 ml with 0.05 *M* triethanolamine buffer (pH 7.8) at 25°.

The data in Table 1 is expressed as units per minute at 25°. A unit of enzyme is defined as that amount necessary to change 1 μmole of substrate per minute at 25°. Control reactions were also incubated which lacked PPi or nucleoside diphosphate sugar for each assay and were read simultaneously using the automatic cuvet positioner.

TABLE 1
COMPARISON OF METHODS OF MEASURING PYROPHOSPHOROLYSIS OF NUCLEOSIDE DIPHOSPHATE GLUCOSE

Preparation	Substrate	Product measured, units/ml	
		XTP	Glc-1-P
Crude extracts:			
Yeast	UDP-Glc	1.29	1.90
Yeast	GDP-Glc	0.09	0.07
Liver	GDP-Glc	0.24	0.54
Liver	IDP-Glc	0.05	0.09
Purified fractions:			
A	UDP-Glc	0.52	0.52
B	UDP-Glc	98.0	98.0
C	UDP-Glc	25.1	23.2
D	UDP-Glc	22.6	23.9
E	GDP-Glc	0.15	0.13
F	GDP-Glc	0.0045	0.0045

RESULTS AND DISCUSSION

Preliminary experiments indicated that the oxidation of NADH was dependent upon addition of 3-P-glycerate, Mg^{++}, nucleoside diphosphate sugar, PPi, and enzyme extract. Oxidation was observed in crude extracts without addition of 3-P-glycerate kinase (EC 2.7.2.3) and glyceraldehyde-3-P dehydrogenase (EC 1.2.1.12) indicating the presence of these enzymes. With some crude extracts (for example plants and the yeast, *Hansenula holstii*) high blanks were obtained in the absence of nucleoside diphosphate sugar substrate. For plants this was attributable to NADH oxidation in the presence of 3-P-glycerate alone. In yeast and some mammalian tissues this was in part due to pyrophosphorolysis of NADH resulting in the formation of ATP, which then reacts with the 3-P-glycerate-kinase system or NADH oxidase. Phosphatases present in crude extracts compete with 3-P-glycerate kinase (EC 2.7.2.3) for nucleoside triphosphates formed and consequently could give a low estimate of the sugar-1-phosphate nucleotidyltransferase levels. Ion-

exchange paper chromatography of these incubation mixtures revealed a concomitant transformation of nucleoside triphosphates into di- and mono-phosphates and even free ribosides.

The further oxidation of 6-P-gluconic acid in the Glc-1-P assay leads to an overestimation of nucleoside diphosphate sugar pyrophosphorolysis in the crude extracts since it was demonstrated that these extracts contain 6-P-gluconate dehydrogenase (EC 1.1.1.44) which will catalyze the formation of a second mole of NADPH per mole of nucleoside diphosphate sugar. Incubation of the crude extracts with ribose-5-P very slowly reduced NADP, thus resulting in the reduction of even more than two moles of NADP in supplemented crude systems. Sugar 1-phosphates may also be formed from nucleoside diphosphate sugars as catalyzed by pyrophosphatases. Controls lacking PPi thus are important with the Glc-1-P assay, whereas controls lacking nucleoside diphosphate sugars are necessary with the nucleoside triphosphate method. Both controls were included with both methods. With GDP-Glc or UDP-Glc as a substrate both spectrophotometric methods may be applied and a comparison is possible (Table 1). In crude extracts the agreement between the two procedures is only approximate. During purification, 6-P-gluconate de-

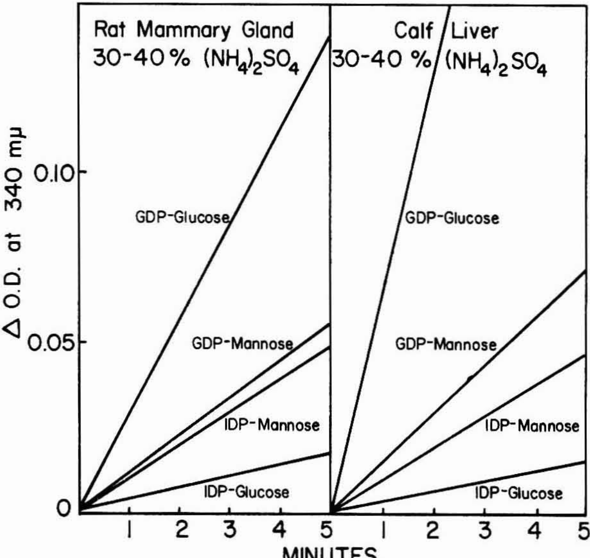

FIG. 1. Comparative rates of pyrophosphorolysis of nucleoside diphosphate hexoses by extracts of mammalian tissues. Reaction rates were followed by measuring formation of nucleoside triphosphates. Lines were drawn from tracings from automatic recorder.

hydrogenase (EC 1.1.1.44), phosphatases, and other interfering enzymes are removed and the correspondence between the methods is greatly improved.

The nucleoside triphosphate method was then used for recording the respective rates of GDP-Glc, GDP-Man, IDP-Glc, and IDP-Man pyrophosphorolysis by calf liver and rat mammary gland extracts (Fig. 1). The liver preparation was homogenized with a Servall Omni-Mixer in 0.1 *M* tris-acetate buffer (pH 7.5) then treated with protamine sulfate followed by solid ammonium sulfate to 40% saturation. Noteworthy is the high rate of pyrophosphorolysis of GDP-Glc and GDP-Man by mammalian tissues. In addition, the nucleoside triphosphate system was found convenient for measuring pyrophosphorolysis by liver extracts of ADP-Man, UDP-Gal, UDP-N-AGlcm, UDP-Man, and UDP-Xyl. The method will not measure directly either CTP or TTP (6, 7). In crude extracts NADH oxidation may sometimes be observed without addition of nucleoside triphosphate. This may be attributable to the presence of nucleoside-diphosphokinase (EC 2.7.4.6) and traces of ADP.

SUMMARY

The enzyme-catalyzed pyrophosphorolysis of nucleoside diphosphate sugars has been followed spectrophotometrically using 3-P-glycerate kinase (EC 2.7.2.3) and glyceraldehyde-3-P dehydrogenase (EC 1.2.1.12) coupled to the oxidation of NADH. The method is rapid, and gives a direct recording of enzyme activity. Some limitations are discussed but these generally apply when crude systems are analyzed. The method has been used for measuring the adenosine, guanosine, inosine, and uridine triphosphates resulting from the pyrophosphorolysis of ADP-Man, GDP-Glc, GDP-Man, IDP-Glc, IDP-Man, UDP-Glc, UDP-Man, UDP-Gal, UDP-N-AGlcm, and UDP-Xyl.

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REFERENCES

1. Verachtert, H., Bass, S. T., and Hansen, R. G., *Biochem. Biophys. Res. Commun.* **15**, 158 (1964).
2. Verachtert, H., Bass, S. T., and Hansen, R. G., *Federation Proc.* **23**, 379 (1964).
3. Verachtert, H., Bass, S. T., and Hansen, R. G., *Biochim. Biophys. Acta* **92**, 482 (1964).
4. Kornberg, A., and Horecker, B. L., *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. I, p. 323. Academic Press, New York, 1955.
5. Verachtert, H., Bass, S. T., Wilder, Janice, and Hansen, R. G., *Anal. Biochem.* **11**, 497 (1965).

6. ADAM, H., *Biochem. Z.* **335**, 25 (1961).
7. HELDT, H. W., *Biochem. Z.* **337**, 397 (1963).
8. OKAZAKI, R., OKAZAKI, T., STROMINGER, J. L., AND MICHELSON, A. M., *J. Biol. Chem.* **237**, 3014 (1962).
9. MICHELSON, A. M., *Biochim. Biophys. Acta* **91**, 1 (1964).
10. WOOD, W. A., AND GILFORD, S. R., *Anal. Biochem.* **2**, 601 (1961).

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Accelerated Chromatographic Analysis of Amino Acids Commonly Found in Physiological Fluids on a Spherical Resin of Specific Design

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In 1958, a two-column chromatographic procedure was announced for the analysis of amino acids and related compounds normally present in physiological fluids (1). This system, using a sulfonated styrene divinylbenzene copolymer cation-exchange resin which had been pulverized from large beads, required 44 hr for a complete analysis of complex samples such as blood plasma, tissue, and urine. Accelerated analysis was reported in 1964 using blended pulverized resin (2).

In addition to the chromatographic goals reported earlier (3, 4), the objectives sought in an improved resin system for the analysis of the amino acids normally found in physiological fluids are as follows:

1. To reduce analysis time in order to complete two physiological fluid analyses in a 24-hr day.
2. To improve resolution obtained by current procedures (1, 5, 6) if possible, or at least maintain the level of resolution of current systems.
3. To maintain ±3% precision and accuracy.
4. To use buffer systems currently employed.
5. To use column flow rates low enough to require only one standard-length reaction coil.

A slight modification of the two-column chromatographic procedure of Spackman, Moore, and Stein (1) was used with the Beckman Model 120 Amino Acid Analyzer, for purposes of comparative column performances.

The characteristics of the comparative systems are outlined below, with a discussion of those areas in each chromatographic procedure which are particularly troublesome for optimum resolution of specific amino acid peaks.

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MATERIALS AND METHODS

Sample Preparation

Synthetic Mixture of Amino Acids and Related Compounds. Amino acids obtained from California Corporation for Biochemical Research were dissolved in pH 2.2 sodium citrate buffer. The quantities analyzed on each column are indicated in parentheses on the chromatograms.

Human Urine.[2] A sample of Hyland Urine Chemistry Control, Lot No. 401U2 was deammoniated as follows:

The pH of the sample was adjusted to 11.7 with 4 *N* NaOH and placed in a desiccator with continuous aspiration for 6 hr. The sample was removed from the desiccator and adjusted to a pH of 2.2 with 6 *N* HCl, and made to final volume with pH 2.2 sodium citrate buffer.

Human Blood Plasma. Whole blood (7) was first centrifuged to obtain plasma and then deproteinized with picric acid. Excess picric acid was removed from the deproteinized sample by passing through a resin bed (Dowex 2-X8, 200–400 mesh) in the chloride form. The sample was adjusted to pH 8.0 and allowed to stand for 4 hr to convert cysteine to cystine. The sample was adjusted to pH 2 and made to volume with pH 2.2 sodium citrate buffer.

Reagents

Sodium Citrate Buffers. The buffers used for the analysis of amino acids using the *pulverized resin* system were (9): pH 3.28 (0.20 *N*) and pH 4.25 (0.20 *N*) sodium citrate for the analysis of the acidic and neutral amino acids. For the analysis of the basic amino acids, pH 4.26 (0.38 *N*) sodium citrate buffer was used.

The preparation of the buffers used in the *spherical resin* system is presented in Table 1.

Analysis of the amino acids using this system required buffers whose pH and ionic concentration were controlled to ±0.005 pH units and ±0.001 sodium ion concentration.

This degree of precision in the preparation of the buffers was necessary to obtain reproducible elution times. In practice it was discovered that the elution behavior of the amino acids functioned as a very sensitive pH metering device. It was possible to detect 0.01 pH unit variation in the buffer solutions by measuring the elution time of glutamic acid relative to that of proline and citrulline on the chromatogram.

[2] For the analysis of urine samples on a comparative research basis, desalting the sample prior to analysis would minimize sample differences due to salt content (8).

TABLE 1
SODIUM CITRATE BUFFERS

	pH[a]			
	3.25 ± 0.005	4.25 ± 0.02	4.26 ± 0.02	5.28 ± 0.02
Sodium concentration, N	0.20	0.20	0.38	0.35
Sodium citrate·$2H_2O$, gm	784.3	784.3	1490	1372.6
Concentrated HCl, ml	493	335	609	260
Thiodiglycol (TG), ml	200	200	—	—
Brij-35 solution (50 gm/100 ml), ml	80	80	80	80
Caprylic acid, ml	4	4	4	4
Final volume, liters	40	40	40	40

[a] Close control of the pH values, among other operating parameters, is critical if consistent elution times and good resolution between analyses are to be achieved.

Ninhydrin Reagent. Prepared according to the method of Spackman, Stein, and Moore (1).

Preparation of Ion-Exchange Columns

A. *Pulverized Resin System.* A 169 × 0.90 cm chromatographic column was used for the analysis of the acidic and neutral amino acids and a 69 × 0.90 cm chromatographic column for the basic amino acids. Before beginning the column packing operation it was necessary to remove the resin fines. These fines, which can be generated by physical and chemical attrition, must be removed to minimize operating back pressure.

The resin was mixed with 2 vol of column packing buffer to produce a slurry. For packing the 169-cm column, the pH 4.25 (0.20 N) sodium citrate buffer was used and for the 69-cm column the pH 5.28 (0.35 N) buffer was used. Neither of these buffers contained Brij-35 or thiodiglycol. The resin slurry was stirred just before pouring and the column filled with slurry. A buffer flow rate of 60 ml/hr was used to pack the column. When the resin bed had packed, buffer was aspirated from above the resin surface and the column was again filled with resin slurry. Several additional packings were necessary to fill the column. All columns were packed at 30°C.

Each column was regenerated with 0.2 N NaOH and the columns were completely equilibrated with the appropriate buffer.

B. *Spherical Resin System.* A 69 × 0.90 cm chromatographic column was used for the analysis of the acidic and neutral amino acids and a 29 × 0.90 cm chromatographic column for the basic amino acids.

A buffer flow rate of 50 ml/hr was used to pack the long column (for analysis of the acidic and neutral amino acids) to a height of 55.5 cm. The short column (for analysis of basic amino acids) was packed to a

height of 22.0 cm, at the same buffer flow rate. The long column was packed at a column temperature of 30°C and the short column at 33°.

Resins

A. *Pulverized Resin System.* (*1*) *Acidic and Neutral Amino Acids.* (Beckman Ion Exchange Resin Type 150 A. Processed from sulfonated styrene divinylbenzene copolymer resin. Nominally 8% cross-linked. Mean particle size 31–41 μ.) Portions of a typical chromatogram (synthetic calibration mixture) on a 159 × 0.9 cm resin column which had been developed at 30 ml/hr buffer flow rate, is represented in Fig. 1A. Only those chromatographic areas which present some difficulty of resolu-

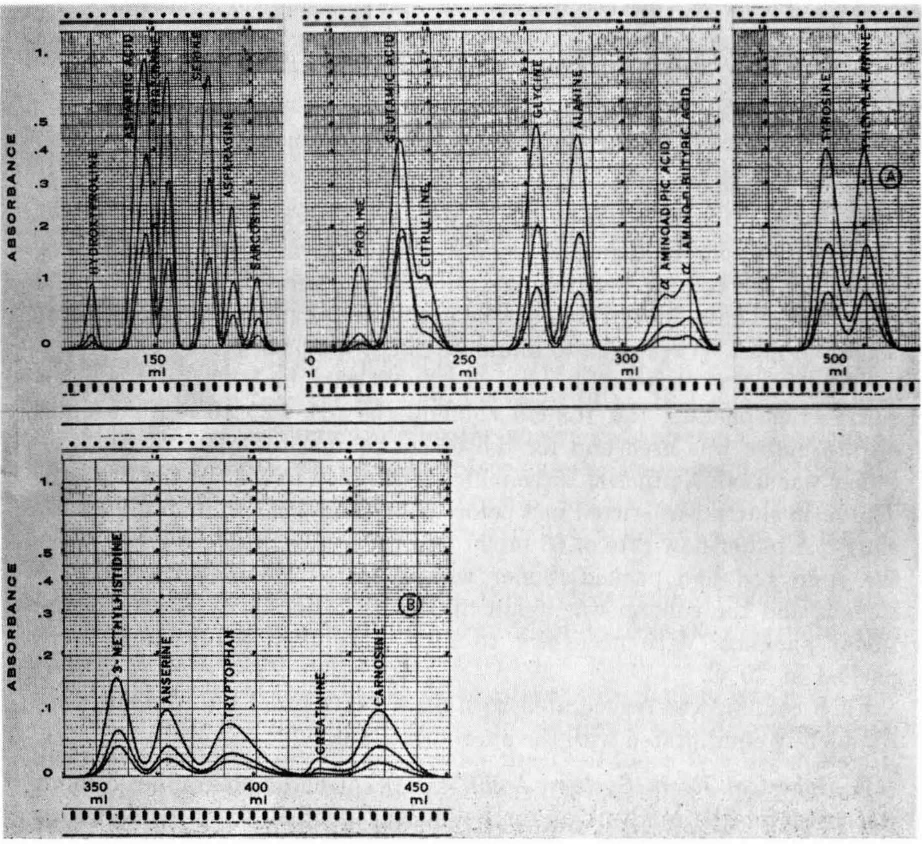

FIG. 1. Portions of a chromatographic analysis, using pulverized resin, on a synthetic mixture of amino acids commonly found in physiological fluids: (A) acidic and neutral amino acids on a 159-cm resin column; (B) determination of basic amino acids on a 56-cm resin column.

tion are shown. All other peaks were well resolved. A programed temperature change from 30° to 50°C was made at 10 hr and a buffer change from pH 3.28 (0.20 N) to pH 4.25 (0.20 N) sodium citrate occurred simultaneously, for an analysis time of 21 hours.

(*2*) *Basic Amino Acids.* (Beckman Ion Exchange Resin Type 50A. Processed from sulfonated styrene divinylbenzene copolymer resin. Nominally 8% cross-linked. Mean particle size 25–31 μ.) Portions of a typical chromatogram (synthetic calibration mixture) on a 56 × 0.9 cm resin column which was also developed at a buffer flow rate of 30 ml/hr are presented in Fig. 1B. Elution was started at 30°C and changed to 50° at 11 hr, 20 min. A pH 4.26 (0.38 N) sodium citrate buffer was used for column development. Total analysis time was 22 hr, 30 min.

Earlier, we had presented a spherical resin of specific design (3) for the analysis of the amino acids normally found in protein or peptide hydrolyzates. The analysis of a synthetic mixture of the amino acids found in physiological fluids was made using this hydrolyzate resin.

B. *Spherical Resin System.* (*1*) *Acidic and Neutral Amino Acids.* (Beckman Custom Research Resin Type AA-15. A sulfonated styrene copolymer resin, nominally 8% cross-linked. The spherical particles have a mean diameter of 22 ± 6 μ. This resin is normally used for the analysis of acidic and neutral amino acids commonly found in the hydrolyzates of proteins or peptides.) A 56 × 0.9 cm resin column was developed at a buffer flow rate of 40 ml/hr. Elution was started at 30°C with pH 3.25 (0.20 N) sodium citrate buffer. A buffer change was made to pH 4.25 (0.20 N) sodium citrate and a temperature change was made to 55°C, to coincide with the 55° temperature used in the hydrolyzate procedure. Using the synthetic calibration mixture, the analysis time was 6 hr, 35 min.

(*2*) *Basic Amino Acids.* (Beckman Custom Research Resin Type AA-27. A sulfonated styrene copolymer resin, nominally 8% cross-linked. The spherical particles have a mean diameter of 15 ± 6 μ. This resin is normally used for the analysis of basic amino acids commonly found in hydrolyzates of proteins or peptides.) A 20 × 0.9 cm resin column was developed at a buffer flow rate of 40 ml/hr, using the synthetic calibration mixture, and changed to 55°C after the elution of 1-methylhistidine. A pH 4.26 (0.38 N) sodium citrate buffer was used throughout the analysis. Total analysis time was 7 hr, 40 min.

C. *Spherical Resin System* (specifically developed for the analysis of the amino acids commonly found in *physiological fluids*).

Beckman Custom Research Resin: For *analysis of acidic and neutral amino acids,* Type PA-28, a sulfonated styrene copolymer resin, nominally 7.5% cross-linked. The spherical particles have a mean diameter of 16 ± 6 μ.

For *analysis of basic amino acids,* Type PA-35, a sulfonated styrene copolymer resin. Nominally 7.5% cross-linked. The diameter of the spherical particles is 13 ± 6 μ.

Figure 2 illustrates the resolution of a synthetic calibration mixture of basic, acidic and neutral amino acids[3] at a buffer flow rate of 50 ml/hr and a ninhydrin flow rate of 25 ml/hr. Analysis of the basic amino acids was started at 33°C and of the acidic and neutral amino acids at 30°. A temperature change to 55° was made in the basic analysis at 185 min, and a buffer change from pH 4.26 (0.38 *N*) to pH 5.28 (0.35 *N*) at the same time (10). A temperature change to 55° was made in the acidic and neutral analysis at 100 min, and a buffer change from pH 3.25 (0.20 *N*) to pH 4.25 (0.20 *N*) at 150 min; recorder chart speed was 6 in./hr with 1 dot/2 sec printing speed. To prevent the recorder dots from cluttering on the chromatogram, the alternate 570 mμ Helipot Potentiometer was adjusted so that the pen printed on the left side of the chart. For the analysis of the basic amino acids at the flow rates indicated above, total analysis time was 5 hr, 45 min. Total analysis time for the acidic and neutral amino acids was 5 hr, 25 min.

A single-length reaction coil was used (11.25 ml volume) and the accuracy and precision in the sample ranges used was 100 ± 3.0%.

A daily schedule is presented in Table 2.

RESULTS

A. *Pulverized Resin System.* (*1*) *Acidic and Neutral Amino Acids.* The aspartic acid-threonine separation was good with a peak height-to-valley ratio[4] of about 0.15. The citrulline peak appeared as a shoulder on the glutamic acid peak and the separation of α-aminoadipic acid and α-amino-*n*-butyric acid was inadequate. The tyrosine-phenylalanine separation had a peak height-to-valley ratio of about 0.25. All the other peaks presented were well resolved.

(*2*) *Basic Amino Acids.* Resolution of the components present in a synthetic calibration mixture was good except for separations in the

[3] Amino acid peaks are eluted in the same sequence as with the pulverized system.

[4] Peak height-to-valley ratio: where two peaks are juxtaposed so that the valley between them does not reach baseline, the peak height-to-valley ratio is a/b, where a is the absorbance read at the minimum of the valley and b is the absorbance read at the maximum of the lower of the two peaks. The absorbance values of the peaks and valleys must be corrected to zero baseline on the chromatogram. These ratios are indicative of the resolving power.

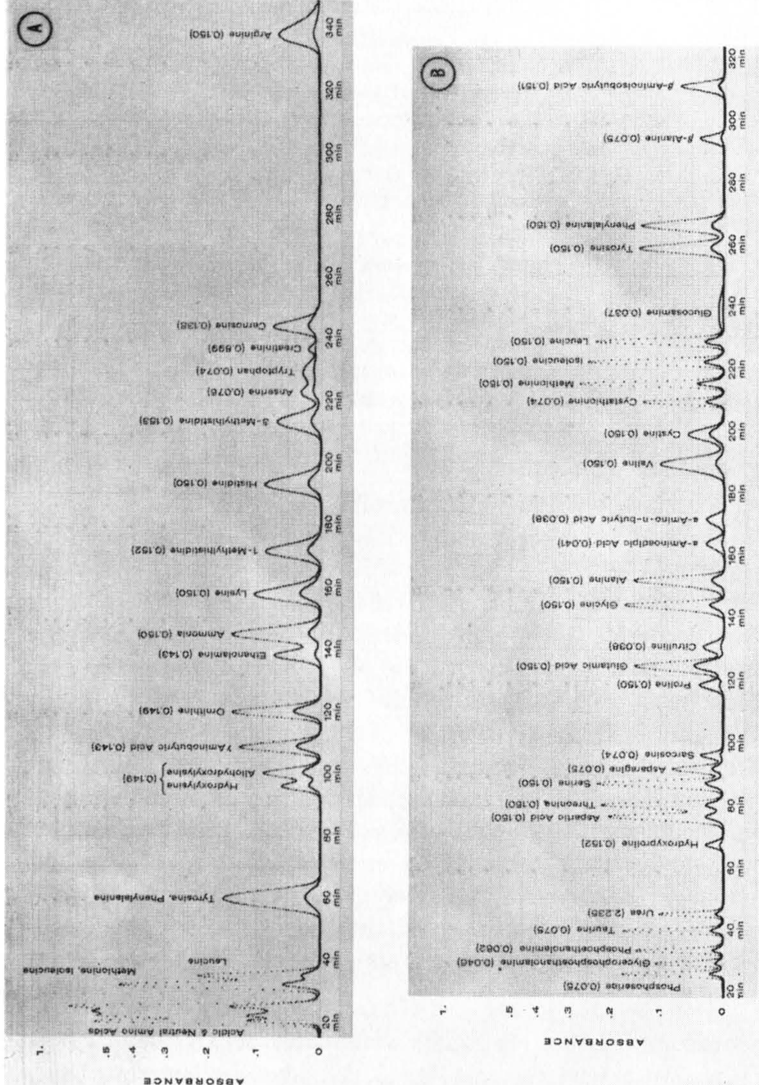

FIG. 2. Analysis of a synthetic mixture of amino acids and related compounds, using spherical resin, found in physiological fluids: (A) determination of the basic amino acids on a 22-cm resin column (PA-35 resin); (B) determination of acidic and neutral amino acids on a 55.5-cm resin column (PA-28 resin). Sample quantities, in parentheses, are in micromoles.

TABLE 2
TYPICAL DAILY SCHEDULE FOR COLUMN ANALYSIS

Time	Lapsed time, min	Operational changes
		Short-Column Analysis (33°C)
8:30 A.M.	0	Start buffer pump (pH 4.26, 0.38 *N*) and ninhydrin pump to coil. Start recorder.
8:45 A.M.	15	Zero baseline on recorder.
11:35 A.M.	185	Buffer change takes place to (pH 5.28, 0.35 *N*).
11:35 A.M.	185	Temperature change to 55°C.
2:15 P.M.	345	Analysis is complete.
		Long-Column Analysis (30°C)[a]
2:30 P.M.	0	Start buffer pump (pH 3.25, 0.20 *N*) and ninhydrin pump to coil. Start recorder.
2:45 P.M.	15	Zero baseline on recorder.
4:10 P.M.	100	Temperature change to 55°C.
5:00 P.M.	150	Buffer change to (pH 4.25, 0.20 *N*).
7:55 P.M.	325	Analysis is complete.

[a] A rapid cooling coil used in circulating water bath.

anserine, tryptophan, creatinine, and carnosine areas. The area under the creatinine peak could be quantitated only with difficulty, using the absorbance method.

In our study of pulverized resin systems, the resolution of basic amino acids has varied considerably with different batches of pulverized resin that quantitative resin tests showed to be identical. Hamilton (11) described these resin variations as minute deviations, probably due to nonuniformity during resin synthesis, or the mixing of different resin lots. The separations of hydroxylysine and allohydroxylysine, tryptophan and creatinine, and ethanolamine and ammonia showed the greatest variations in resolution. Increasing the flow rates to achieve a reduction in analysis time produced poorer peak separations on basic as well as acidic and neutral amino acid analyses. The higher flow rates reduced the plate contact time, decreasing the number of theoretical plates of the resin column. In effect this increase in flow rate reduced the effective length of the column.

B. *Spherical Resin System. (1) Acidic and Neutral Amino Acids (Type AA-15)*. The aspartic acid-threonine peak height-to-valley absorbance ratio was 0.48. The valley between serine and asparagine did not reach baseline. Although proline was well separated from aspartic acid, the citrulline peak appeared as a shoulder on the glutamic acid peak. α-Aminoadipic acid and α-amino-n-butyric acid were incompletely separated. The tyrosine-phenylalanine peak height-to-valley absorbance

ratio was 0.137. All peaks were broad, which was thought to be caused by an overload of column capacity, and the total run time was extended. Therefore, the sample load was decreased by a factor of 3. No improvement in resolution was noted.

(*2*) *Basic Amino Acids* (*Type AA-27*). The resolution on most of the peaks was unacceptable, and, in those areas where resolution was quite critical, the absorbance ratios of peak height-to-valley exceeded 0.50.

It was apparent that the spherical resin which had been developed for the analysis of those amino acids normally found in protein or peptide hydrolyzates did not give the desired resolution of complex physiological mixtures under these conditions.

C. *Spherical Resin System.* (*1*) *Acidic and Neutral Amino Acids* (*Type PA-28*). The acidic and neutral amino acids of our synthetic mixture are shown in Fig. 2B, and the analysis conditions are outlined in Table 3. The sample size (micromoles) is shown on the chromatogram. The aspartic acid-threonine peak height-to-valley absorbance ratio was 0.26 and the tyrosine-phenylalanine ratio was 0.074. The separation of proline from glutamic acid and citrulline from glutamic acid was almost complete. α-Aminoadipic acid and α-amino-*n*-butyric acid were almost completely separated. In the long column analysis there are also several amino acids whose movement is influenced by a temperature or buffer change made either too early or too late. As column temperature increases, the resolution of α-aminoadipic acid and α-amino-*n*-butyric acid improves; however, the separation between valine and cystine decreases. The temperature change should occur at the time which impairs both separations as little as possible. The buffer change should take effect just after cystine is eluted to obtain the best resolution of cystathionine, methionine, and the leucines.

(*2*) *Basic Amino Acids* (*Type PA-35*). The analysis of the basic amino acids of a synthetic mixture is shown in Fig. 2A. The analysis conditions are outlined in Table 3 and the sample size, in micromoles of each component, is indicated on each peak on the chromatogram. There are several peak areas which are affected by slight variations in temperature and buffer changes. The amino acids whose elution times are affected by temperature variations are carnosine, arginine, and tryptophan. An increase in the column temperature will cause carnosine and arginine to be eluted sooner, resulting in improved resolution. A temperature change which takes place too early will cause tryptophan to be eluted with anserine.

If the buffer change takes place just after anserine is eluted, the

TABLE 3

COLUMN DATA FOR PULVERIZED AND SPHERICAL RESINS USED FOR PHYSIOLOGICAL ANALYSIS

Parameter	Basic amino acids		Acidic and neutral amino acids	
	Pulverized	Spherical	Pulverized	Spherical
Resin column length, cm	56–58	22.0	159	55.5
Column diameter, cm	0.9	0.9	0.9	0.9
Temperature (°C) and times[a]	30°, 0–680 min 50°, 680–1350 min	33°, 0–185 min 55°, 185–345 min	30°, 0–600 min 50°, 600–1260 min	30°, 0–100 min 55°, 100–325 min
Resin type	50A	PA-35	150A	PA-28
Buffers and times	pH 4.26 (0.38 *N*), 0–1350 min	pH 4.26 (0.38 *N*), 0–185 min pH 5.28 (0.35 *N*), 185–345 min	pH 3.28 (0.20 *N*), 0–600 min pH 4.25 (0.20 *N*), 600–1260 min	pH 3.25 (0.20 *N*), 0–150 min pH 4.25 (0.20 *N*), 150–325 min
Buffer flow rate, ml/hr	30	50	30	50
Ninhydrin flow rate, ml/hr	15	25	15	25
Back-pressure, psi	50 (30°C)	210 (33°C)	60 (30°C)	240 (30°C)
Analysis time	22 hr, 30 min	5 hr, 45 min	21 hr	5 hr, 25 min

[a] It is imperative that the water level in the column circulating water bath be kept at the "full" mark to prevent variation of temperature gradients in subsequent analysis.

remaining peaks will be sharper and arginine will be eluted from the column sooner. A buffer change which takes effect too early will elute tryptophan and carnosine too soon, causing these peaks to overlap those of anserine and creatinine.

The chromatographic analysis of human urine (12) is presented in Fig. 3. A 0.6 ml sample of urine was chromatographed on the short column for the basic amino acids and a 0.5 ml sample was chromatographed on the long column for the acidic and neutral components.

When human plasma is analyzed as shown in Fig. 4, excellent resolution is obtained. A sample size corresponding to 1.7 ml of protein-free normal human plasma was used for analysis on both the short and long columns.

DISCUSSION

Experimentation on our earlier polymer systems revealed problems in the separation of actual physiological materials, although resolution of synthetic mixtures was acceptable. These problem areas were:

1. *Acidic and neutral amino acids.* The resolution of glutamic acid in a urine sample was inadequate, presenting quantitation difficulties.
2. *Basic amino acids.* Separations of ethanolamine and ammonia, of lysine and 1-methylhistidine, and of tryptophan and creatinine in a urine sample were inadequate.

An empirically designed resin enabled these areas, which can be used as "yardsticks" in evaluating complete chromatographic systems for physiological fluid analysis, to be resolved.

Differences in peak heights (absorbance units) between the spherical and pulverized resin systems were quite dramatic. When using the spherical resin for the basic amino acid analysis, several peak heights (absorbance units) increased 2.6-fold over those obtained with the pulverized system at the same sample load and the same length of cuvet light path.

In the acidic and neutral amino acid analysis many of the peaks obtained using the spherical resin system showed an increase in height (absorbance units) of 2.2-fold over those obtained with the pulverized resin system.

This would indicate that the amino acids are being eluted from the spherical resin column in narrower bands, resulting in improved resolution of the individual amino acid peaks.

Figure 3A presents the chromatographic results for the analysis of urine. The ethanolamine-ammonia separation is improved, as is the integrity of the lysine peak, over that of the pulverized system. This

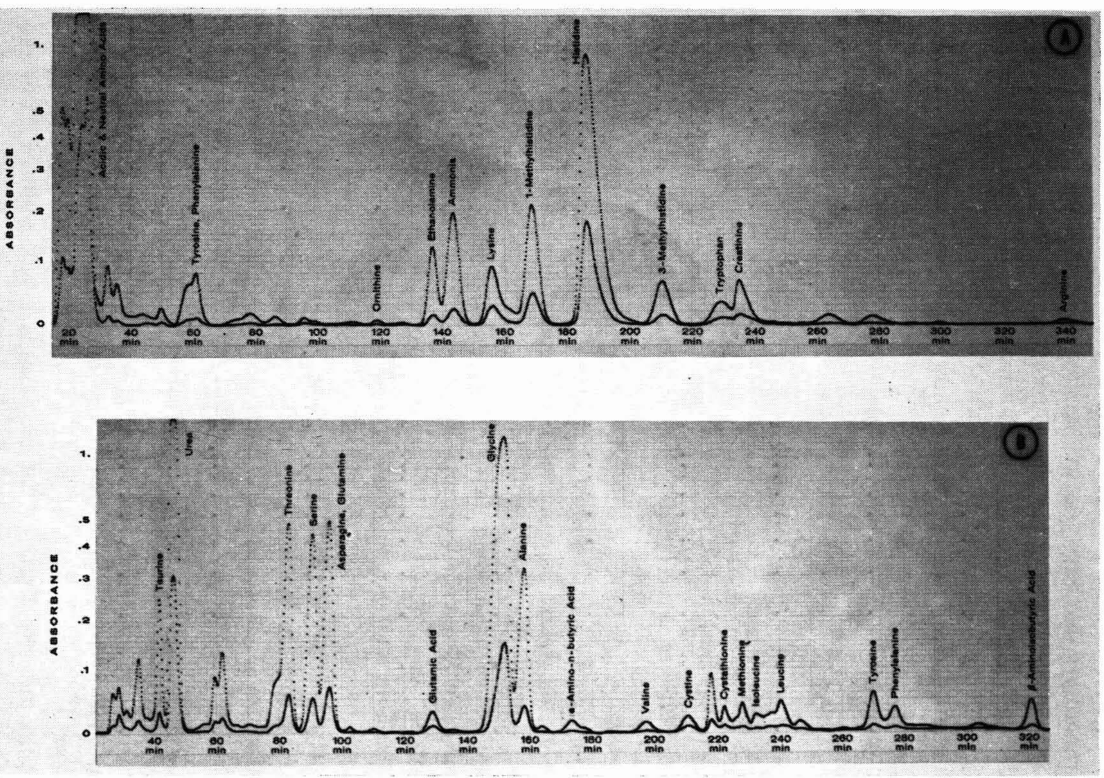

FIG. 3. Analysis of normal human urine on spherical resin: (A) determination of the basic amino acids on a 22-cm resin column (PA-35 resin); (B) determination of acidic and neutral amino acids on a 55.5-cm resin column (PA-28 resin). Samples applied to the 22-cm and 55.5-cm columns were 0.6 ml and 0.5 ml of urine, respectively.

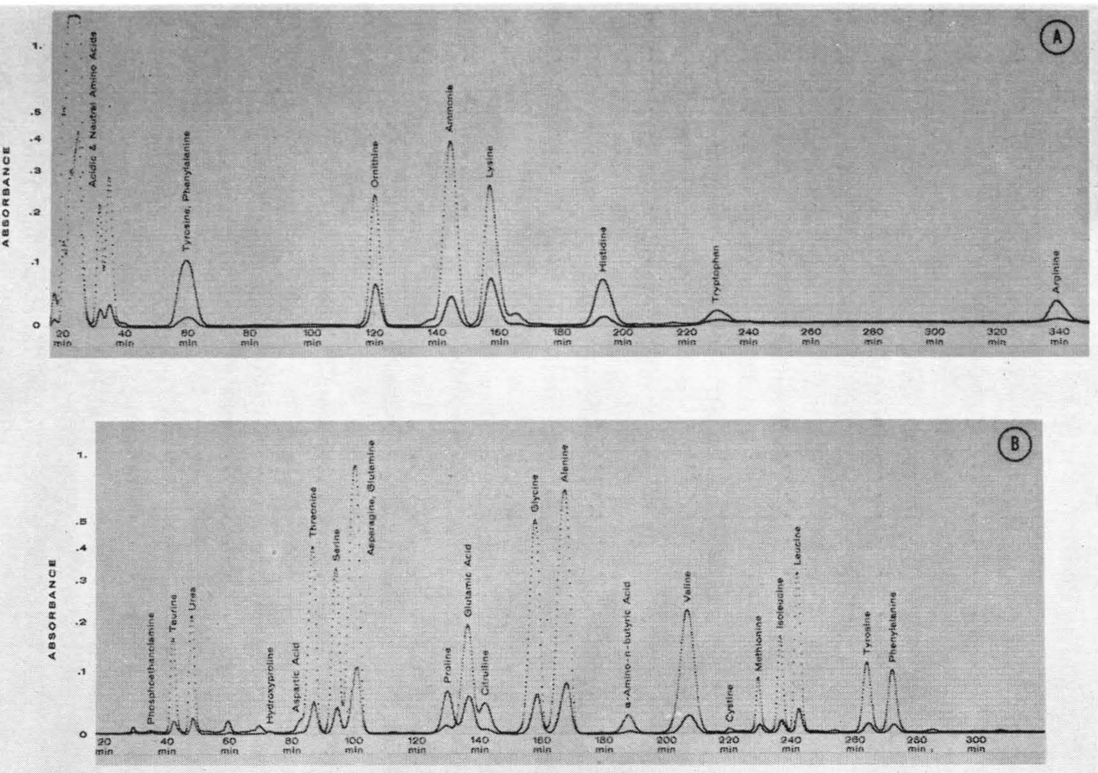

FIG. 4. Amino acid composition of protein-free human blood plasma, on spherical resin: (A) determination of the basic amino acids on a 22-cm resin column (PA-35 resin); (B) determination of acidic and neutral amino acids on a 55.5-cm resin column (PA-28 resin). A sample corresponding to 1.7 ml of plasma was added to each column.

improved resolution makes it possible to detect and quantitate an unknown ninhydrin-positive substance eluted between lysine and 1-methylhistidine. Also, the separation of tryptophan and creatinine is improved.

Figure 3B, acidic and neutral amino acids of urine, shows the separation of serine and the asparagine-glutamine peaks to be sharper and the valley to be lower in absorbance units. It is now possible to calculate the area under the glutamic acid peak by the height-width method. The peak height-to-valley ratio between tyrosine and phenylalanine is reduced below 0.50, which facilitates area quantitation.

The ability to carry out the chromatographic column development at higher flow rates, allowing low operating back-pressures, with improved peak resolution is largely due to an optimally designed resin column system. The spherical form of the resin minimizes liquid flow channeling and eddy dispersion in the column. Polymerization of the resin was controlled to minimize cross-linking variations among particles. Physical and chemical properties of the resin were controlled to give a uniform polymer matrix. Although the use of a slightly lower nominally cross-linked resin was helpful in some of the peak separations, success is in large part due to the controls mentioned above.

The resolution of acidic and neutral amino acids at higher flow rates was investigated. The same 55.5-cm long column, as indicated above, was operated (using the same buffer and ninhydrin system previously mentioned) at 60 ml/hr [97.8 linear cm/hr (13)] buffer flow rate and 30 ml/hr ninhydrin reagent flow rate. The operating back-pressure was 285 psi at 30°C. A sample load of a synthetic amino acid mixture contained the same quantities as shown in Fig. 2B of each amino acid tested. The temperature was changed from 30° to 55° at 85 min and the buffer changed from pH 3.25 (0.20 N) to pH 4.25 (0.20 N) at 125 min. The aspartic acid-threonine peak height-to-valley absorbance ratio was 0.270. The tyrosine-phenylalanine peak height-to-valley absorbance ratio was 0.138. The analysis time was 4 hr, 45 min, through the analysis of β-aminoisobutyric acid. There did not appear to be any sacrifice of resolution between the other amino acids. The slower 50 ml/hr flow rate for the long column eliminates the need to change the ninhydrin pump stroke setting between the short and long column analyses. However, if analysis time is an important consideration the faster flow rates will give satisfactory chromatographic results. This resin system can be a means of faster analysis times as improvements in elution techniques are developed.

Eighteen analyses have been made on each of the spherical resin columns with the final back-pressures reaching 245 psi (30°) on the long

column and 212 psi (33°) on the short column. Each column was operated at the usual 50 ml/hr buffer flow rate. However, several chromatographic glass columns which had been recently obtained exhibited abnormally high back-pressures (300 psi) during the long column analysis. When the same resin was repacked in the columns originally used, the previously observed 240 psi back-pressure was obtained. In our opinion this increase in back-pressure is due to differences in the glass surfaces. This phenomenon may be present in most chromatographic operations, using glass columns, and may be a functional part of the chromatographic process. It has been evident that the resolution of some amino acids has been affected by the glass surface. It was also noted that there was a 2-cm resin bed compression (usual compression was about 0.5 cm during first analysis) and that this compression was accompanied by a sharp rise in back-pressure. The resin bed had apparently undergone an irreversible compression which could be remedied only by repacking the columns. A report on the effects of glass surfaces on the chromatographic process is in preparation.

The detergent Brij-35 was used by Moore and Stein (14) in the sodium citrate buffers. They found that they were then able to operate their columns at faster flow rates without broadening the peaks on the effluent curves. Preliminary investigation as to the effects of this detergent in our chromatographic system revealed that high back-pressures, as has been previously stated, could often be attributed to glass surface effects on the column. It has been possible to eliminate the Brij-35 detergent to obtain an average of 20 psi decrease in column operating pressures. However, where high concentrations of dissolved gases are present in the buffers, the addition of a surfactant might be desirable.

SUMMARY

Chromatographic analysis and improved resolution of the amino acids commonly found in physiological fluids have been accelerated by improved resin technology. The acidic and neutral amino acids are analyzed on a 55.5 × 0.9 cm resin column and the basic amino acids on a 22.0 × 0.9 cm resin column. Buffer flow rates of 50 ml/hr (79 linear cm/hr) are pumped through both columns under pressures of 240 psi and 210 psi for the long and short columns, respectively. This system reduces the analysis time from the previously reported 44 hr of a physiological-type amino acid mixture, urine, or blood plasma to 11 hr, 10 min (5 hr, 25 min, for the acidic and neutral amino acids and 5 hr, 45 min, for the basic amino acids). Two complete physiological fluid analyses are possible in a 24-hr day.

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REFERENCES

1. Spackman, D. H., Stein, W. H., and Moore, S., *Anal. Chem.* **30,** 1190 (1958).
2. Spackman, D. H., *Federation Proc.* **23,** 371 (1964).
3. Benson, J. V., and Patterson, J. A., *Federation Proc.* **23,** 371 (1964).
4. Benson, J. V., and Patterson, J. A., *Anal. Chem.* **37,** 1108 (1965).
5. Moore, S., Spackman, D. H., and Stein, W. H., *Federation Proc.* **17,** 1107 (1958).
6. Moore, S., Spackman, D. H., and Stein, W. H., *Anal. Chem.* **30,** 1185 (1958).
7. Stein, W. H., and Moore, S., *J. Biol. Chem.* **211,** 915 (1954).
8. Drèze, A., Moore, S., and Bigwood, E. J., *Anal. Chim. Acta* **11,** 554 (1954).
9. "Beckman Technical Bulletin," TB 6028A, March, 1962.
10. Elwyn, D., personal communication.
11. Hamilton, P. B., *Anal. Chem.* **35,** 2055 (1963).
12. Stein, W. H., *J. Biol. Chem.* **201,** 45 (1953).
13. Hamilton, P. B., *Anal. Chem.* **32,** 1779 (1960).
14. Moore, S., and Stein, W. H., *J. Biol. Chem.* **192,** 663 (1951).

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Factors Affecting the Estimation of Nucleic Acids in *Euglena gracilis*

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Most methods used for the extraction and estimation of nucleic acids from biological materials are based on procedures which were developed by Schneider (1) or Schmidt and Thannhauser (2). Both procedures were principally used with animal tissues, although they have been applied to different kinds of organisms. It is evident from recent investigations that, in addition to the RNA and DNA fractions, both procedures extract numerous contaminants which interfere with nucleic acid determination by ultraviolet absorption, pentose color reactions, and phosphorus analysis. This is discussed critically in a review of nucleic acid analytical procedures by Hutchison and Munro (3). The Ogur and Rosen (4) method was especially used for the estimation of nucleic acids in plant tissues. It is a method based on the differential extraction of RNA and DNA by PCA[3] at different concentrations and temperatures. This procedure has been adopted for nucleic acid estimation in *Euglena* (5).

Smillie and Krotkov (6) tested the Schneider, Schmidt-Thannhauser, and Ogur-Rosen methods for *Euglena* cells. They specifically studied the efficiency of preliminary extractions for acid- and lipid-soluble phosphorus compounds.

The usual preliminary treatment is to remove acid-soluble and lipid-soluble substances in order to eliminate any possible interfering contaminants. Removal of acid-soluble substances is commonly carried out by washing with either cold TCA or cold PCA. The usual concentration of TCA employed is 5–10%, and 1.2 *N* (12%) for PCA, as recommended by Schneider, Hogeboom, and Ross (7) or 0.2 *N* employed by Ogur and Rosen (4). For routine analysis Hutchison, Downie, and

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[3] Abbreviations used in this paper: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; PCA, perchloric acid; TCA, trichloroacetic acid.

Munro (8) recommend 5% cold TCA. Although no acid precipitant leads to large losses of nucleic acids, Venkataraman and Lowe (9) and Venkataraman (10) have reported losses of RNA from the acid precipitate when the tissue is put in contact with ethanol as a lipid solvent. Hallinan, Fleck, and Munro (11) demonstrated that the acid concentration is critical in causing solubilization of RNA when lipid solvents are later used. Smillie and Krotkov (6) reported that for *Euglena* cells 10% PCA and 15% TCA remove a small amount of RNA, and in order to take off any interfering substances without RNA solubilization they recommended a first extraction with methanol followed by methanolic 0.05 N formic acid at 4°C and afterward washing with 5% TCA, i.e., a modification of the Ogur and Rosen preliminary extraction procedure employed for plant tissue. Cooper and Loring (12) reported that RNA recovery from tobacco-leaf chloroplasts is not affected when preliminary treatment is omitted. It would thus appear that the preliminary treatment requires critical re-evaluation. It is apparent that any of the strong acid treatments and defatting procedures is likely to produce some loss of nucleic acids and not ensure complete elimination of contaminants.

RNA and DNA can be estimated by differential extraction—Schmidt-Thannhauser (2) or Ogur-Rosen (4) methods—or by the Schneider (1) procedure that extracts RNA and DNA together. In the first two methods, color reactions, phosphorus analysis, or ultraviolet absorption photometry are applicable for both nucleic acid estimations if no interfering substances contaminate the fractions. In the Schneider procedure, the specific color reactions provide the only method available.

The elimination of interfering substances was overcome by different techniques: absorption on ion-exchange resin (13), paper electrophoresis (14), or paper chromatography (15)—all are methods too laborious for multiple analyses.

After taking into consideration the advantages of the spectrophotometric determination and the possibility for determining the correction due to interfering substances by absorption measurement at additional wavelengths (16, 17), the present investigation was undertaken in order to seek a suitable method for RNA estimation in *Euglena* cells. The high specificity of the deoxypentose color reaction makes the determination of DNA less troublesome than that of RNA in the presence of contaminants. However, Chiba and Sugahara (18) found that there is a chloroplast DNA fraction in spinach leaves extracted only in high PCA concentration. Since *Euglena* cells contain such a DNA chloroplast fraction (19) our investigation was also focused on the evaluation of DNA extraction by hot acid methods.

Our present investigation deals with: (*a*) study of the contaminants obtained after alkali hydrolysis of the RNA fraction; (*b*) the suitable conditions for this alkaline digestion; (*c*) choice of the best preliminary extraction procedures; and (*d*) the satisfactory conditions for extraction and determination of the DNA.

METHODS

Euglena gracilis strain z was used. Cells were grown in semisynthetic medium (20) in the light or in the dark, at 24°C. They were collected, washed twice with distilled water, and frozen at —20°. Cell number was determined in a hemocytometer after fixation with 5% formaldehyde. Each determination was made in quadruplicate after suspending the freshly collected cells in a known volume of distilled water.

I. Treatment of the Samples

(*a*) *Extraction of Acid-Soluble and Alcohol-Soluble Substances.* Preliminary treatments employed the following solvents at 10 to 20 times the volume (0.5–1.0 ml) of packed cells.

Procedure I: Packed cells were extracted several times with 10 ml of ethanol at 0°C for 10-min periods until a pale yellow residue was obtained. Usually with dark grown cells two extractions were necessary but green cells needed three or four cold alcohol treatments. The residue was then extracted twice with 10 ml of cold 5% TCA for 1 hr, followed by extraction with cold ethanol, ethanol at room temperature, ethanol-ether (3:1) at room temperature, ethanol-ether (3:1) in a boiling water bath for 1–2 min, and ether, and was finally dried at room temperature before digestion in alkali. Sometimes the alkaline digestion was performed directly after the hot ethanol-ether extraction.

Procedure II: Similar to Procedure I, except that the initial alcohol extractions were omitted.

Procedure III: Same as Smillie and Krotkov's (6) preliminary method for acid and alcohol extraction—cold methanol with 0.05 N formic acid followed by cold 5% TCA and lipid solvents.

Procedure IV: Cells were extracted twice with 1.0 N PCA at 0°C for 1 hr. The residue was resuspended in distilled water, neutralized with KOH in the cold, made up to 0.3 N with 10 N KOH, and subjected to alkaline digestion.

Procedure V: Cells were extracted twice with 10 ml of cold 5% TCA for 1 hr. The residue was resuspended in cold distilled water, neutralized and made up to 0.3 N with 10 N KOH, and subjected to alkaline digestion. Extraction of the TCA residue with ether (11) was found to be unnecessary.

(*b*) *Alkaline Digestion.* The pretreated samples were digested in a water bath at 37°C with 0.3 *N* KOH. The volumes used were 12 ml in the procedure where lipid solvents were omitted and 10 ml for the lipid-extraction materials. At the end of the incubation the samples were chilled, neutralized with concentrated PCA and brought up to 0.5 *N* PCA. The resulting precipitate was removed by centrifugation and extracted with 10–12 ml of cold 0.5 *N* PCA. The two extracts were combined and analyzed for RNA.

(*c*) *Isolation of the DNA Fraction with Hot PCA.* After the alkali hydrolysis, the precipitate was resuspended in 8 ml of 0.5 *N* PCA and incubated in a water bath at 70°C for 20 min. This step was repeated three times and the combined supernates were analyzed for DNA. The residue was examined for the presence of DNA resistant to PCA extraction by repeating the extraction with higher PCA concentration.

II. Purification of Euglena RNA

RNA was prepared by phenol extraction (21). Light grown cells were washed twice with distilled water and resuspended in 0.01 *M* acetate buffer pH 5.1 (ten times the packed cell volume) and 1% sodium dodecyl sulfate. To this suspension an equal volume of freshly redistilled phenol (Merck) saturated with water was added and stirred for 5 min at 60°C and quickly chilled in an iced water bath. The phenol and water phases were separated by centrifugation. The phenol phase was reextracted once more with fresh acetate buffer. The water phases were combined and the RNA precipitated by addition of 2 vol of ethanol in the cold. After washing with ethanol, the precipitate was resuspended in 0.01 *M* acetate, pH 5.1, 0.05 *M* NaCl, and 0.001 *M* MgCl, and dialyzed against the same buffer for 48 hr at 4°C. Insoluble material was eliminated by centrifugation at 10,000 $\times$ *g* for 10 min. The RNA was obtained from the clear supernatant solution by addition of 2 vol of ethanol. The precipitate was washed with cold 5% TCA, cold ethanol, ethanol-ether (3:1), and ether, and dried at room temperature. The dried RNA fraction was hydrolyzed with 0.3 *N* KOH for 18 hr at 37° and the alkali hydrolyzate was further purified from non-nucleotide phosphorus compounds with the use of a small Dowex 1 (13) column. The mixture of 2′,3′-ribonucleotides from *Euglena* RNA have ϵ(P) of 10,300 at pH 2.

III. Chemical Analysis

The pentose of RNA was estimated by the phloroglucinol procedure of Bolognani, Coppi, and Zambotti (22). The ribose used as standard was calibrated against the *Euglena* 2′- and 3′-ribonucleotide mixture and yeast 2′- and 3′-ribonucleotides. To convert μg ribose into μg RNA-P,

the factor 0.608 was used. DNA was estimated by the Burton (23) procedure, using as a standard herring sperm DNA (California Biochemical Research) purified by the Sevag procedure (24). Since the color yield of the diphenylamine reaction varies with the concentration of PCA, the standard was used at the same PCA concentration as that of the samples. Protein was assayed by a modified biuret (25) method. Phosphorus was estimated by the method of Allen (26).

RESULTS AND DISCUSSION

Isolation and Estimation of Breakdown Products Resulting from Alkali Treatment. Dark grown *Euglena* cells treated with Procedure I were used. Alkali hydrolysis for this purpose employed 1 N KOH at 37°C for 18 hr. The acid-soluble, nondialyzable material was lyophilized. It contained, by dry weight, 2% ribose, 92% protein, and 2.5% phosphorus. Its UV absorption spectrum (Fig. 1) did not have a maximum

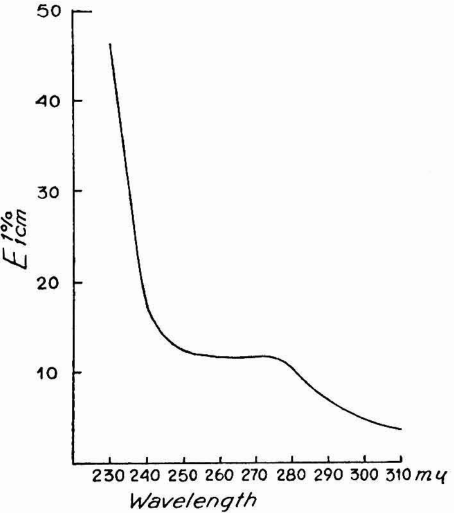

FIG. 1. Ultraviolet spectrum of acid-soluble breakdown products produced by alkali hydrolysis of *Euglena* cells in 0.5 N PCA.

at 275 mμ but a plateau from 260 to 275 mμ. $E^{1\%}_{1\,\text{cm}}$ at 260 mμ was 11.43 in 0.5 N PCA. Thus it appears that alkaline hydrolysis releases a significant amount of peptide material.

The concentration of RNA in the presence of the preceding contaminants is given by:

$$C_{\text{RNA-P}}(\mu\text{g/ml}) = 13.6(A_{260} - A_{275})$$

where A_{260} and A_{275} are the absorbancies of the hydrolyzate in 0.5 N PCA at the respective wavelengths. The effect of the contaminant is eliminated, since it has the same absorbancy at these two wavelengths. The factor 13.6 was calculated from the molar extinction values of the purified mixture of 2′- and 3′-ribonucleotides. Purified 2′- and 3′-ribonucleotides and the contaminants prepared from the *Euglena* cells were mixed in various proportions (Table 1), and absorption measured. There was good agreement, within the experimental error.

TABLE 1

ADDITIVITY OF ULTRAVIOLET ABSORPTION FOR *Euglena* RIBONUCLEOTIDES AND THE MATERIAL RELEASED BY ALKALINE DIGESTION

Pure *Euglena* 2′- and 3′-ribonucleotides and the nondialyzable acid-soluble product of alkaline digestion were mixed in 0.5 N PCA and from the optical density at 260 and 275 mμ the RNA content was calculated according to the formula. Each mixture was prepared in duplicate.

Components of mixture		Expected OD for mixture at 260 mμ	OD found		μg RNA-P/ml, calculated by formula	Difference
μg RNA-P/ml	μg UK[a]/ml		260 mμ	275 mμ		
			0.400	0.332	0.938	
0.949	61.5	0.386	0.397	0.328	0.925	0.8%
			0.260	0.220	0.544	
0.541	61.5	0.252	0.260	0.221	0.530	0.8%
			0.500	0.419		
1.082	102.5	0.477	0.500	0.419	1.100	1.5%

[a] UK, unknown substances.

Kinetics of the Alkali Hydrolysis. Dark grown *Euglena* cells were washed and extracted by Procedure I followed by 0.3 N KOH at 37°C. Samples were removed at different times and the acid-soluble fractions were prepared as indicated and analyzed (Fig. 2). From the RNA and ribose values it appears that RNA is fully extracted in about 3–4 hr, that during this interval there is also release of non-nucleotide ribose and peptide material, and that further alkaline digestion only produces an increase in the amount of protein in the acid-soluble fraction.

The release of RNA during alkaline digestion was also followed by absorption of the acid-soluble material on Dowex 1 and elution of the 2′- and 3′-ribonucleotides (Table 2). The results obtained in this manner are in excellent agreement with those obtained by differential UV absorbancy measurements of the crude acid-soluble fraction. Thus the absorption correction can be applied throughout the duration of the alkaline hydrolysis.

Evaluation of Preliminary Treatments of Cell Samples. In order to

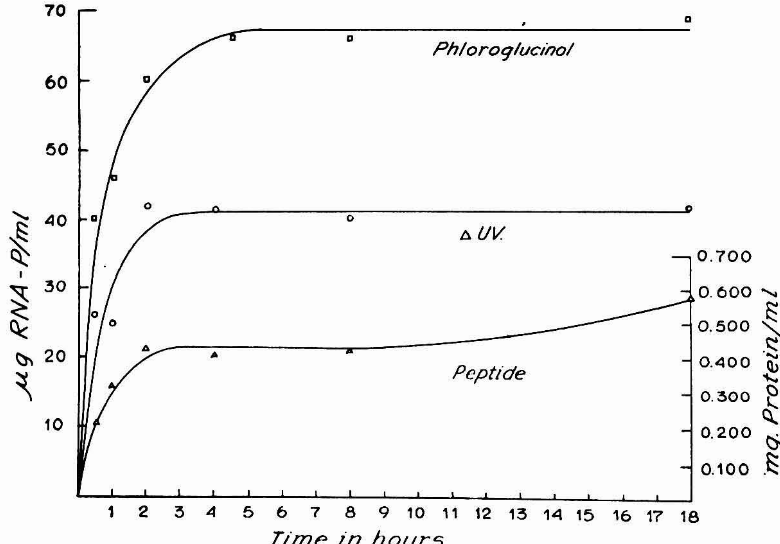

FIG. 2. Kinetics of alkaline digestion. RNA was determined from corrected absorption, ribose by the phloroglucinol test and protein by the biuret test.

test the cold acid extraction and the treatments with lipid solvents on *Euglena* RNA estimation, five different preliminary treatments were used with known amounts of cells. Quadruplicate cell counts were made on samples of not less than 500 (Table 3). It is evident that the recovery

TABLE 2

CALCULATED RNA ON THE ACID-SOLUBLE FRACTION WITH DIFFERENT AMOUNT OF CONTAMINANTS

The acid-soluble mixture was neutralized with KOH and the pH was maintained at 7.8 with 0.025 *N* tris buffer. The solution was absorbed in a Dowex 1 small column. After washing with the tris buffer, pH 7.8, the 2′- and 3′-ribonucleotides were eluted with 1.0 *N* HCl. The optical density at 260 mμ of the contaminants was measured after the 2′- and 3′-ribonucleotides were absorbed in the column.

Duration 0.3 *N* KOH digestion, hr	μg RNA-P/ml: Calculated in acid-soluble fraction before ion-exchange purification[a]	μg RNA-P/ml: Amount of 2′,3′-ribonucleotides in eluate[b]	Contaminants (measurement by OD)
4	15.0	14.4	5%
18	17.0	16.7	11%

[a] RNA content was computed according to formula given in text.

[b] Estimated by reading at 260 mμ.

TABLE 3

RIBONUCLEIC ACID RECOVERED FROM CELL SAMPLES SUBJECTED TO DIFFERENT PRELIMINARY COLD ACID AND LIPID EXTRACTIONS (RNA DETERMINATIONS BY DIFFERENTIAL UV ABSORBANCY[a])

Light grown *Euglena* cells in the logarithmic phase of growth were collected and washed twice with distilled water. Five different duplicate aliquots were made from the same cell culture, and submitted to five different preliminary treatments (see text). Cell counts were made in quadruplicate.

Preliminary treatments			μg RNA-P/10^7 cells
Lipid solvent extraction after TCA treatment	I	(ethanol before TCA)	45.4
	II	(no ethanol before TCA)	45.5
Methanol and formic acid	III		39.5
No extraction with lipid solvent	IV	(1 *N* PCA)	43.2
	V	(5% TCA)	44.2

[a] See methods in the text.

of RNA is not affected by the use of a preliminary cold ethanol extraction (Procedures I, II). A significant amount of RNA is lost if methanol-formic acid is used as recommended by Smillie and Krotkov (6) (Procedure III). The use of cold 1 *N* PCA appears to produce a slight loss of RNA (Procedure IV).

In order to determine the optimal conditions for the cold acid extraction, samples of *Euglena* cells were treated with 5% TCA for varying lengths of time and other samples with different concentrations of PCA. Increasing the duration of the cold TCA extraction from 15 min to 1 hr had little effect on the yields of either DNA or RNA (Table 4). A 15-min extraction is not sufficient to release all cold acid-soluble phosphorus and ribose compounds. Since a 1-hr preliminary extraction also failed to remove these contaminants (see Table 5), 15 min was used as

TABLE 4

AMOUNT OF RNA AND DNA IN *Euglena* CELLS AFTER EXTRACTION WITH ACID

Light grown *Euglena* cells in the stationary phase of growth were collected and washed with distilled water. Duplicate aliquots were taken from the same cell culture. Cell counts were made in quadruplicate.

Acid used in preliminary treatment		μg RNA-P/10^7 cells	μg DNA-P/10^7 cells
5% TCA	15 min	32.9	2.75
	30 min	33.4	2.80
	60 min	34.1	2.90
PCA	0.2 *M*	34.5	2.80
	0.5 *M*	33.2	2.72
	1.0 *M*	31.7	2.71

TABLE 5

PURIFICATION OF ACID-SOLUBLE MIXTURE AFTER ALKALI HYDROLYSIS OF GREEN *Euglena* CELLS

Light grown *Euglena* cells were treated by Procedure V for 1 hr. The acid precipitate was washed and incubated with 0.3 *N* KOH at 37°C for 4 hr. The neutralized acid-soluble mixture was passed through a Dowex 1 column and the 2′- and 3′-ribonucleotides were eluted with 1 *N* HCl. The amount of RNA in the acid-soluble mixture was calculated by formula and in the eluate by optical density at 260 mμ using ε(P) of 10,300. The phloroglucinol test was made using D-ribose as standard.

	μg RNA-P/ml	
	UV	Phloroglucinol
Acid-soluble mixture	34.3	43.1
Substances not absorbed on Dowex 1	—	9.4
2′- and 3′-ribonucleotides eluted with 1 *N* HCl	34.5	34.8

maximum interval of cold acid extraction. Cold PCA treatment is another standard preliminary extraction, but, as was demonstrated by Ogur and Rosen, 1 *N* PCA produces the solubilization of RNA (Table 4). With 0.2 *N* or 0.5 *N* PCA the amounts of RNA and DNA agree, within the experimental error, with those obtained with TCA. These experiments were performed with the same cell batch.

DNA Determination by Hot Acid Extraction. The most common procedure for DNA extraction is hot 0.5 *N* PCA at 70°C for 20 min. The concentration of the PCA was critically evaluated by Hutchison, Downie, and Munro (8). They agree with De Deken Grenson and De Deken (13) that 90° produces destruction of deoxypentose and these results are in accord with those from purified DNA heated with TCA solution (23). Increasing concentration of PCA also produces low values with rat liver DNA, presumably due to deoxypentose destruction (23). But Chiba and Shugahara (18) claim that higher PCA concentrations are required in order to release all the DNA from the chloroplasts. Kirk (27) found the same situation in broad bean chloroplasts. From these various observations it can be concluded that conditions for adequate extraction of DNA in *Euglena* cells requires critical exploration. Two parameters, PCA molarity and duration of hot acid extraction, were tested. The temperature was maintained at 70°. Figure 3 shows that between 1 and 1.5 *N* all the DNA is extracted. Ultraviolet absorption spectra (Fig. 4) gave evidence that upon increasing the PCA concentration there occurs a shift of the maximum from 260 to 280 mμ. At 1.0 *N* PCA the spectrum represents a nucleic acid fraction highly contaminated and the diphenylamine reaction becomes negative at 1.5 *N* PCA. With 0.5 *N* PCA there is no

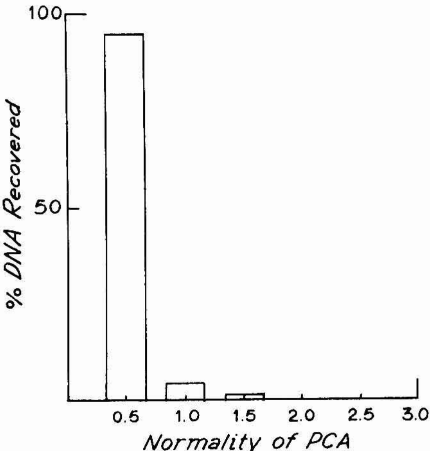

FIG. 3. *Euglena* cells were treated by Procedure I and after alkali hydrolysis the acid precipitate was submitted to successive hot extraction at different PCA molarities for 20 min each.

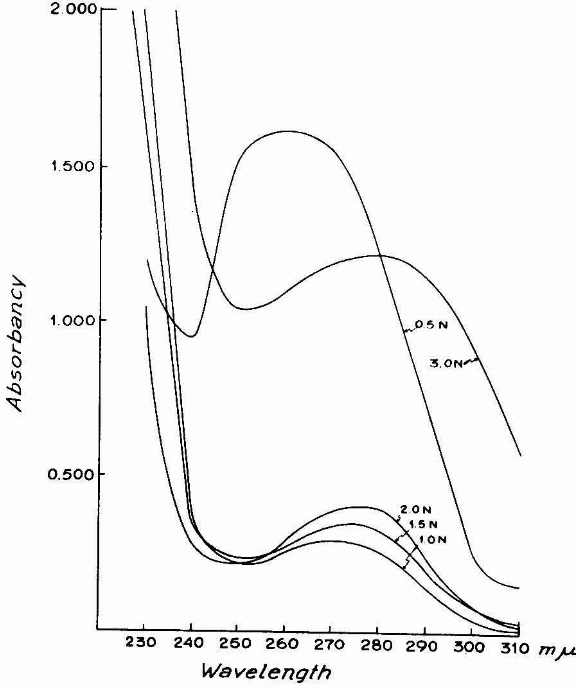

FIG. 4. Ultraviolet spectra of successive hot PCA extractions. See Fig. 3 for experimental conditions.

shown by Ogur and Rosen (4) the peak of the DNA spectrum after hot acid extraction shifts to 268 mμ. In four hot 0.5 *N* PCA extractions the *Euglena* DNA recovered was successively, 77, 18, 4, and 0.6% of the total.

It is apparent from our results that all *Euglena* DNA is released with 0.5 *N* PCA at 70°C. It would therefore seem that the chloroplast DNA fraction of *Euglena* was not resistant to the 0.5 *N* hot PCA extraction (5). However, this should be specifically studied with pure chloroplast fractions. Three 20-min periods are necessary to recover all the DNA. By prolonging the time of hot acid extraction there is some loss in deoxypentose content (23). The substance released in 1 *N* PCA or higher concentrations did not have a nucleic acid spectrum; most probably it is peptide material with high aromatic amino acid content. The presence of UV-absorbing substances in the hot acid extract makes the estimation of DNA by optical density more indirect. Tsanev and Markov (16) also applied a two-wave length method for DNA estimation. However, it is obvious that without an exact evaluation of the absorption properties of the contaminants, the deoxypentose test is the only correct method for DNA determination.

Evaluation of Procedures and Conclusion. Table 6 provides a comparison of different extractions of both nucleic acids from *Euglena* cells. The

TABLE 6

NUCLEIC ACID CONTENT OF GREEN *Euglena* CELLS ESTIMATED BY VARIOUS METHODS

Analyses were carried out in the same cell batch, in the stationary phase growth, by duplicate. Cell counts were made by quadruplicate.

	Procedure	μg RNA-P/10^7 cells		μg DNA-P/10^7 cells
		UV	Phloroglucinol	Diphenylamine
A	72 hr cold 1 *N* PCA extraction for RNA, followed by three hot 0.5 *N* PCA extractions, 20 min at 70°C for DNA (Procedure I as preliminary treatment)	28.2[a] 28.7[b]	29.2	2.70
B	Hot 0.5 *N* PCA twice for 15 min at 90°C (Smillie and Krotkov preliminary treatment)	—	43.2	2.72
C	4 hr alkaline hydrolysis for RNA, followed by three hot 0.5 *N* PCA extractions, 20 min at 70°C for DNA (5% cold TCA without lipid solvents as preliminary treatment)	34.0[a]	—	2.63

[a] Calculated by differential absorption; see text.

[b] Calculated by reading at 260 mμ.

three procedures were applied to the same cell batch. It is evident that 72 hr of cold acid extraction (Procedure A) did not recover all *Euglena* RNA; this fraction, estimated by color reaction and UV methods, agrees within the experimental error. It is assumed that 1.0 N cold PCA extracted *Euglena* RNA without contaminants that affect RNA estimation by UV or color reaction. Although this procedure, as modified by Brawerman, Pogo, and Chargaff (5), appeared adequate for the quantitative extraction of the RNA, our results indicate that some of the RNA remains insoluble. The hot TCA extraction used by Smillie and Krotkov (6) (Procedure B) gave a very high RNA value by the ribose test. Since phloroglucinol is highly specific for ribose (21) it is assumed that this high value was due not to the deoxyribose but to non-nucleotide contaminants. Procedure C appears to be the most reliable for both RNA and DNA.

SUMMARY

1. Ultraviolet absorption at two wavelengths for the estimation of RNA in the acid-soluble fraction after alkaline digestion has been examined in *Euglena* cells. An appreciable amount of ultraviolet-absorbing and ribose-positive non-nucleotide components is released in alkaline digestion. The consequent error in ultraviolet absorption is adequately corrected by taking readings at two wavelengths and applying an equation.

2. It is not necessary to remove lipids and pigments in order to estimate the nucleic acid content of *Euglena* cells. On the other hand, lipid and pigment solvents do not dissolve the ribonucleic acid of the acid precipitate.

3. Total *Euglena* DNA can be recovered after three hot 0.5 N $HClO_4$ extraction of 20 min each. Apparently the *Euglena* plastid DNA is also included in this hot acid extract.

ACKNOWLEDGMENT

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REFERENCES

1. Schneider, W. C., *J. Biol. Chem.* **161**, 293 (1945).
2. Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.* **161**, 83 (1945).
3. Hutchison, W. C., and Munro, H. N., *Analyst* **86**, 768 (1961).
4. Ogur, M., and Rosen, G., *Arch. Biochem.* **25**, 262 (1950).
5. Brawerman, G., Pogo, A. O., and Chargaff, E., *Biochim. Biophys. Acta* **55**, 326 (1961).
6. Smillie, R. M., and Krotkov, G., *Can. J. Bot.* **38**, 31 (1960).

7. Schneider, W. C., Hogeboom, G. H., and Ross, H. E., *J. Natl. Cancer Inst.* **10,** 977 (1950).
8. Hutchison, W. C., Downie, E. D., and Munro, H. N., *Biochim. Biophys. Acta* **55,** 561 (1962).
9. Venkataraman, P. R., and Lowe, C. U., *Biochem. J.* **72,** 430 (1959).
10. Venkataraman, P. R., *Biochim. Biophys. Acta* **39,** 352 (1960).
11. Hallinan, T., Fleck, A., and Munro, H. N., *Biochim. Biophys. Acta* **68,** 131 (1963).
12. Cooper, W. D., and Loring, H. S., *J. Biol. Chem.* **228,** 813 (1957).
13. De Deken-Grenson, M., and De Deken, R. H., *Biochim. Biophys. Acta* **31,** 195 (1959).
14. Davidson, J. N., and Smellie, R. N., *Biochem. J.* **52,** 599 (1952).
15. Kleinschmidt, W. J., and Manthey, J. A., *Arch. Biochem. Biophys.* **73,** 52 (1958).
16. Tsanev, R., and Markov, G. G., *Biochim. Biophys. Acta* **42,** 442 (1960).
17. Fleck, A., and Munro, H. N., *Biochim. Biophys. Acta* **55,** 571 (1962).
18. Chiba, Y., and Shugahara, K., *Arch. Biochem. Biophys.* **71,** 367 (1957).
19. Brawerman, G., *Biochim. Biophys. Acta* **91,** 477 (1964).
20. Hutner, S. H., Bach, M. K., and Ross, G. I. M., *J. Protozool.* **3,** 101 (1956).
21. Pogo, B. G. T., Ubero, I., and Pogo, A. O., to be published.
22. Bolognani, L., Coppi, G., and Zambotti, V., *Experientia* **17,** 67 (1961).
23. Burton, K., *Biochem. J.* **62,** 315 (1953).
24. Sevag, M. G., Lackman, D. B., and Smolens, J., *J. Biol. Chem.* **124,** 425 (1938).
25. Crampton, C. F., Lipshitz, R., and Chargaff, E., *J. Biol. Chem.* **206,** 499 (1954).
26. Allen, R. J. L., *Biochem. J.* **34,** 858 (1940).
27. Kirk, J. T. O., *Biochim. Biophys. Acta* **76,** 417 (1963).

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Polyacrylamide Gel Electrophoresis

A Simple System Using Gel Columns

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Studies in this laboratory, initiated for a more detailed understanding of the Ornstein-Davis polyacrylamide gel electrophoretic procedure, have revealed that extensive simplification of operating conditions can be made while still obtaining good resolutions. Modification and devising of specialized equipment permits easy, rapid manipulation of large numbers of specimens with good reproducibility. The purpose of this article is to present a procedure utilizing what appears to be unique characteristics of polyacrylamide gel as an electrophoretic separatory media.

MATERIALS AND METHODS

Buffer Baths and Electrodes: Polyethylene storage containers, either round or rectangular, make good buffer baths. I use (*a*) 2-qt round containers as described by Ornstein (1), (*b*) rectangular containers, $12 \times 5 \times 4\frac{1}{2}$ in., or (*c*) rectangular containers $12\frac{1}{8} \times 8 \times 4$ inches. Containers *b* are prepared with totals of 12, 24, 36, or 48 holes in four rows. Containers *c* are prepared with totals of 36, 48, 60, and 72 holes in six rows. The limitation of the number of gel columns which can be handled is set by the power supply, since 12 columns will require about 30 ma at the beginning of the run. I have available a Spinco Duostat for 50 ma, a Heath IP-32 for 100 ma, and a Gates G-94F for up to 1 amp.

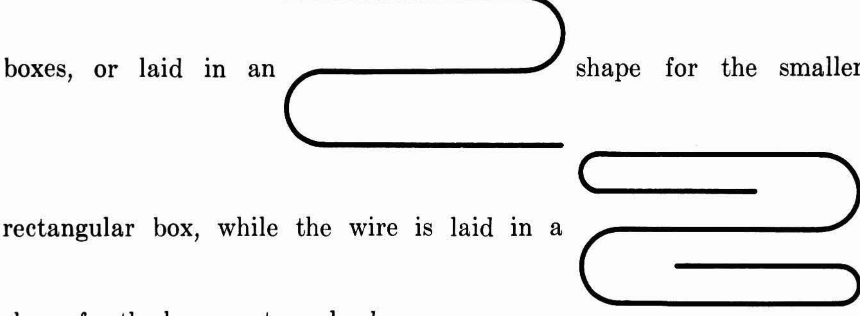
The upper buffer box is prepared by drilling the desired number of holes with a heated cork borer, trimming with a razor blade, and smoothing with a round file. Stoppers for Becton-Dickinson 10-ml Vacutainers are prepared by drilling 6-mm holes through them, trimming the top to the desired thickness, and inserting into the holes as required.

The lower box is cut down at the ends to permit placement of Plexiglas crossbeams which hold the upper box in such a position that the gel column ends are approximately 6 mm from the bottom of the lower bath.

The glass cylinders are cut from 7-mm o.d., 5-mm i.d. soft glass tubing

in 2½-in. lengths. The ends are lightly flamed. On cooling, they are washed with detergent, rinsed well with distilled water, and dried 1 hr at 110°C. After using, the insides are wiped out with wet gauze wrapped around an applicator stick and the tubes rinsed several times with distilled water. For easiest removal of gel columns, the tubes should be air-dried or dried for not more than 1 hr at 110°. This procedure cuts down on the affinity of the polyacrylamide gel for the glass.

The electrodes are 24-gage platinum wire set in the middle of the round boxes, or laid in an



shape for the smaller rectangular box, while the wire is laid in a shape for the large rectangular box.

Contact with the power supply is through a banana jack in contact with the middle of the wire in the rectangular boxes. This wire is supported approximately 1 in. from the bottom of the box by a Plexiglas frame, which is identical for the upper and lower baths (Fig. 1).

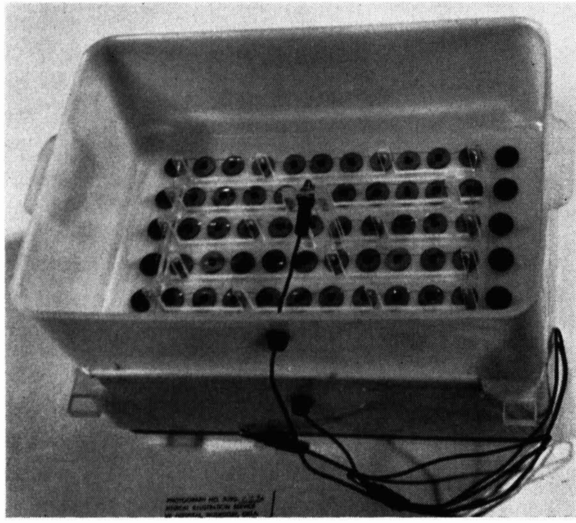


Fig. 1. Buffer bath arrangement for 72 columns. The electrodes are removable and can be moved from one bath to another.

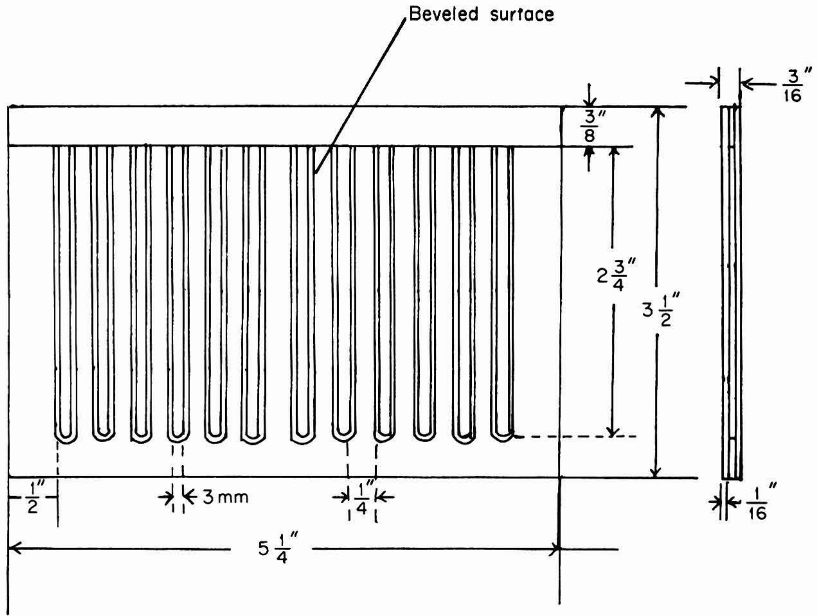


FIG. 2. Schematic diagram of gel column holders described. A strip ⅜ in. wide by 1/16 in. thick is glued along one edge of the holder as shown. Placing these strips at opposite ends, as shown in side view at right, provides sufficient gap to hold the gel column in place.

Destaining Unit: The destaining unit (Figs. 2 and 3) consists of the following components: (*a*) holders for maintaining the gel columns in place (Fig. 2), (*b*) rack for maintaining the holders and electrodes in an upright position, (*c*) 24-gage stainless-steel electrodes with dimensions of $5\frac{1}{4} \times 3$ in., (*d*) a bath permitting circulation of the destaining solution through a decolorizing charcoal bed held in place by a cellulose sponge placed about 1 in. from the bottom of the bath, and (*e*) a circulating pump.

The rack is removable, and it is possible to design it so four holders (each containing 12 gel columns) can be slipped into place and a destaining DC current of 14 volts, 500 ma, applied through the width of the columns. The destaining solution is circulated downward for optimal removal of stain. A combination of electric current and good circulation is required for best results. Thirty-six columns can easily be destained in about 30 min: poorer circulation prolongs destaining time of 48 columns to about 1 hr, at time of writing. Cocoanut charcoal is the decolorizing charcoal of choice.

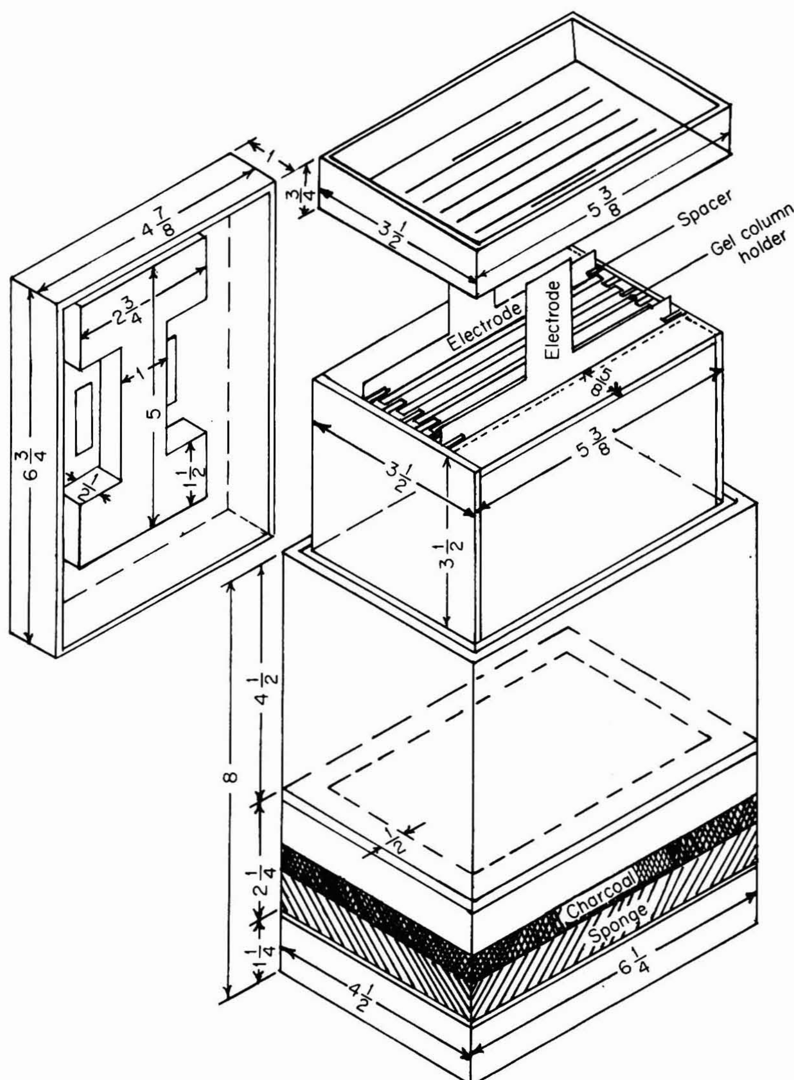


FIG. 3. General schematic view of destainer. The destaining rack sits on the ½ in. wide rib shown 3½ in. from bottom. The spacers are ⅛ in. thick. The upper slotted box slides into place over the electrode projections. The slots are aligned to fit between the gel column holders, which forces the destaining solution to flow between the gel columns. Holes are drilled into the bottom of the hollow I-box in the lid to permit dispersed flow of the destaining solution into the rest of the unit. A circulating pump and rubber tubing have been omitted, for convenience. Holes are centered and drilled in the middle of the top and along one side beneath the sponge. Quick disconnect halves are glued into the respective holes with chloroform. The circulation is forced from the top of the bath through the charcoal bed.

Scanning: An adaptor[1] for holding the gel columns in place was constructed of black, opaque Plexiglas, $9 \times 2 \times 1/4$ in. A 2.5–3.0 mm slit was cut lengthwise down the middle of the Plexiglas piece and the sides gouged out on each lip so the top width of the groove was about 15 mm across. The lip at the bottom of the groove is 2 mm thick to prevent transmission of light. A Photovolt model 530 recording densitometer, using a 520M photomultiplier unit, is used with the thin-layer chromatography scanning adaptor.

The Plexiglas adaptor described above is clamped into place after ascertaining where maximum transmission of light is obtained. The 0.1×6 mm slit, located in the photomultiplier holder, was shortened to 3 mm by use of black electrician's tape. This adjustment of the slit length cuts down on stray light without actually having much effect on the total light transmitted. The filter used is a 505 mμ filter.

Reagents:

1. *Acrylamide solution—for 7% total, 2% cross linkage.*
   28 gm acrylamide.[2]
   0.56 gm *N,N'*-methylenebisacrylamide (BIS).[2]
   Dissolve and bring to 100 ml with distilled water.
2. *Stock buffer.*
   38.8 gm tris(hydroxymethyl)aminomethane.
   13.5 gm glycine.
   Dissolve and bring to 2 liters with distilled water. (This gives a 0.16 *M* tris—0.09 *M* glycine solution with a pH of about 9.2. Before use, 1 part is diluted with 3 parts distilled water for working buffer).
3. *Ammonium persulfate.*
   140 mg dissolved in 100 ml distilled water.
4. *N,N,N',N'*-Tetramethylethylenediamine (TEMED).[2]
5. *Stain.*
   0.5 gm Buffalo black NBR in 2.5% acetic acid.
6. *Destaining solution.*
   2.5 ml glacial acetic acid diluted to 100 ml in distilled water.

Preparation of the Gel Column: Buffer solution—add 0.23 ml TEMED to 100 ml working buffer; then combine 1 part buffer-TEMED solution, 1 part acrylamide solution, and mix; now add 2 parts ammonium persulfate solution and mix by swirling.

This gel solution is poured into a hypodermic syringe barrel, (10, 20, or 30 ml, depending on the volume poured) attached to a 15-gage flat-

[1] The Photovolt Corporation has a similar adaptor available for use with their model 542 densitometer.

[2] Available from Distillation Products Industries.

beveled needle held against a Becton-Dickinson stopper. The glass cylinders are held in place as described by Ornstein and Davis (1). The needle is long enough to pass through the glass cylinder. A one-holed rubber stopper is placed over the top of the syringe and the gel solution pipetted into each glass cylinder in orthodox fashion. Air bubbles are tapped out and the columns adjusted to about 3 mm from the top. Distilled water is added through a 25-gage needle held at a right angle to the top of the cylinders. Best results are obtained by forcing the water directly against the opposite side of the cylinders by means of a hypodermic syringe. This solution is allowed to gel 30 min.

Electrophoresis: The excess solution is shaken out of the tops of the cylinders and the cylinders are inserted into the holders in the bottom of the upper bath. The lower electrode is placed in the lower bath, buffer added to cover the wire but not the banana jack, and the upper bath with gel columns slipped into place. The upper electrode is then placed in the upper bath.

Serum (0.03 ml) is diluted with 2 ml of working buffer and pipetted onto the top of the gel column until even with the top of the stopper. Three $3\frac{1}{4} \times 4$ in. photographic glass plates are leaned against the electrode along one lengthwise edge of the upper bath, and buffer is

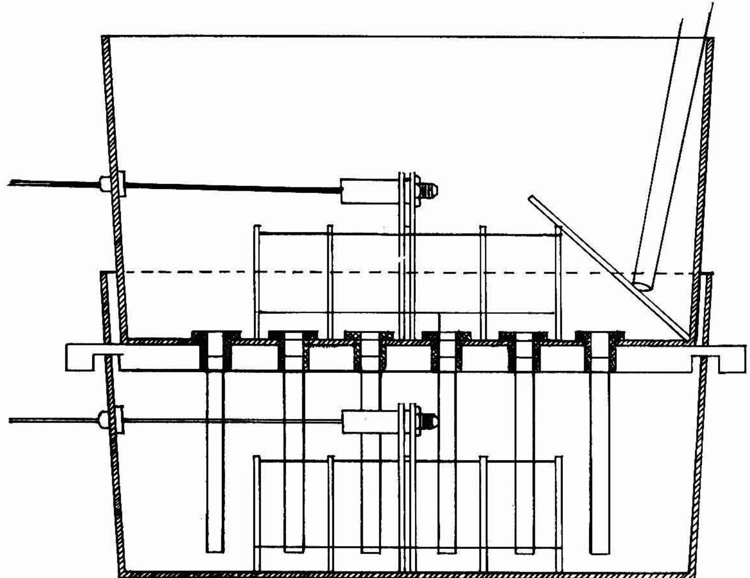


FIG. 4. Schematic end view of baths shown in Fig. 1, illustrating placing of photographic slides for filling the upper bath and general positioning of the platinum electrodes about 1 in. from the bottoms, relative to the gel columns.

poured through a 65-mm filtering funnel against a glass plate. If properly done, little trouble is encountered with washing out the protein solution. This is the procedure for the rectangular baths (Fig. 4).

The round baths have platinum electrodes surrounded by 1-in. high circular baffles, and in this case the working buffer is added by means of a funnel into the middle of the bath (Fig. 5).

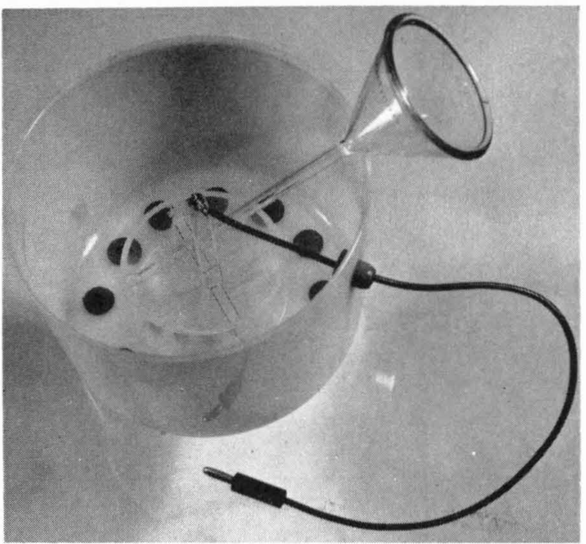


FIG. 5. Filling the round bath. The circular baffle is cut from 3-in. diameter Plexiglas tubing. The lower end of the funnel should be pointed toward the middle of the electrode, which is not glued into place.

For the electrophoretic run, 180 volts is used for 30 min with the 7% total, 2% cross-linkage gel used.

The gel columns are reamed out of the glass tubing by use of 2-in. long, 22-gage regular beveled needles attached to a hypodermic syringe. Water is forced in as desired and the gel columns slipped into 13-mm test tubes held at a 45° angle. The gel columns will now adhere to the walls of the tubes when they are uprighted. The tubes are filled with staining solution, rotated to detach the gel columns, and placed in a 60°C water bath for 45 min or 1 hr (whichever is most convenient).

The stained columns are now placed in the gel column holders, washed free of excess stain, and destained in the destaining unit described above. The destained gel columns are scanned in a routine manner, with air as a blank.

RESULTS

Figures 6, 7, and 8 indicate the general type of results I have obtained with this system. These gel columns were picked at random from a single 72-gel column run using a single set of baths. Twelve serum specimens were done in replicates of six. Despite the relative lack of anticonvection measures, all 72 gel columns showed essentially equal retention of protein fraction, as shown in Figure 6. Actually, with the baffling systems described, loss of specimens is a minor problem. It was possible to go for several weeks at a time without a single loss. In using rectangular buffer baths, the more elaborate baffle systems created more difficulties than the simple one described.

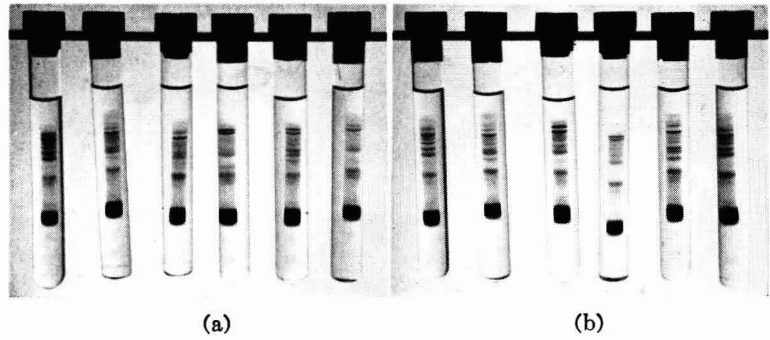


FIG. 6. (a) Six individual serum specimens from 72-column run performed in bath shown in Fig. 1. (b) Six individual serum specimens from 72-column run.

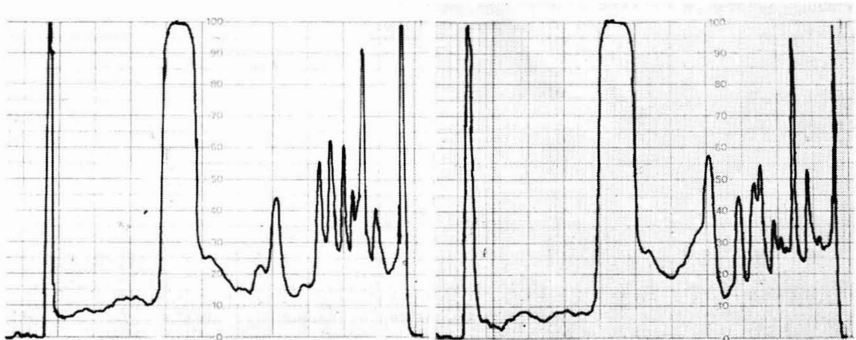


FIG. 7 (*at left*). Tracing of column 1 in Fig. 6a.

FIG. 8 (*at right*). Tracing of column 5 in Fig. 6b. Note small diffraction peak at posterior base of the last globulin fraction.

Figure 6 shows differing protein fractions found in 12 individual serums, and demonstrates the over-all reproducibility of the distance migrated by the albumin in a single run. The distance will vary somewhat from day to day and from power supply to power supply. The gel columns in this run were prepared in groups of 36. If prepared in groups of 24, still better reproducibility would have been obtained, apparently because of more consistency in the gel column preparation.

It is to be noted that all protein fractions migrate into the gel columns. The distance of penetration of the slowest band is governed by the thickness of the soft gel at the top. Under these circumstances, the single band found by other procedures may become two or three. It is best to avoid too much penetration because of destructive effects on the slow bands. They evidently break down quite easily if permitted to migrate too far.

The sharp bands at each end of the tracings in Figs. 7 and 8 are due to diffraction of light at the end of the gel columns and they serve to demarcate the ends of these gel columns. The 7%, 2% cross-linkage gel now permits the tracing to go to 10% transmission or less. On occasion, I have been able to get the gel and the air on the same baseline, but not consistently.

The complexity of the globulin fractions is well demonstrated in all these illustrations. Figures 2 and 4 do not show the large number of fine bands observed after the next-to-last globulin fraction. I have been able to observe up to 25 to 28 individual fractions in a single specimen.

On scanning the gel columns, a diffraction point will often be observed a short distance from the top, within the gel, as in Fig. 8. This can be recognized as such in this procedure, as well as the false peak at the end of the gel column.

In Fig. 8, the tracings at each end of the protein peaks manage to correlate fairly well on a baseline basis. This is not always the case, and demonstrates that absolute values assigned to slower moving fractions may be misleading.

Efforts to observe enhancement of staining by the globulin fractions due to presence of lactate in the gel column or in the staining solutions, as observed by Ferris, Easterling, and Budd (3), have been unsuccessful.

DISCUSSION

Polyacrylamide gels may be prepared with a wide variety of buffers, of pore sizes, of degrees of clarity, of pH, and of degrees of flexibility or brittleness. This flexibility in preparation makes it difficult to prescribe optimal operating conditions, since this may vary slightly from serum to serum. At this time, a 7% total gel with 2% cross linkage appears to offer the best compromise in separatory powers at pH 9.2.

The variety of buffers used in analytical procedures (1–7) indicate that this point alone is quite unsettled. I have used tris-boric acid buffers to obtain good separations, but with no improvement over the tris-glycine buffer. Preliminary experiments in progress in this laboratory indicate that most of these buffers are interchangeable, but that separations are markedly affected by pH changes between 8.4 and 9.2, especially in the relative migration rate of some globulin fractions as compared to the albumin.

The results of Hjertén, Jerstedt, and Tiselius (7) indicate that the Ornstein-Davis procedure will not necessarily produce superior resolution to that produced by a single 0.37 *M* tris-glycine buffer at pH 9.5. An essential feature of the Ornstein-Davis procedure is that the tris-HCl buffer in the gel columns has a higher current carrying capacity than does the tris-glycine combination in the buffer baths. These operating conditions, coupled with shortened migration distances normally found with single tris-HCl buffer system mean that the serum proteins will migrate only about one-fourth the distance under equivalent voltage-current conditions, as compared to the 0.0625 *M* tris-glycine buffer. This brings about unnecessary compression of the protein bands.

I have also found that gel stiffness or pore size apparently reaches an optimum around 7% total, 2% cross linkage. Better resolution of some globulin fractions occurs with larger pore size, while others resolve better with smaller pore sizes. The albumin band shows strong bimodality in a large percentage of serums where a stiffer gel is used at the same pH, or with the same gel concentration (7%, 2% cross linkage) if the tris-glycine buffer pH is lowered below 8.8. The variability of bimodality observed indicates that this is not an artifact.

Another feature of this method is that the gel at the top of the column is soft enough to permit penetration by the larger or slower moving proteins, and they will often split into two or more fractions. Leaving some of the protein at the point of origin is a common difficulty. This tends to crowd the slower moving fractions together. Also, the gels may have a line of diffraction at the upper end, giving spurious peaks, or enhancing a peak already present. Lorber (5) avoids this to a large extent in a manner similar to that described, but still leaves some material at the point of origin because of the lack of a softer gel at the top of his preparation.

SUMMARY

A polyacrylamide gel electrophoretic system based on the use of 2½-in. gel columns with a tris-glycine buffer system is described. It features relatively low voltage and amperage conditions with high migration rates,

and operates at room temperature. Equipment of electrophoresis for destaining and for scanning the developed patterns is described. This system is adapted for easy, simultaneous handling of large numbers of gel columns.

REFERENCES

1. ORNSTEIN, L., AND DAVIS, B. J., preprint, Distillation Products Industries, Rochester, N. Y., 1962.
2. FERRIS, T. G., EASTERLING, R. E., AND BUDD, R. E., *Am. J. Clin. Path.* **39,** 193 (1963).
3. FERRIS, T. G., EASTERLING, R. E., AND BUDD, R. E., *Anal. Biochem.* **8,** 477 (1964).
4. RAYMOND, S., AND NAKAMICHI, M., *Anal. Biochem.* **3,** 23 (1962).
5. LORBER, A., *J. Lab. Clin. Med.* **64,** 133 (1964).
6. CLARKE, J. T., *Ann. N. Y. Acad. Sci.* **121,** 428 (1964).
7. HJERTÉN, S., JERSTEDT, S., AND TISELIUS, A., *Anal. Biochem.* **11,** 219 (1965).

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Studies of Binding by Macromolecules

A New Dialysis Technique for Obtaining Quantitative Data

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Equilibrium dialysis (1, 2) is a proved technique for obtaining quantitative data concerning the reversible interaction between a dialyzable species and a macromolecule. Upon proper treatment of the binding data, it is possible to determine the equilibrium constant for the formation of the complex and to estimate the number of sites on the macromolecule to which the small species is bound (3–5).

A significant disadvantage of the experimental procedure is the long periods of time required for attainment of equilibrium. Frequently, periods of 24 hr or more are involved, and if a labile material is being studied decomposition products can yield misleading results.

The purpose of this paper is to describe a dynamic dialysis technique which yields the same information as the classical procedure, but which requires only minutes to perform since equilibrium is not required. Used in conjunction with an automatic analytical system, it is possible to generate the requisite data for a complete binding profile within an 8-hr period.

DEVELOPMENT OF METHOD

The new method is based on the difference in the dynamic, short-term dialysis behavior of the species of interest in the presence and in the absence of a nondialyzable macromolecule. Dialysis is carried out in a closed flow system at constant conditions chosen so that the quantity of material which diffuses across the membrane is directly proportional to the free concentration in the original solution stream. An instrument designed to carry out experiments based on this concept is the AutoAnalyzer® (Technicon Instrument Corporation, Chauncey, N. Y.), a closed, time-flow dependent system actuated by a constant-speed, peristaltic pump (6). The unit contains a dialyzer module which possesses the necessary characteristics and, in addition, it contains the analytical potential to perform the required assays automatically.

The dialyzer module of the AutoAnalyzer is composed of two horizontally positioned matched grooved plates separated by a semipermeable membrane. The solution containing the dialyzable material is pumped along a prescribed path through the top plate while a second stream is propelled along an identical path in the bottom plate and receives the diffusing species. Transfer takes place in both directions and is in no sense allowed to reach equilibrium. However, because of the constancy of the experimental conditions, the difference in the amount of dialyzable material diffusing across the membrane in both the absence and the presence of a macromolecule can be used to obtain the parameters necessary to describe the binding behavior. These are r, the moles bound per mole of macromolecule, and D, the free concentration of the binding species (2).

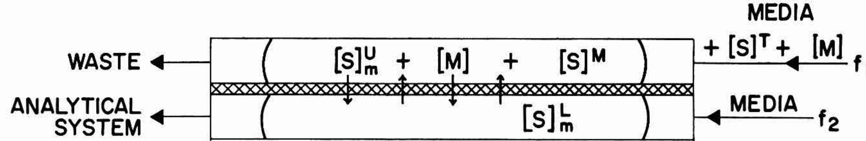

FIG. 1. Schematic representation of dialysis system.

A representation of the ideal situation is shown in Fig. 1. The notations used are defined below:

$[S]^T$ = total concentration of S, a dialyzable species
$[M]$ = concentration of macromolecule
$[S]^U$ = concentration of free S in the upper layer
$[S]^M$ = concentration of S bound to M
$[S]^L$ = concentration of S diffused into the bottom stream
f_1 = flow rate of top stream
f_2 = flow rate of bottom stream

Flow rates are expressed in ml/min and concentrations in molarity. The subscript m, when used, refers to concentrations in the presence of M in the upper stream.

If the analytical system is based on a colorimetric method which obeys Beer's law, the following relationships apply:

$$A = K_a[S]^T \quad (1)$$

$$A_m = K_a([S]^T{}_m - [S]^M) \quad (2)$$

where A is the absorbance and K_a is the proportionality constant relating $[S]^T$ and A.

Rearranging, one obtains:

$$[S]^T = \frac{A}{K_a} \quad (3)$$

$$[S]^T_m = \frac{A_m}{K_a} + [S]^M \quad (4)$$

If the same total concentration of S is chosen in the presence as well as in the absence of macromolecule, i.e.:

$$[S]^T = [S]^T_m \quad (5)$$

then:

$$[S]^M = \frac{(A - A_m)}{K_a} \quad (6)$$

In order to determine all of the binding parameters, $[S]^U_m$ must be known explicitly. This value is obtained by first defining:

$$k_d = \frac{[S]^U}{[S]^L} = \frac{[S]^U_m}{[S]^L_m} \quad (7)$$

which is a function of flow rate, temperature, membrane material, dialyzing species, sampling rate, solution composition, etc., but presumably independent of $[S]^T$ and the presence of M.

A mass balance yields:

$$[S]^T = [S]^U + \frac{f_2}{f_1}[S]^L \quad (8)$$

$$[S]^T_m = [S]^U_m + \frac{f_2}{f_1}[S]^L_m + [S]^M \quad (9)$$

Substituting for $[S]^L_m$ in Eq. (9) from Eq. (7) and rearranging, one obtains:

$$[S]^U_m = \frac{[S]^T_m - [S]^M}{1 + \frac{f_2}{f_1 k_d}} \quad (10)$$

or

$$[S]^U_m = \frac{A_m}{K_a\left(1 + \frac{f_2}{f_1 k_d}\right)} \quad (11)$$

The value of k_d can be determined by combining Eq. (7) and Eq. (8) and rearranging to yield:

$$k_d = \frac{[S]^T}{[S]^L} - \frac{f_2}{f_1} \quad (12)$$

Substitution for the concentration terms from Eq. (1) gives:

$$k_d = \frac{(A)^T}{(A)^L} - \frac{f_2}{f_1} \quad (13)$$

$(A)^L$ is the value normally obtained in the AutoAnalyzer, while $(A)^T$ is the value obtained by connecting the usual upper dialyzer line directly into the analytical system.

Equations (1), (6), (11), and (13) thus provide the means to evaluate the parameters necessary to describe the binding behavior, namely, the free concentration of the small species and that bound to the macromolecule. The concentration of the macromolecule does not change during the experiment since it does not cross the membrane and it is simply the original amount taken corrected for dilution in the incubation mixture.

The new technique was tested by repeating the work of Klotz *et al.* (3) on the binding of methyl orange to bovine serum albumin.

EXPERIMENTAL

All solutions were prepared in a phosphate buffer, pH 6.9, containing 7.56 gm KH_2PO_4 and 18.72 gm $Na_2HPO_4 \cdot 7H_2O$ per liter. Methyl orange (sodium salt, Distillation Products Industries, Rochester, N. Y.) and crystallized bovine albumin (Pentex Inc., Kankakee, Ill.) solutions were prepared by dissolving appropriate quantities in buffer and diluting aliquots to the required concentrations. Both solids were corrected for water content by determining the loss in weight at 110°C on separate samples not used in the binding studies.

A typical AutoAnalyzer flow diagram utilized is shown in Fig. 2. The flow rates listed were determined experimentally. Periodic checks revealed a change of only a few per cent after several hundred hours usage.

Operationally, a graded concentration series of duplicate methyl orange solutions were sampled at a rate of 20 per hour; a buffer solution blank was inserted between the samples. In the first run the methyl orange samples were diluted approximately one-half with buffer, incubated for 10 min, and dialyzed. The dye which diffused across the membrane was picked up in another buffer stream which was then diluted with more buffer to lower the concentration, and the absorbance was measured and recorded. This procedure yielded the standard curve, that is, K_a. The second part of the experiment was essentially a duplication of the first, except that a protein solution was substituted for the initial buffer diluent.

Data for the determination of k_d were obtained by connecting the inlet stream to the top side of the dialyzer, with no albumin present, to the connection receiving the flow from the outlet of the bottom side. Several of the lowest methyl orange concentrations were sampled, and k_d was determined by means of Eq. (13) for each of the levels. An average value was used in the subsequent calculations.

The concentration of methyl orange and albumin in the incubation

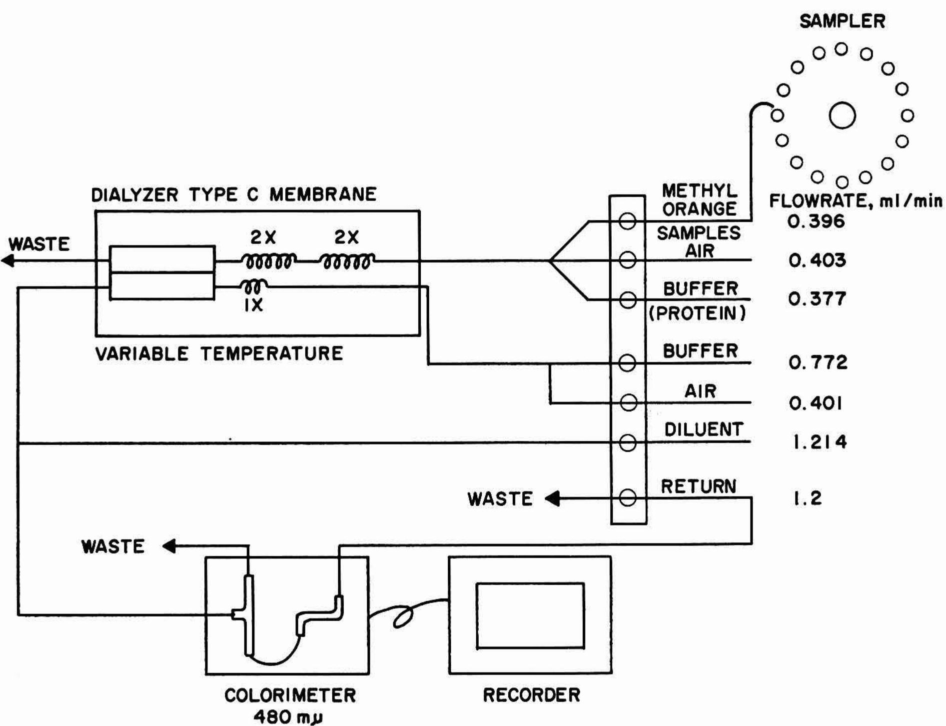

FIG. 2. Flow diagram.

coils was calculated by correcting the initial value for dilution resulting from the joining of the two streams; this factor is simply the ratio of the respective flow rates to the total solution flow in the stream.

Different flow rates and concentrations were employed to test the derived equations. Data were also obtained at two temperatures so that the thermodynamic data could be compared with previously published results.

RESULTS

A complete set of data at 36.7°C obtained with the flow diagram illustrated in Fig. 2 is listed in Table 1. The standard deviation of the duplicates, 0.005 absorbance unit, is a typical value for the precision of the system. Extrapolation of a graph of r/D vs r to $r = 0$ (2) yielded a value of 4.2×10^4 for k_1. Another experiment at 36.7° at different flow rates which changed k_d to 2.23, and in which the albumin concentration was increased to 1.30×10^{-5} M, gave a value of $k_1 = 4.0 \times 10^4$. A composite plot of the values is shown in Fig. 3.

TABLE 1

CALCULATION OF BINDING PARAMETERS FROM AUTOANALYZER DATA AT 36.7°C

Methyl orange concn. in sample cup, $M \times 10^5$	Methyl orange concn. in incubation coils[a] $M \times 10^5$, $[S]^T$	Absorbance with buffer diluent	Absorbance with protein diluent[b]	$[S]^M$, $M \times 10^5 = \frac{A - A_m}{K_a}$ [c]	$\frac{\text{moles S}}{\text{mole protein}}$ (r)	$[S]^U_m$, $M \times 10^5 = \frac{A_m}{K_a\left(1 + \frac{f_2}{f_1 k_d}\right)}$ [d] (D)	$\frac{r}{D} \times 10^{-4}$
4.35	2.23	0.077, 0.079	0.066, 0.068	0.31	0.51	1.34	3.81
13.06	6.69	0.242, 0.240	0.203, 0.217	0.87	1.44	4.18	3.44
21.76	11.15	0.394, 0.404	0.352, 0.355	1.28	2.12	7.04	3.01
32.65	16.73	0.590, 0.594	0.535, 0.529	1.69	2.79	10.62	2.63
43.53	22.30	0.796, 0.790	0.714, 0.718	2.17	3.59	14.26	2.52

[a] The dilution factor is 0.396/0.773 = 0.512.

[b] Stock protein concentration is 1.24×10^{-5} M assuming MW = 7.0×10^4. Concentration in incubation coils is 6.05×10^{-6} M.

[c] $K_a = 3.55 \times 10^3$; standard deviation = 0.04×10^3.

[d] $k_d = 2.41$; standard deviation = 0.03; the average of 4 trials with stock methyl orange concentrations ranging from 2.18 to 21.8×10^{-5} M.

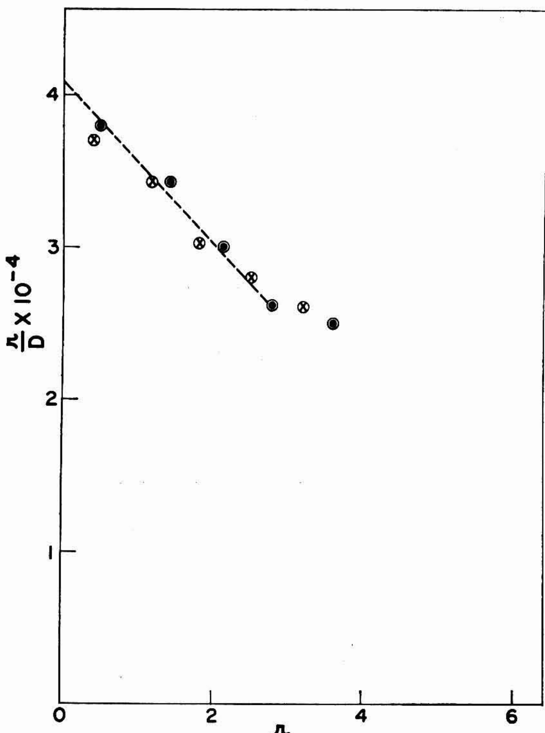

FIG. 3. Binding data, 36.7°C.
⊙ Data from Table 1
Albumin concentration—6.05×10^{-6} M
$f_1 = f_2 = 0.773$ ml/min.
⊗ Albumin concentration—1.30×10^{-5} M
$f_1 = 0.741$ ml/min.
$f_2 = 0.567$ ml/min.

It can be seen that, in spite of the scatter, it is possible to obtain a good average value for k_1, the intercept on the ordinate. It is theoretically possible to determine n, the number of binding sites, by extrapolating the graph to the abscissa intercept, but it is obvious that in this instance the lack of data at high r values would make such a procedure very inexact. A similar conclusion was also reached in previous work with this system (7).

Data at 28.2° extrapolated to a k_1 value of 4.6×10^4. The corresponding thermodynamic parameters calculated from the two k_1 values are given in Table 2.

TABLE 2
THERMODYNAMIC VALUES FOR BINDING OF METHYL ORANGE TO BOVINE ALBUMIN

Data source	$-\Delta F^0$, cal/mole			$-\Delta H^0$, cal/mole	ΔS^0, cal/mole-degree
	25.0	28.2	36.7		
Ref. (7)	6411	—	—	2100	14.5
This study	—	6430	6560	2000	15

DISCUSSION

The agreement between the k_1 and other related thermodynamic values reported in the literature (7) with those obtained here substantiates the premises implicit in the new method. It would be expected that departures from an ideal flow pattern would cause the largest error, but evidently they occur to a minor extent or randomly so that their net effect on the final data is not significant.

Another source of error would be introduced if in Eq. (7) k_d were incorrectly presumed to be independent of $[S]^T$ and the presence of the macromolecule. However, k_d was constant over a tenfold change in methyl orange concentration, and there was agreement between k_1 values obtained at different albumin levels. Thus, the assumptions inherent in Eq. (7) were shown to be valid.

The derivation of the necessary mathematical relationships in this study was based on the concept of an isolated solution segment in a continuous stream. However, it would not be practical to use a continuous stream because of the contamination which would occur from one sample to the other. Air was purposely introduced to isolate the individual segments so that each maintained its identity; this procedure is ordinarily used with the AutoAnalyzer.

Although it is possible to work with different flow rates in the top and bottom sides of the dialyzer and make the requisite calculations, it is more convenient if the rates are made the same. This is especially true if dilutions occur in the analytical system since a difference must then be accounted for in the absorbance ratio in the determination of k_d, as well as D.

Calibration of flow rates is accomplished most accurately under load conditions, i.e., by preparing a manifold and measuring the volume aspirated in a given time period. Tubes that had been used for several hours prior to calibration were not observed to change significantly for the next several hundred hours. It is imperative that the nominal flow rate value of a tube, as cited by the manufacturer, not be used for calculations since the actual rates vary considerably.

The methyl orange system obeyed Beer's law in the concentration

range studied so that the equations developed could be used directly. If for some reason the analytical system does not yield a linear relationship between $[S]^T$ and absorbance, it is possible to account for the deviation by substituting for A_m/K_a the equivalent concentration as determined from a calibration curve. Such a procedure has been used successfully in another study which will be reported elsewhere.

The standard deviation of duplicate measurements was of the order of 0.005 absorbance unit. Similar reproducibility has been observed in other systems which required a chemical reaction for color development. This high degree of precision, coupled with the ease of experimental manipulation and the automatic analytical instrumentation, makes the method a very useful tool for binding studies.

SUMMARY

A dialysis technique utilized with a constant-flow system has been described which yields the same binding information as the classical equilibrium dialysis procedure. The new method is based on the difference in the short-term dialysis behavior of the species of interest in both the presence and the absence of a macromolecule. Used in conjunction with an automatic analytical system, it is possible to elucidate the binding profile in a matter of hours. The speed, reproducibility, and general applicability of the method make it a very useful tool for these studies.

ACKNOWLEDGMENT

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REFERENCES

1. Osborne, W. A., *J. Physiol.* **34**, 84 (1906).
2. Rosenberg, R. M., and Klotz, I. M., *in* "A Laboratory Manual of Analytical Methods of Protein Chemistry" (P. Alexander and R. J. Block, eds.), Vol. 2, p. 131. Pergamon Press, New York, 1960.
3. Klotz, I. M., Walker, F. M., and Pivan, R. B., *J. Am. Chem. Soc.* **68**, 1486 (1946).
4. Scatchard, G., *Ann. N. Y. Acad. Sci.* **51**, 660 (1949).
5. Karush, F., *J. Am. Chem. Soc.* **72**, 2705 (1950).
6. Skeggs, L. T., *Am. J. Clin. Pathol.* **28**, 311 (1957).
7. Klotz, I. M., and Urquhart, J. M., *J. Am. Chem. Soc.* **71**, 847 (1949).

ANALYTICAL BIOCHEMISTRY 13, 314–321 (1965)

Determination of Nucleic Acids with Concentrated H_2SO_4

I. Deoxyribonucleic Acid[1]

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In 1962, Staron *et al* (1) described a quantitative method for the determination of deoxyribonucleic acid (DNA) with concentrated sulfuric acid. According to their procedure, 1.5 ml of concentrated H_2SO_4 is added to 100 μl of an extract of DNA obtained by heating a DNA extract in 5% trichloroacetic acid (TCA) at 90°C for 30 min. After 30 min at room temperature, the deoxyribose in the extract is converted into a yellow colored compound which is measured at 480 mμ. Under these conditions only fructose, raffinose, inositol, and tryptophan produced an absorbance at this wavelength, which however is small if compared with the absorbance produced by equal concentrations of deoxyribose. Dische, who described this reaction in 1955 (2), pointed out that arabinal produces a yellow color too. DNA preparations obtained by phenol extraction procedures or by the Schmidt-Thannhauser method (3) are in general minimally contaminated with other sugars than deoxyribose. In view of its simplicity, as well as its sensitivity, the H_2SO_4 reaction was studied, and found to be a satisfactory method. A few modifications, however, were introduced to increase its sensitivity and accuracy.

EXPERIMENTAL

Apparatus: The Beckman Model DU ultraviolet spectrophotometer, the Coleman Junior spectrophotometer, and the Cary recording ultraviolet spectrophotometer, model 15, were used in these studies.

Materials: 2′-Deoxy-D-ribose, A grade, Calbiochem; D-ribose, A grade, Calbiochem; sperm deoxyribonucleate, Nutritional Biochemicals; yeast ribonucleic acid (RNA), Schwartz BioResearch. All other chemicals were of analytical grade.

Procedure: In general the reaction was carried out by adding 1.5 or 3 ml concentrated H_2SO_4 (specific gravity 1.84) to a 100-μl sample in

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5% TCA and reading the absorption at 470 mμ after 30 min at room temperature.

RESULTS

Spectra of Ribose and Deoxyribose in Concentrated H_2SO_4: To 100 μl of a 5% TCA solution, containing either 100 μg deoxyribose or 200 μg ribose, was added 3 ml concentrated H_2SO_4. The spectra of these solutions shown in Fig. 1 indicate that the absorption maximum of

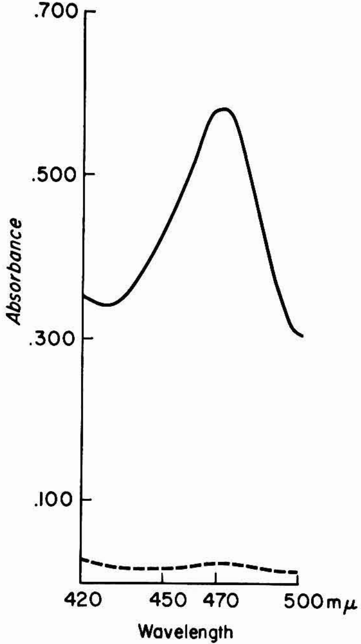

FIG. 1. Absorption spectra of deoxyribose (solid line) and ribose (broken line) in concentrated H_2SO_4.

deoxyribose under these conditions occurs at 470 mμ rather than at 480 mμ. It appears that ribose will not interfere with the determination of deoxyribose at this wavelength. In order to establish the effect of various conditions prior to the addition of sulfuric acid, the spectra of deoxyribose in concentrated sulfuric acid were studied after dissolving the deoxyribose in 5% TCA, 1 *N* HCl, or 0.1 *N* NaOH. The alkaline solution was heated at 60°C for 30 min. The spectra recorded in Fig. 2 show that heating in alkali diminishes the maximum at 470 mμ, with the development of a peak with a maximum at 290 mμ. Although DNA develops a bright yellow color if treated with concentrated sulfuric acid, deoxyribose produces a brownish yellow color under the same conditions.

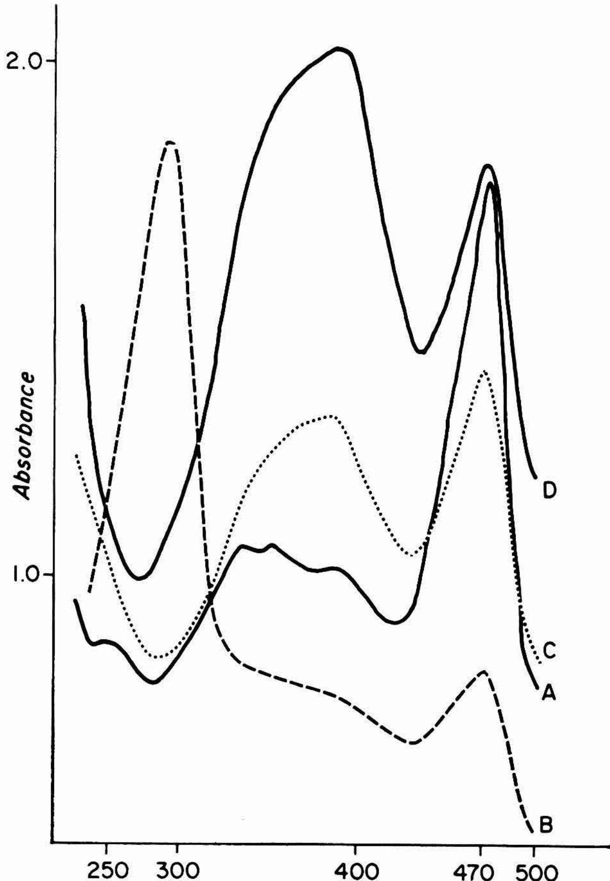

FIG. 2. Absorption spectra of 400 μg deoxyribose in 3 ml concentrated H_2SO_4. Before addition of H_2SO_4 the deoxyribose was dissolved in 1 *N* HCl (A), in 0.1 *N* NaOH and heated at 60°C for 60 min (B), in water (C), and in 5% TCA (D).

This is probably due to the absorbance in the area between 340 and 420 mμ, which is always slight in the case of DNA. As can be seen, these maxima disappear if the deoxyribose is pretreated with 1 *N* HCl, but become more pronounced if the deoxyribose is pretreated with TCA. Accordingly, a bright yellow color is produced after pretreatment with HCl, whereas TCA tends to cause a brown tinge. It is conceivable that the maxima at 365 and 385 mμ represent deoxyribose derivatives. Heating in 1 *N* HCl destroys deoxyribose rapidly, with the development of a chromophore with $\lambda_{max} = 261$ mμ (4).

Spectra of DNA and RNA in Concentrated H_2SO_4: DNA and RNA (3 mg/ml) were hydrolyzed in 5% TCA at 90°C for 30 min. To 100 μl

of these hydrolyzates was added 3 ml concentrated H_2SO_4. The spectra shown in Fig. 3 indicate that the absorption maximum of the DNA is also situated at 470 mμ, and that no interference is caused by RNA at this wavelength. The concentration of DNA in solutions containing

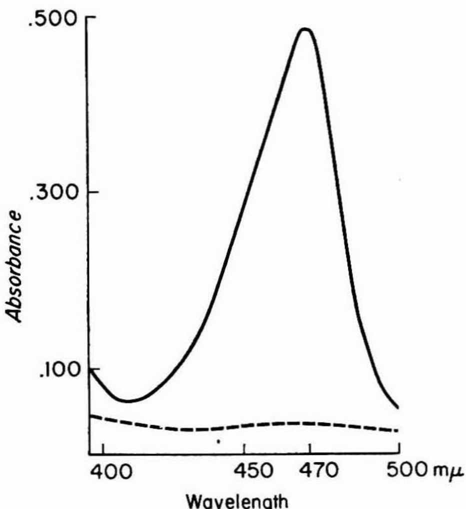

FIG. 3. Absorption spectra of DNA (solid line) and RNA (broken line) in concentrated H_2SO_4. Both samples were treated with 5% TCA at 90°C for 30 min.

DNA as well as RNA can therefore also be determined accurately.

Linearity of the Reaction: Increasing volumes of solutions containing untreated DNA and DNA heated in 5% TCA were brought to a final volume of 100 μl with a final concentration of 5% TCA. After the addition of 1.5 ml of concentrated H_2SO_4, the resulting absorptions were plotted against the amounts of DNA in the samples, as shown in Fig. 4. It appears that the relation between the absorbances and the concentrations is not linear, but slightly curved. As can be seen, preheating of the DNA in 5% TCA hardly affected the yield of chromogen.

Development of Color with Time: Staron *et al.* reported that maximal color is obtained after 20–30 min at room temperature, as is shown in Fig. 5. Deoxyribose, untreated DNA or DNA heated in 5% TCA behaved in a similar fashion. The heat which is produced when the concentrated H_2SO_4 is added to the sample probably accelerates the development of color. Indeed, as is shown in Fig. 4, the development of the maximal absorbance was delayed considerably if the tube containing the hydrolyzed DNA sample was cooled in ice during the addition of the concentrated H_2SO_4.

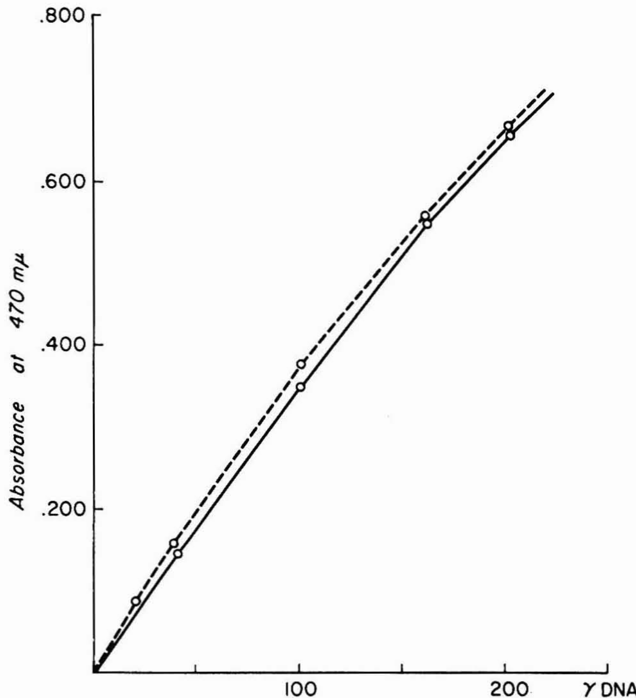

FIG. 4. Absorbances at 470 mμ of various concentrations of DNA treated with 5% TCA at 90°C for 30 min (broken line) and untreated DNA (solid line) 30 min after addition of 1.5 ml concentrated H_2SO_4 to 100-μl samples.

Effect of Total Volume of the Sample: As can be seen from the data recorded in Table 1, the absorbance at 470 mμ is decreased sharply by

TABLE 1
EFFECT OF SAMPLE VOLUME ON OPTICAL DENSITY
(Volume of H_2SO_4 added = 3 ml).

DNA solution (concentration 1 mg/ml), μl	Volume of distilled water added, μl	E_{470}
100	0	0.750[a]
100	100	0.395
100	200	0.185

[a] Averages of duplicate determinations.

addition of small amounts of distilled water. The total volume of the sample should therefore always be kept constant and should not exceed 100 μl.

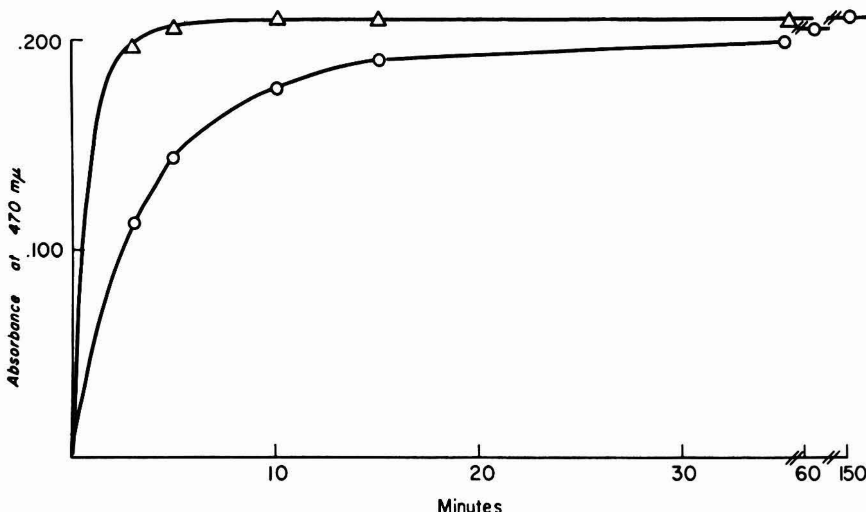

FIG. 5. Development of absorbance at 470 mμ with time after addition of concentrated H_2SO_4 to a sample containing DNA heated in 5% TCA. Untreated DNA as well as deoxyribose yielded the same curve. When the sample was cooled in ice during addition of concentrated H_2SO_4 the curve with the open circles was obtained.

Effect of TCA Concentration of the Sample: 50 μl of TCA solutions containing increasing concentrations of TCA were added to 50 μl of a solution containing either 1 mg/ml DNA or 1 mg/ml deoxyribose; 1.5 ml concentrated H_2SO_4 was added and the absorptions measured at 470 mμ. The results recorded in Table 2 indicate that a slight increase in

TABLE 2
EFFECT OF TCA CONCENTRATION ON OPTICAL DENSITY
(Volume of H_2SO_4 added = 1.5 ml)

Sample	Added	E_{470}
50 μl DNA soln. in 5% TCA (1 mg/ml)	50 μl distilled water	0.174[a]
	50 μl 10% TCA	0.176
	50 μl 20% TCA	0.242
	50 μl 40% TCA	0.272
50 μl deoxyribose soln. in H_2O (1 mg/ml)	50 μl 10% TCA	0.400
	50 μl 20% TCA	0.455
	50 μl 40% TCA	0.482

[a] Average of triplicate determinations.

absorption results from the increase of the TCA concentration.

Comparison with the Diphenylamine Reaction: DNA samples were prepared from the livers of 12 different rats by blending the tissues in

isotonic saline and subsequently carrying the sediments through the routine extraction procedures with acid and organic solvents (5). The material was then subjected to the Schmidt-Thannhauser procedure (3), precipitated, and extracted with 5% TCA at 90°C. All samples were assayed for deoxyribose with the diphenylamine reaction (2) as well as the H_2SO_4 reaction. The results recorded in Table 3 show that the

TABLE 3
COMPARISON OF H_2SO_4 REACTION AND THE DIPHENYLAMINE REACTION

Rat liver DNA Sample No.	H_2SO_4 Reaction		Diphenylamine reaction		
	E_{470}	μg DNA	E_{595}	$E_{595-650}$	μg DNA
1	0.465[a]	87	0.228[a]	0.109[a]	96
2	0.460	86	0.212	0.100	88
3	0.500	95	0.246	0.118	104
4	0.580	115	0.295	0.141	125
5	0.465	87	0.214	0.098	86
6	0.565	111	0.264	0.127	112
7	0.435	80	0.200	0.093	81
8	0.395	71	0.177	0.083	73
9	0.580	115	0.277	0.137	120
10	0.445	82	0.208	0.096	85
11	0.428	78	0.205	0.094	83
12	0.550	108	0.206	0.121	112

[a] Averages of duplicate determinations.

absorbancy values observed in the H_2SO_4 reaction are approximately twice those obtained by the diphenylamine reaction.

It appears that the values obtained by the two methods agree reasonably well. The values for the H_2SO_4 reaction were calculated from a calibration curve similar to the one shown in Fig. 3.

DISCUSSION

Although the reaction with concentrated H_2SO_4 appeared to be only about twice as sensitive as the diphenylamine reaction in our hands, its simplicity is probably unequaled by other methods designed for the determination of DNA. The procedure as described by Staron *et al.* (1) seems satisfactory, provided that the absorbances are measured at 470 mμ, and the corresponding DNA concentrations are read from a calibration curve. In addition, the volume as well as TCA concentration of the samples and the DNA standard with which they are compared, have to be rigorously standardized. Neither heating of the DNA in 5% TCA nor addition of TCA to the sample was found to be essential to the reaction.

SUMMARY

A simple reaction described by Staron *et al.* (1) for the determination of DNA was studied, and found to be useful. A few modifications were introduced.

ACKNOWLEDGMENT

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REFERENCES

1. Staron, T., Allard, C., and Chambre, M., *Compt. Rend. Acad. Sci.* **254,** 765 (1962).
2. Dische, Z., *in* "The Nucleic Acids" (E. Chargaff and J. N. Davidson, eds.), Vol. 1, p. 285. Academic Press, New York, 1955.
3. Schmidt, G., and Thannhauser, S. T., *J. Biol. Chem.* **161,** 83 (1945).
4. Seydel, J. K., and Garrett, E. R., *Anal. Chem.* **37,** 271 (1965).
5. Schneider, W. C., *J. Biol. Chem.* **164,** 747 (1946).

ANALYTICAL BIOCHEMISTRY 13, 322–335 (1965)

Automated Assay of Lactate Dehydrogenase in Urine[1,2]

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As part of a program under way in our laboratory to assess the significance of certain enzyme activities in urine as a diagnostic aid in urinary tract disease, we were prompted to seek a rapid, convenient method for determination of urinary lactate dehydrogenase (LDH). The procedure to be described is a modification of an automated enzymic procedure developed by us for determination of L-lactic acid in body fluids and tissues (1) utilizing the AutoAnalyzer.[3]

The oxidation of L-lactate by NAD^+ catalyzed by lactate dehydrogenase is coupled, via diaphorase, to the reduction of a tetrazolium dye, and the absorbance is measured at 500 mμ. Under the conditions to be described, the reaction rate is strictly proportional to the enzyme activity, and the method is convenient also for determination of the enzyme in body fluids and tissues. The method is similar in principle but differs in detail from a manual procedure described by Nachlas *et al.* (2) and an automated method published recently by Hicks and Updike (3) while this work was in progress.

METHOD

Reagents

3-p-Nitrophenyl-2-p-iodophenyl-5-phenyltetrazolium Chloride–INT. The dye, obtained from Dajac Laboratories, Division of the Borden Chemical Company, Philadelphia, is dissolved in water in a concentration of 1.08 gm/liter and then filtered through Whatman No. 1 filter paper. It is kept in a brown bottle at room temperature.

[1] This work was supported by a contract with the National Cancer Institute, Contract No. 43-64-552, supplemented by partial support from Grants Ca-07174 and AM-05487 from the National Institutes of Health, U. S. Public Health Service.

[2] The aid of Dr. Richard Stambaugh and Dr. Henry Altschuler in some phases of this work is gratefully acknowledged.

[3] Technicon Instruments Corporation, Chauncey, New York.

Bio-Rad AG1-X8, 100–200 Mesh, Analytical-Grade Anion-Exchange Resin. This resin, obtained in the chloride form, is washed three times with 0.1 *M* pH 7.4 sodium phosphate buffer solution and is filtered with suction. It is not allowed to dry out, but is transferred in the moist form to a wide mouth bottle, from which it is dispensed with a spatula.

Diaphorase-NAD⁺ Solution. To 9 ml 0.067 *M* sodium phosphate buffer, pH 7.4, are added: sufficient albumin powder, human fraction V, obtained from Calbiochem, New York, to give a final 0.15% solution; 1.0 ml diaphorase solution, Worthington Biochemical Corporation, Freehold, N. J., containing 100 units/ml; and 200 mg NAD^+, A grade, from Calbiochem, New York. The solution is filtered through Whatman No. 1 filter paper and is kept in an ice bath during the assays. This amount is sufficient for 30 samples and should be prepared fresh daily.

Glycine-Lactate Solution. To 75 ml water containing 9.1 gm NaOH is added 20 ml Merck 85% lactic acid, and the solution is brought to 100° for 5 min and cooled. It is then added to 700 ml water containing 20 ml Triton X-100, a detergent obtained from Rohm and Haas, Philadelphia, and 7.5 gm glycine, obtained from Matheson, Coleman and Bell, East Rutherford, N. J. The pH is adjusted to 9.6 with 10% NaOH solution, and diluted to 1 liter. It should be kept in the refrigerator when not in use to retard growth of microorganisms. It has a final concentration of 0.20 *M* lactate in 0.1 *M* glycine.

Blank Solution. Phosphate buffer, 0.067 *M*, pH 7.4, containing 0.15% human serum albumin. It is kept in an ice bath and prepared fresh each day.

Automation

The flow diagram is shown in Fig. 1. The sample is drawn in at a rate of 0.32 ml/min (tube A), using sampler II,[3] air at 2.00 ml/min (tube B), and glycine-lactate solution at 3.90 ml/min (tube C), and the streams are joined by means of a bubbler cactus. The combined stream is led through a 5-ft mixing coil and then through the larger diameter arm of a bubbler cactus. Through the other arms of this cactus there are drawn in the INT solution at 0.60 ml/min (tube D) and the diaphorase-NAD^+ (or blank) solution at 0.159 ml/min (tube E); and the combined stream is passed first through a 5-ft mixing coil, then through 2 standard-length coils (40 ft) in a bath kept at 32°C. The time of reaction is 8.0 min. Finally, it enters the colorimeter equipped with a 10-mm cholesterol-type flow cell[3] and 500-mμ filters.

Samples are run consecutively at a rate of 40 per hour. Between 0 and 1.4 absorbance units corresponding to enzyme activities of 0 to 60 munits/ml the error due to "carryover" is negligible.

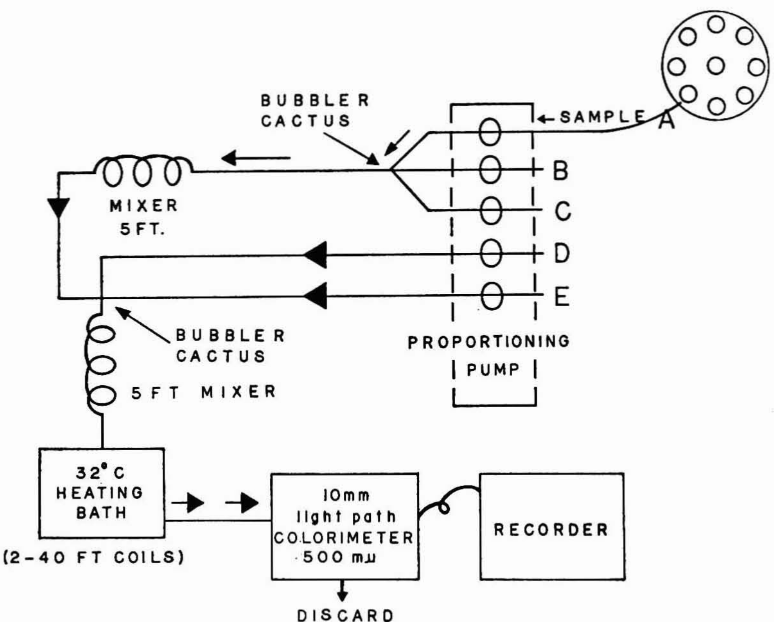

FIG. 1. Flow diagram for automation of LDH assay. The aspiration tubes are designated as follows: A, sample; B, air; C, glycine buffer solution containing lactate; D, INT dye solution; E, NAD⁺-diaphorase solution (or blank solution).

Preparation of Standards

One-tenth ml crystalline rabbit muscle lactic dehydrogenase suspension, A grade, 500 enzyme units/mg, 5 mg/ml, obtained from Calbiochem, New York, is diluted to 10 ml with 0.067 *M* phosphate buffer solution, pH 7.4, containing 0.15% human serum albumin fraction V; and 0.4 ml of this solution is diluted further with the same buffer solution to 50 ml, thus giving a stock enzyme solution representing 80 mμl of the original solution, corresponding to an activity of approximately 70 munits/ml.

Further dilutions of this stock solution are made, and these are assayed by the manual procedure of Dorfman *et al.* (4) using either a Zeiss PMQ II spectrophotometer or a Gilford spectrophotometer with automatic cuvet positioning. With a 3-ml sample in cuvets of 1-cm light path an absorbance of 1 corresponds to 0.48 μmole DPNH (see "Calculations"). The same dilutions of the stock solution, corresponding to activities ranging from 0 to 20 munits/ml, are placed in sample cups and assayed in the AutoAnalyzer. A typical plot of absorbance of the standards in the automated procedure against enzyme assayed manually is shown in Fig. 2. By reference to this curve, absorbances observed in the automated assay are converted directly to enzyme activity in munits/ml.

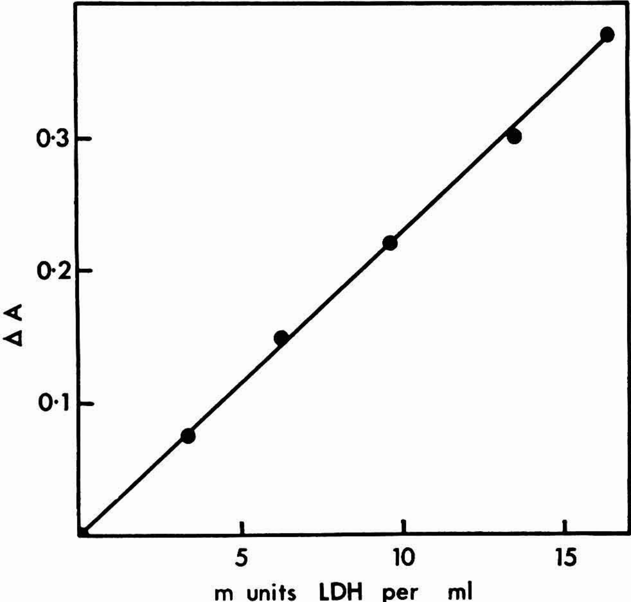

FIG. 2. Standard curve for automated LDH assay. Ordinate is in absorbance units of the AutoAnalyzer recorder, and abscissa is in munits of enzyme activity per ml, as determined manually.

An alternative procedure would be to use commercially available lactic dehydrogenase standards to standardize the instrument, but we prefer to prepare our own standards in the aforementioned manner. If one uses a commercially available standard whose activity has been assayed in the direction pyruvate to lactate, it is important to keep in mind that its activity is likely to be about 2.5 times higher than the values obtained when assayed in the reverse direction, as is done in this procedure (see "Calculations").

Measurement of LDH in Urine

Approximately 10 ml urine is placed in a 25-ml test tube with a screw-cap closure, and a quantity of the suspension of AG1-X8 resin added, equivalent to about 5 gm of the dry resin. The tube is capped and placed on a mechanical shaker[4] which imparts a rocking motion at a rate of about 60 oscillations/min. After 5 min of shaking at room temperature, the urine is filtered or carefully decanted from the resin, and samples are placed in AutoAnalyzer cups.

The following sequence of assays is followed: first, a series of stand-

[4] Built by Mr. Joseph Hochella, Hazleton, Pennsylvania.

ards; then two identical series of urine specimens. The first set is run with the complete set of reagents as shown in Fig. 1. The second set is run immediately following and exactly like the first set, except that the blank solution is substituted for the diaphorase-NAD^+ solution. The absorbance of each blank, which is in the vicinity 0.2 absorbance unit, is subtracted from the corresponding sample, to give the net absorbance, from which the activity is obtained by reference to Fig. 2.

Calculations

Because of the wide variety of units employed for measurement of lactate dehydrogenase activities, it is becoming increasingly difficult to compare results from different laboratories. We feel it is highly desirable, therefore, to express results in terms of International Units, as recommended by the Enzyme Commission of the International Union of Biochemistry (5). An enzyme unit is that amount of enzyme which under stated conditions converts one μmole of substrate per minute.

Using the procedure of Dorfman, Amador, and Wacker (4) for standardization of lactate dehydrogenase, and assuming a molar absorbancy of 6.22×10^3 for NADH, an absorbance change of 1 unit in a 3-ml volume and 1-cm light path is equivalent to 0.482 μmole NADH. Thus, lactate dehydrogenase activity in International Milliunits = $(\Delta A/\text{min}) \times 482$. These are the values given in the ordinate in Fig. 2. The activity expressed in Wacker Units (4), the amount of enzyme giving a ΔA of 0.001, is therefore equivalent to International Milliunits/0.482, an International Munit being equivalent to 2.07 Wacker Units. Under the conditions here described, normal urinary LDH levels of 2 to 5 munits/ml give Δ absorbances of between 0.06 and 0.12. The sensitivity of this urine assay could be increased by increasing the proportion of urine flowing through the manifold, but this also raises the background absorbance and is therefore considered inadvisable, even though the accuracy of assay of the low activities in normal urines is not as high as would be desired (*vide infra*).

RESULTS

In general, such conditions as temperature, concentration of reagents, and automation setup were adopted directly from our previously described enzymic assay of L-lactate (1). The considerations which led to the procedure described here are described in the following paragraphs.

Removal of Interfering Substances. When normal urine is assayed directly by the procedure outlined here, the blank absorbance is extremely high; it ranges from 1.5 to 2 or above, owing to the presence of a substance that acts directly on the tetrazolium dye to produce the colored

formazan. According to Dorfman *et al.* (4) urine is reported to contain inhibitors of LDH which can be removed by 80 min of dialysis in running tap water. By applying this procedure to the automated assay, the blank absorbance can be reduced to between 0.3 and 0.5, and assays of LDH can be obtained which agree well with the manual assay. In seeking more efficient means of reducing the blank it was found that nearly all of the blank absorbance could be removed easily and rapidly, without significant loss of enzymic activity, by treatment with the ion-exchange resin, Bio-Rad AG1-X8. This is a strongly basic anion-exchange resin composed of quaternary ammonium groups attached to a styrene-divinylbenzene polymer lattice. This resin is available in the chloride form, but it is desirable to equilibrate it with phosphate buffer as described earlier. By a 2-min shaking of urine with this resin at room temperature, the blank absorbance is brought to approximately 0.2 or less. As shown in Fig. 3,

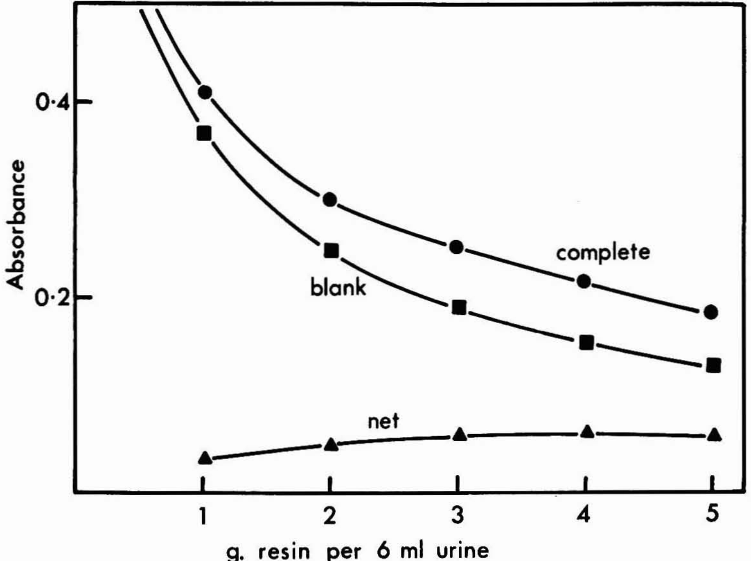

FIG. 3. Removal of interfering absorbance by resin treatment. Each specimen was shaken with the designated quantity of resin for 20 min.

the degree of removal of the interfering material is dependent upon the quantity of resin employed, but it is interesting to note that despite differences in background absorption, the net ΔA remained constant. Figure 4 shows that, in the presence of 5 gm resin, color removal is nearly maximal in as short a period as 2 min; only a slight further decrease was

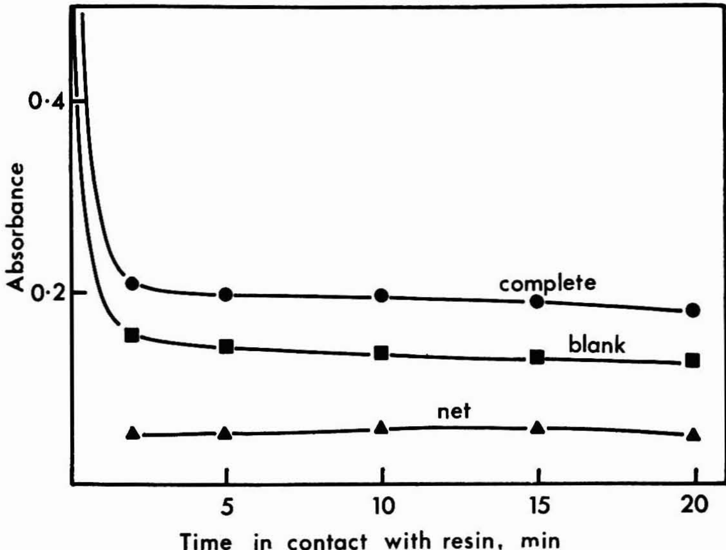

FIG. 4. Time course of removal of interfering absorbance. Each sample was shaken with 5 gm resin for the designated times.

obtained by increasing the contact time to 20 min. Again, net absorbance was invariably constant throughout the 20-min period.

The background absorption may be reduced further by repeated resin treatment, a procedure which may be desirable when the initial absorption is extremely high. However, even four successive treatments reduced the background only to about 50% of that after a single treatment; hence we felt the decreased absorption was not worth the additional manipulations, which increase the time and effort, as well as the opportunity for error.

To test the reproducibility of the assays after resin treatment, twelve samples of the same urine were run simultaneously and the results are shown in Table 1. Despite some variability in the background absorption, which averaged 0.232 with a standard deviation of 0.009, the net absorbances were quite reproducible, with an average of 0.010 and a standard deviation of only 0.002. Despite the unusually low activity of this urine sample, the highly satisfactory reproducibility lends confidence in the reliability of the resin treatment.

To test whether the resin itself will adsorb LDH or otherwise have a deleterious effect on LDH assay, several experiments, of the type shown in Fig. 5, were carried out. To a sample of normal urine, an equal volume of a standard LDH solution (M subunit, C. Boehringer, New York) was added, and the mixture was treated with resin and assayed. Before resin

TABLE 1

REPRODUCIBILITY OF URINARY LDH ASSAY AFTER RESIN TREATMENT

A sample of normal urine was separated into twelve fractions, and these were assayed after resin treatment as described in the text.

Sample No.	Absorbance		
	Blank	Complete	Net
1	0.228	0.237	0.009
2	0.233	0.245	0.012
3	0.220	0.228	0.008
4	0.226	0.236	0.010
5	0.243	0.254	0.011
6	0.240	0.250	0.010
7	0.247	0.251	0.004
8	0.217	0.228	0.011
9	0.222	0.234	0.012
10	0.237	0.247	0.012
11	0.237	0.245	0.008
12	0.233	0.242	0.009
Mean	0.232	0.241	0.010
S.D.	0.009 (4%)	0.009 (4%)	0.002 (20%)

treatment, the net absorbance was 1.23 — 0.91 = 0.32. After 2 min of resin treatment, the background dropped to 0.04, and the net absorbance increased to 0.38, at which level it remained essentially constant over the

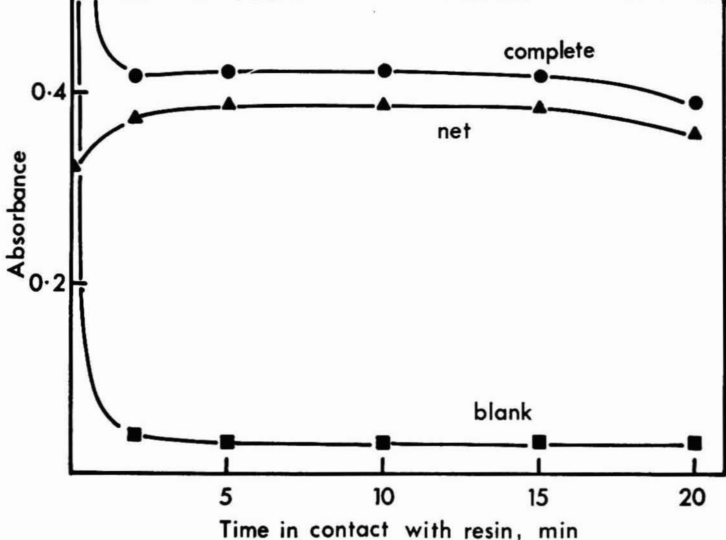

FIG. 5. Effect of resin treatment on activity of M subunit of rabbit muscle LDH, shaken for the designated periods with 5 gm resin.

remaining 18 min. Similar results have been obtained with other urines mixed with crystalline rabbit muscle LDH or with purified beef heart H subunit. These results demonstrate that purified LDH is not appreciably affected by the resin treatment, and they indicate also that if inhibitors of LDH are present in urine their quantitative effects are not great. Our experience in this respect is in agreement with that of Hicks and Updike (3), who reported a maximum inhibition of 20% before resin treatment in their automated procedure.

To compare resin treatment with dialysis, a series of urine assays was conducted over several months with samples assayed respectively after a 2-hr dialysis in flowing tap water and after 5 min of treatment with resin. The results, shown in Table 2, demonstrate a very satisfactory agreement

TABLE 2
COMPARISON OF DIALYSIS AND RESIN TREATMENT FOR URINARY LACTATE DEHYDROGENASE ASSAY

Ninety-one samples of normal urine were assayed after resin treatment and dialysis and are divided below into three categories on the basis of the specific activity—values ranging from below 2 munits, between 2 and 5 munits, and greater than 5 munits/ml.

Range of values	No. of samples	Resin-treated, % of dialyzed	Standard deviation, %
Below 2	43	89	26
2 to 5	20	94	19
Above 5	28	101	11

between the two procedures. With low activity samples, that is below 2 munits/ml, the resin treatment gave values that were on the average 89% of the dialysis values, with a standard deviation between the values of 26%. With higher activity samples, the agreement was even closer. These results make it clear that resin treatment is fully as effective as dialysis for sample preparation, and is, of course, far superior in ease and convenience.

Separation of Chromogenic Material in Urine from LDH. Although the interfering substance(s) was not identified, it could be separated from LDH by column chromatography. A column of Bio-Rad P-60 (50–150 mesh) of the dimensions 23 $\times$ 133 mm, was prepared, and after thorough washing with pH 7.4 phosphate buffer a mixture of 1 ml urine and 1 ml commercial purified rabbit muscle LDH (49 munits/ml) was placed on the column and developed by slow addition (about 1 ml/min) of 0.1 *M* pH 7.4 phosphate buffer, while 2-ml fractions were collected. Thirty-one such fractions were assayed with the AutoAnalyzer, and the results are shown in Fig. 6. The squares show the absorbances obtained on assay with

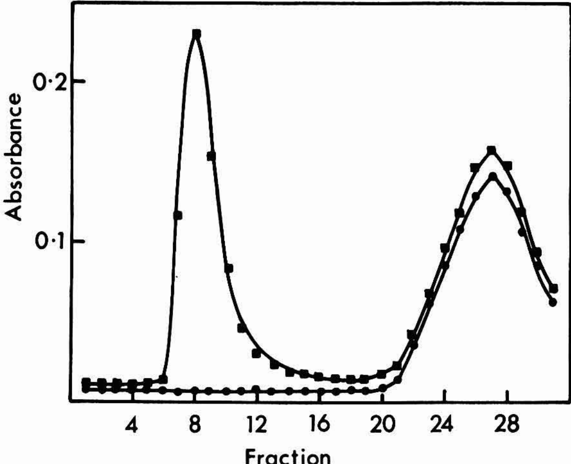

FIG. 6. Separation of urinary LDH from interfering chromogen by chromatography on Bio-Rad gel P-60. Squares represent assays with the complete system; circles represent blank assays without NAD^+ and diaphorase.

the complete system, whereas the circles give the blank absorbances without the NAD^+-diaphorase solution. All of the nonenzymic chromogen appeared in tubes 20 to 31, completely separated from LDH. Similar separation of LDH from the interfering chromogen was reported by Hicks and Updike (3) using Sephadex. Although not identified, ascorbic acid seems a likely possibility, since it reacts directly with the tetrazolium dye under our assay conditions and is present in sufficient quantity in urine to give the color development observed (6).

Choice of Blank. In view of the appreciable background color development, even after resin treatment, it was important to choose for the blank determination a system that would most nearly approximate the true, nonenzymic color. To compare various "blanks," five urine samples were assayed in the complete system, with the following omissions: lactate, NAD^+, diaphorase, and both NAD^+ and diaphorase. The results are shown in Table 3. With all five samples the blank with lactate omitted was considerably higher than the others, owing possibly to some enzymic activity because of endogenous lactate, which is normally present in urine (6). However, the differences between all of the possible blanks were small compared with the total net absorbance. Consequently, for economy of reagents we adopted the omission of both diaphorase and NAD^+ for the blank, and have found it satisfactory to run the sample and blank determinations consecutively. Automatic elimination of the blank can no doubt be achieved by splitting the sample and utilizing a differential

TABLE 3

COMPARISON OF BLANKS IN URINARY LACTATE DEHYDROGENASE ASSAY (values are given in absorbance)

		Omissions			
Sample No.	Complete system	Lactate	NAD^+-diaphorase	Diaphorase	NAD^+
1	0.263	0.100	0.063	0.064	0.067
2	0.280	0.211	0.188	0.182	0.203
3	1.430	0.187	0.078	0.085	0.089
4	0.267	0.092	0.052	0.063	0.063
5	0.348	0.278	0.231	0.233	0.257

colorimeter, but such equipment is not now available to us.

Lactic Acid Concentration. Although large and differential effects of pyruvate concentration on the activities of the different LDH subunits are well known (7), it is not widely appreciated that the same is true for lactate concentration as well. In work to be reported separately from our laboratory, Stambaugh[5] found that isolated LDH subunits exhibit widely different lactate concentration optima, as well as differential inhibitory effects of high concentrations. This prompted a study of lactate concentration in the automated LDH assay. Experiments were conducted with the crystalline rabbit muscle enzyme, known to contain mainly the M subunit, with purified rabbit muscle M subunit, and pig heart H subunit obtained from the Boehringer Mannheim Corporation, New York; and with beef heart H subunit purified by Dr. Stambaugh by gel electrophoresis. Typical curves relating activity to concentration are reproduced in Fig. 7. The pure H form has a lactate concentration optimum of 60 m*M* from which there is a gradual decline, reaching 57% of the optimum at 500 m*M*. In contrast, the M subunit at 60 m*M* has only about 61% of its maximal activity which is reached at 300 m*M*; and in further contrast, the M form is not inhibited with increase in lactate concentration to 500 m*M*. From Fig. 7 it appears that a concentration of 200 m*M* is the best compromise for the efficient assay of both subunits, giving about 90% of maximal activity of both subunits, and this has determined our choice of this concentration of lactate.

The differential effects of high and low lactate concentrations on the activities of the subunits makes it possible to assay for the subunits by measuring absorbances at two lactate levels. Such experiments are now under way and will be reported separately.

[5] Stambaugh, R., private communication.

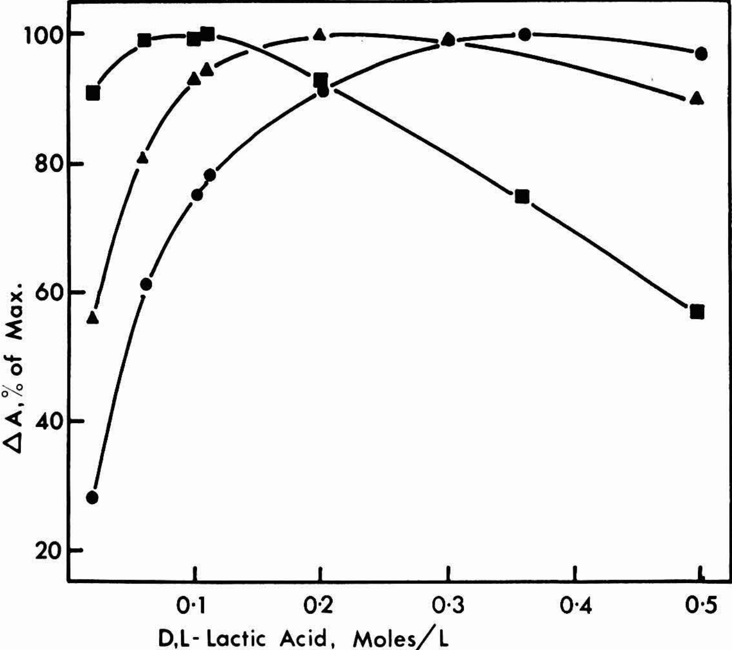

FIG. 7. Effect of lactate concentration on the automated enzyme assay. Circles give the activities of the "M" subunit, squares the activities of the "H" subunit, and triangles the activities of a mixture of 50% "M" and 50% "H" subunit. Lactate concentrations in the abscissa are those of the lactate-glycine buffer solution entering at tube C, Fig. 1.

DISCUSSION

Automated LDH assays have been reported by Schwartz, Kessler, and Bodansky (8) using direct measurement of NADH at 340 mμ. Blaedel and Hicks (9) and Hicks and Updike (3) described automated procedures in the visible range by coupling NAD reduction with dye reduction. In the former study, designed primarily for serum, the reduction of 2,6-dichlorophenolindophenol is coupled to NADH via diaphorase; in the latter, designed primarily for urinary LDH assay, the coupling is mediated via phenazine methosulfate. The present procedure, which uses commercially available equipment, is similar in principle to these, the NADH formation being coupled via diaphorase to a tetrazolium dye. This procedure is also highly satisfactory for serum LDH assay, where no interferences have been observed and the "blanks" are negligible. The automated procedure is sensitive, the curves are smooth and with no "noise," and the reproducibility is excellent. There seems little doubt

that the limiting factors in the accuracy of LDH assays in the urine or serum would not involve the determination itself, but rather the stability of the enzyme, or the presence of inhibitors, or other interfering substances. In this connection it is important to be aware of the possibility that some substances in urine might directly reduce NAD^+ nonenzymically. Although very rare, several such instances have been observed using the manual procedure,[6] and the prevalence of such occurrences needs further investigation before the automated LDH assay can be accepted at face value, without corroboration by a manual assay in the spectrophotometer.

Since the assay method depends on NAD^+ reduction, evidently any NAD^+ (or $NADP^+$) specific dehydrogenase should be determinable by suitable modification, and this should be true also for assay of enzymes which can be coupled to such dehydrogenases. By use of the appropriate enzymes, a host of substrate assays could also be automated, using modifications of the many enzymic procedures now available for this purpose (10).

The methodology of urinary LDH assay and its use as a diagnostic aid is now under intensive investigation in several laboratories (3, 4, 8, 9, 11–17). After thorough study we have found the spectrophotometric method of Dorfman, Amador, and Wacker (4) to be reliable, and by use of this manual procedure for standardization the automated procedure as described here should prove to be useful and reliable for the rapid assay of large numbers of urine samples.

REFERENCES

1. Hochella, N. J., and Weinhouse, S., *Anal. Biochem.* **10,** 304–317 (1965).
2. Nachlas, M. V., Margulies, S. I., Goldberg, J. D., and Seligmann, A. D., *Anal. Biochem.* **1,** 317–327 (1960).
3. Hicks, G. P., and Updike, S. J., *Anal. Biochem.* **10,** 290–303 (1965).
4. Dorfman, L. E., Amador, E., and Wacker, W. E. C., *J. Am. Med. Assoc.* **184,** 1 (1963).
5. "Report of the Commission on Enzymes of the International Union of Biochemistry." Pergamon Press, New York, 1961.
6. Long, C., Ed., "Biochemists' Handbook." Van Nostrand, Princeton, N. J., 1961.
7. Cahn, R. D., Kaplan, N. O., Levine, L., and Zeilling, E., *Science* **136,** 962–969 (1962).
8. Schwartz, M. K., Kessler, G., and Bodansky, O., *J. Biol. Chem.* **236,** 1207 (1961).
9. Blaedel, W. J., and Hicks, G. P., *Anal. Biochem.* **4,** 476–488 (1962).
10. "Methods of Enzymatic Analysis" (Bergmeyer, H. U., ed.). Academic Press, New York, 1963.
11. Wacker, W. E. C., and Dorfman, L. E., *J. Am. Med. Assoc.* **181,** 972–978 (1962).

[6] Altschuler, H., private communication.

12. Wacker, W. E. C., Dorfman, L. E., and Amador, E., *J. Am. Med. Assoc.* **188**, 671 (1964).
13. Riggins, R. S., and Kiser, W. S., *J. Urol.* **90**, 594–603 (1963).
14. Gelderman, A. H., Gelboin, H. V., and Peacock, A. C., *J. Lab. Clin. Med.* **65**, 132–142 (1964).
15. Dubach, U. C., and Rediger, L., *Urol. Intern.* **17**, 65–83 (1964).
16. Kern, W. H., and Webster, W. W., *Acta Cytol.* **8**, 302–305 (1964).
17. Massod, M. F., Franey, R. J., Therrien, M. E., Rideout, P. T., and Babcock, M. T., *Am. J. Clin. Pathol.* **42**, 623–625 (1964).

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An Acrylamide Gel Soluble in Scintillation Fluid: Its Application to Electrophoresis at Neutral and Low pH[1]

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In using the gel electrophoresis method of Ornstein (1) and Davis (2) on fragments of antibody molecules, we encountered two problems that led to useful modifications of the technique. The first was the difficulty of obtaining an accurate measure of the radioactivity of labeled protein in the acrylamide gel. We have solved this difficulty by the use of the cross-linking agent, ethylene diacrylate (I), in place of the usual *N,N'*-methylenebisacrylamide (II); the new gel is soluble in Kinard's scintillation-counting fluid (3) after treatment with piperidine. The second problem was the difficulty of polymerizing the acrylamide with the usual initiating system at low pH. This was circumvented by the use of the modified initiator described below, which allows polymerization to proceed rapidly in the presence of 1 *M* propionic acid.

(I)

$$H_2C{=}CH{-}\overset{\overset{O}{\|}}{C}{-}O{-}CH_2{-}CH_2{-}O{-}\overset{\overset{O}{\|}}{C}{-}CH{=}CH_2$$

(II)

$$H_2C{=}CH{-}\overset{\overset{O}{\|}}{C}{-}NH{-}CH_2{-}NH{-}\overset{\overset{O}{\|}}{C}{-}CH{=}CH_2$$

The new gel obtained with the ethylene diacrylate cross linker does not appear to differ significantly from the old gel in either its filtration properties or in its mechanical strength. Its solubility in counting fluid results from the susceptibility of the ester bond in the cross linker to hydrolysis in the presence of piperidine (4).

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Fleischman *et al.* (5) have demonstrated the utility of 1 *M* propionic acid for the separation of the heavy and light chains of gamma globulin, by Sephadex gel filtration, which would otherwise associate with each other. The combined effects of maximizing the positive charge of a protein or peptide, minimizing the ionic strength, and providing some detergent action make dilute propionic acid an attractive medium for handling systems with tendencies to associate. Data are presented here which show the separation of trypsin and chymotrypsin, proteins stable at low pH but self-digesting at pH values much above 4.

The modified gels may be used with either continuous or discontinuous buffer systems. However, in the experiments described below, a continuous-pH electrophoresis system was employed (buffer bath, sample, and gel all at the same pH), rather than the discontinuous system of Ornstein (1) or of Poulik (6). In order to obtain high resolution, a modification of the mobility spectrum technique of Kolin (7, 8) was utilized. The initial liquid sample layer is made low in ionic strength, and, as a result of the high mobility of the protein in this layer, the sample is "focused" as it encounters the relatively high-ionic-strength low-porosity gel. The narrow band thus formed can be resolved into its components in minimal time.

APPARATUS

The apparatus is pictured in Fig. 1. The upper and lower buffer baths are connected to their respective electrode baths through acrylamide salt bridges. Silver-silver chloride electrodes are employed for maximum pH stabilization. The level of the lower bath is controlled with a pair of screw jacks (Micro Lab-Jack obtained from Central Scientific Company). A constant-voltage power supply is used. The novel feature of this apparatus is that the 5-mm i.d. glass tubes containing the gel are held in place by "O" ring seals which make it easy to push them up and down, greatly facilitating gelling and loading operations. The tubes are readied for pouring the gel by pushing the tubes down into soft rubber pads. Following gel formation the rubber pads are removed and, with the tubes in the up position, the density-stabilized liquid samples are layered in on top of the gel. The tubes are then simply pushed down into contact with the lower bath with little risk of stirring up the sample zone. Shop blueprints can be obtained on request from the first author.

MATERIALS

The acrylamide monomer, *N,N,N',N'*-tetramethylethylenediamine (TEMED), piperidine, methyl acrylate, and aniline blue black dye were obtained from Matheson, Coleman and Bell. Baker Analyzed ammonium persulfate and Fisher purified (low in iron) sodium hydrosulfite were

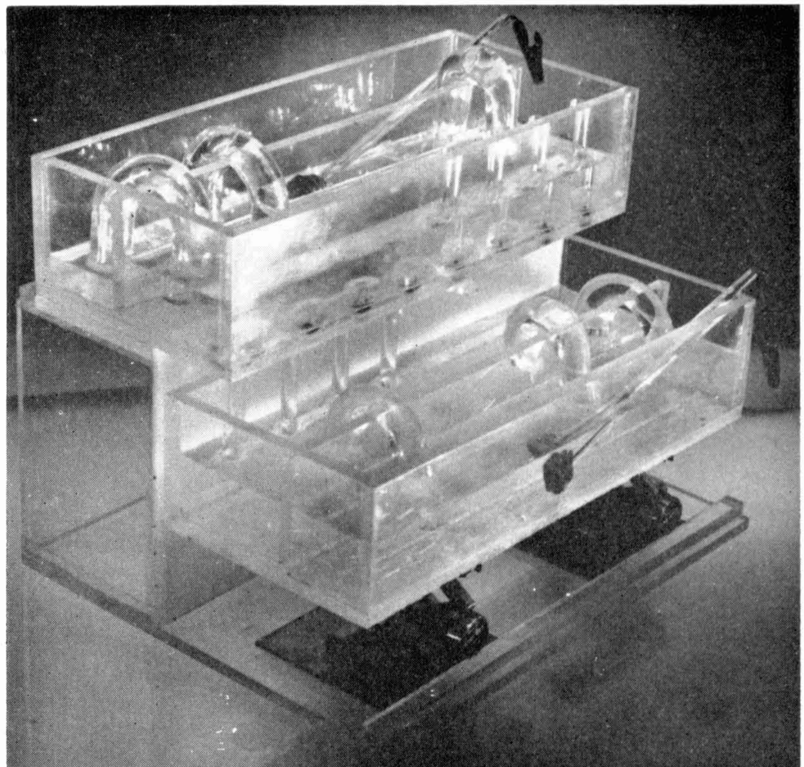

FIG. 1. Electrophoresis apparatus.

used. Hydroxide of Hyamine 10-X was obtained as a 1 *M* solution in methanol from Packard Instrument Company.

Ethylene diacrylate[2] was prepared by alcoholysis of methyl acrylate with ethylene glycol. Methyl acrylate (90 ml) was mixed with 14 ml ethylene glycol, and about 0.5 gm solid potassium hydroxide was added as catalyst; 20 ml of fluid was slowly distilled off at 65°–67°C as the reaction progressed. Following this, the temperature of the vapor slowly rose to 74° while a further 10 ml of distillate was collected. Air was bubbled through the boiling mixture constantly to inhibit polymerization. Unreacted methyl acrylate was distilled off under moderate vacuum and then the product was collected at 95°–100° at 6 mm pressure. The yield was approximately 30%.

Rabbit anti *m*-dinitrophenol antibody was affinity labeled with *m*-nitrophenyldiazonium fluoroborate (m-NBDF-^{3}H) by the method of

[2] Ethylene diacrylate is now available from the Borden Chemical Co., Monomer-Dimer Laboratory, 5000 Langdon Street, P. O. Box 9522, Philadelphia, Pennsylvania.

Wofsy, Metzger, and Singer (9) as described by Good *et al.* (10). This was used to prepare the light chains by the method of Fleischman *et al.* (5).

Purified trypsin and chymotrypsin were obtained from Sigma Chemical Company. Labeled trypsin was prepared by mixing it with a tenfold molar excess of m-NBDF-^{3}H in ammonium acetate buffer at pH 5.0. The mixture was allowed to react overnight and then dialyzed against several changes of the acetate buffer.

Scintillation fluid was prepared according to the recipe of Kinard (3). The fluid contained 576 ml xylene, 576 ml dioxane, 348 ml ethanol (absolute), 120 gm naphthalene, 7.5 gm 2,5-diphenyloxazole (PPO), and 75 mg 1,4-di-2-(5-phenyloxazolyl)benzene (POPOP). The total volume was 1.5 liters. PPO and POPOP were obtained from Calbiochem, Los Angeles.

GEL PREPARATION

The pH 6.5 gels are prepared much as in the method of Davis (2) for his "standard" gel except for a change in the buffer and the substitution of ethylene diacrylate for methylenebisacrylamide. The same modified acrylamide stock solution is employed in making the pH 2.1 gels containing 1 M propionic acid in place of buffer, but here it is also necessary to change to a persulfate-hydrosulfite catalyst system.

Stock Solutions

Solution A: Buffer, ionic strength 0.10 M, measured pH 6.5; containing 0.079 M sodium hydroxide, 0.0071 M TEMED (0.115 ml/100 ml solution), and 0.136 M cacodylic acid.

Solution A′: 4 M propionic acid.

Solution B: 30 gm acrylamide plus 2 gm ethylene diacrylate per 100 ml.

Solution C: 0.0031 M ammonium persulfate (70 mg/100 ml).

Solution C′: 0.001 M ammonium persulfate (23 mg/100 ml).

Solution D: 0.002 M sodium hydrosulfite (35 mg/100 ml), made fresh just before use.

pH 6.5 Gel

Solutions are mixed in the ratio of 1 part A, 1 part B, and 2 parts C. The mixture is pulled up into a glass disposable syringe, degassed, and poured to a depth of 5.3 cm in 5-mm i.d. glass tubes 6.5 cm long. Degassing is accomplished by first clamping off the flexible plastic (Intermedic) tubing attached to the delivery end of the syringe and then pulling back the plunger and shaking to bring the air out of solution. Water is layered on top of the gel mixture with a Krogh-Keys syringe

pipet[3] and, after 30 min at room temperature, the water and unpolymerized gel solution are decanted, leaving a flat-topped gel column 5 cm long. The remaining space in the top of the glass tubes is rinsed with water and filled with 5% sucrose to keep buffer out of the sample zone.

Gel Containing 1 M Propionic Acid

Solutions are mixed in the ratio of 1 part A', 1 part B, and 1 part C'. Solid hydrosulfite is added to cold water making solution D, and 1 part D is added to the above mixture. The measured pH was 2.1. After the mixture has been carefully degassed, glass tubes are filled as above, but only to a depth of 5.0 cm. More solution D is layered over the gel solution in place of the water used above. This excludes oxygen so that the gel line forms right at the liquid junction, instead of below it, and ensures the formation of a flat-topped gel. After 1 hr, the columns are rinsed and the remaining space is filled with 1 *M* propionic acid. The tubes are positioned in the electrophoresis apparatus and prerun at 80 volts and 2 ma per tube for 30 min to remove excess catalyst. The 5% sucrose is layered in as above.

STAINING AND COUNTING PROCEDURES

Following electrophoresis, gels are removed from the tubes by the method of Davis (2) and half of them are stained for 20 min in a solution of 1% aniline blue black dye and 10% acetic acid. Columns are destained overnight by soaking them in two changes of 10% acetic acid with constant stirring. After destaining the columns are scanned in a Joyce-Loebl microdensitometer. The dye intensity is assumed to be proportional to the protein concentration since the proteins involved are very similar.

Duplicate columns are frozen quickly (within 5 min by dry ice) and then cut into 1-mm slices with slicer made of fifty razor blades. One or two slices are placed in the bottom of each of an appropriate number of 20-ml counting vials, and the digestion is started by adding 0.5 ml of a solution containing 9 vol 1 *M* piperidine and 1 vol alcoholic Hyamine solution. The gel dissolves in 1 to 4 hr depending on the amount of gel and the extent of cross linking. The process is hastened by placing the vials on a shaker in a 37°C room. Following this digestion, 10 ml of Kinard's scintillating fluid (3) is added to each vial and mixed briefly; a clear homogeneous solution results. The above proportions work best with one 1-mm slice of gel, but up to two slices can be dissolved with difficulty. Counting is done in a Nuclear-Chicago model 725 scintillation counter.

[3] Krogh-Keys syringe pipets were obtained from Aloe Scientific Division of the Brunswick Corporation, 1150 S. Flower Street, Los Angeles 15, California.

In one experiment the piperidine was half neutralized with HCl to give a pH of 11.0 with no serious change in the rate of dissolution of the gel. It might thus be possible to recover a base-sensitive material, such as DNA, intact from the gel.

METHODS AND RESULTS

Electrophoresis of Rabbit Anti m-dinitrophenol Light Chains at pH 6.5. The gels were prepared and layered with 5% sucrose as described above. Samples were dialyzed against buffer and then for 2 hr against 10% sucrose to increase the density and reduce the ionic strength. The 50 μl of sample containing 0.17 mg of rabbit anti m-DNP material were pipetted into each of four tubes under the 5% sucrose with a Krogh-Keys adaptor equipped with a Hamilton microliter syringe. Electrophoresis was allowed to proceed 5 min at 70 volts (14 volts/cm) and about 1 ma per tube to "focus" the sample material into a sharp band just below the gel surface. The sucrose solution was then flushed out of the tubes with buffer from the bath, establishing good electrical contact between bath and gel surface and eliminating irregularities in the resistance in this region. The current was thus increased to about 3 ma per tube and the run was continued for 4 hr. This long running time was necessitated by the very low mobility of these peptides and is not typical of the method.

The results are shown in Figs. 2 and 3. In this case two of the duplicate columns were stained prior to scanning them with the densitometer. Two other columns were cut up and two 1-mm slices were counted in each vial. The resulting curves are shown in Fig. 2. One of the curves is

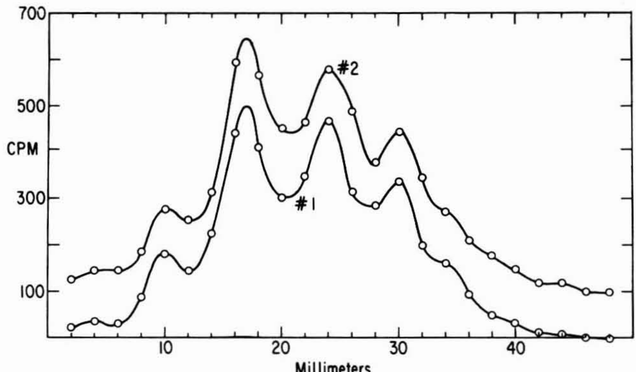

FIG. 2. Electrophoresis at pH 6.5 of the light chains derived from anti dinitrophenol antibody which was affinity labeled with m-NBDF-^{3}H. Each point represents cpm derived from 2-mm slices of the polyacrylamide columns. Curve 2 is displaced upward by 100 cpm for clarity.

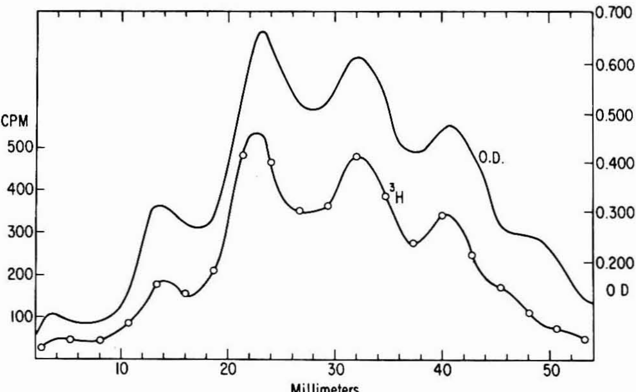

FIG. 3. A counting curve from Fig. 2 is compared with a densitometer tracing of another column run at the same time and subsequently stained.

displaced upward by 100 counts per minute (cpm) to be shown more clearly. The counting efficiency was 10% so that the central peak at 24 mm represents 4700 disintegrations per minute. The background was approximately 40 cpm. As can be seen, the two curves are in agreement. Counts from the lower curve were divided by the upper for points between 8 and 38 mm on the horizontal axis. The resulting mean and standard deviations for the 17 ratios were 1.07 and 0.08, respectively. This indicates that there is about 7% difference between the amount of sample originally added to each column and the coefficient of variation is 7.5%. Recovery of total counts was 102% for #1 and 93% for #2. Figure 3 shows a densitometer tracing of one of the stained gels compared with one of the counting data curves. The gels swelled from 5 to 7 cm while staining so the abscissa for the counting curve had to be multiplied by 7/5.

Electrophoresis of Trypsin and Chymotrypsin at pH 2.5 in 1 M Propionic Acid. Samples were dissolved in a solution of 0.2 *M* propionic acid and 10% sucrose; 25 μl of sample containing 100 μg of m-NBDF-^{3}H labeled trypsin was pipetted into each of two tubes containing the pH 2.5 gel. Two more tubes were loaded with 25 μl of the same labeled trypsin solution plus 25 μl of another sample solution containing 100 μg of cold chymotrypsin. Loading and electrophoresis procedures were the same as for the pH 6.5 gel above. This electrophoresis required 80 volts and about 1 ma per tube for 5 min before flushing out the sucrose, and 80 volts and about 2.2 ma per tube for only 40 min thereafter. One of each pair of tubes was stained and the other sliced and counted. Only one 1-mm slice was placed in each counting vial.

The results are shown in Figs. 4 and 5. A densitometric comparison

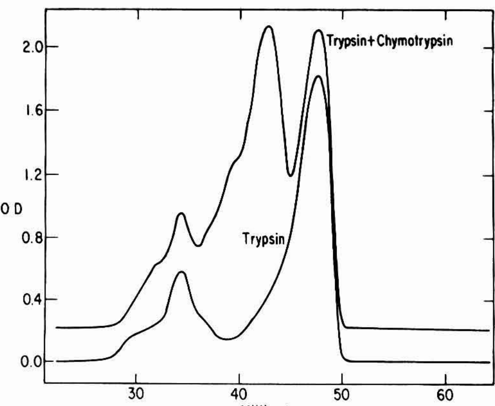

FIG. 4. Electrophoresis of trypsin and chymotrypsin in 1 M propionic acid. Densitometer tracings are shown of two columns electrophoresed at the same time: one had trypsin alone and the other had a mixture of trypsin and chymotrypsin. One curve is displaced upward for clarity.

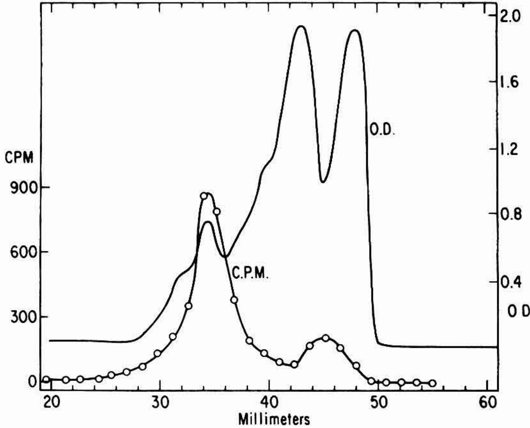

FIG. 5. Densitometer tracing from Fig. 4 compared with one of the counting curves from Fig. 5. Labeled trypsin and unlabeled chymotrypsin were present in each case.

of the two stained columns is shown in Fig. 4. The trypsin shows a single major band with two or three minor components. Addition of chymotrypsin to the second column adds the expected second major band plus its own minor components. A comparison of the counting and densitometer data from duplicate columns containing labeled trypsin and unlabeled chymotrypsin is shown in Fig. 5. The counting data from the

column containing trypsin alone yielded an essentially indistinguishable curve (not shown). The striking result here is that most of the counts are in one of the minor trypsin bands. The remainder of the counts seem to be in the trailing edge of the major trypsin band, possibly due to impurity or to denatured trypsin. A discussion of the possibilities is beyond the scope of this paper. The objective here is to show how much additional information the counting data can offer over that shown by the densitometer tracings alone.

SUMMARY

Two additions to diacrylamide gel techniques are presented. First the gel structure can be modified by substituting diester cross links for the diacrylamide cross links. This modified gel is soluble in dioxane scintillation fluid after digestion in 1 *M* piperidine at room temperature. Reasonably efficient and accurate tritium counting can be done on column slices subjected to the procedure described. The second modification is the development of a catalyst system suitable for making pH 2.5 gels containing 1 *M* propionic acid as the electrolyte. The low pH gels are of value in obtaining good separation of proteins and peptides which associate at higher pH values or are stable only at low pH. Densitometric and counting data are cited to illustrate the value of combining these techniques.

ACKNOWLEDGMENT

The authors are indebted to Dr. John Lenard for suggesting the use of piperidine which made possible the rapid hydrolysis of our modified gels, and to Dr. S. J. Singer, in whose laboratory this work was done, for providing the antibody and labeling compound used in these experiments.

REFERENCES

1. Ornstein, L., *Ann. N. Y. Acad. Sci.* **121**, 321 (1964).
2. Davis, B. J., *Ann. N. Y. Acad. Sci.* **121**, 404 (1964).
3. Kinard, F. E., *Rev. Sci. Instr.* **28**, 293 (1957).
4. Lenard, J., and Hess, G. P., *J. Biol. Chem.* **239**, 3275 (1964).
5. Fleischman, J. B., Porter, R. R., and Press, E. M., *Biochem. J.* **88**, 220 (1963).
6. Poulik, M. D., *Nature* **180**, 1477 (1957).
7. Kolin, A., *J. Chem. Phys.* **22**, 1628 (1954).
8. Kolin, A., *Proc. Natl. Acad. Sci. U. S.* **41**, 101 (1955).
9. Wofsy, L., Metzger, H., and Singer, S. J., *Biochemistry* **2**, 979 (1963).
10. Good, A. H., Traylor, P. S., and Singer, S. J., in preparation.

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A Colorimetric Method for Estimation of Micro-quantities of Tartaric Acid Isomers

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Several methods for the quantitative estimation of the optically active isomers of tartaric acid are known (1–3). The smallest amounts of material which can be detected with these methods range from 1 to 3 μmoles. For the determination of *m*-tartaric acid, the only method described in the literature cannot detect amounts less than about 400 μmoles (4). The method described in the present paper allows estimation of any of the three isomers of tartaric acid in amounts of 0.05 μmole or more. The method is based on the formation of a tartrate-iron (Fe^{++}) complex, which on addition of dinitrophenylhydrazine and alkali forms a purple colored compound.

METHODS

Materials and Apparatus

Acetate buffer—0.1 *M*, pH 5.4 (B.D.H. Analar).

$Fe(NH_4)_2(SO_4)_2$ reagent—a solution of 0.015 *M* $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (B.D.H. Analar) in 0.2 *N* HCl (Baker Analyzed).

NaH_2PO_4 solution—0.01 *M* (Mallinckrodt A.R.).

2,4-*Dinitrophenylhydrazine reagent*—a solution of 0.05% DNPH (Eastman-Kodak) in 1 *N* HCl.

NaOH solution—6 *N* (Baker USP).

m-Tartaric acid (Calbiochem, C Grade).

l-Tartaric acid (Calbiochem, A Grade).

d-Tartaric acid (B.D.H. Analar).

Klett-Summerson photoelectric colorimeter—Model 800-3, filter 54.

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Procedure

To a 1-ml sample add 2 ml of the acetate buffer, 0.1 ml of the $Fe(NH_4)_2(SO_4)_2$ reagent, and 0.3 ml of NaH_2PO_4 solution. Make up the volume to 4 ml with distilled water. Swirl and incubate 75 min in a water bath at 60°. Add 0.5 ml of dinitrophenylhydrazine reagent and continue incubation for 15 min at the same temperature. Cool in ice water for 5 min and add 0.5 ml of the NaOH solution. Swirl and centrifuge for 2 min at 3000 rpm. Read the color intensity of the clear supernatant in a Klett-Summerson colorimeter with filter 54. The color is stable for at least 1 hr at room temperature.

EXPERIMENTAL

Light Absorption of the Color Compound

The absorption curve of the color compound, formed by the reaction of the tartaric acid isomers with ferrous ions and dinitrophenylhydrazine, shows a maximum between 520 and 600 mμ (Fig. 1). For routine tests with the Klett-Summerson colorimeter, filter 54 was therefore used.

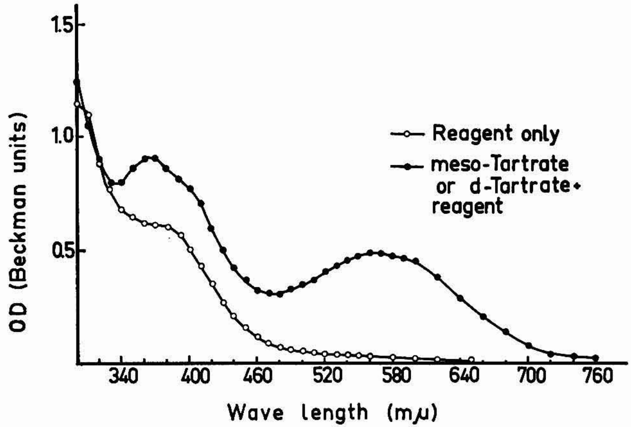

FIG. 1. Absorption curve of the complex of tartrate, ferrous ions, and dinitrophenylhydrazine and of the dinitrophenylhydrazine reagent. The color reaction was performed as described under "Procedure," using 0.3 μmole *d*-tartrate or *m*-tartrate.

Components of the Color Reaction

As mentioned above, the intensity of the color formed in the reaction was found to be proportional to the amounts of tartaric acid isomers in the range of 0.05 to 0.35 μmole; Beer's law is obeyed over this range of concentrations.

An equivalent amount of malic acid gave only one-sixth, and of citric acid one-eighth, of the color intensity obtained with tartrate. At a pH value higher than 5.4, citrate formed a compound similar in color to that formed by the tartrates. Isocitric acid, salicylic acid, succinic acid, and fumaric acid did not interfere. Under the same conditions keto acids, such as pyruvic, oxaloacetic or ketoglutaric, gave a red-brown color. The intensities of the colors developed by these acids and by the tartaric acid isomers under the specified conditions were additive. In the absence of ferrous ions only keto acids gave a color reaction; and tartrate isomers

TABLE 1
DETERMINATION OF TARTRATE AND PYRUVATE IN MIXTURE

Tartrate added, μmole	Pyruvate added, μmole	Molar percentage of pyruvate in mixture	Fe^{++} present		Without Fe^{++}	
			Klett units	Total % recovery	Klett units	Total % recovery
0.1	—	0	120	100	5	0
0.1	0.1	50	250	94	145	54
0.1	0.2	66.6	390	98	270	67
0.1	0.3	75	515	97	415	78
0.2	—	0	245	100	0	0
0.2	0.1	33.3	375	96	150	38
0.2	0.2	50	520	97	270	53
0.3	—	0	370	100	0	0
0.3	0.1	25	505	98	150	29
—	0.1	100	145	100	145	100
—	0.2	100	280	100	285	100
—	0.3	100	410	100	410	100

did not. Accordingly, tartrates may be estimated in the presence of keto acids by subtracting the values obtained in the presence of Fe^{++} ions from those obtained in its absence. It is also possible to estimate the keto acids alone, even in presence of ferrous ions, by performing the second step of the color reaction (incubation with dinitrophenylhydrazine), at either a pH higher than pH 2 or a low temperature (4–6°). Under either condition the color reaction with the tartrates practically does not proceed.

Ferrous ions were essential for the color reaction. Other metal ions such as Co^{++}, Ni^{++}, Cu^{++}, Mg^{++}, and Fe^{+++} could not replace it. Moreover, Fe^{+++} ions were found to inhibit the color formation (Fig. 2). To overcome the inhibition by these ions, which are formed under the conditions of the color reaction (pH 5.4, 60°), a limited amount (3–10 μmoles) of phosphate, which is known to form complexes with ferric ions (11), was added (Fig. 2). Excess of phosphate caused the formation of a precipitate and decreased the color intensity.

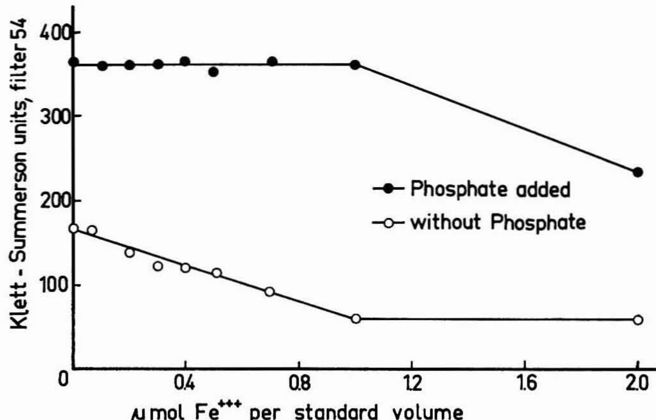

FIG. 2. Effect of phosphate on inhibition of color formation by ferric ions. The color reaction was performed as described under "Procedure," using 0.3 μmole *m*-tartrate and 3 μmole phosphate. The various amounts of ferric ions were added at the first step of the reaction.

As can be seen in Fig. 3, the color intensity was found to be proportional to the quantity of the ferrous ions present in the range of 0.05 to 0.3 μmole. Maximal values were obtained with 0.5–2.0 μmole, while the color intensity actually decreased when more than 2.0 μmoles was added. This reaction can therefore be used for the estimation of ferrous ions in the range of 0.05 to 0.3 μmole.

Dinitrophenylhydrazine (DNPH) was found to be essential for color formation. Neither phenylhydrazine nor 2,4-dinitroaniline could replace it. The color intensity was found to be proportional to the amount of

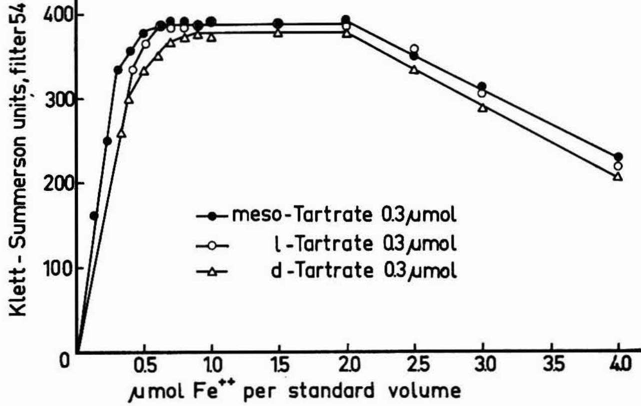

FIG. 3. Effect of various concentrations of ferrous ions on color intensity.

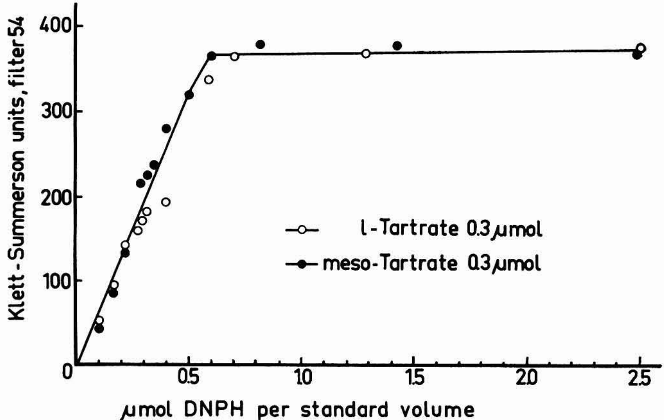

FIG. 4. Effect of various concentrations of dinitrophenylhydrazine (DNPH) on color intensity.

DNPH in the range of 0.1 to 0.6 μmole (Fig. 4). This reaction can therefore be used for the estimation of this reactant. Excess of DNPH reagent must, of course, be provided when the reaction is used to determine tartrate. As seen in Fig. 4, for determination of 0.3 μmole of tartrate at least 0.6 μmole of DNPH should be present.

The purple color, the intensity of which was measured by a photocolorimeter, developed only after addition of the NaOH solution.

Conditions Required for the Color Reaction

1. *Order of addition of components of the color reaction*

It was found that the reaction must be performed in the following sequence: (*A*) Incubation of the tartaric acid isomers with ferrous ions, at 60°C. (*B*) Addition of dinitrophenylhydrazine and a second incubation, at 60°C. (*C*) Addition of alkali. No color was formed if the components were all added together at the same time, or were added in an order different from that indicated above.

2. *Conditions of pH and temperature. Time of reaction*

To obtain reproducible results, precise conditions of pH, temperature, and time of incubation were found to be required for each step.

Step A: Incubation of tartrate with ferrous ions

Effect of pH on color intensity. In Fig. 5 it can be seen that the color intensity increased with the rise of pH, reaching a plateau in the pH

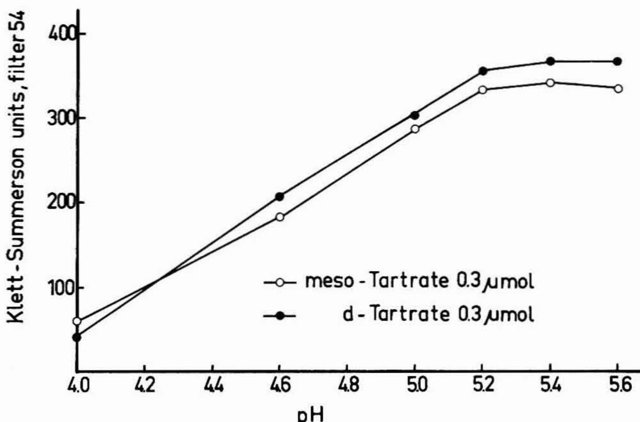

FIG. 5. Effect of pH during first step of the reaction on color intensity.

range of 5.2 to 5.6. For standard tests the pH of 5.4, which was obtained by the use of acetate buffer (0.05 M), was chosen.

Effect of temperature and time of incubation. This step proceeds slowly (maximal color development is reached after 3–4 hr) at a temperature of 20 to 30°C. At 100° the time of the reaction is shortened to 15–20 min, but the values obtained under these conditions are variable. Accordingly a temperature of 60°, which was found to give reproducible results in a reasonable time, was chosen for test conditions. As can be seen from

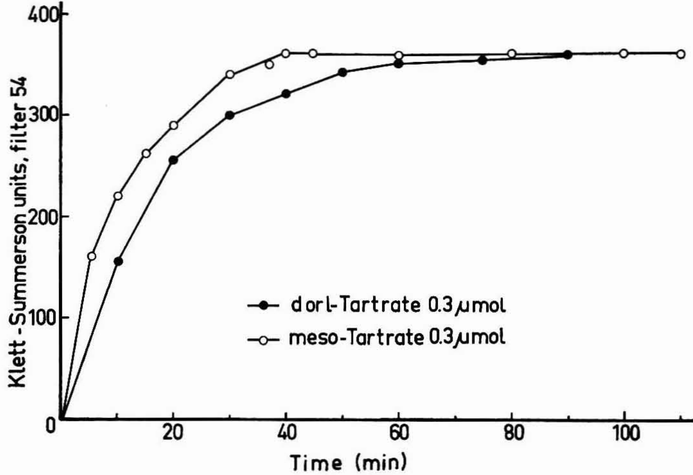

FIG. 6. Kinetics of first step of the color reaction. This step was stopped at various times by addition of the reagent of the second step (DNPH reagent). Other conditions of the reaction were as described under "Procedure."

Fig. 6, the rate of the reaction with *m*-tartrate was faster than with the other isomers (45 instead of 75 min to obtain maximal color intensities).

Step B: Incubation with dinitrophenylhydrazine

Effect of pH. Maximal color development was found when this step was performed at a pH lower than 2. In less acidic solutions no color was formed at all.

Effect of temperature and time of incubation. This step was also affected by temperature. However, for reasons of convenience it was performed again at 60°C. At this temperature about 20 min was required to reach maximal color intensity.

Step C: Addition of alkali

Before the addition of alkali the samples were cooled in ice water. The iron hydroxide precipitate, formed when the alkali was added, had to be removed by centrifugation for about 2 min, at 3000 rpm, before reading the color intensity. The color formed in this reaction was found to be quite stable; its intensity decreased only about 2% after standing 1 hr at room temperature.

DISCUSSION

The pH dependence of the first step of the color reaction suggests that the ferrous ions form a chelated compound (5) with the tartaric acid molecule.

It is interesting to note that ferric ions prevent the color formation. These ions may perhaps compete with the divalent ions for the binding sites of the tartaric acid molecule, and form a different complex that does not develop the purple color on the addition of the other reagents (6–9). Inclusion of phosphate in the reaction mixture prevented the inhibitory effect of the trivalent iron, possibly by binding the iron selectively, by complex formation or by precipitation (10, 11).

The different pH conditions needed for the various steps of the color reaction explain the need of the addition of the various reactants in a definite order.

Although some metabolic products, as well as some metal ions, large amounts of phosphate, oxidizing materials, and certain chelating agents, interfere with the estimation of the tartaric acids by this method, it can be used for determinations in biological and biochemical systems provided the concentration of these materials is low relative to the amount of tartrate. Because of the high sensitivity of the method, the dilution of the sample needed for the tartrate estimation eliminates in this case the effect of these materials. Otherwise these materials must be removed before the estimation.

The method described here has been used for the estimation of tartaric acid isomers in a bacterial growth medium (12) that contained small amounts of Ca^{++} and Fe^{++} salts (0.0005%), higher amounts of $MgSO_4$ (0.0005%) and NH_4Cl (0.1%), yeast extract (0.0025%), and phosphate buffer (0.007 *M*). The samples were diluted 5–10 times. The method was also used for the estimation of tartrates in the presence of bacterial extracts containing 0.4 mg protein per milliliter.

By simultaneous use of this method and the previously described methods for the estimation of the optically active isomers, *m*-tartrate and one of the other isomers can be estimated in mixtures.

SUMMARY

(*1*) A colorimetric micro method for estimation of the tartaric acid isomers is described. The principle of the method consists in the formation of a tartrate-iron (Fe^{++}) complex, which on addition of dinitrophenylhydrazine followed by addition of alkali forms a purple color. The intensity of this color is proportional to the amount of the tartaric acid isomers in the range of 0.05 to 0.35 μmole.

(*2*) The various stages and the optimal conditions of the color reaction were investigated and are described in detail.

(*3*) Certain substances, including malic acid, citric acid, materials which bind ferrous ions, oxidizing agents, ferric ions, and possibly also other metallic ions, interfere with estimation of the tartaric acids by this method. However, in cases in which the relative concentration (with respect to tartrate) of such substances is low, the dilution generally required to bring the concentration of the tartaric acid into the appropriate range is usually sufficient to eliminate interfering effects. Under the assay conditions described keto acids also develop a color, but the tartaric acids can be estimated in their presence by a subtraction method.

(*4*) It is possible to use the method described for estimation of the other components of the color reaction, the intensity of the color being proportional to the amounts of ferrous ions in the range of 0.1 to 0.3 μmole, and to the amounts of dinitrophenylhydrazine in the range of 0.1 to 0.6 μmole.

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REFERENCES

1. Underhill, P., Peterman, F. I., and Krause, A. G., *J. Pharmacol. Exptl. Therap.* **43**, 351 (1931).
2. Matchett, J. R., Leagault, R. R., Nimmo, C. C., and Notter, G. K., *Ind. Eng. Chem.* **36**, 851 (1944).

3. GORSKI, F., *Bull. Intern. Acad. Polon, Sci. Classe. Sci. Math. Nat.* A239 (1937).
4. ANDERSON, A. K., ROWSE, A. H., AND LETNOFF, T. V., *Ind. Eng. Chem., Anal. Ed.* **5,** 19 (1933).
5. MARTELL, A. E., AND CALVIN, M., "Chemistry of the Metal Chelate Compounds," pp. 39–50. Prentice Hall Inc., Englewood Cliffs, New Jersey, 1952.
6. DELSAL, J. L., *J. Chim. Phys.* **35,** 314 (1938).
7. DELSAL, J. L., *J. Chim. Phys.* **35,** 350 (1938).
8. PAIRA, AD., *Ber.* **47,** 1773 (1914).
9. FRANKE, W., *Ann. Chem.* **486,** 242 (1931).
10. VAN WAZER, J. R., "Phosphorus and Its Compounds," Vol. 1, pp. 556–7. Interscience, New York, 1958.
11. VAN WAZER, J. R., AND CAMPONELLA, D. H., *J. Am. Chem. Soc.* **72,** 655 (1950).
12. YASHPHE, J., Ph.D. Thesis, The Hebrew University, Jerusalem, Israel, 1961.

ANALYTICAL BIOCHEMISTRY 13, 354–365 (1965)

SHORT COMMUNICATIONS

An Improved Electrolytic Desalter[1]

The desalting of relatively large volumes of solutions containing "dialyzable" proteins, peptides, and amino acids has been accomplished with the aid of a simple electrolytic cell. Blainey and Yardley (1) demonstrated that small volumes (3 ml) of plasma, urine, and cerebrospinal fluid could be desalted in an electrolytic cell equipped with ion-exchange membranes. The losses of amino acids desalted at their isoelectric point were insignificant, and the resulting solutions could be applied directly to paper for chromatographic separation.

The electrolytic cell described here is essentially identical to the cell described by Blainey and Yardley except for the increased dimensions and the use of mechanical stirring. Construction of the cell was facilitated by using a 1-in. thick Plexiglas (Lucite) sheet from which three 6-in. squares were cut. The center section of the cell was formed by cutting a 5-in. square out of the 6-in. square, leaving a ½-in. thick frame. The

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FIG. 1. Cell assembly: (A) Anode compartment. (B) Sample compartment. (C) Cathode compartment. (D) Membrane support, cut from ¹⁄₁₆-in. Plexiglas (Lucite) sheet; 16 evenly spaced 1-in. diameter holes cut in support. (E) Anion membrane, Permaplex A-20 anion-exchange membrane, purchased from Permutit, Ltd., London. (F) Cation membrane, Permaplex C-20 cation-exchange membrane, purchased from Permutit, Ltd., London. (G) Platinum electrodes, cemented at intervals to wall of compartment. (H) Electrical socket plug, color coded. (I) Polyethylene spigots. (J) Reciprocator. (K) Brass shaft, ³⁄₁₆-in. diameter. (L) Teflon bearing, ½-in. diameter. (M) Brass coupling, ⅝-in. diameter. (N) Plexiglas (Lucite) drive shaft, ½-in. diameter. (O) Drive shaft support. (P) Stirring rods, ⅛-in. diameter Plexiglas (Lucite). (Q) Stirring motor assembly support. (R) Set screws. (S) Screw, ⅛-in. diameter. (T) Filling holes, ⅜-in. diameter. (U) Slot holes for stirring rods. (V) Stirring motor assembly plate. (W) Fly wheel, 2-in. diameter. (X) Ball bearing, ⅜-in. diameter, fastened ¾ in. from center of fly wheel. (Y) Bodine motor, type KCl-23RB, 115 volts, 8.5 watts input, 56 rpm. (Z) Stainless-steel screws (12), ⅛-in. diameter, 3½-in. length, and stainless-steel nuts (24).

[1] This investigation was supported by grant funds from the National Institutes of Health of the Public Health Service (Grant No. AM-05851).

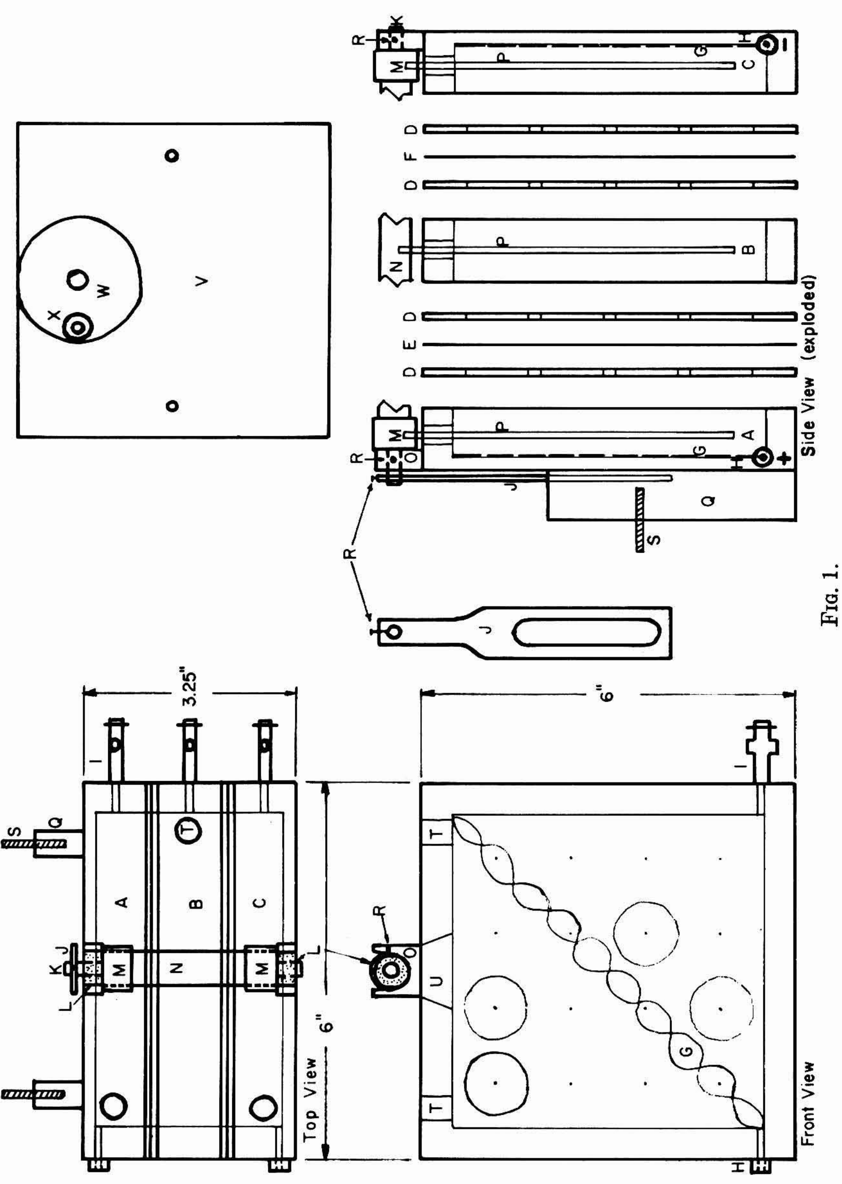

FIG. 1.

outer sections of the cell were formed by cutting similar sections out, but leaving the $\frac{1}{4}$-in. thick outer wall of the cell intact. Maximum volumes of 400 ml of sample in the center cell compartment and 300 ml of dilute acid and base solutions in the outer sections were obtained.

Platinum wire electrodes were fixed to the outer cell wall and connected to color coded plugs. Additional holes were drilled in the cell for the stirrer and for filling and emptying, and for the bolts used to assemble the cell. Membrane frames were prepared from $\frac{1}{16}$-in. thick Plexiglas (Lucite), and a like series of 1-in. holes were cut in the frames to allow the solutions to have maximum exposure to the membrane. Membranes used in the cell were obtained from Permutit, Ltd., London, W4, and were as follows: Permaplex A-20 Anion Resin Membrane and Permaplex C-20 Cation Resin Membrane. The membranes were soaked in water overnight to allow stretching to the full 6-in. size.

Assembly of the cell is illustrated in Fig. 1. The assembled cell is held secure by $\frac{1}{8}$-in. diameter stainless-steel screws and nuts as shown in

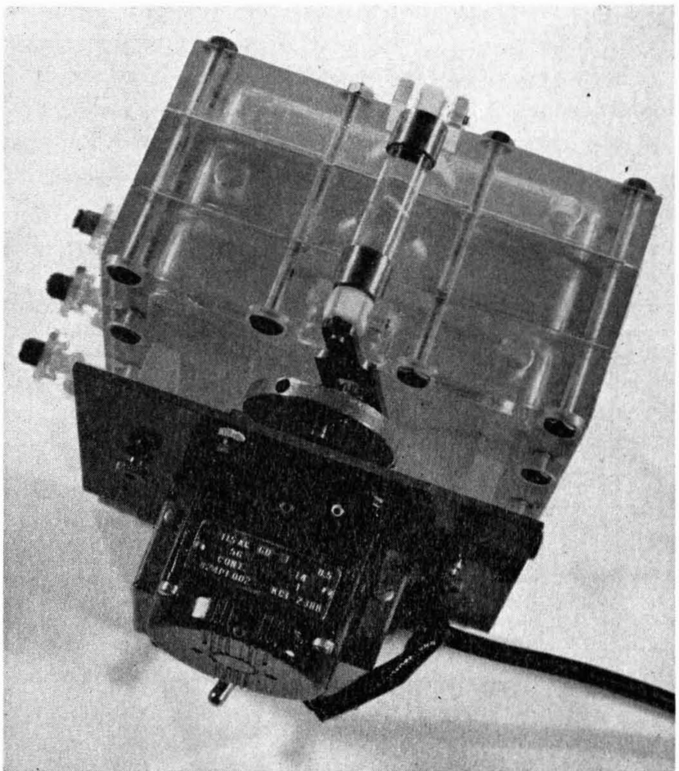

FIG. 2. Assembled cell.

Fig. 2. A reciprocating stirrer was constructed from a Bodine motor, type KCl-23RB, and an eccentric ball-bearing drive. Once the cell is assembled, the three compartments must be kept filled with distilled water to prevent the membranes from deteriorating.

The cell is prepared for use by washing the compartments several times with distilled water. The anode compartment is then filled with 0.2 N NaOH and the cathode compartment with 0.2 N H_2SO_4. It is convenient to label the compartments with the solutions added to prevent errors in further use. The sample compartment is filled, or partially filled, with the sample. It is wise to adjust the amino acid, peptide, protein, etc. to its isoelectric point to minimize losses of the sample. Also, an approximate calculation of the salt concentration and the equivalents of acid and base in the electrode compartments will indicate whether the acid and base will need replenishment. In desalting solutions of bovine pancreatic ribonuclease in 0.01 M borate buffer, pH 8.0, containing 0.1 M NaCl, the direct-current power supply is adjusted to 100 volts output before connecting the cell. This setting should not be changed and, once the cell is connected, this voltage drops and the amperage rises to about 250 ma. After stirring the solution for about 3 hr, the voltage returns to nearly 100 volts and the amperage drops to less than one-tenth its original reading. The sample solution was found to be free of chloride ions by adding an aliquot to silver nitrate solution. The solutions are removed from the desalter via the polyethylene spigots and replaced with distilled water or fresh solutions. Only a few degrees of heating was observed in the solutions during desalting at room temperature. The desalting may be accomplished more rapidly in a cold room at a higher voltage. The exact best conditions depend upon the sample, the salt and its concentration, the surface area of the platinum electrodes, and the power supply.

REFERENCE

1. Blainey, J. D., and Yardley, H. J., *Nature* **177**, 83 (1956).

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Dispenser for Addition of Internal Standard in Liquid Scintillation Counting

The internal standard method for efficiency determination in liquid scintillation counting involves adding a precisely known amount of the appropriate isotope to a previously counted sample. The standard is usually in the form of a volatile liquid such as toluene. It is desirable to add as small a volume as practicable to avoid appreciable change in the composition of the counting solvent. Usually a large number of samples is involved. There has thus been need for a method of rapidly adding a small, precisely known volume of a volatile material to a large number of samples.

We have found that a Hamilton repeating dispenser and gastight syringe[1] (Fig. 1) meet these requirements very well. This dispenser is designed to deliver one-fiftieth the syringe volume each time its button is depressed. It is thus possible to add 49 aliquots of internal standard before having to refill the syringe. A No. 28, blunt-end needle[2] is attached to the 2.5-ml syringe with epoxy cement. Larger or tapered-end needles do not give as precise delivery as the one specified. If the needle is not cemented to the syringe, air bubbles develop when the syringe is filled and leakage may occur during delivery. Such a system has been in routine use in our laboratory for nearly two years. No difficulties have been encountered even though the syringe has been kept full of tritiated toluene.

The precision of adding internal standard with the dispenser was compared with that of a pipet. Forty-nine 50-μl aliquots of tritium-labeled toluene were measured from the dispenser into individual vials containing scintillation counting solvent. A similar set of 30 samples was prepared by measuring 0.5-ml aliquots of a more dilute tritium-labeled toluene standard from a 1-ml measuring pipet having a drawn-out tip. In each case an aliquot contained approximately 10^4 counts per minute. The two sets of samples were counted with a Packard Tri-Carb[3] liquid scintillation spectrometer, model 314EX2A. Each sample was counted 8 times, taking 10^5 total counts each time, in order to make the counting error insignificant (standard error approximately 0.11%). Statistical analysis of the resulting data showed delivery from the dispenser to have a per

[1] Hamilton Company, Inc., Whittier, Calif., No. 1002 2.5-ml gastight syringe specially fitted to a PB-600 repeating dispenser.

[2] Hamilton No. N728, 2-in. length, point No. 3.

[3] Packard Instrument Co., Inc., Downers Grove, Ill.

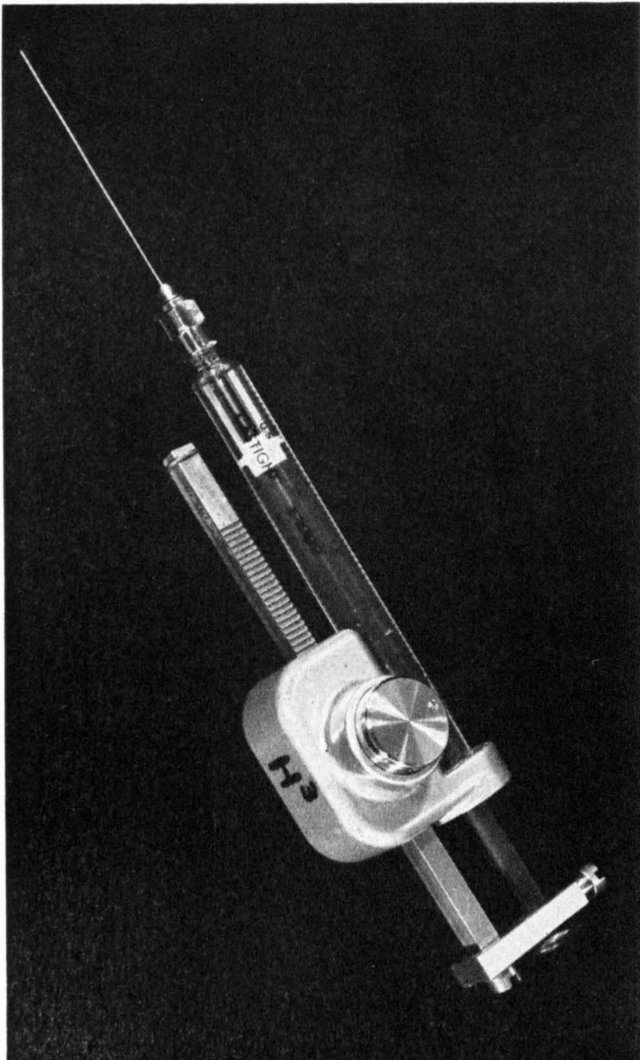

Fig. 1. Hamilton repeating dispenser.

cent standard deviation of 1.01% for a single addition of internal standard. The corresponding value for the pipet was 1.35%. Three additional dispenser assemblies have been evaluated in a similar manner and found to be capable of the same precision. A little experience in using the dispenser is required to attain the specified precision. Along with good precision in adding a small volume of volatile liquid the dispenser allows rapid addition of internal standard and is always ready for use.

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The Detection of Aldosterone by Borate Paper Electrophoresis

The detection and measurement of aldosterone involves the use of several chromatographic procedures (1–6). The frequent manipulation and lengthy development times required for separation of aldosterone from other adrenocorticoids are unattractive aspects of these methods. We would like to report a rapid process we feel may assume a role in these analytical schemes.

Aldosterone may exist in any of the three forms shown here (7, 8).

HO, CHO, CH_2OH, C=O, O (I) ⇌ OH, CH_2OH, O—CH, C=O, O (II) ⇌ H, O, CH_2OH, O—C, C—OH, O (III)

It seemed possible that a complex with borate could form either with the C-18,21 hydroxyl groups of the hemiacetal (II), or with the C-20,21

hydroxyl groups of the full acetal (III). Paper electrophoresis of such a borate complex would lead to anodic migration away from other noncomplexing steroids (9).

When aldosterone was subjected to electrophoresis in pH 9.3 borate buffer, as previously described (9), it migrated toward the anode at a rate of 0.6–0.7 cm/hr. At pH 8.6 the migration was reduced to about 0.25 cm/hr. Since aldosterone-21-acetate did not migrate under these conditions, it is probable that the complex involves the 21-hydroxy group. The nonborate complexing steroids such as hydrocortisone, cortisone, corticosterone, and cortexone, which are metabolic companions of aldosterone, showed only a slight electro-osmotic flow toward the cathode. The C-18 hydroxylated derivatives of cortexone and corticosterone might also be expected to complex with borate and form a migrating species. Unfortunately, these steroids were not available to us for testing. Steroids having a $17\alpha,20\beta,21$-glycerol type of side chain, e.g., 20β-dihydro-17α-hydroxycortexone, also complex with borate and migrate toward the anode (9, 10) to the same degree as aldosterone. $16\alpha,17\alpha$-Dihydroxyprogesterone, reported to be produced from progesterone by hyperplastic adrenal tissue (11), migrates to the anode at one and a half times the rate of aldosterone (9).

Because alkaline conditions can cause inversion of the side chain of aldosterone to form isoaldosterone (12), it might be questioned whether the alkaline conditions of borate electrophoresis (pH 9.3) are sufficient to catalyze this inversion. Such is not believed to be the case because aldosterone migrated as a single discrete zone, whereas a by-product resulting from the alkaline hydrolysis of 21-acetylaldosterone, and thought to be isoaldosterone, apparently did not complex with borate, since no migration was observed.

REFERENCES

1. NEHER, R., AND WETTSTEIN, A., *J. Clin. Invest.* **35,** 800 (1956).
2. HERNANDO, L., CRABBÉ, J., ROSS, E. J., REDDY, W. J., RENOLD, A. E., NESON, D. H., AND THORN, G. W., *Metabolism, Clin. and Exptl.* **6,** 518 (1957).
3. NOWACZYNSKI, W., KOIW, E., AND GENEST, J., *Can. J. Biochem. Physiol.* **35,** 425 (1957).
4. MATTOX, V. R., AND LEWBART, M. L., *J. Clin. Endocrinol. Metab.* **19,** 1151 (1959).
5. SOBEL, C., HENRY, R. J., GOLUB, O. J., AND RUDY, M., *J. Clin. Endocrinol. Metab.* **19,** 1303 (1959).
6. AYRES, P. J., GARROD, O., SIMPSON, S. A., AND TAIT, J. F., *Biochem. J.* **65,** 639 (1957).
7. SIMPSON, S. A., TAIT, J. F., WETTSTEIN, A., NEHER, R., V. EUW, J., SCHINDLER, O., AND REICHSTEIN, T., *Helv. Chim. Acta* **37,** 1200 (1954).
8. HAM, E. A., HARMAN, R. E., BRINK, N. G., AND SARETT, L. H., *J. Am. Chem. Soc.* **77,** 1637 (1955).

9. BLANK, R. H., HAUSMANN, W. K., HOLMLUND, C. E., AND BOHONOS, N., *J. Chromatog.* **17**, 528 (1965).
10. BULASCHENKO, H., RICHARDSON, E. M., AND DOHAN, F. C., *Arch. Biochem. Biophys.* **87**, 81 (1960).
11. VILLEE, D. B., *J. Clin. Endocrinol. Metab.* **24**, 442 (1964).
12. SCHMIDLIN, J., ANNER, G., BILLETER, J. R., HEUSLER, K., UEBERWASSER, H., WIELAND, P., AND WETTSTEIN, A., *Helv. Chim. Acta* **40**, 2291 (1957).

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A Quantitative Assay for Residual Selenite in Bacteriological Media[1]

In a comprehensive study of selenite reduction by the enterococci (1) it was found necessary to develop a simple technique for the quantitative assay of residual selenite in bacteriological media. A method for the determination of tellurite in bacteriological media (2) was modified to measure selenite. The principle of this method is the precipitation of elemental tellurium with hydrogen sulfide generated by thioacetamide. It was found that elemental selenium can also be precipitated and quantitated by this method.

A stock solution of selenite was prepared by dissolving 5.004 gm of crystalline sodium selenite[2] in approximately 100 ml of distilled water. This was further diluted to a volume of 1000 ml to yield a final concentration of 1:250 or 4000 μg/ml. A 10% solution of reagent-grade thioacetamide[3] was prepared and stored in the refrigerator. Prior to use, the reagent was redissolved by holding at 37°C for 10 min.

The standards were prepared in 20×150 mm screw-capped Pyrex test tubes and adjusted to a final volume of 5.0 ml. Each tube contained 2.0 ml of distilled water, 1.0 ml of bacteriological medium, 1.0 ml of

[1] A contribution from the Massachusetts Agricultural Experiment Station, Amherst.
[2] Obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.
[3] Obtained from Fisher Scientific Company, Medford, Massachusetts.

selenite standard (diluted so as to fall within the limits of the standard curve), and 1.0 ml of thioacetamide reagent.

Samples which contained microorganisms and deposited selenium metal were centrifuged at 12,000 $\times$ g for 20 min. The unknown sample to be tested was diluted so that a 1.0-ml aliquot contained 5–100 μg selenite. To a screw-capped test tube, 3.0 ml of distilled water, 1.0 ml of test solution, and 1.0 ml of thioacetamide reagent were added. The tubes, containing either standards or unknown, were closed and mixed well, and the cap was reopened half a turn. The tubes were placed in a boiling water bath for 10 min and then cooled to room temperature before reading. Both standards and unknown were read at 525 mμ on a Bausch & Lomb Spectronic-20 against a reagent blank (total system minus selenite). Controls (total system minus selenite, total system minus thioacetamide reagent) indicated that the change in optical density of the assay system was due solely to the precipitation of colloidal selenium. A standard curve is shown in Fig. 1. Assays of selenite-free culture

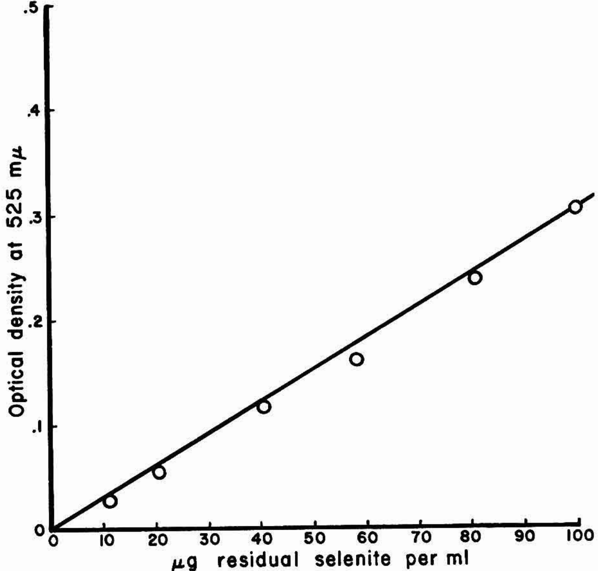

FIG. 1. Standard curve for determination of residual selenite by the thioacetamide method.

medium in which bacteria had been grown showed that no bacterial excretory products occurred which might react with the thioacetamide and contribute to the change in optical density. Repeated trials on growth medium failed to yield selenium-containing metabolites or complexes

formed between selenite and excretory products which would give indications of elemental selenium under test conditions. Tests with selenate in the medium showed it to remain unreduced.

From the results of the standards, a plot of absorbance versus concentration was found to follow the Beer-Lambert law, over a range of 5–100 μg/ml of residual selenite. A 50.0-μg sample of selenite was assayed 20 times and the results analyzed statistically. Results in Table 1 indicate that, at one standard deviation, the coefficient of variation was 4.57%. At varying levels of selenite within the range of 5–100 μg/ml, the variation remained constant.

TABLE 1
STATISTICAL ANALYSIS OF THIOACETAMIDE TEST FOR QUANTITATION OF RESIDUAL SELENITE

Absorbance (525 mμ)	Calculation
0.200	
0.194	
0.235	
0.208	
0.188	Mean – 0.202
0.195	$s = \frac{n\Sigma\chi^2 - (\Sigma\chi)^2}{n(n-1)}$
0.188	
0.187	
0.199	$s = 0.00924$
0.206	
0.193	Coefficient of variation is 4.57% at 50 μg of selenite per ml.
0.194	
0.223	
0.202	
0.216	
0.191	
0.222	
0.197	
0.201	
0.200	

This test proved rapid, especially for enzyme assay, accurate, specific, and relatively sensitive to changes in microgram quantities of residual selenite.

ACKNOWLEDGMENTS

This investigation was supported in part by Public Health Service research grant EF 00011 from the Division of Environmental Engineering and Food Protection. Portions of this work were completed while the senior author was a U. S. Public Health Service Trainee.

REFERENCES

1. Tilton, R. C., Doctoral Dissertation, "The Reduction of Selenite by Enterococci," University of Massachusetts, 1965.
2. Walper, J. F., Tucker, F. L., and Appleman, M. D., *Anal. Biochem.* **3**, 298–301 (1962).

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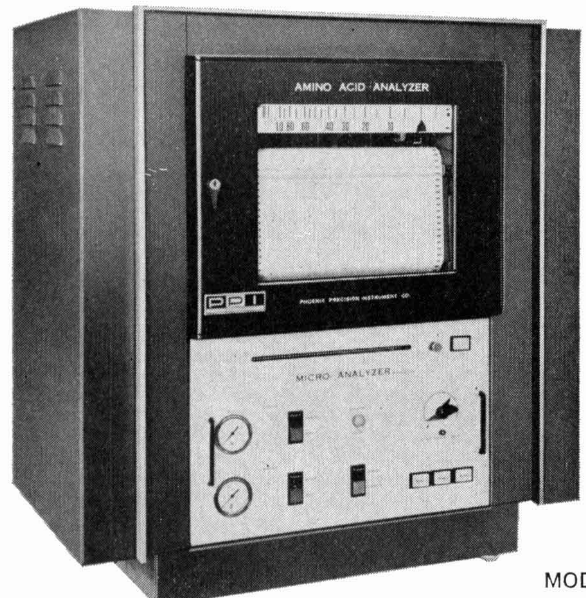

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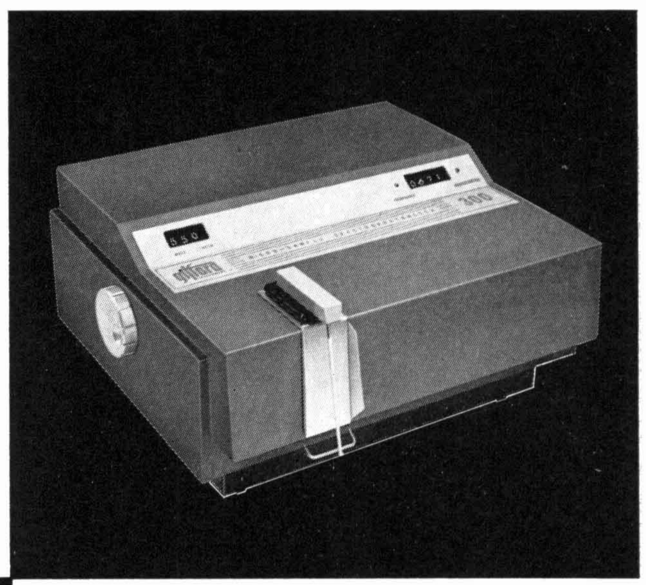

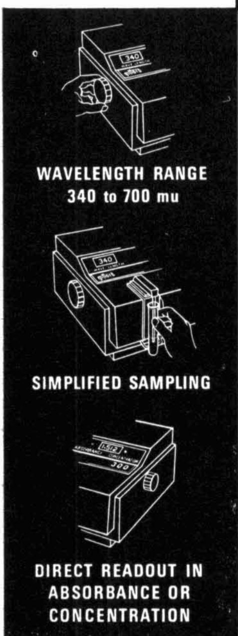

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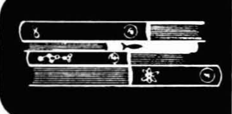

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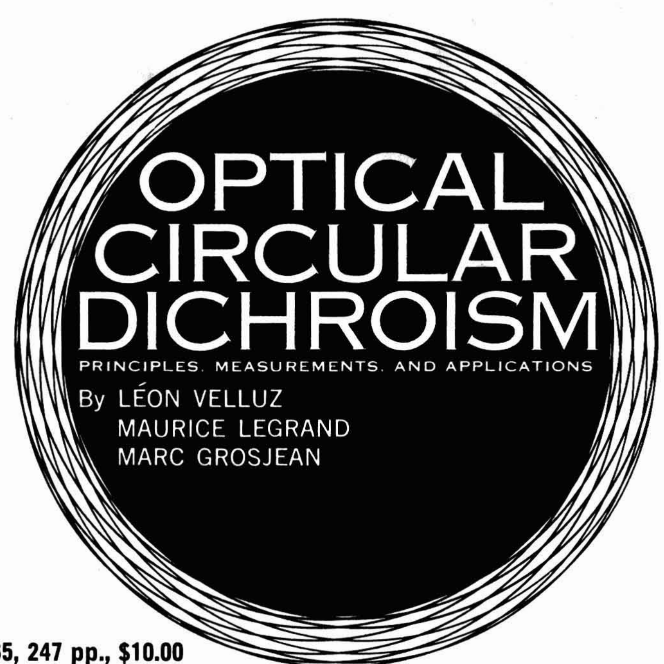

March 1965, 247 pp., $10.00

This book, for the first time, describes optical circular dichroism in comprehensive terms. The first part is devoted to the principles of circular dichroism and its relationship to rotary dispersion. The latter provides similar information, but is inferior to optical circular dichroism in its precision, ease of application, and suitability for colored compounds.

The second part deals with techniques of measurement, and includes extensive descriptions of polarizers and recording procedures. Measuring equipment, developed by the authors themselves, and approved in many centers of research, is described in detail.

The third part discusses the fields of application. For such a recently developed method, a surprisingly large number of examples is given from among the steroid ketones, cobalmines, hydroxyanthraquinones, and other classes of compounds. These examples provide clear evidence of the value of optical circular dichroism in structural determination.

The book will be of special interest to analytical chemists, organic chemists, optical physicists, and physical chemists.

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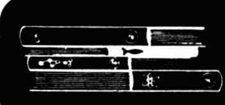

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