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An International Journal

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Statement of Purpose

Analytical Biochemistry has originated as a result of a definite requirement for a central organ devoted to methodological papers in experimental biology. There is an urgent need for a journal principally concerned with analytical and preparative procedures because many periodicals in the field of general biochemistry and related subjects prefer not to publish papers whose main emphasis is on new or modified analytical methods.

This new journal is primarily intended to serve as a central, international source of information on new and improved methods in the various fields of biochemistry, biophysics, and related areas of experimental biology. It is designed to meet the need for a medium devoted solely to papers describing methods in these areas. In so doing, it is our hope that it will also stimulate and encourage the publication of new and useful methods that might otherwise never appear in print for want of a proper journal. *Analytical Biochemistry* will publish detailed accounts of fundamental contributions on analytical and preparative procedures. It will include papers on qualitative and quantitative techniques based on chemical, physical, and biological principles; methods of preparation, purification, characterization, isolation and separation of biological substances and related materials; and instrumentation. Short communications will also be considered.

We have been extremely fortunate in obtaining the cooperation of an outstanding and distinguished group of scientists from the United States and abroad as members of our Advisory Board. Their own high standards will reflect the standards of the new journal. It is hoped that the international nature of our Board will further emphasize the international scope of *Analytical Biochemistry;* and that it will serve as added encouragement for the contribution of articles by our colleagues in other countries.

ALVIN NASON

June, 1960

ANALYTICAL BIOCHEMISTRY 1, 1–7 (1960)

Analysis for Potassium, Sodium, Chloride, and Water in a 2-μl Sample of Extracellular Fluid[1]

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Received January 7, 1960

INTRODUCTION

This paper describes a system of analysis for measuring K, Na, Cl, and water in a 2-μl sample of mammalian extracellular fluid. The methods were developed in the course of a study (2) of the fluid formed by the choroid plexus of the cat.

Various micro methods for determining these several electrolytes singly are already available. Micro Cl analyses, using both colorimetric and electrometric techniques, have been described; and, when carried out with microvolumetric glassware, some of these are capable of measuring amounts of Cl as low as 4×10^{-10} mole (5) or even 3×10^{-12} mole (6). Solomon and Caton (7) have described a micro flame photometer capable of determining Na and K at levels similar to those reported here. Keynes and Lewis (4) have measured comparable amounts of Na by activation analysis; but this technique has been less successful for the measurement of K, particularly in the presence of appreciable amounts of Na.

The methods described in this paper can be carried out with conventional glassware, a standard spectrophotometer, and a flame photometer adapted to micro levels. The individual analyses are coordinated to permit the measurement of the three major extracellular electrolytes and water on a single specimen. We believe this system provides a useful combination of ease and accuracy of performance with smallness of specimen size.

METHOD

The samples to be analyzed consisted of from 2 to 8 μl of extracellular fluid (serum or cerebrospinal fluid) collected in fine Pyrex pipets. They were expressed from the collect-

[1] This investigation was supported in part by a research grant (M-1230) from the National Institute of Mental Health, Public Health Service, and aided by a grant from the American Cancer Society.

[2] Rockwell Levy Associate Biochemist in Neurosurgery at the Massachusetts General Hospital.

ing pipets into small (15 × 60 mm), tared Vycor tubes; tightly sealed with plastic stoppers; and their wet weight immediately determined. They were then dried for 4 hr at 105°C in a mechanical convection oven, restoppered, allowed to cool, and reweighed. The difference in weights represented the water content. It was found that, unless the stoppers fitted the glass tubes perfectly, significant amounts of water were rapidly lost from the wet samples or gained by the dry samples.

An extracting solution was then added to each tube in an amount determined by the water content of the original specimen, but greater by a factor of a thousand. The accuracy of the addition was such that the volume, in milliliters, of extracting fluid corresponded to the volume, in microliters, of the original water to within 1%. The extracting solution contained $AgNO_3$ 0.220 mmole/l, Li_2SO_4 5 mmoles/l, and HNO_3 0.1 *N*. It served to provide: sufficient volume for aliquoting for the subsequent analyses; Ag^+ ion as the first step in the Cl analysis; and Li^+ ion as internal standard for the flame photometry. Since the electrolyte composition of extracellular fluids is rather uniform and since the extracting solution was added on the basis of the volume of the sample, the final concentrations of the ions to be analyzed were similar in different specimens and could be read between closely spaced standard solutions.

Chloride was determined indirectly according to the method of Lowry *et al.* (5) by measuring the free Ag^+ remaining after precipitating the Cl^- in the sample with a known amount of $AgNO_3$, added in the extracting solution. A 0.1-ml aliquot of the supernatant was added to 1-½ ml of the freshly prepared Rhodanine reagent in a Beckman cuvette and read, between 70 and 100 min after mixing, in a Beckman model DU spectrophotometer at 570 mμ. Unknown solutions were compared with standard solutions containing the same concentrations of Li_2SO_4 and HNO_3, and with $AgNO_3$ concentrations of 0.005, 0.008, and 0.012 μmole/.1 ml. Standard Pyrex cuvettes were used with an 8-mm. filler in the holder under the cuvette to raise the column of fluid to the level of the light path.

Sodium and potassium were determined by internal standard flame photometry. K, being in low concentration in the samples, was the limiting ion; and it was necessary to obtain an accurate reading with as little as 1.2 mμmoles (0.06 μg) of K; i.e. with 0.5 ml of a solution containing 2.5 μmoles/l. For this purpose, we used an instrument specially designed for microanalysis by Baird-Atomic Inc. of Cambridge, Massachusetts. It utilizes a Meker-type burner with a relatively cool propane–air flame in a "closed system," that is, the chimney of the burner fits tightly at the base so that the only source of air for the flame is that which comes through the atomizer.

A relatively large solid angle of the light from the flame is collected by a 2-in. convex lens located 2-½ in. from the center of the flame. The light then passes through one of several exchangeable filters mounted on a disk, and is focused by a second lens onto the cathode of an R.C.A. 1 P22 photomultiplier tube. Since the flame is a rather large source, the image is diffuse and covers all of the sensitive surface of the photomultiplier. The Na and K filters are 2-in. square, multilayer interference filters, of which a circular aperture of 1-⅞ in. is utilized. The Na filter has a peak transmission of over 60% at 5893 A with a half width of about 50 A. The K filter has a wider band of transmission with a half width of about 200 A in order to utilize both the emission line at 7665 A and that at 7699 A. Its peak transmission is over 70%. A second optical system, located at right angles to the first, utilizes a fixed Li filter and a second R.C.A. 1 P22 photomultiplier tube whose output signal serves as an internal standard. The Li filter is a circular, ½-in., multilayer interference filter, with a peak transmission of over 60% at 6708 A and a half width of about 50 A.

The high voltage power supply for the photomultipliers is regulated for stability so that a 10% change in the input voltage produces less than a ½% change in output volt-

age and the drift is less than 1% in 72 hr. The output currents from the two photomultipliers are amplified by duplicate, matched d.c. amplifiers and fed onto opposite sides of a microammeter. When used with internal standard, the circuits are balanced by means of a 15-turn potentiometer incorporated into the Li side; and the instrument is read at null point. Direct readings of Na or K light may be obtained as deflections of the ammeter with the output from the Li photomultiplier excluded.

The sample to be analyzed is introduced into a small (0.5 ml) Pyrex glass funnel from whence it is atomized by a simple home-made atomizer into a heated, 4 × 20 cm, Pyrex cylinder which is connected by a short length of 1-cm polyethylene tubing to the base of the burner.

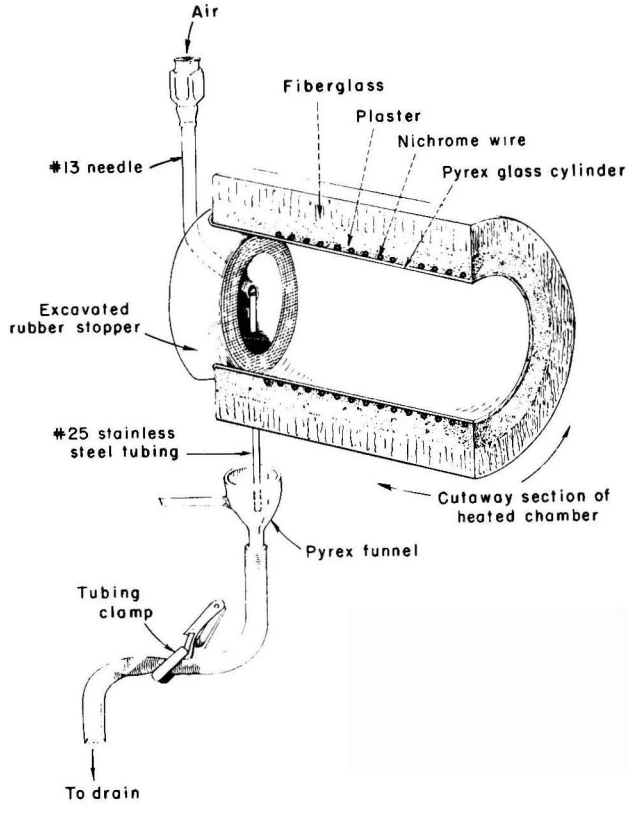


FIG. 1. Drawing of atomizer and heated chamber used in the flame photometry.

The atomizer consists merely of a 5-cm length of #25 stainless steel tubing, across the top of which is blown a horizontal stream of air from a #13 needle (Fig. 1). The two lengths of tubing are held in a partially excavated rubber stopper which fits tightly into the end of the Pyrex cylinder. Used with 3.6 lb/sq in. of air pressure, it atomizes about 0.5 ml/min in a mist so fine as to be barely visible. The Pyrex cylinder is heated by a winding with #26 nichrome wire and insulated with plaster covered with fiber glass

[cf. (3)]. The heat is controlled by a rheostat in series so that it is just sufficient (about 40 watts) to vaporize the mist and to prevent the appearance of fluid in the cylinder. If overheated, the chamber was found to develop a memory for preceding samples.[1]

Standards are delivered from polyethylene wash bottles. Unknowns are delivered with Pyrex glass droppers, bent through 180° at their tops to prevent particulate matter which might originate in the 1-ml. rubber bulbs from falling into the solution. The funnel which receives the samples has a run-off tube from the bottom, controlled by a clamp which can be opened to empty the funnel for the next sample. Retention in the funnel is slight; and, when using internal standard, if the system is rinsed once with distilled water, none of the solution to be analyzed need be used for rinsing.

In the current study, with the instrument operated at somewhat less than maximal sensitivity, the atomization of 1 μmole/l of K at the rate of 0.5 ml/min gave about a 25 μa response (or ¼ of the full scale) when read by the "direct" method and a reading of 153 divisions of the potentiometer (or about 1/10 of its full scale) when read against 10 mmoles/l of Li as internal standard. The data to be reported were obtained using internal standard. Two readings for both Na and K were usually obtained on each unknown solution, using about 0.5 ml per reading. The readings for unknowns were compared with those obtained on standards run at about the same time. A typical standard curve for K is shown in Fig. 2.

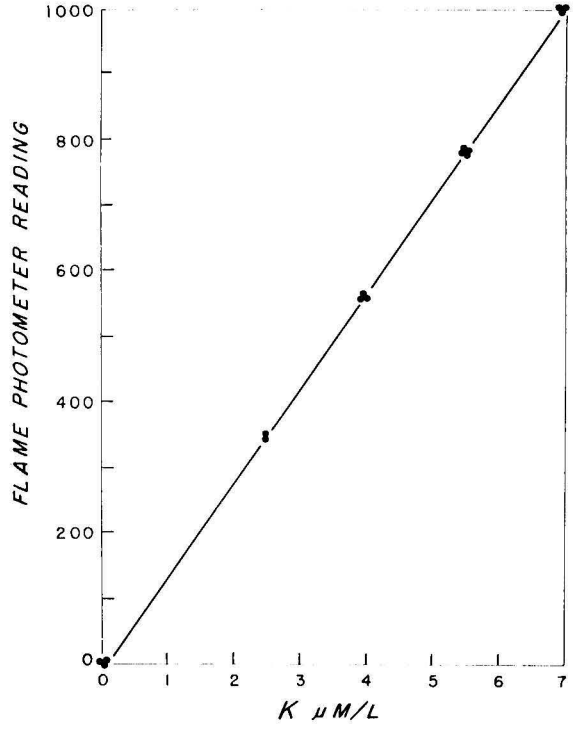


FIG. 2. Standard curve for K, obtained by internal standard flame photo

RESULTS

The accuracy and reproducibility of the complete system were tested by performing replicate analyses on aliquots of a salt solution of known composition containing Na, Cl, and K in amounts similar to those expected in a 2-μl specimen of extracellular fluid. These analyses were performed in conjunction with a study (2) of the fluid formed by the choroid plexus of the cat. One sample of the salt solution of known composition was analyzed concurrently with the six specimens of serum and cerebrospinal fluid obtained from each cat. This study was conducted over a period of 5 weeks and included six cats. The results of the six analyses on the solution of known composition are presented in Table 1. The data were quite reproducible, the standard deviation being, for Cl, K, and Na respectively: 0.9%, 1.8%, and 0.7% of the means. By performing analyses on the contents of tubes which received no samples but were carried through the entire procedure as blanks, we found evidence of a small contamination by Na and

TABLE 1

RESULTS OF ANALYSES ON SIX SAMPLES OF A SOLUTION OF KNOWN COMPOSITION, EACH SAMPLE CONTAINING 0.560 μMOLE OF NaCl AND 0.0160 μMOLE OF KCl

Sample	Cl μmole	K μmole	Na μmole
1	0.573	0.0169	0.564
2	0.577	0.0168	0.560
3	0.578	0.0164	0.564
4	0.574	0.0165	0.560
5	0.567	0.0162	0.558
6	0.567	0.0164	0.554
Average ± S.D.	0.572 ± .005	0.0165 ± .0003	0.560 ± .0004
Average Corrected for blank	0.572	0.0163	0.554
Theory	0.576	0.0160	0.560

TABLE 2

RESULTS OF ANALYSES ON SAMPLES CONTAINING 0.560 μMOLE OF NaCl AND 0.0160 μMOLE OF KCl, WITH AND WITHOUT 0.05 ML OF ETHYL IODOPHENYLUNDECYLATE (PANTOPAQUE)

Sample	Cl, μmole		K, μmole		Na, μmole	
	No oil	Oil	No oil	Oil	No oil	Oil
1	0.562	0.566	0.0164	0.0170	0.556	0.548
2	0.563	0.560	0.0167	0.0165	0.556	0.560
Average	0.563	0.563	0.0166	0.0168	0.556	0.554

K, amounting to 1.1% and 1.2%, respectively, of the amounts being analyzed. When the results were corrected for these blanks, the final averages were: for Cl 99.3% of theory; for K 101.4% of theory; and for Na 98.9% of theory.

In some applications of these methods iodinated oil (Ethyl Iodophenylundecylate or Pantopaque) was used in the process of sampling and was present in small amounts in the specimens collected. We tested its possible effect on the analyses by adding to aliquots of the known solution, a volume of oil of 0.05 ml. or 25-fold that of the specimen. As shown in Table 2, this had no significant effect on the results.

DISCUSSION

A major consideration in conducting these studies was the avoidance of contamination of the specimens by any of the ions being analyzed for. This is a particular problem when analyzing such small amounts of commonly found ions. Our precautions against contamination have been described elsewhere (1), and Thiers (8) has recently published an extended discussion of the problem. Contamination in reverse (called by Thiers, negative contamination), in the form of a loss of ions from the sample into the walls of its containers, may also be a problem. We have observed a loss of 35% of the K from a 2.7-μmole/l solution of KCl which had remained for 1 week in a 250-ml volumetric flask, the only other solute being 65 μmoles/l of NaCl. The presence, in relatively enormous concentrations of H^+ and Li^+ in the solution used in the studies described above protects against such a loss, probably by competing for adsorption sites in the glass.

Since the system described above was more than sufficiently micro to handle the biological specimens we were studying, we made no systematic attempt to determine how small a sample of extracellular fluid could be analyzed. It seems likely that the size could be further reduced by a factor of 4 (to 0.5 μl), or possibly of 8, without a prohibitive loss in accuracy. The amount of specimen allocated to Na and K analysis, now 95%, could be considerably reduced by using single rather than duplicate aliquots for the flame photometer. Furthermore, K could be read at concentrations $\frac{1}{2}$ or even $\frac{1}{4}$ of those used above; and Na could be read at very much lower concentrations, though this would require a separate further dilution of the Na aliquot. Some further reduction could be effected in the aliquot used for Cl analysis, even without resorting to microvolumetrics, though precautions must be taken not to perform the AgCl precipitation at concentrations too low relative to the solubility product of AgCl (10^{-10}). The gravimetric determination of the water is probably the measurement that would be most difficult to perform accurately in smaller specimens.

SUMMARY

Methods are described for analyzing Na, K, Cl, and water on a single 2-μl sample of extracellular fluid, with an accuracy and reproducibility of better than 2%. Conventional glassware was used throughout. Cl was determined colorimetrically; Na and K were measured by a flame photometer of special design (Baird-Atomic Inc. of Cambridge, Mass.) with a modified system for atomization. Considerable further reduction in sample size is probably possible with slight modifications of these methods.

REFERENCES

1. AMES, A., AND NESBETT, F. B., *J. Neurochem.* **3,** 116 (1958).
2. DE ROUGEMONT, J., AMES, A., NESBETT, F. B., AND HOFMANN, H. F., *J. Neurophysiol.* (in press).
3. DUBBS, C. A., *Anal. Chem.* **24,** 1654 (1952).
4. KEYNES, R. D., AND LEWIS, P. R., *J. Physiol. (London)* **114,** 151 (1951).
5. LOWRY, O. H., ROBERTS, N. R., LEINER, K. Y., WU, M. L., AND FARR, A. L., *J. Biol. Chem.* **207,** 1 (1954).
6. RAMSAY, J. A., BROWN, R. H. J., AND CROGHAN, P. C., *J. Exptl. Biol.* **32,** 822 (1955).
7. SOLOMON, A. K., AND CATON, D. C., *Anal. Chem.* **27,** 1849 (1955).
8. THIERS, R. E., *Methods of Biochem. Anal.* **5,** 273 (1957).

ANALYTICAL BIOCHEMISTRY 1, 8–16 (1960)

Chemical Synthesis of β-Ketooctanoyl Coenzyme A

P. ROY VAGELOS AND A. W. ALBERTS

From the Section on Enzymes, Laboratory of Cellular Physiology, National Heart Institute, National Institutes of Health, Bethesda, Maryland

Received January 10, 1960

INTRODUCTION

The synthesis of β-keto thiolesters has been achieved only by enzymic means (1–4) with the notable exception of acetoacetate thiolesters, which have been synthesized by a procedure utilizing diketene (5). Enzymically prepared β-keto thiolesters are generally impure and also have the disadvantage of requiring the preparation of enzymes for their synthesis. Chemical synthesis of β-keto thiolesters from β-keto acids or β-keto acid derivatives has thus far been impossible due to interference by the keto group in the usual thiolester preparative procedures.

A stimulus to prepare pure β-ketooctanoyl coenzyme A (CoA) was provided by recent experiments in our laboratory suggesting that it might be a product of an enzymic reaction between malonyl-CoA and caproyl-CoA (6). A search for a procedure that could be used on β-keto acids in general resulted in the development of a synthesis of acetoacetyl thiolesters starting with readily available ethyl acetoacetate. In this procedure the β-keto group was blocked by converting it to the ethyleneketal. When it was established that the ethyleneketal of ethyl acetoacetate could be readily converted to acetoacetyl thiolesters, a similar procedure was carried out with ethyl β-ketooctanoate as the starting material.

This paper will present the procedure for the chemical synthesis of β-ketooctanoyl-CoA as well as give evidence that the product is enzymically active in the β-ketothiolase reaction. The chemical procedure described should be applicable to the synthesis of other keto thiolesters.

METHODS

The enzyme, β-ketothiolase, was found to be present in an enzyme preparation from *Clostridium kluyveri* previously found to contain β-hydroxypropionyl-CoA dehydrogenase (7).

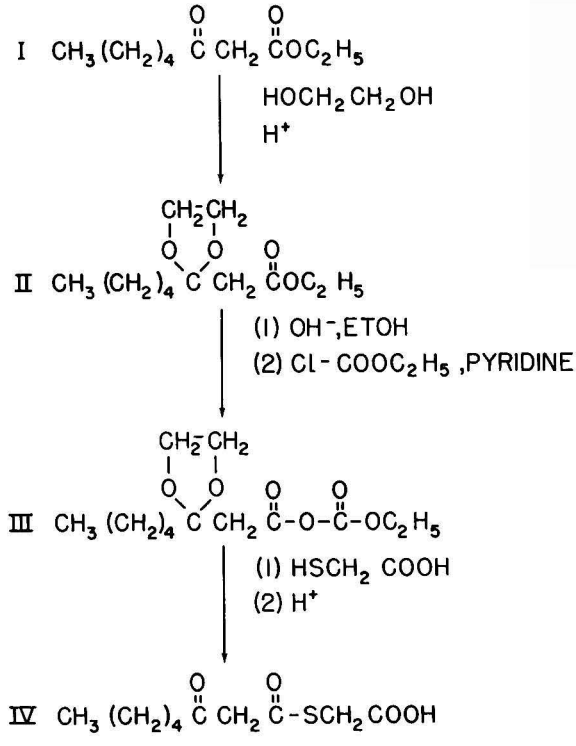
Hydroxamic acid derivatives of thiolesters (8) were compared chromatographically in two solvent systems: *n*-butanol–water (100:18) and *n*-propanol–ammonium hydroxide–water (80:10:10). The R_f values of acetyl hydroxamate were 0.55 and 0.34 in these two solvents, respectively; the R_f values of caproyl hydroxamate were 0.90 and 0.75, respectively.

Infrared absorption spectra[1] were determined with a Perkin-Elmer model 21 double-beam spectrophotometer; the solids were dissolved in chloroform. Ultraviolet spectra were determined in a Cary recording spectrophotometer. Elementary analyses were performed by Joseph F. Alicino, Metuchen, New Jersey.

Ethyl β-ketooctanoate was purchased from Farchan Research Laboratories, thioglycolic acid from Fisher Scientific Company, and CoA from Pabst Laboratories.

EXPERIMENTAL

β-Ketooctanoyl-*S*-thioglycolic acid, a crystalline intermediate that could be converted to β-ketooctanoyl-CoA, was synthesized by the following series of reactions.



Ethyleneketal of Ethyl β-Ketooctanoate (II)

Ethyl β-ketooctanoate (I) (18.6 gm, 0.1 mole), ethylene glycol (6.8 gm), and *p*-toluenesulfonic acid (190 mg) were mixed with 120 ml of toluene. The mixture was refluxed under

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

a take-off condenser for about 3 hr or until water stopped accumulating. The reaction mixture was cooled in ice, and 1 *M* potassium bicarbonate was added cautiously with continuous stirring to neutralize the *p*-toluenesulfonic acid. The toluene layer was separated and washed twice with 50-ml portions of water and then dried over anhydrous magnesium sulfate. The toluene was removed by distillation, and the residue was subjected to vacuum distillation. The fraction containing the ethyleneketal or ethyl β-ketooctanoate distilled at 81–95°C (0.5 to 0.6 mm) and weighed 6.0 gm. An infrared spectrum showed that the band due to the β-keto function had largely disappeared, although there was a little contamination by the unreacted keto ester. This fraction was not further purified or characterized.

Mixed Anhydride of Ethyl Hydrogen Carbonate and Ethyleneketal of Ethyl β-Ketooctanoate (III)

To 5.0 gm of the above fraction were added 50 ml ethanol and 50 ml of 1 *N* potassium hydroxide. This solution was heated over a steam bath for 1½ hr to hydrolyze the ethyl ester, and then concentrated under a stream of air to about 10 ml. The remaining solution was cooled in ice and acidified to pH 1 by dropwise addition of 6 *N* sulfuric acid with continuous stirring. The mixture, from which a white precipitate had separated, was extracted three times with 5-ml portions of ether. The combined ether layers, containing ethyleneketal β-ketooctanoic acid, were dried over anhydrous magnesium sulfate. Titration of an aliquot with standard alkali indicated that a total of 12.67 mmoles of free acid was present in the ether. The mixed anhydride was then formed essentially by the method of Wieland and Rueff (9). To the ether solution, cooled in ice, were added anhydrous pyridine (1.21 ml, 15.2 mmoles) and anhydrous ethyl chloroformate (1.47 ml, 15.2 mmoles). After 2 hr at 0°C, the white precipitate that had formed was removed by centrifugation and discarded. The total amount of hydroxamate-forming material (5.12 mmoles), presumably the mixed anhydride of ethyleneketal β-ketooctanoate and ethyl hydrogen carbonate, was measured by the method of Lipmann and Tuttle (10).

β-Ketooctanoyl-S-Thioglycolic Acid (IV)

After the ether was blown off with a stream of dry helium, the mixed anhydride preparation was dissolved in 20 ml tetrahydrofuran (freshly distilled over lithium aluminum hydride). This solution (cooled to 0°C) was added to an ice-cold solution containing 5.1 ml of a 1 *M* potassium thioglycolate, 2.5 ml of 1 *M* potassium bicarbonate, and 5 ml water in 10 ml tetrahydrofuran. The pH of the resulting solution was maintained at 7.5 (pHydrion) by dropwise addition of 5 *N* potassium hydroxide. Esterification of the mercaptan was complete in 10 min as indicated by a negative nitroprusside spot test (11). To this thiolester solution were added 5.7 ml of concentrated sulfuric acid, and the white precipitate which formed was separated by centrifugation and discarded. The solution was incubated at 30°C in a stoppered flask for 14 hr. At the end of that time the tetrahydrofuran was removed by blowing a jet of air onto the solution at 30°C. The remaining aqueous solution was extracted three times with 5-ml portions of ether, which were combined and dried over anhydrous magnesium sulfate. When the ether was evaporated, a yellowish sirup containing some crystalline material remained. The crystalline mixture was washed six times with 0.5-ml portions of *n*-hexane (cooled to 0°C). The addition of cold *n*-hexane caused many more crystals to form in the sirup. The crystals were collected by filtration and recrystallized from hot *n*-hexane. Two additional crystallizations yielded white, thin plates of β-ketooctanoyl-*S*-thioglycolic acid, 362.4 mg

(30.4% yield based on mixed anhydride). An analytical sample was dried under high vacuum at room temperature for 12 hr. This product had a melting point of 73.5–74.5°C.

Anal. Calcd. for $C_{10}H_{16}O_4S$: C 51.70, H 6.95, S 13.80; found: C 51.59, H 6.88, S 13.70.

Properties of β-Ketooctanoyl-S-Thioglycolic Acid

The infrared spectrum of this compound is shown in Fig. 1. The ultraviolet absorption spectrum was characteristic of other reported β-keto thiolesters with a major peak at 303 mμ due to the formation of an enolate ion (1) and a lesser peak at 230–240 mμ due to the thiolester linkage. There was a characteristic dependence of the absorbancy at 303 mμ upon alkaline pH and the presence of magnesium ions as shown in Fig. 2. It can be seen that the molar extinction coefficient increased sharply as the pH was increased above 7.5. In the presence of magnesium ion, the extinction coefficient was higher at each pH and increased sharply from pH 6.35 to about 8.8 where it reached a plateau. Such spectral changes due to alkaline pH or the presence of magnesium ions have been

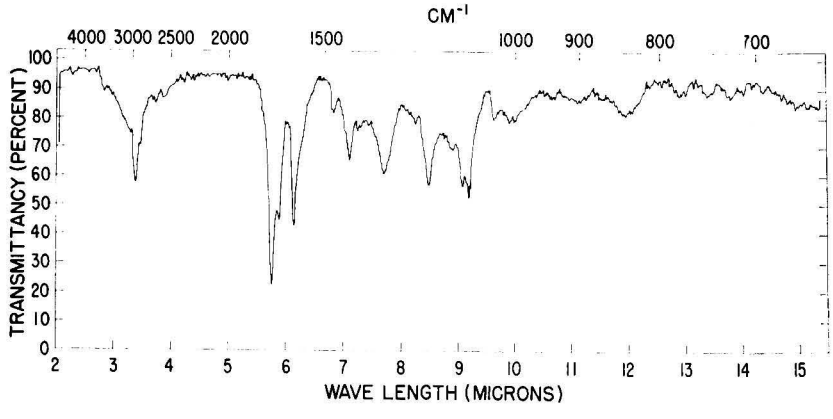


FIG. 1. Infrared spectrum of β-ketooctanoyl-*S*-thioglycolic acid.

described for other β-keto thiolesters (1, 2) and are accounted for by enolate-ion formation. The effect of increasing magnesium-ion concentration on the extinction coefficient at 303 mμ at pH 8 is shown in Fig. 3. It can be seen that maximum absorbancy was reached at about 0.5 *M* magnesium chloride.

Because an ester interchange reaction between β-ketooctanoyl-*S*-thioglycolate and CoA-SH was to be used as the final step in the synthesis of β-ketooctanoyl-CoA (see below) and because of the susceptibility of similar β-keto thiolesters to alkaline hydrolysis (4), the stability of this compound was tested at alkaline pH values. At 24°C β-ketooctanoyl-*S*-thioglycolate had a half-life of 100 min at pH 8 and 33 min at pH 8.75. The addition of magnesium ion (0.5 *M* final concentration) increased the half-life to 11.6 hr and 14 hr at the two pH values, respectively.

Conversion of β-Ketooctanoyl-S-Thioglycolate to β-Ketooctanoyl CoA by Ester Interchange

The ability of thiolesters to undergo non-enzymic ester interchange has been reported (12, 13). This reaction proceeds rapidly at pH values above 7. Because β-ketooctanoyl

thiolesters would undergo hydrolysis at alkaline pH values, the interchange reaction was studied at both pH's 8 and 8.75 in order to find optimal conditions. The reaction was carried out by incubating a solution of CoA-SH with a buffered solution of the β-keto thiolester at 24°C. At the end of the reaction, the solution was acidified to pH 1 by dropwise addition of 5 *N* hydrochloric acid and then extracted three times with an equal volume of ether. The ether extraction quantitatively removed the residual β-keto-octanoyl-*S*-thioglycolic acid, β-ketooctanoic acid, and thioglycolic acid, leaving in the aqueous layer all the β-ketooctanoyl-CoA (and unesterified CoA-SH) which could be assayed spectrophotometrically using the molar extinction coefficient derived by Beinert

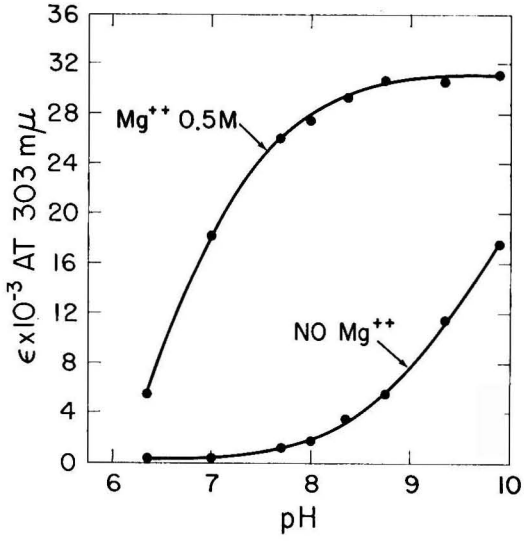


FIG. 2. Effect of pH and magnesium ion on molar extinction coefficient of β-keto-octanoyl-*S*-thioglycolic acid. The cuvettes contained 200 μmoles Tris-hydrochloride[2] buffer at the indicated pH values, 0.05 μmole β-ketooctanoyl-*S*-thioglycolate, and where indicated 500 μmoles of magnesium chloride in a total volume of 1.0 ml. A reference cuvette contained only buffer and water. Absorbancy was read at 303 mμ.

(4). The results of an experiment utilizing two different concentrations of β-keto thiolester at pH's 8.75 and 8.0 are shown in Table 1. It can be seen that, in the absence of magnesium chloride, net synthesis of β-ketooctanoyl-CoA was higher with 0.01 *M* than with 0.005 *M* β-ketooctanoyl-*S*-thioglycolate. Also, higher yields were obtained at pH 8.75 than at pH 8 in the absence of magnesium chloride. Since magnesium chloride (0.5 *M* final concentration) was found to stabilize the β-ketooctanoyl-*S*-thioglycolate against alkaline hydrolysis, another set of similar experiments was done with the addition of magnesium chloride in an attempt to increase the net synthesis of the CoA derivative. It can be seen in Table 1 that the addition of magnesium decreased the yield in every set of conditions tested, although this decrease was greater at pH 8.75 than at pH 8. Magnesium ion apparently stabilized the thiolester against both hydrolysis and ester

[2] The abbreviation used is: Tris, tris(hydroxymethyl)aminomethane.

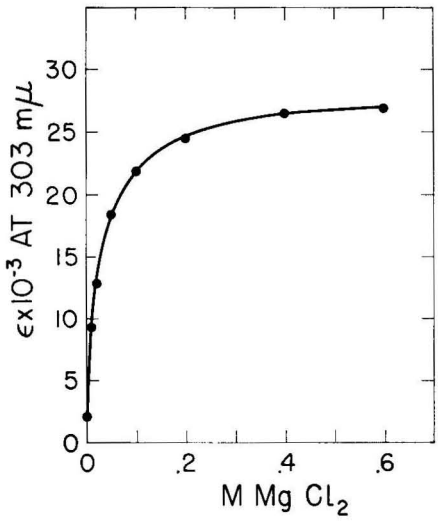


FIG. 3. Effect of increasing magnesium-ion concentration on the molar extinction coefficient of β-ketooctanoyl-*S*-thioglycolate at pH 8. Cuvettes contained 200 μmoles Tris-hydrochloride buffer, pH 8; 0.05 μmole β-ketooctanoyl-*S*-thioglycolate; and magnesium chloride solution to give the final concentration indicated above in a total volume of 1.0 ml. A reference cuvette contained only buffer and water. Absorbancy was read at 303 mμ.

TABLE 1

ESTER INTERCHANGE BETWEEN CoA-SH AND β-KETOOCTANOYL-*S*-THIOGLYCOLATE[a]

β-Ketooctanoyl-*S*-thioglycolate	pH 8.75 β-Ketooctanoyl-CoA		pH 8.0 β-Ketooctanoyl-CoA	
(*M*)	μMoles	Yield (%)	μMoles	Yield (%)
0.005	0.30	51	0.27	46
0.005 + $MgCl_2$	0.20	34	0.25	42
0.01	0.41	71	0.37	63
0.01 + $MgCl_2$	0.30	51	0.36	62

[a] Incubation mixtures contained 200 μmoles of Tris-hydrochloride buffer, pH 8.75 or pH 8.0; 500 μmoles $MgCl_2$ where indicated; β-ketooctanoyl-*S*-thioglycolate as indicated; and 0.58 μmole of CoA-SH in a total volume of 1.0 ml. Incubations were done at 24°C for 30 min. Reactions were stopped by acidifying to pH 1 with 5 *N* hydrochloric acid, diluted to 2.5 ml with water, and extracted three times with an equal volume of ether. The aqueous layers were assayed for β-ketooctanoyl-CoA by reading the absorbancy at 303 mμ and using the molar extinction coefficient derived by Beinert (4). The yield of β-ketooctanoyl-CoA and the per cent yield based upon the added CoA-SH were recorded.

interchange. The time course of the ester interchange reaction at pH 8.75 is shown in Fig. 4. It can be seen that maximal esterification of the added CoA-SH had occurred within 30 min under these experimental conditions.

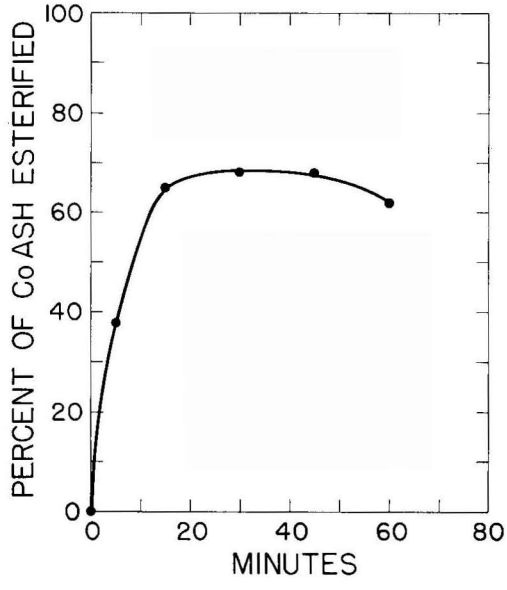


FIG. 4. Time course of the ester interchange reaction. Reaction mixtures contained 200 μmoles Tris-hydrochloride buffer, pH 8.75; 10 μmoles β-ketooctanoyl-*S*-thioglycolate; and 0.58 μmole CoA-SH in a total volume of 1.0 ml. All were incubated at 24°C for the indicated time intervals. Experiments were stopped by acidifying to pH 1 with 5 *N* hydrochloric acid. β-Ketooctanoyl-CoA was assayed as in Table 1. The per cent of CoA-SH esterified was recorded.

Large-scale syntheses of β-ketooctanoyl-CoA utilizing 60 μmoles of β-keto thiolester (buffered at pH 8.75) and 3.0 μmoles of CoA-SH per milliliter and a 10-min incubation period at 24°C resulted in yields of 95% or better based on CoA-SH. Such high yields were attainable because at these high substrate concentrations the rate of the ester interchange reaction was so rapid that shorter incubations could be employed, thus decreasing the per cent of thiolester hydrolyzed. β-Ketooctanoyl-CoA was stored at pH 5 and at −20°C.

Enzymic Decomposition of Synthetic β-Ketooctanoyl-CoA by β-Ketothiolase

In order to establish the enzymic reactivity of the synthetic β-ketooctanoyl-CoA, the thiolester was incubated with CoA-SH and a preparation of β-ketothiolase from *C. kluyveri*. The reaction was followed spectrophotometrically by noting the decrease in absorbancy at 303 mμ, as shown in Fig. 5. Under the conditions of these experiments, disappearance of β-ketooctanoyl-CoA was dependent upon both enzyme and CoA-SH. β-Ketooctanoyl-*S*-thioglycolate was completely inactive when tested under similar conditions. A larger scale experiment, utilizing 2 μmoles of the CoA derivative and 2 μmoles

f CoA-SH was done in order to identify the products of the enzymatic reaction. When the reaction had gone to completion (as noted by the absorbancy decrease at 303 mμ), neutral hydroxylamine was added and the hydroxamic acids were chromatographed in two solvent systems. The hydroxamate derivatives of acetyl-CoA and caproyl-CoA, the expected products of the reaction catalyzed by β-ketothiolase, were identified in both chromatograms by comparison with authentic acetyl hydroxamate and caproyl hydroxamate.

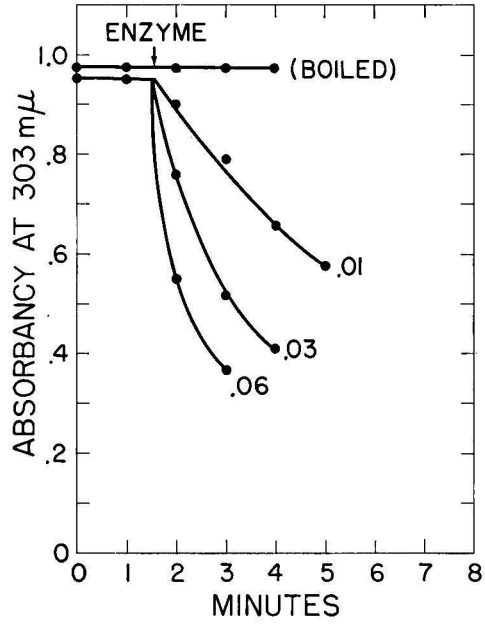


FIG. 5. Enzymic decomposition of β-ketooctanoyl-CoA by β-ketothiolase. Cuvettes contained 200 μmoles Tris-hydrochloride buffer, pH 8.75; 0.034 μmole β-ketooctanoyl-CoA; 0.05 μmole CoA-SH; 100 μmoles magnesium chloride; and the indicated volumes of a β-ketothiolase preparation (18 mg protein/ml) in a total volume of 1.0 ml. Reference cuvettes for all experiments lacked only the thiolester. A control experiment with 0.06 ml of boiled enzyme was included. The absorbancy was followed at 303 mμ.

SUMMARY

β-Ketooctanoyl coenzyme A was synthesized from ethyl β-ketooctanoate. β-Ketooctanoyl-*S*-thioglycolic acid, a crystalline intermediate that was converted to the coenzyme A ester by an ester interchange reaction, was shown to have properties similar to those of other β-keto thiolesters previously described. Synthetic β-ketooctanoyl coenzyme A was shown to be enzymically active with β-ketothiolase, which catalyzed its coenzyme A-dependent conversion to caproyl coenzyme A and acetyl coenzyme A.

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ACKNOWLEDGMENTS

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REFERENCES

1. LYNEN, F., WESSELY, L., WIELAND, O., AND RUEFF, L., *Angew. Chem.* **64,** 687 (1952).
2. STERN, J. R., COON, M. J., AND DEL CAMPILLO, A., *J. Am. Chem. Soc.* **75,** 1517 (1953).
3. STERN, J. R., AND DEL CAMPILLO, A., *J. Am. Chem. Soc.* **75,** 2277 (1953).
4. BEINERT, H., *J. Biol. Chem.* **205,** 575 (1953).
5. LYNEN, F., AND WIELAND, O., *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. I, p. 566. Academic Press, New York, 1955.
6. VAGELOS, P. R., *J. Am. Chem. Soc.* **81,** 4119 (1959).
7. VAGELOS, P. R., AND EARL, J. M., *J. Biol. Chem.* **234,** 2272 (1959).
8. STADTMAN, E. R., AND BARKER, H. A., *J. Biol. Chem.* **184,** 769 (1950).
9. WIELAND, T., AND RUEFF, L., *Angew. Chem.* **65,** 186 (1953).
10. LIPMANN, F., AND TUTTLE, L. C., *J. Biol. Chem.* **159,** 21 (1945).
11. TOENNIES, G., AND KOLB, J. J., *Anal. Chem.* **23,** 823 (1951).
12. STADTMAN, E. R., *J. Biol. Chem.* **196,** 535 (1952).
13. WIELAND, T., AND BOKELMANN, E., *Angew. Chem.* **64,** 59 (1952).

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The Determination of Sodium and Potassium in Biological Fluids with the Dual Channel Ultramicro Flame Photometer[1, 2]

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INTRODUCTION

The present trend of biochemical and pathological inquiry is to demand the analysis of smaller and smaller amounts of material. The necessity for determining sodium and potassium in very small amounts led us to attempt analyses by ultramicro flame photometer. Although our immediate problem dealt with analyses of renal tubule fluid in amphibia as well as in mammals (1–3), we wished to have an instrument capable of analyzing biological fluids in general. Since the instrument described by Ramsay, Brown, and Falloon (4) in England was designed and used for the simultaneous determination of micromicromole (less than millimicrogram) amounts of sodium and potassium in biological fluids, it was decided to try a modification of this instrument for our work in which, in addition to tubule fluid, blood serum and urine were to be analyzed.

In Ramsay's procedure an ultramicro sample of fluid was placed upon a platinum loop, dried in a heated coil of wire, and the platinum loop then thrust into an air-gas flame. The emission from the flame entered a spectrometer, the spectrum from which fell upon a mask having two slits, one passing the potassium doublet and the other the sodium doublet. These emissions reached photomultiplier tubes which were connected to integrating circuits for meter presentation. The maximum deflections were maintained sufficiently long to obtain readings.

METHOD

Apparatus and Manipulation

General. Our modified instrument was built by the American Electronic Laboratories of Philadelphia. It involves the same principles as that described by Ramsay *et al.* (4)

[1] Reported in part before American Physiological Society, Chicago, Ill., April 1957. See also Refs. (1–3).

[2] This work was supported by a grant from the Life Insurance Medical Research Fund.

but differs in some detail. A complete description of the instrument proper will be published elsewhere by Mr. John Busser[3] of that company. Figure 1 shows the instrument, complete except for the air pump. To the left are the pressure gauges for propane and air which are kept at 4 ± 0.02 in. water by a series of regulators. Meters for potassium and sodium readings are at the right-hand end of the case, and below each meter are controls for drift, dark current, and sensitivity adjustments. Above the right-hand meter is the multiposition switch which controls the operation of the instrument.

In Fig. 2 the motor-driven sample-carrier and cover arms are shown spread apart, in position to push the platinum loop into the heating coil. On signal from the multiposition switch, the arms move together until they reach the flame where the sample-carrier is stopped and the cover proceeds on, allowing a flash of light to be received through the quartz condensing lens. The arms then reverse their direction under the control of micro switches which work automatically once motion has been started.

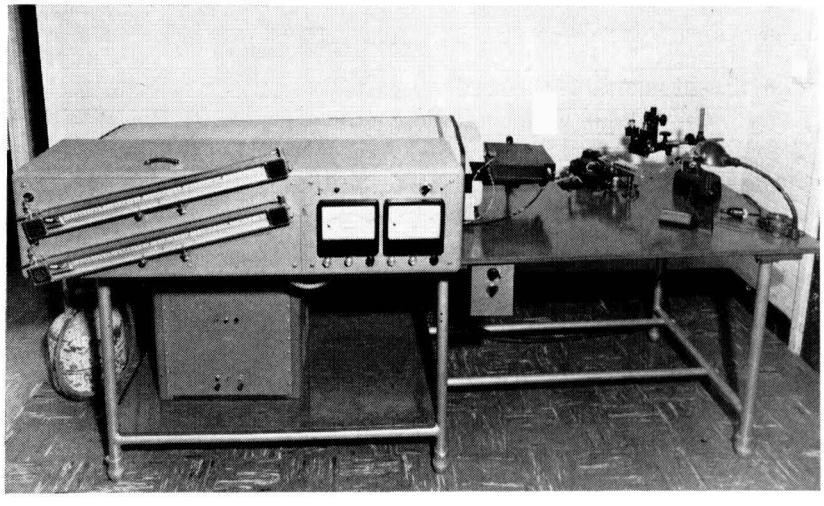

FIG. 1. Wide-angle photograph of dual channel flame photometer with power supplies on the shelf below. See text for description.

The condensing lens centers the light on the 50-μ entrance slit of a spectrograph containing a special grating from which the spectrum is reflected. Slits in a mask, which is installed in place of the film strip of the original spectrograph, allow the light to reach the two photomultiplier tubes and current from these is lead to the integrating circuits.

Platinum Loop and Burning Time. With the 26-gauge platinum wire loop and a 1-sec burning time used in our earlier work, there was a pronounced effect of sodium on potassium readings. At this stage it was necessary to use an elaborate set of standardization curves in order to correct for sodium content (2). Later Dr. Ramsay informed us that he was using a finer wire and a longer burning time than first described in order to prevent accumulation of salts on the wire. In our experience, changing to a loop (approximately 0.7 mm I.D.) of 31 or 36-gauge wire attached to a heavier stem and increasing the burning time to 2 sec not only markedly decreased the accumulation of salts on the wire but

[3] Present address: Franklin Institute, Philadelphia, Pa.

also eliminated, essentially, the effect of sodium upon potassium readings. However, when wires became roughened from long use they began to absorb salt and had to be discarded.

Accurate positioning of the wire was found to be of great importance. Centering the wire inside of the cover served as a control. We are now using 31-gauge wire loops, the stems of which are pushed through platinum–ruthenium tubing[4] for stability.

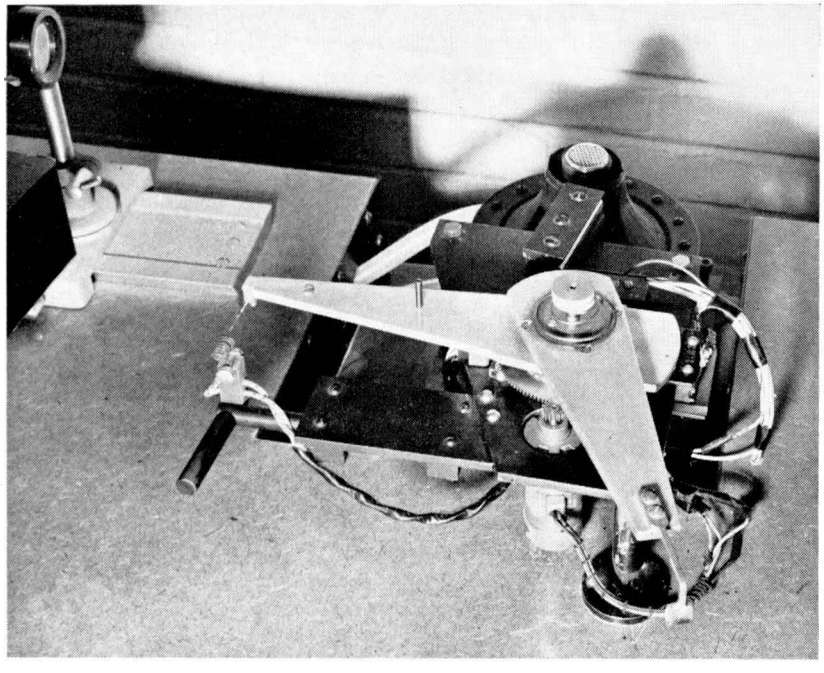

FIG. 2. Close view of motor assembly and burner. Drying unit is at left and quartz condensing lens is in upper left-hand corner.

Delivery of Samples to the Loop. Samples of standard solutions or diluted serum, tubule fluid, or urine were delivered to the loop from Wigglesworth type pipets (5) made of quartz and delivering, usually, approximately 0.2 μl. The pipet was attached to a microsyringe which was in turn fastened to a micromanipulator. It was filled by capillarity, either by dipping into a solution or from a storage pipet.

Delivery of the sample was made either to a dry wire or into a larger drop of water or solution. When the material to be analyzed contained much protein, best results were obtained by pulling some of the mixture back into the pipet in order to rinse it. This, of course, necessitated the use of the same technique for all standards and unknown fluids used in the series. When substances which interfered with the readings for sodium and/or potassium were present in the biological solutions 1 mmolar diabasic ammonium phosphate solution was substituted for the water drop. This technique was found neces-

[4] These items may be obtained from the Bishop Platinum Works, Malvern, Pa.

sary in analysis of fluid from mammals even when these were considerably diluted. Although separate standardizations were necessary because of the interfering effect of the phosphate, errors due to phosphate, sulfate, and other substances were found to be "swamped" in this way as suggested by Ramsay *et al.* (4).

RESULTS AND DISCUSSION

On standard solutions of pure potassium and sodium chloride, standardization curves were linear over ranges varying almost 1000-fold, although ranges usually used in analysis of biological fluids required curves with only 1–100-fold variation in concentration. In order to test the reproducibility of readings at lowest levels, a number of trials were made with solutions just distinguishable from water[5] in the amounts of pipetted to the bare wire. Figures for a typical trial series of seven consecutive readings on a solution containing 0.01 mmole each of potassium and sodium per liter were obtained using a pipet delivering approximately 0.2 μl, the total amount per sample therefore being approximately 2 $\mu\mu$moles each of potassium and sodium or 8×10^{-8} mg potassium and 4.6×10^{-8} mg sodium. Readings for potassium and sodium were made simultaneously. For the potassium meter readings a mean of 3.3 was obtained, with a standard deviation ± 0.27 amounting to about 8%. The mean for distilled water was 0.5, and the difference which was due to potassium alone, 2.8. Putting the figures in a form similar to that used by Ramsay for comparison this indicates that the standard deviation in terms of potassium was

$$8 \times 10^{-8} \times \frac{0.27}{2.8} = \pm 7.7 \times 10^{-9} \text{ mg.}$$

In the case of sodium, although the difference between readings on water (mean 3.2) and a 0.01 mmole per liter solution (mean 37.0) was much greater than that for potassium, the series was marred by one anomalous reading. Even with this the standard deviation (± 3.10) in terms of milligrams was

$$4.6 \times 10^{-8} \times \frac{3.10}{33.8} = \pm 4.2 \times 10^{-9}.$$

With these extremely small amounts the deviations were similar to those of Ramsay *et al.* for sodium and about half as great in the case of potassium. In this low range the factor producing the largest errors was the signal-to-noise ratio. Our experimental work on renal tubule fluid did not require the determination of these extremely small amounts.

With the larger amounts usually used in experiments (often 0.1–3 mμg potassium and 1–50 mμg sodium) this ratio improved and the standard

[5] This limit applies to potassium as the sensitivity to sodium could have been made greater.

deviation of individual readings for solutions of potassium and sodium chloride was approximately 3%. Since readings were made at least in duplicate, the errors were less than this. Delivery of the sample to the wire was here the largest source of error. In the case of protein-containing solutions like serum this was especially troublesome and rinsing technique was used.

Table 1 shows a comparison of results obtained on various biological fluids by the ultramicro and macro methods. Macro determinations were made by technical assistants using the instrument and method described by Fox (6). For the ultramicro analysis *micro-diluted* 0.2-μl samples were used. The dilution for amphibian (*Necturus*) serum was 20 times and that for pooled mammalian (rat) serum was 20 times or more. Bladder urine of *Necturus* was so low in salt content that it was used without dilution. The figures presented are the averages of two or three readings. Samples of

TABLE 1

COMPARISONS OF ANALYSIS BY MACRO AND ULTRAMICRO METHODS

Fluid	Potassium		Sodium	
	mmole/l		*mmole/l*	
	Ultramicro	Macro	Ultramicro	Macro
Necturus serum #1	3.3	3.2	88	90
#2	3.7	3.7	91	91
#3	3.5	3.4	90	93
Necturus urine	1.16	1.12	2.2	2.0
Rat serum #1; shows hemolysis	6.0	6.0	124	126
#2; shows hemolysis	6.2	5.9	134	138
#3; shows hemolysis	6.9	7.2	124	121
#4	4.7	4.5	132	128

pooled serum from *Necturus* were analyzed using a 31- or 36-gauge wire loop and a 2-sec burning time. For rat serum the fine wire loop plus the ammonium phosphate rinsing technique were used. On serum the greatest difference between results of macro and ultramicro methods was 5% for potassium and 3% for sodium. A sodium determination made on rat serum #3 by Dr. Judith Litchfield of this department with the electrode described by Eisenman *et al.* (7) yielded a value of 125 mmole per liter. On urine the percentage difference was larger but the absolute difference was exceedingly small (0.04 mmole per liter for potassium and 0.2 for sodium). Ramsay and his co-workers did not show comparisons of macro and ultramicro analyses nor did they deal with solutions containing as much protein as mammalian serum. For this reason a comparison of results cannot be made completely, but it is probable that stability and control have been improved in the modified instrument. The apparatus offers no advantage over standard flame photometers for routine work in which material is abundant.

However, in those cases where only minute amounts of material are available, the dual channel ultramicro flame photometer should be generally useful. It has been used in scores of determinations of renal tubule fluid and serum and should lend itself equally well to the analysis of other body fluids limited in amount. This applies also to extracts of bits of tissue such as are obtained in biopsies.

ACKNOWLEDGMENTS

The author wishes to acknowledge the assistance of Miss Jane Cohen and Miss Sarah Mills who prepared many solutions and ran macro analyses as controls.

SUMMARY

A modification of a dual-channel ultramicro flame photometer is described. It was built for analysis of very small amounts of biological fluids including that from renal tubules and determines potassium and sodium simultaneously in amounts as low as 2 $\mu\mu$moles. The author's experience in the use of the modified instrument is described. It should be generally useful for analysis wherever the sample supply is very limited.

REFERENCES

1. Bott, P. A., *Federation Proc.* **14,** 185 (1955).
2. Bott, P. A., *Federation Proc.* **15,** 223 (1956).
3. Bott, P. A., *in* "8th Ann. Conf. on the Nephrotic Syndrome, 1956" (J. Metcoff, ed.), p. 39. National Nephrosis Foundation, New York, 1957.
4. Ramsay, J. A., Brown, R. H. J., and Falloon, S. W. H. W., *J. Exptl. Biol.* **30,** 1 (1953).
5. Wigglesworth, V. B., *Biochem. J.* **31,** 1719 (1937).
6. Fox, C. L. Jr., *Anal. Chem.* **23,** 137 (1951).
7. Eisenman, G., Rudin, D. O., and Casby, J. U., *Science* **126,** 831 (1957).

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A New Approach to Radioassay of Aqueous Solutions in the Liquid Scintillation Spectrometer

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INTRODUCTION

Liquid scintillation systems of practical efficiency depend on the use of a relatively nonpolar solvent system. Since many, if not most, of the compounds of biochemical and medical interest are polar compounds, the problem of how to incorporate them into the available highly nonpolar systems is an important one and it has received considerable attention. Three of the approaches which have been successfully used are: (*a*) conversion of the compound under study to a new form soluble in organic solvents (1, 2); (*b*) inclusion of a secondary, more polar solvent in the system to permit incorporation of small amounts of polar solute (3, 4); and (*c*) counting of fine suspensions of the solid polar compound in one of the standard nonpolar systems (5, 6). In the present communication we describe a new approach which permits the direct assay of labeled compounds *in aqueous solution.*

The method is based on the principle of using a two-phase system: a solid phase consisting of a finely divided fluor and a liquid phase containing the radioactive material to be assayed. The effectiveness of the method depends on the intimate mixing of the two phases so that beta particles emitted in the liquid phase do not have to travel too far before they strike a solid fluor particle; i.e., the mean free path in the liquid phase is small relative to the average range of the beta particles. A second requirement is that the light emitted when the fluor is activated by beta radiation must be able to escape the counting sample and reach the photomultiplier tubes with reasonable efficiency. In order to maximize light transmission, our our earlier studies employed particles of clear plastic in which a fluor was embedded. More recent studies show that masses of fluor crystals, although they scatter light and thus appear to be "opaque" in the usual sense, do not in fact absorb a very large fraction of the light emitted within them. Actually, higher efficiencies are obtained with several common fluors in crystal-

line form than are obtained with the clear scintillating plastic. Highly purified anthracene is the most effective of those so far tested. Preliminary reports of these methods have been published elsewhere (7–9).

MATERIALS AND METHODS

Plastic Scintillator

Small cylindrical filaments (3.2 cm. long, 0.043 cm. in diameter) of Pilot Scintillator B were obtained from Pilot Chemicals, Inc. through the courtesy of Mr. Mark Hyman. Pilot Scintillator B is a clear, colorless plastic containing diphenylstilbene, giving it a blue–violet daylight fluorescence. Its properties are as follows: sp gr 1.02; refractive index 1.58; softening temperature 70–75°C; insoluble in water and lower alcohols, soluble in most other organic solvents; peak of fluorescence spectrum approximately 4050 A; decay time 3×10^{-9} sec. In the earlier studies the filaments were used as such or cut with scissors into short segments about 1 mm long. In later experiments machine-cut segments (0.5 mm long) prepared especially for this purpose were used, and these segments are now available from Pilot Chemicals, Inc., Watertown, Mass., under the name *B-beads*.

Solid Fluors

Several standard commercial grades of anthracene were tested, but best results were obtained with Distillation Products Industries, blue–violet fluorescence grade anthracene (Catalog No. X-480). The fluorescence spectrum of highly purified anthracene shows strong peaks at 4220 A and 4450 A, secondary peaks at 4025 A and 4375 A, and a very weak output at 5090 A (10). The peak sensitivity of the photomultiplier tubes in the Packard Liquid Scintillation Spectrometer used in the present studies is in the neighborhood of 4000 A and thus matches the anthracene output spectrum very well. The fluorescence spectrum of anthracene preparations is highly sensitive to traces of impurities such as chrysene and naphthacene. The lower efficiency of some of the anthracene preparations described below is in part at least attributable to shifts in the fluorescence spectrum due to such impurities. Results with any single lot of anthracene were highly reproducible. However, as discussed in more detail below, different lots, even of the highly pure blue–violet fluorescence grade anthracene, showed somewhat different efficiencies. It is not yet clear whether this is entirely attributable to differences in the size and shape of the crystals or whether it is due in part to trace impurities. Dr. William West and Dr. L. J. Rall of Eastman Kodak Company Research Laboratories have kindly undertaken to explore this point in the hope of eliminating variability between lots.

2,5-Diphenyloxazole (PPO), *p*-bis[2-(5-*p*-biphenylyloxazolyl)]benzene (BOPOB), *p,p'*-diphenylstilbene, and *trans*-stilbene were obtained from Pilot Chemicals, Inc.

Labeled Compounds and Counting Equipment

Absolute efficiencies for C^{14} and H^3 were determined using National Bureau of Standards reference sources of $Na_2C^{14}O_3$ and $H_2{}^3O$ at appropriate dilutions. Alanine-1-C^{14}, lysine-1-C^{14}, and palmitic acid-1-C^{14} were obtained from Nuclear Chicago, Inc. All solutions counted on crystalline fluors were made up to contain 1:1000 (*v/v*) Triton GR-5 (Rohm and Haas, Inc., Philadelphia, Pa.), an anionic detergent. Unless the detergent is added, the crystals do not wet satisfactorily and a nonhomogeneous counting sample results. Triton GR-7 has also been used, and probably any nonquenching detergent that will make the crystals wettable can be substituted.

All radioassays were carried out using a Packard Liquid Scintillation Spectrometer equipped with automatic sample changer. Efficiencies obtained with the manual instrument of the same manufacture were somewhat lower, probably due to the fact that the photomultiplier tubes are spaced further apart in the manual instrument. To avoid freezing of aqueous samples, the temperature of the counting chamber was adjusted to approximately +2°C. Except where otherwise indicated, weighed amounts of the solid fluor were added to a 5-dram Wheaton Glass Co. Crystallite vial and the solution of the labeled compound (containing detergent) was pipetted onto the surface of the solid fluor. All count rates, unless otherwise indicated, have been corrected for background measured with a blank vial containing water and fluor in amounts matching the volume of liquid and mass of fluor in the samples.

RESULTS

Plastic Scintillator Filaments

As previously reported (7), an estimate of the maximum efficiency obtainable with the plastic scintillator was first made using a bundle of filaments, packed side by side like Roman fasces, in a segment of glass tubing (4.4 cm × 0.8 cm) sealed at the ends with rubber stoppers. Labeled solutions were added at the top of the bundle and allowed to seep down into the fine interstices by capillary action. The tubing was then placed upright inside a standard counting vial and introduced into the liquid scintillation counter in this way.

The observed count rate, corrected for the background of 104 counts/min, was a linear function of the volume of $Na_2C^{14}O_3$ added from 10 to 80 μl. Absolute efficiency was 35%. Tritiated water introduced in the same way was counted at 0.7% efficiency. By opening both ends of the glass tube and allowing first dilute acetic acid, then water, and finally alcohol to drip through the packed filaments, all of the labeled carbonate was removed and the filament bundle could, after drying, be reused without disturbing the packing.

For special purposes this technique using filaments may be useful, particularly since much larger bundles of filaments can be used to increase the volume of sample counted. One potentially useful application might be in the continuous monitoring of the outflow from chromatographic columns. An important limitation lies in the difficulty of correcting for quenching effects since complete mixing of internal standard with sample would be almost impossible because of the geometry of the system. Such a correction might be made with a second aliquot of sample independently counted after addition of internal standard. However, the methods employing fragments of fluor as described below appear to be more suitable for routine use.

General Characteristics of the Two-phase System

When increasing volumes of a solution of $Na_2O^{14}O_3$ (75,000 d/min/ml.)

were added to a constant weight of B-beads (1 gm), the count rate increased as a linear function of sample volume up to 0.8 ml, then sloped off and reached a plateau value at about 1.5 ml (Fig. 1). Further additions of sample beyond this point did not further increase the count rate. Similar results were obtained using crystalline anthracene except that the linear portion of the curve was more limited (Fig. 2). These results can be inter-

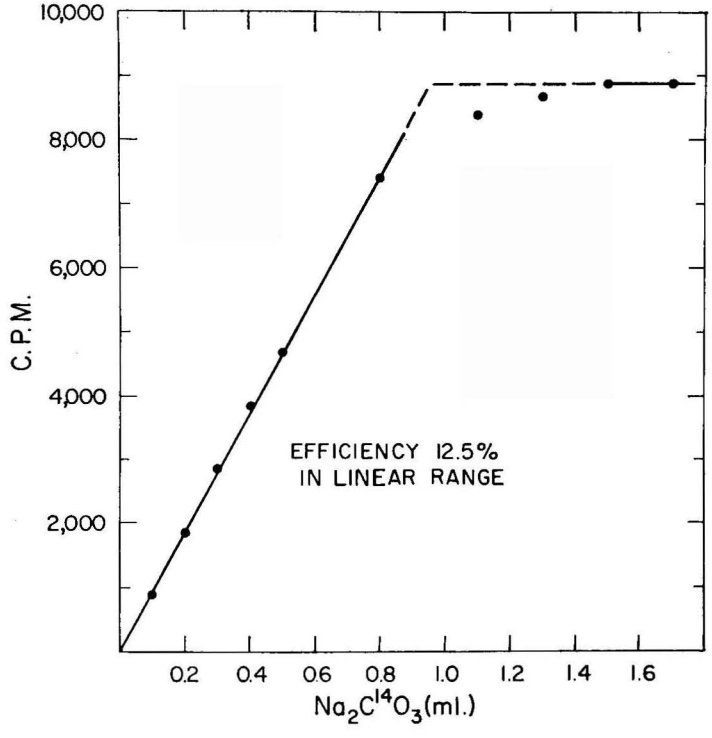

FIG. 1. Observed counting rate as a function of volume of sample added to Pilot B plastic scintillator (1 gm) in a standard counting vial.

preted in the following way. Over the linear portion of the curve each added increment of sample sinks into the interstices of the mass of fluor leaving no excess above it. True linearity in this first part of the curve would indicate that the packing of fluor particles is, on the average, quite uniform, so that the mean free path for beta particles in the liquid phase remains constant as more and more of the interstices are filled with radioactive solution. It would also indicate that the fraction of light lost from the successive layers of fluor wetted by the sample is reasonably constant. The beads

closely approach this ideal behavior; the anthracene deviates somewhat from it.

As the level of the liquid sample begins to rise above the top of the fluor mass, more and more of the radiation from the uppermost layers is absorbed within the liquid sample before it can reach a fluor particle. Finally, at the plateau, *none* of the radiation from the uppermost layer of liquid sample is reaching the fluor and thus it contributes nothing to the observed count. Further additions of sample cannot further increase the count rate. The volume of sample actually being counted then is determined by the amount

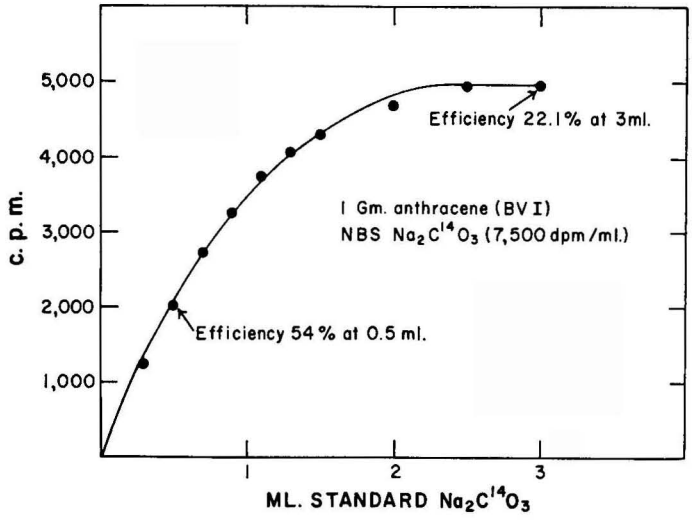

FIG. 2. Observed counting rate as a function of volume of sample added to anthracene (1 gm) in a standard counting vial.

of solid fluor and, more particularly, by the volume of fluid held in its interstices. With any amount of solution in excess of that needed to cover the fluor, the observed count rate sould be proportional to the *concentration* of radioactivity in the solution (*specific radioactivity* in counts per minute per milliliter). This was confirmed experimentally both for plastic beads and for anthracene by adding to 1 gm of the solid fluor in each of a series of bottles an excess of several different dilutions of $Na_2C^{14}O_3$. The count rate decreased exactly in proportion to the dilution. Thus, counting with an excess of liquid phase and a constant amount of fluor can be considered analogous to the counting of solid samples at infinite thickness.

Conversely, if an excess volume of the same sample (enough to completely cover the fluor) is added to varying amounts of solid fluor, the observed

count rate should be directly proportional to the mass of fluor used, provided there is no change in the fraction of light lost within the counting sample with increasing volumes of solid fluor. This was confirmed experimentally for B-beads by adding an excess of a standard solution of $Na_2C^{14}O_3$ to vials containing 0.25, 0.50, and 1.0 gm of beads. The observed count rates were, respectively, 2166, 4410, and 8987 counts/min. In the case of anthracene, when an excess of sample (alanine-1-C^{14}) was added to increasing amounts of solid phase, the observed count rate at first increased linearly with the amount of solid phase but the curve began to slope off above 0.5 gm (Fig. 3). This deviation from linearity is probably due to light losses

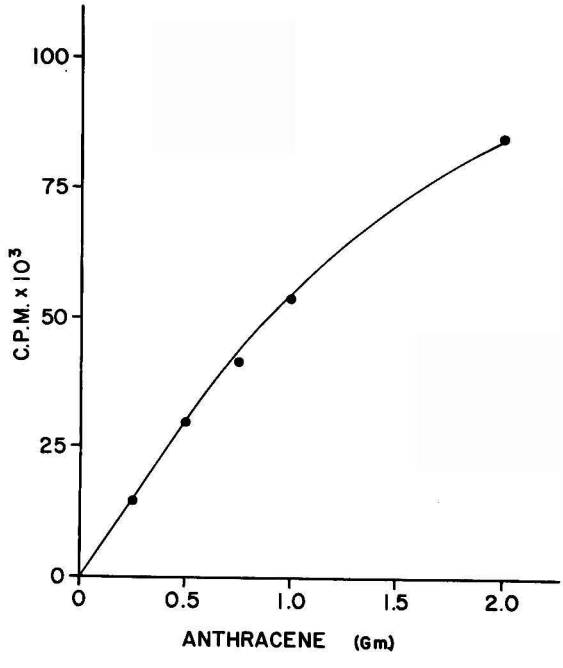

FIG. 3. Observed counting rate as a function of increasing mass of anthracene in a standard vial containing an excess (10 ml) of an aqueous solution of alanine-1-C^{14}.

within the mass of anthracene. It should be noted that the curve over any short range between 0 and 1 gm is close enough to a straight line to indicate that error due to small differences in the amounts of anthracene weighed into a series of vials during routine studies will be very nearly proportional to the percentage error in the weighing.

In summary, two general methods of counting suggest themselves: (*a*) in the linear range, where the count rate is proportional to *total radioactivity* independent of sample volume; (*b*) in the plateau region, where the

count rate is proportional to the *specific radioactivity* in counts per minute per milliliter of solution (concentration of radioactivity) provided a constant mass of fluor is used for counting all samples. If in the latter method the same volume of liquid sample is always used, the count rate is then proportional to the total radioactivity in that volume. We have found that the first method does not always give reproducible results with anthracene unless particular care is taken to pipet the sample slowly and to distribute it very uniformly over the surface of the crystals. The results with beads are more nearly reproducible in the linear range, but still there is the important drawback that a quenching correction is very difficult to make accurately because internal standard cannot be easily mixed with the small volume of sample wetting the fluor. Because the absolute efficiency is higher and because the fluor is much cheaper, anthracene is best suited for routine counting. The technique for its use will be described in detail, then, with occasional reference to the beads where relevant.

Suggested Procedure for Routine Counting with Anthracene

There are many different ways in which this two-phase system can be applied. The choice of procedure will depend upon the isotope to be assayed, the nature of the sample, the amount and specific radioactivity of available sample, and the geometry and counting characteristics of the scintillation counter used. The procedure described below is one which has been tested and in use in our laboratory for over a year for assay of C^{14} in aqueous solutions.

Exactly 1.00 gm anthracene is added to each of the standard counting vials. Then 3 ml of the aqueous sample to be assayed, containing a final concentration of 1:1000 (*v/v*) Triton GR-5, is pipetted slowly onto the surface of the anthracene so that the crystals are wetted and the solution sinks down into the crystal mass. The suspension is gently stirred to break up lumps, and any anthracene adhering to the walls above the fluid level is scraped down (paper clips with one "arm" straightened out are convenient). The suspension is allowed to stand for 3 hr so that the crystals can settle completely to the bottom of the vial and the sample is then ready for counting. A vial to which 3 ml of a 1:1000 solution of Triton GR-5 in water has been added is counted with each set of samples to obtain the appropriate background correction. A vial to which 3 ml of a standard reference solution of radioactive material has been added is counted to check on possible variations in the efficiency of the counter or variations in the anthracene preparations.

After the initial count, a known amount of the reference solution of radioactive material in a small volume (10–50 μl) is added to each vial (including

the blank), the contents are stirred, and, after settling, the vials are counted again to check for and correct for quenching. This is discussed more fully below.

Response and Background as a Function of Voltage

A voltage response curve obtained for a vial containing 3 ml of a solution of alanine-1-C^{14} and 1 gm anthracene is shown in Fig. 4. Maximum response was obtained counting integrally (10-∞ window) at the highest photomultiplier voltages (taps 10 and 11). For samples counting well above background, the higher efficiencies obtained here are desirable. Unless otherwise specified, the data reported in this paper have been obtained counting integrally at voltage tap 10. It will be noted, however, that the background

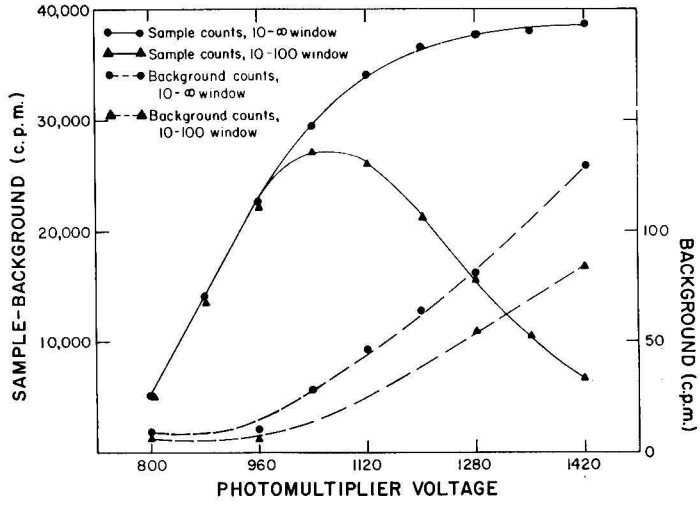

FIG. 4. Observed counting rate (corrected for background) and background counting rate as a function of photomultiplier voltage. Background data were collected for a standard vial containing 1 gm anthracene and 3 ml water; counting rates were determined with a similar vial containing an aqueous solution of alanine-1-C^{14}.

count rate rises sharply at the higher voltages. For weakly radioactive samples, operation at lower voltages may be preferable, depending on the experimental background and efficiency of the instrument used. It should be emphasized that different instruments, even of the same manufacture, will vary in spectral sensitivity and optimal operating voltages and discriminator settings. Optimal operating conditions should be explored for each instrument, and the data presented here are meant only to serve as a guide.

Absolute Efficiency for C^{14}

The absolute efficiency for counting C^{14} on anthracene is 54% in the linear range, that is, with very small volumes applied to the anthracene crystals (see Fig. 2). The efficiency falls off with increasing volumes as discussed above. For the routine procedure just described, the efficiency is 22% for that amount of C^{14} radioactivity contained in a 3-ml sample. These values were obtained on a particular sample of Distillation Products Industries blue–violet fluorescence grade anthracene (Lot No. 61). The efficiency for different samples of anthracene taken from this particular lot was highly uniform. On the other hand, different lots of the same material have differed by as much as 10% in counting efficiency. It should be stressed that the ordinary chemically pure (CP) anthracene is decidedly inferior to this highly purified fluorescence grade material. Chemically pure anthracene samples gave only about one-third the efficiency observed with the fluorescence grade anthracene.

When larger volumes of sample are available they can be counted either by increasing the amount of anthracene added to the standard vial or by using bottles of larger diameter so as to accommodate more anthracene. The efficiency with which the larger volumes can be counted in the standard vial is somewhat less than that obtained in the routine procedure (see Fig. 3). Still, the higher absolute count obtained by using a larger volume of sample can be useful under some circumstances. Four grams of anthracene plus 12 ml sample can be handled reproducibly in a manner analogous to that described for the routine procedure with efficiencies of about 16%.

Using larger size counting bottles (4.2 cm outside diameter) it was possible to count the C^{14} in 8 ml. solution added to 4 gm anthracene with an efficiency of 20%.[1] There is no theoretical reason why, with the use of these large bottles, it should not be possible to go to much larger sample volumes with corresponding increases in the weight of anthracene used.

Reproducibility

As shown in Fig. 5, excellent reproducibility is obtained with aliquots of anthracene weighed out directly from the stock bottle with no further grading or special handling. The vertical lines at each of the mean points for four samples indicate the *range* for the four samples. For a group of 12 identically prepared 3-ml samples counted by the routine procedure, the standard deviation was $\pm 4\%$ (counting error $\pm 0.7\%$).

If the anthracene is not allowed to settle completely after stirring, a false

[1] These data were obtained with an automatic Packard Scintillation Spectrometer specially modified to accommodate the larger vials.

count may be obtained (too high if the sample does not quench, too low if it is a highly quenching sample). Allowing the sample to stand for 3 hr brings the observed count rate to a stable value. Anthracene as supplied contains some finely powdered crystals. By stirring in a Triton solution and decanting when the crystals have partially settled, these "fines" can be removed and the settling time required for samples can be decreased.

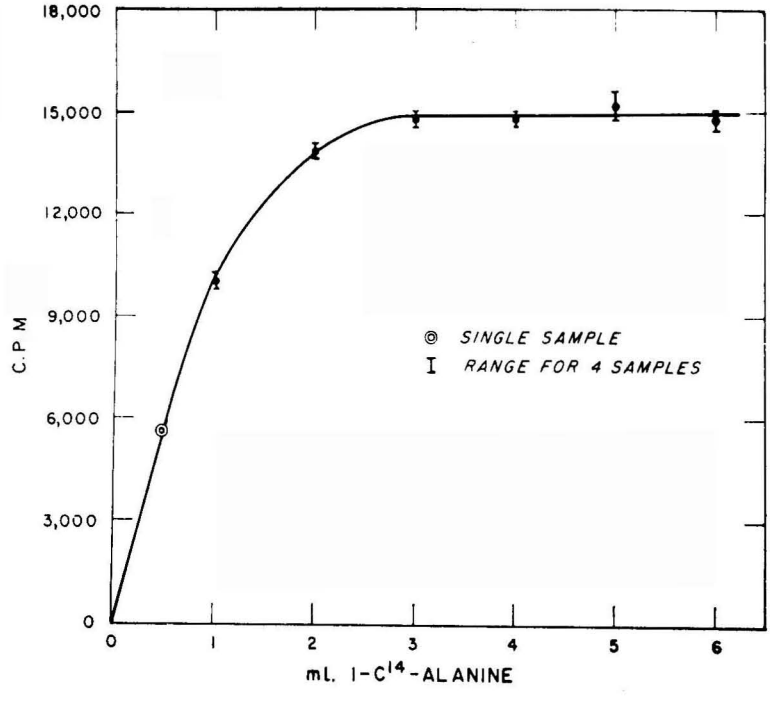

FIG. 5. Reproducibility for counting C^{14} by the suggested routine procedure. The vertical bars represent the range of observed counting rates and the solid circles the mean counting rates for four vials containing 1 gm anthracene and 3 ml of an aqueous solution of alanine-1-C^{14}.

Error due to variations in the exact weight of anthracene in the vial will be, over a narrow range, just proportional to the percentage error in the weighing. Thus it is generally not necessary to weigh the 1 gm anthracene more exactly than ± 10 mg. Occasionally a clump of dry anthracene crystals will be trapped in the middle of the crystal mass, and for this reason stirring is important.

Efficiency for Other Radioisotopes

The absolute efficiencies for several other radioisotopes counted by the

routine procedure (i.e., sample in 3 ml, 1 gm anthracene) are given in Table 1. While the efficiency for tritium is relatively low (0.5%), the larger sample size allowable and the simplicity of the procedure may make it useful even at this low efficiency. For example, counting 3 ml at 0.5% efficiency would be equivalent to counting 0.1 ml at 15% efficiency. Dr. George Bray of the National Heart Institute has applied the method successfully in the counting of tritiated acetazolamide in the course of studies on the binding of this compound to proteins. Ca^{45} (E_{max} 0.255 Mev), gave an absolute efficiency a little more than twice that of C^{14}. It is in the counting of soft beta emitters such as these that the method may be particularly helpful.

TABLE 1
REPRESENTATIVE EFFICIENCIES FOR SEVERAL ISOTOPES

Isotope	Voltage tap	Window	Background counts/min	Absolute efficiency[a] %
H^3	10	10–∞	99	0.5
C^{14}	10	10–∞	99	16 to 20
Ca^{45}	10	10–∞	99	49
	10	10–∞	99	93
P^{32}	5	10–∞	11	78
	3	10–100	7	56
I^{131}	10	10–∞	99	58
	5	10–∞	11	46

[a] These values represent the absolute efficiency with which the total radioactivity in 3 ml sample was measured. Except in the case of C^{14}, efficiency was only determined once, using Lot No. 61 of Eastman Kodak Blue–Violet Fluorescence Grade anthracene. The range of values found for C^{14} with different batches of anthracene is given.

The efficiency for P^{32} (93%) is remarkably high. Even at low photomultiplier voltages where the background is extremely low, the efficiency is still well above 50% (Table 1). Even though this strong beta emitter can be readily counted in a number of other ways, the simplicity of sample preparation plus the high efficiency make the present method attractive.

The efficiency for I^{131} is somewhat higher than that obtained using well-scintillation counters. The latter method is simple and satisfactory but the present procedure, utilizing an instrument applicable also to the counting of weak beta emitters, offers advantages of economy as well as convenience. We are currently exploring the application of this method to the counting of pure gamma and x-ray emitters. It would seem that weak x-ray radiation could be efficiently counted with this system.

Correction of Quenching

Quenching in the conventional liquid scintillation systems is of two

kinds: (*a*) that due to simple absorption of fluorescence radiation by components having appreciable absorption in the corresponding spectral region; (*b*) that due to interactions with solvent or solute components of the scintillation system that cause interference with the efficiency of energy transfer or fluorescence. In the present system the fluor is in a separate insoluble phase, and once a beta particle or gamma ray has reached a fluor particle it should be able to excite fluorescence within it exactly as it would in the absence of the liquid phase. Except under unusual circumstances, then, we need only consider the light-absorbing properties of the liquid phase in relation to the emission spectrum of the fluor to evaluate the quenching properties of a sample.

Pragmatically, quenching can be tested for and corrected for just as in conventional liquid scintillation counting, i.e., by the use of an *internal standard*. After the sample has been counted, a known amount of radioactivity in a small volume (10–50 μl) is added, and the mixture is thoroughly stirred and then recounted. If the increment in count rate is less than that observed when the same amount of internal standard is added to a water blank, the initial count rate is appropriately corrected. The validity of this quench correction is indicated by the results in Table 2.

If a stable standard reference sample of labeled material is used as in-

TABLE 2
QUENCHING CORRECTION[a]

Quencher added	Glucose-U-C^{14} added μl	Observed counts/min	Specific Activity (counts/min/μl glucose-U-C^{14})	
			Uncorrected	Corrected[b]
None	50	34,870	(698)	—
	100	68,326	(683)	—
	150	100,014	(667)	—
			Mean: 683	
0.6 ml. of 10^{-3} *M* dinitrophenol	150	73,542	490	683
	200	97,722	489	682
	250	120,868	484	678
			Mean: 488	Mean: 681

[a] Successive small increments of glucose-U-C^{14} were added as indicated to 10 ml. of 1:1000 Triton and 1 gm. anthracene in a standard counting vial. The volumes added were small enough relative to the initial 10-ml volume to be ignored in calculation of the count per microliter of labeled solution.

[b] The corrected values represent the calculated values assuming that the same percentage quenching observed on first adding the yellow dinitrophenol solution would apply to any concentration of radioactivity in the vial.

ternal standard throughout the course of a study, it will automatically provide correction for: (*a*) changes in the efficiency of the counting device, (*b*) differences in the efficiency of anthracene samples, (*c*) losses due to quenching.

Temperature Effects

The efficiency of the two-phase system, both with anthracene and B-beads, remains essentially constant in the range between 0°C. and 25°C. This is a distinct advantage in that careful temperature equilibration, required when using conventional systems, is not essential. The samples should not, however, be allowed to freeze. They will count when frozen despite their apparent opacity, but the count rate observed relative to that

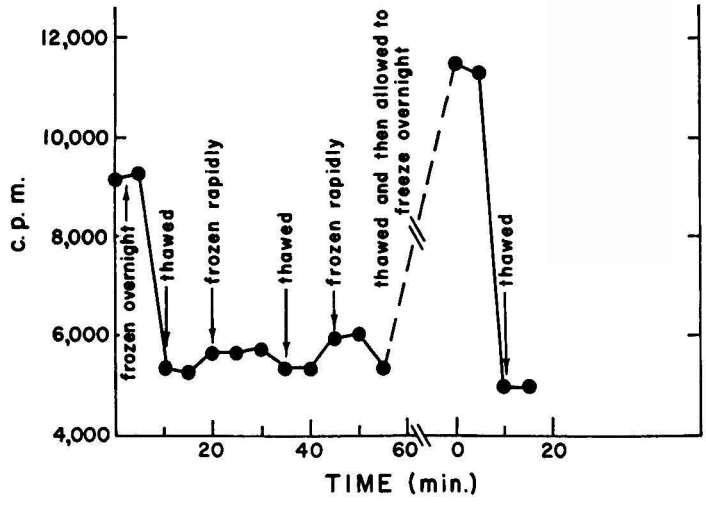

FIG. 6. Effects of freezing and thawing on observed counting rate. The counting vial contained 1 gm of finely divided Pilot B scintillator plastic and an aqueous solution (3 ml) of $Na_2C^{14}O_3$.

of the liquid sample will depend on the nature of the radioactive solute and the rate of freezing. This is illustrated by the experiments shown in Fig. 6. An aqueous solution of $Na_2C^{14}O_3$ was added to finely divided Pilot B plastic scintillator and allowed to freeze overnight in the sample changer (−10°C). A count rate of slightly over 9000 was observed on the frozen sample. When the sample was thawed the count rate fell to about 5000. When refrozen rapidly, by immersion in a solid CO_2–acetone mixture, the count rate rose only very slightly. Allowed to thaw and then slowly freeze

overnight once more, the sample counted at a considerably higher rate, approximately 12,000. These variable increases in count rate on freezing probably reflect the separation of solute occurring as ice crystals form. The radioactive solute becomes progressively more concentrated in the remaining liquid phase adjacent to the fluor particles, which are also being "frozen out." Thus in the frozen sample the labeled solute is, on the average, much closer to the fluor particles and the mean free path for beta particles is much reduced, hence the higher efficiency. Unfortunately, as seen in Fig. 6, the exact degree of "freezing out" of solute depends on the rate of freezing and will vary according to the nature of the solutes and their concentrations. For routine counting it is best to set the temperature of the counting chamber slightly above the freezing point of the liquid samples.

Recovery of Sample

The solubility of anthracene in water at 25°C. is negligible and its chemical reactivity is very low. Consequently aqueous samples counted by the routine procedure can be recovered without significant contamination with fluor. Recoveries have been checked for $Na_2C^{14}O_3$ and for alanine-1-C^{14}. Anthracene and liquid sample were transferred quantitatively to a 15-ml conical centrifuge tube with the aid of several water washes, and the anthracene was centrifuged down at low speed. The supernatant was drawn off, and the sedimented anthracene crystals were washed twice with water. The pooled supernatant solutions contained all of the radioactivity in the original sample and the anthracene, reused in a blank vial, gave a background count rate no higher than that found originally. However, when amino acid samples were allowed to stand in contact with anthracene or with the plastic beads for long periods of time, it was found that some of the radioactivity was tightly bound. Since some compounds may adsorb on the fluor particles, the completeness of recovery should be checked for each new material studied.

The option of recovering sample for subsequent chromatography, chemical identification, or use in biological experiments can be valuable when only minute amounts of material are available for study: It also makes it possible to conserve anthracene by washing thoroughly, drying, and reusing.

Although nonpolar compounds are readily counted in conventional organic solvent systems, they are difficult to recover. Since B-beads are insoluble in lower aliphatic alcohols, nonpolar compounds can be counted in ethanolic solution and recovered. Ethanolic solutions can also be counted on anthracene, but the recovered sample will contain dissolved anthracene (e.g., solubility 19 mg/ml in ethanol).

If the low concentration of detergent used in the routine procedure seriously interferes with further study of the sample, its use can be elim-

inated by adding the sample to the anthracene in an evacuated counting bottle through a vaccine-port seal.

Other Crystalline Fluors

A number of other crystalline fluors were tested for efficiency in counting aqueous solutions of C^{14}-labeled compounds. The results are shown in Table 3. 2,5-Diphenyloxazole (PPO) and *p*-bis[2-(5-*p*-biphenylyloxazolyl)] benzene (BOPOB) gave maximum efficiencies comparable to that of anthracene. Diphenylstilbene and *trans*-stilbene were considerably less efficient than anthracene. By appropriate recrystallization procedures, the packing properties and hence the efficiencies of these other fluors might be improved. In view of their cost, however, they are not likely to be as desirable as anthracene for general use.

TABLE 3
COMPARISON OF SEVERAL CRYSTALLINE FLUORS

Fluor	Mass of fluor gm	Volume of sample ml	Efficiency relative to anthracene[a]
Anthracene	1	3	100
PPO[b]	1	3	102
trans-Stilbene	1	3	45
Anthracene	0.25	0.75	100
BOPOB[c]	0.25	0.75	81
p,p'-Diphenylstilbene	0.25	0.75	63

[a] Samples were counted at voltage tap 11 in the 10–∞ window.
[b] 2,5-Diphenyloxazole.
[c] *p*-bis[2-(5-*p*-biphenylyloxazolyl)]benzene.

Attempts were made to improve the efficiency of anthracene by recrystallization from several different solvents and also by grinding the commercial preparation more finely. None of these treated samples were superior to the anthracene as supplied.

Range of Compounds Assayable

An outstanding advantage of the present method is its wide generality. Except for limitations imposed by excessive quenching of colored compounds in high concentration, the routine procedure should be applicable to *any* material soluble in water, salt solution, dilute acid, or dilute alkali. A partial list of compounds in water solution for which it has already been proved in practice at the National Institutes of Health includes: $H_2{}^3O$, acetazolamide-H^3, $Na_2C^{14}O_3$, C^{14} amino acids, C^{14} peptides, C^{14} proteins, glucose-C^{14}, and Ca^{45} salts.

Because of the insolubility and inertness of anthracene, one is not limited to neutral water solutions. Dilute acid or dilute alkali may be used instead, thus broadening further the range of materials that can be assayed. For example, $C^{14}O_2$ can be trapped in NaOH during metabolic experiments *in vitro* or *in vivo*, and aliquots can be counted directly by pipetting onto anthracene. [Alkali does not wet the crystals readily, and here it is best to use a higher concentration of detergent (1:100)]. Denatured proteins can be solubilized in dilute alkali for counting. Dilute acids can also be used as a medium, and we have found the method valuable for determining total radioactivity in unfractionated trichloroacetic acid supernatant solutions.

SUMMARY

1. A new two-phase counting method is described that permits radioassay of aqueous solutions in the liquid scintillation spectrometer. The sample in solution is pipetted onto crystals of anthracene in a standard counting bottle, and the fluorescent emission is counted. The general features of the technique are discussed from a theoretical and practical point of view.

2. Efficiencies as high as 54% for small volumes of C^{14} samples can be obtained. In the routine procedure described the C^{14} in 3 ml of sample is counted with approximately 20% efficiency. Efficiencies for H^3, Ca^{45}, P^{32}, and I^{131} are presented.

3. The efficiencies for C^{14} obtained with several other solid fluors, including a plastic scintillator, are presented.

4. The advantages of the method over existing methods are several:

a. It is general and applicable to virtually any compound soluble in water, salt solutions, dilute acid, or dilute alkali.

b. No chemical conversion or other preliminary handling is necessary, and sample preparation is simple and rapid.

c. Samples can be recovered unaltered after radioassay and used for further characterization.

d. Large quantities of sample can be counted, limited only by their solubility in the liquid phase, and, in the case of colored compounds, by quenching effects.

REFERENCES

1. PASSMANN, J. M., RADIN, N. S., AND COOPER, J. A. D., *Anal. Chem.* **28,** 484 (1956).
2. STEINBERG, D., VAUGHAN, M., ANFINSEN, C. B., GORRY, J. D., AND LOGAN, J., *in* "Liquid Scintillation Counting" (C. G. Bell and F. N. Hayes, eds.), p. 230. Pergamon Press, New York, 1958.
3. HAYES, F. N., AND GOULD, R. G., *Science* **117,** 480 (1953).
4. DAVIDSON, J. D., AND FEIGELSON, P., *Intern J. Appl. Radiation and Isotopes* **2,** 1 (1957).

5. HAYES, F. N., ROGERS, B. S., AND LANGHAM, W. H., *Nucleonics* **14,** 48 (1956).
6. WHITE, C. G., AND HELF, S., *Nucleonics* **14,** 46 (1956).
7. STEINBERG, D., *Nature* **182,** 740 (1958).
8. STEINBERG, D., *in* "Proceedings of the Symposium on Advances in Tracer Applications of Tritium," New York City, October 31, 1958.
9. STEINBERG, D., *Nature* **183,** 1253 (1959).
10. PRINGSHEIM, P., *Trans. Faraday Soc.* **35,** 28 (1939).

ANALYTICAL BIOCHEMISTRY 1, 40–43 (1960)

A Micro Method for the Determination of Levulinic Acid[1]

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INTRODUCTION

Previously published methods for the determination of levulinic acid are as follows: (*a*) titration (1), (*b*) cuprometric analysis (1), (*c*) picric acid complex formation (2), and (*d*) iodometric analysis (3). All these methods have one common defect which is the lack of sensitivity. The most sensitive is the iodometric method which is useful to analyze samples containing from 1 to 20 mg levulinic acid.

A method for determining levulinic acid in microgram quantities is described in the present communication. The principle of the method is based upon the formation of the 2,4-dinitrophenylhydrazone of levulinic acid in the presence of sulfuric acid as catalyst, and the extraction of the resulting 2,4-dinitrophenylhydrazone by alkali. Samples containing 2.5–25 μg levulinic acid can be rapidly determined by this method.

EXPERIMENTAL

Materials

2,4-Dinitrophenylhydrazine and levulinic acid were purchased from the Matheson Company and the Eastman Kodak Company, respectively.

DNP Reagent. 2,4-Dinitrophenylhydrazine, 125 mg, was refluxed with 250 ml benzene for 15 min to form a clear solution. The solution is stable indefinitely at room temperature.

Ten per Cent H_2SO_4 in Ethyl Alcohol. Concentrated H_2SO_4, 5 ml, was slowly added with cooling to 30 ml of 95% ethyl alcohol. The final volume of the solution was adjusted to 50 ml with 95% alcohol.

Procedure for Color Development

Alcoholic solutions of levulinic acid were added to 0.05 ml of 10% H_2SO_4 in alcohol, and diluted with 95% ethyl alcohol to a final volume of 0.2 ml. One milliliter of DNP reagent was added, and the mixture was allowed to stand at room temperature for 20

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min with occasional shaking. Four milliliters of 0.5 *N* NaOH was added to each tube. The mixture was shaken vigorously and centrifuged for 3 min. The optical density of the alkaline layer at 540 mμ was determined in a Beckman DU spectrophotometer. When aqueous samples of levulinic acid were determined, the solutions were adjusted to pH 8 and evaporated to dryness *in vacuo*. Then 0.15 ml of 95% ethyl alcohol and 0.05 ml. of 10% H_2SO_4 in alcohol were added to the dried samples, and the amounts of Levulinic acid were determined as described above.

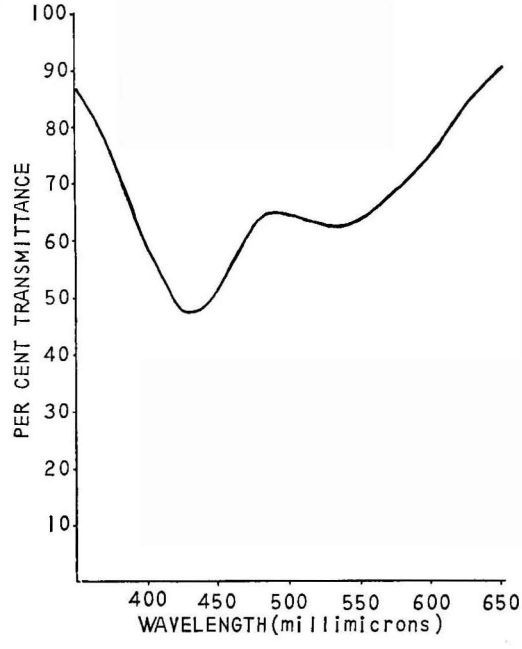

FIG. 1. Spectrum of 2.5 μg levulinic acid 2,4-dinitrophenylhydrazone/ml of 0.5 *N* NaOH. The absorption peak at 540 mμ is more characteristic of levulinic acid dinitrophenylhydrazone than the peak at 430 mμ (4). Curve prepared with a Beckman DK-2 recording spectrophotometer.

RESULTS

Figure 1 shows the spectrum of levulinic acid 2,4-dinitrophenylhydrazone in 0.5 *N* NaOH. The absorption peak at 540 mμ is proportional to concentration in the range of 0.5–6.0 μg/ml (Fig. 2). Under the conditions which are used, the molar extinction coefficient is 10.24×10^3.

The reaction conditions which were chosen are optimal. Reaction times shorter than 20 min will give variable results. Slight changes in sulfuric acid concentration do not appreciably affect the net yield of levulinic acid 2,4-dinitrophenylhydrazone. An increase in the amount of 95% ethyl alcohol will decrease the sensitivity. A concentration of NaOH lower than

0.5 N decreases the sensitivity of the method. A higher concentration of NaOH will increase the sensitivity, but the color stability decreases. When 1 N NaOH is used, the loss of color is so rapid as to make the method impracticable. In 0.5 N NaOH the color fades slowly at room temperature. Therefore the optical density must be determined within 20 min after extraction by alkali.

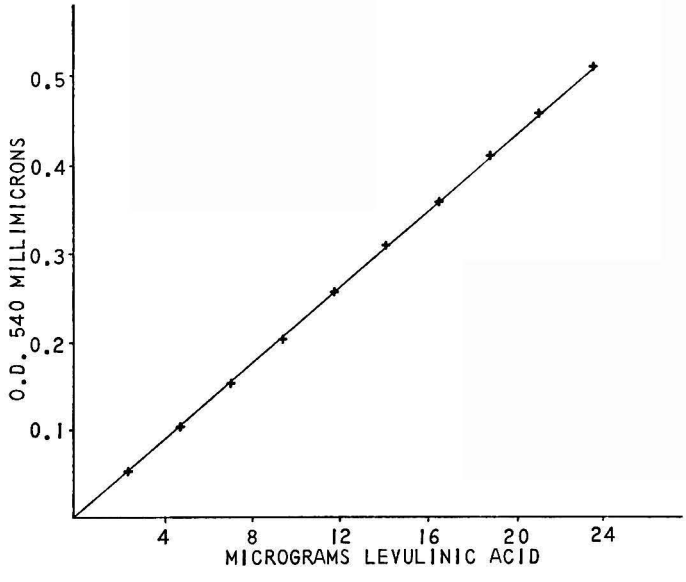

FIG. 2. Standard curve for levulinic acid 2,4-dinitrophenylhydrazone plotted in micrograms of levulinic acid used against the optical density measured at 540 mμ in a 1.0-cm cuvette in a Beckman DU spectrophotometer.

DISCUSSION

The method described in the present communication is the most sensitive one for determining levulinic acid that has ever been recorded. Since under the same conditions other keto acids will react with 2,4-dinitrophenylhydrazine as well, and the resulting 2,4-dinitrophenylhydrazones are also extractable by alkali (4), all the keto acids which are possibly present in the sample must be removed before the determination of levulinic acid can be made.

A disadvantage of this method is that it does not yield the levulinic acid 2,4-dinitrophenylhydrazone when aqueous solutions of levulinic acid are employed. Attempts were made without success to form the 2,4-dinitrophenylhydrazone in aqueous solution.

SUMMARY

A method for the determination of levulinic acid in 2.5- to 25-microgram quantities is presented. The method includes the formation of levulinic acid 2,4-dinitrophenylhydrazone in benzene in the presence of H_2SO_4, and the extraction of the resulting 2,4-dinitrophenylhydrazone by 0.5 *N* NaOH. The optical density of the alkaline layer at 540 millimicrons is then determined.

REFERENCES

1. Boinon, R., *Ing. chim.* (Milan) **27,** No. 161, 8 (1945).
2. Staub, M., *Mitt. Lebensm. u. Hyg.* **35,** 59 (1944).
3. Ploetz, T., and Bartelo, H., *Ber.* **74B,** 1456 (1941).
4. Friedmann, T. E., and Haugen, G. E., *J. Biol. Chem.* **147,** 415 (1943).

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The Separation of Human Serum Lipoproteins by Conventional Descending and Centrifugally Accelerated Paper Chromatography

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INTRODUCTION

In 1958, Michalec and co-workers (1) reported briefly on the separation of serum lipoproteins by paper chromatography but did not give the composition of the developing solution or specific data regarding the nature of the fractions obtained. In a later personal communication with the first author of this paper, Michalec gave the composition of the Veronal–oxalate–citrate buffer solution used, in admixture with isopropyl alcohol (60:40, *v/v*), to develop the chromatograms (2). Using this buffer, which will be referred to hereafter simply as the VOC buffer, the authors have reported preliminary results on chromatographic separations of human serum lipoproteins (3). This article deals more extensively with the paper chromatographic separation of serum lipoproteins using the classical descending technique as well as the newer centrifugally accelerated procedure (4). A phosphate buffer, which is simpler to prepare then the VOC buffer, was used in some of the experiments.

MATERIALS AND METHODS

Serum

The pooled human serum generally represented samples drawn in early morning from approximately 50 unselected patients. For the preparation of ultracentrifugal fractions, the serum was used the same day it was drawn; for chromatography, it was used for several days after it was taken. All samples including those obtained from the ultracentrifuge and prestained samples were stored at 0–3°C. Prestained serum was prepared according to the method of McDonald and Ribeiro (5).

[1] National Science Foundation Cooperative Research Fellow, 1959–60.

[2] Research Assistant.

Ultracentrifugal Preparation of Lipoprotein Fractions

The ultracentrifugal method used was fundamentally that of Havel *et al.* (6). Some modifications in the preparation of the solutions of various densities in which the ultracentrifugation was carried out, were previously utilized by Ray, Davisson, and Crespi (7) and by Bermes (8). The modifications consist of the removal of heavy-metal ions, especially copper, from the stock solution.

The solutions of various densities were prepared as follows: A stock salt solution of density 1.346 was prepared by dissolving 153.0 gm sodium chloride in 1 l water. This solution was extracted with a saturated solution of dithizone (diphenylthiocarbazone, Eastman Kodak) in CCl_4. The stock salt solution was then extracted five times with a total of 500 ml of CCl_4. Traces of the CCl_4 were removed by boiling the salt solution for 30 min. After boiling, it was necessary to readjust the density, the final value of which was checked with a 5-ml pycnometer. The various solvent densities were prepared by dilution of the stock solution with standard distilled water which had been redistilled in an all-glass apparatus. Throughout the ultracentrifugal preparation, all glassware used in the various manipulations was subjected to scrupulous cleaning and was rinsed with the double-distilled water.

TABLE 1

LIPOPROTEIN FRACTIONS OBTAINED FROM THE ULTRACENTRIFUGE

Fraction	Density range	Method
(1) Low density	<1.019	II
(2) Low density, β-lipoproteins	1.019–1.063	II
(3) Combined low density (1) and (2)	<1.063	I
(4) High density, α-lipoproteins	1.063–1.21	I

The density of the serum or serum fraction was then adjusted by pipetting the desired amount of the serum and salt solutions directly into the Lusteroid ultracentrifuge tubes and filling the tube with a salt solution of the final density. Final filling and capping were performed using the technique of Bermes (8). All ultracentrifugations were carried out in a Spinco model L ultracentrifuge employing the No. 40 rotor. The average centrifugal force was 105,400 $\times$ *g*. Temperature in the head chamber was maintained at 10–12°C. After centrifuging for about 18 hr, the floated lipoproteins appeared as a layer on top of the salt solution. The tube was sliced directly below the supernatant lipoprotein concentrate by means of a tube slicer.

Essentially two distinct isolation procedures were followed for preparing the α- and β-lipoproteins. In theory, the fractions obtained should be the same as those of Havel (6).

Method I. To six tubes were added 2.0 ml of the salt solution of density 1.346 and 10.0 ml serum. The tubes were then filled with a solution of density 1.063. After centrifuging for about 18 hr at 40,000 rpm, the combined fractions containing the chylomicrons and β-lipoproteins, density less than 1.063, floated to the top. The α-lipoproteins and the other serum proteins remained in the bottom portion of the tube. The infranatant was adjusted to a density of 1.210 by adding 1.12 gm potassium bromide, recrystallized three times, and 0.15 ml of glass-distilled water for every 4.50 ml infranatant. Repeating the ultracentrifugation once again for 18 hr at 40,000 rpm, the fraction containing the lipoproteins floated to the top and was removed.

Method II. This procedure was carried out simultaneously with Method I. To six tubes were added 2.0 ml of salt solution, having a density of 1.085, and 10.0 ml serum. The tubes were capped, filled with a salt solution of density 1.019, and centrifuged. After removing the lipoproteins, of density class less than 1.019, as supernatants, the solutions in the lower portions of the tubes were pooled. For part II, 9.0 ml of the infranatant from part I was added to 3.0 ml of a salt solution having a density of 1.196.The tubes were capped and filled with a salt solution of density 1.063. After again centrifuging for about 18 hr at 40,000 rpm, the β-lipoproteins floated to the top and were collected. The fractions obtained from both methods I and II are listed in Table I. The fraction labeled *low density* consisted of both β-lipoproteins and, possibly, chylomicrons.

Conventional Descending Paper Chromatography

Whatman 3MM paper was used in practically all the chromatography experiments. Strips or sheets, 43 cm in length, were cut from the stock paper 46 × 57 cm, in a manner such that the direction of development was parallel to the machine or grain direction. The strips or sheets were developed in a chromatography jar, 60 cm in height and 30 cm in diameter. For conventional descending development, the migrant was placed 7 cm from the top end of the paper. Unless otherwise indicated, the chromatographic experiments were carried out at 25°C.

For the early experiments, the VOC buffer (2) was used. It was prepared by dissolving in 1 l of distilled water, 10.3 gm sodium Veronal, 0.04 gm oxalic acid, $(COOH)_2 \cdot 2H_2O$, and 0.61 gm citric acid, $H_3C_6H_5O_7 \cdot H_2O$, to yield a solution having a pH between 8.5 and 8.6 and an ionic strength of 0.06. Later, it was found that a phosphate buffer gave equally good results. It was prepared by dissolving 7.08 gm $Na_2HPO_4 \cdot 12H_2O$ and 0.033 gm $NaH_2PO_4 \cdot H_2O$ in 1 l of distilled later, to yield a solution having a pH between 8.5 and 8.6 and an ionic strength of 0.05. Unless otherwise indicated, the developing solution, i.e., the mobile phase in the chromatographic development, contained buffer–isopropyl alcohol, 60:40, *v/v;* the VOC developing solution, as referred to in the subsequent text, consists of VOC buffer–isopropyl alcohol in the same proportions.

Prior to the application of the migrant, the paper was prewetted with the buffer solution by submerging the paper completely in the buffer and then blotting between two sheets of Whatman 3MM paper. Preliminary experiments had shown that about 40 μl of the ultracentrifuge fractions and/or serum were necessary to give reasonably detectable final Sudan Black B spots. The migrant was applied from a 0.1-ml pipet as a streak across the paper strip.

After development, the chromatograms were dried for 30 min at 105–110°C. They were stained for 1 hr at 40°C in an ethanol solution, saturated with Sudan Black B. The staining solution was prepared by heating 1 l of 60% ethanol to boiling and adding 1 gm of Sudan Black B slowly with stirring. The ethanol solution was cooled to room temperature and filtered twice through Whatman No. 1 filter paper. After the chromatograms were stained, excess Sudan Black B was removed by three rinses in 50% ethanol. The stained chromatograms were dried at room temperature or in an oven for 15 min at 105–110°C.

The bromophenol blue solution used for staining proteins was made by dissolving 2.0 gm bromophenol blue in 190 ml of 95% ethanol saturated with mercuric chloride and 1.0 ml of glacial acetic acid. Chromatograms to be stained for protein were dipped in the bromophenol blue stain solution and then rinsed three times with a dilute acetic acid solution. The solution of dilute acetic acid consisted of 1 ml of glacial acetic acid in 99 ml water.

Ionographic Separation of Lipoproteins

Veronal buffer, pH 8.6, with an ionic strength of 0.05 was used throughout the ionographic experiments. A stock solution with an ionic strength of 0.10 was prepared by dissolving 20.6 gm sodium Veronal and 2.80 gm Veronal in 1 l of distilled water. This buffer was diluted with distilled water to give the buffer solution used in the ionographic experiments.

Ionographic separation of the lipoproteins was done with a Precision Scientific Co.[3] Ionograph, using strips of Whatman 3MM paper, 2.5 cm in width and 40 cm in length. The details of construction and operation of this apparatus have been discussed previously (9). The strips were wetted with Veronal buffer from a 10-ml graduated pipet and allowed to equilibrate for 1 hr with an applied potential of 200 v. The prestained serum, or serum, was streaked across the center of the paper strip and a potential of 5.0 v/cm was applied. With a potential of this magnitude, good separation of α- and β-lipoproteins was attained in 3 hr. The strips were then dried and developed as described in the section on paper chromatography.

Centrifugally Accelerated Paper Chromatography

The apparatus and principles involved in centrifugally accelerated paper chromatography have been described in a recent publication (4). The apparatus used in this investigation was the Chromatofuge, manufactured by Labline, Inc.[4]

Circular sheets of Whatmann 3MM paper, 18 in. in diameter, were used. The paper was prewetted with phosphate buffer. After blotting the paper disk between two sheets of Whatman 3MM paper, it was rotated for 5 min at 200–800 rpm to distribute the buffer on the paper evenly and to remove any excess buffer solution. After application of the migrant, the rotor of the Chromatofuge and the solvent flow were started simultaneously. Chromatograms were developed, for periods between 5 and 30 min, with a solution of VOC or phosphate buffer–isopropyl alcohol, 60:40, *v/v*, as the mobile phase. Drying and staining of the lipoprotein fractions were performed as previously described.

Densitometric Techniques

The scanning device used to measure the color intensity of the Sudan Black B stained chromatograms consisted of a Bausch and Lomb grating monochromator, a Welch Densichron with a blue-sensitive photoelectric probe, a Welch Logarithmic Converter, and a Minneapolis-Honeywell Brown strip-chart recorder. The theory and use of this scanning device have been described elsewhere (10). The blue probe had a maximum sensitivity at 400 mμ. The scanning was performed at a wavelength of 600 mμ, since it was shown that Sudan Black B has a maximum light absorption approximately at that point.

Chromatograms and ionograms were scanned as soon as possible after staining with Sudan Black B. When prestained serum samples were used, the scanning was performed within 2 hr following development. The area under the densitometric recording was measured with a Keuffel and Esser compensating planimeter.

EXPERIMENTAL RESULTS

Conventional Paper Chromatographic Separations

If serum is subjected to paper chromatography using the VOC buffer–

[3] 3737 W. Cortland St., Chicago 47, Ill.

[4] 3070 W. Grand Ave., Chicago 22, Ill.

isopropyl alcohol, 60:40, *v/v*, two fractions, each of which may be stained with Sudan Black B, are obtained. In order to determine the nature of these lipoproteins, a comparison with the known fractions obtained by some other analytical technique had to be made. The ultracentrifugal fractionation procedure was chosen because it yields lipoprotein fractions in comparatively large quantities. Furthermore, the lipoprotein fractions obtained by ultracentrifugation have been carefully studied by means of ionography (11) so that a comparison between paper chromatographic and ionographic lipoproteins could be made.

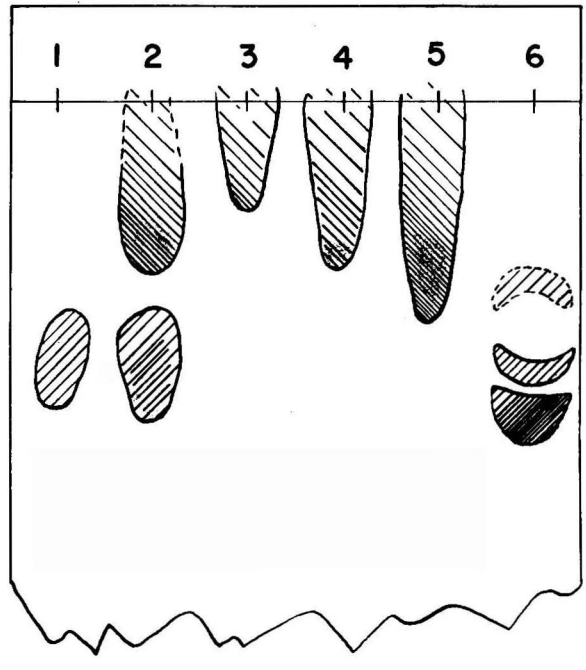

FIG. 1. Chromatographic diagram of lipoprotein fractions separated by ultracentrifugation: (1) 10 μl bromophenol blue; (2) serum; (3) chylomicrons, density fraction less than 1.019; (4) β-lipoproteins, density fraction 1.019–1.063; (5) combined low-density lipoproteins, density fraction less than 1.063; (6) α-lipoproteins, density fraction 1.063–1.21. Developed for 2 hr with VOC buffer–isopropyl alcohol (60:40, *v/v*). Whatman 3MM paper prewetted with VOC buffer.

Figures 1, 2, and 3 illustrate the results of the paper chromatographic studies of ultracentrifugally prepared lipoproteins and serum, using the descending solvent technique described earlier. The figures were prepared by tracing the spots directly from the chromatograms. Each figure represents the upper one-third of one chromatogram with the exception that

certain portions have been omitted to avoid repetition. While these chromatograms represent only one series of experiments, similar results were obtained by repeating both the ultracentrifugal fractionation and the subsequent paper chromatographic separation.

Numbers 1, 7, and 12 on Figs. 1, 2, and 3, respectively, are developed spots of 10 μl of a 0.1% bromophenol blue solution. One spot of bromophenol blue was used on each chromatogram so that a comparison of lipoprotein spots could be made from different chromatograms. R_f values were not determined because the developing solvent front could not be located with sufficient accuracy on the prewetted paper. Since the experimental

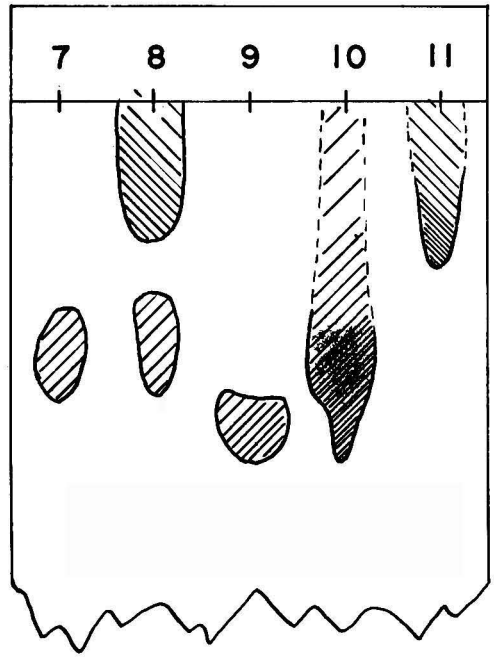

FIG. 2. Chromatographic diagram of lipoprotein fractions separated by ultracentrifugation: (7) 10 μl bromophenol blue; (8) serum prestained with Sudan Black B; (9) α-lipoproteins stained with bromophenol blue; (10) serum, stained with bromophenol blue; (11) β-lipoproteins stained with bromophenol blue. Prepared as in Fig. 1.

data required for R_f calculations were not obtainable, it was decided to compare spots from different chromatograms by comparing the ratio of the distance moved by the migrant divided by the distance moved by a reference substance, bromophenol blue. The R_b value of a particular lipoprotein fraction is defined as the distance moved by the lipoprotein fraction divided by the distance moved by bromophenol blue. The distances were measured

from the origin to the leading edge of the final spots. Relatively large volumes, 10 μl, of the bromophenol blue were used to give an initial spot commensurate with the initial protein spots. The chromatograms for Figs. 1, 2, and 3 were developed under the same conditions so that a comparison of the results might be made.

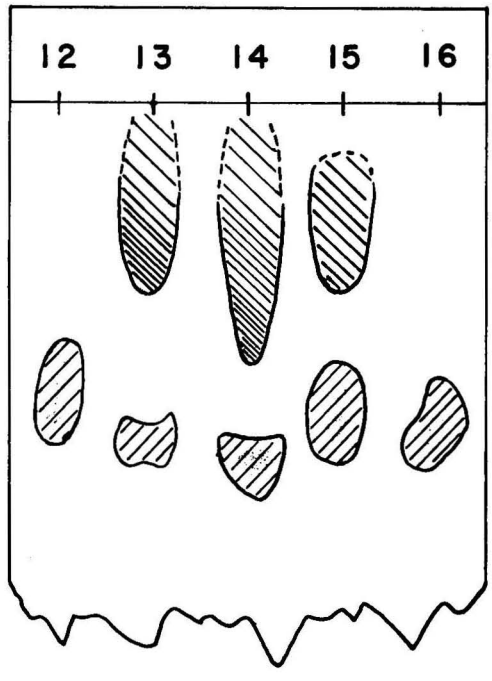

FIG. 3. Chromatographic diagram of lipoprotein fractions separated by ultracentrifugation: (12) 10 μl bromophenol blue; (13) reconstituted mixture of Fractions (1), (2), and (4), Table 1; (14) α- and β-lipoproteins, reconstituted mixture; (15) serum minus Fraction (1); (16) serum minus Fractions (1) and (2). Prepared as in Fig. 1.

The portions numbered 2 and 8 represent the lipoprotein fractions obtained from serum by chromatography. Number 2 is serum which had been stained with Sudan Black B after development, and No. 8 is the pattern resulting from the chromatography of prestained serum. From a comparison of the chromatograms, it was obvious that the two fractions obtained were the same in both cases. When prestained serum was used, the question arose as to whether or not Sudan Black B was being "washed" from the lipoprotein molecules. If that were the case, one spot on the chromatogram of the prestained serum might be the Sudan Black B. That this is not the case was evident because Sudan Black B, spotted and devel-

oped under the same conditions as the prestained serum, did not move from the origin.

Under the Nos. 3, 4, and 5 are the lipoprotein fractions obtained from the ultracentrifuge which represent what many investigators refer to as "low-density lipoproteins." It is apparent that the slower moving lipoprotein component of serum represents the low-density lipoproteins.

Chromatography of ultracentrifugal α-lipoproteins, No. 6, results in three spots. The very faint spot closest to the origin may represent a trace of low-density lipoprotein. The other two fractions which separate during the paper chromatography of the density class 1.063 to 1.21 from the ultracentrifuge do not separate from serum under the same conditions. However, the farthest moving component of serum does correspond to the ultracentrifugal α-lipoproteins. Numbers 13 and 14 are reconstituted mixtures of ultracentrifugally separated lipoprotein preparations. A reconstituted mixture consisted of mixing equal portions of the lipoprotein fractions isolated ultracentrifugally. These mixtures do not contain the same concentrations of the lipoproteins as is found in serum. The α-lipoproteins appear as a single spot. If the chromatogram of the α-lipoproteins is stained for protein with bromophenol blue, No. 9, again only one fraction appears. This seems to indicate that the ultracentrifugal preparation containing the lipoproteins is contaminated with other serum proteins which are not resolved by this chromatographic procedure.

Protein staining with bromophenol blue of a serum chromatogram, No. 10, indicates that practically all of the serum proteins move with the α-lipoproteins.

R_b Values of Serum Lipoproteins

In order to make a more accurate comparison between lipoproteins contained in serum and the corresponding fractions obtained from the ultracentrifuge, a series of experiments were performed to determine the R_b values of these determinations. The standard deviation (S.D.) was calculated from the formula

$$\text{S.D.} = \frac{\sum (x - m)^2}{n}$$

where x is an experimental R_b value and m is the arithmetic mean of the corresponding series of n R_b determinations.

The β-lipoproteins listed in Table 2 in reference to serum chromatograms represent all the low-density lipoproteins. They are represented by the slower moving spot which appears on the chromatogram of serum stained with Sudan Black B. It has an average R_b value of 0.55 for prestained serum and 0.62 for poststained serum. In contrast, the R_b of the low-density lipo-

TABLE 2
R_b VALUES OF LIPOPROTEINS

Lipoprotein		Average R_b	S.D.	Number of determinations
Prestained serum	α	1.03	0.03	6
	β	0.55	0.03	6
Poststained serum	α	1.13	0.05	6
	β	0.62	0.05	6
Ultracentrifugal low density Table 1, fraction 1		0.39	0.03	3
Ultracentrifugal β-lipoproteins, Table 1, fraction 2		0.57	0.04	3
Ultracentrifugal combined low-density lipoproteins and chylomicrons, Table 1, fraction 3		0.74	0.05	3
Ultracentrifugal α-lipoproteins, Table 1, fraction 4	α_1	0.97	0.04	3
	α_2	1.21	0.05	3

proteins isolated by ultracentrifugation is 0.74. The α-lipoproteins on the serum chromatogram have an R_b midway between the two zones which appear on the chromatogram representing the α-lipoproteins which were separated by ultracentrifugation. Both fractions resulting from the chromatography of serum prestained with Sudan Black B had an R_b value slightly less than the corresponding fractions determined on chromatograms of serum poststained with Sudan Black B.

Since the R_b values are determined by measuring to the leading edge of the lipoprotein spot, the initial volume of migrant applied to the paper can influence the value. This was therefore fixed at 40 μl for all lipoprotein solutions used in these experiments. It is well to remember, of course, that either the lipid or protein concentration could possibly influence this value.

Densitometry of Lipoprotein Chromatograms

Densitometric scanning of the chromatographic strips was chosen in preference to the ethyl alcohol–acetic acid extraction of Sudan Black B bound to each fraction (1). Preliminary experiments had shown that complete extraction of the Sudan Black B associated with the lipoproteins was difficult to accomplish. Furthermore, paper fragments remaining in the solution after extraction often interfered with the colorimetric determination of the Sudan Black B.

Figure 4 represents the density distribution on a chromatogram and an ionogram prepared under similar conditions from serum lipoprotein prestained with Sudan Black B. Two main peaks appear in both instances; the peak farthest from the origin represents the α-lipoproteins while that closest to the origin consists of the low-density lipoproteins. In the case of

the low density lipoproteins, the gradual slope on the densitometric recording of the chromatogram is the result of tailing. Similarly, on the ionogram, the color of the Sudan Black B streaks from the origin out to the crest of the low-density lipoprotein peak.

In the range 10–50 μl, the area under the densitometric recording was found to be proportional to the amount of prestained serum applied to the paper. When the chromatograms were poststained with Sudan Black B, instead of using prestained serum, the results were found to be erratic. This can probably be attributed to the relatively high absorbance and uneven background of the poststained chromatograms.

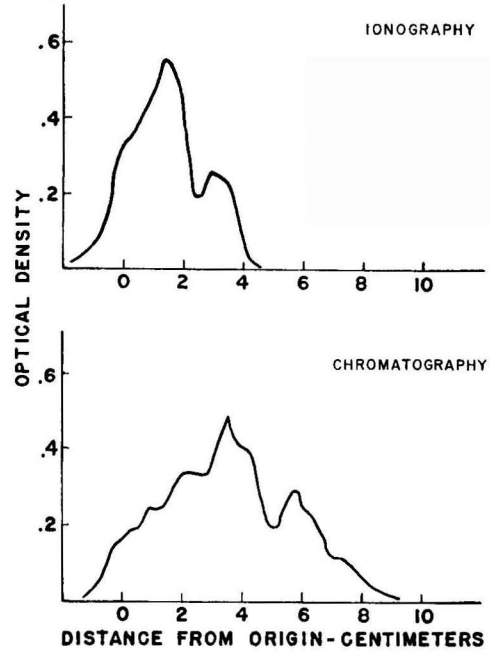

FIG. 4. Densitometric recordings of serum lipoprotein separations. Forty microliters of prestained serum on Whatman 3MM paper. Ionography: Veronal buffer, pH 8.6, ionic strength 0.05, run for 3 hr, 5.0 v/cm. Chromatography: paper prewetted with phosphate buffer, pH 8.6, ionic strength 0.05, developed for 2 hr with isopropyl alcohol–phosphate buffer, 40:60, *v/v*.

Investigators studying alterations in lipoprotein levels often express their results in terms of lipoprotein ratios, e.g., the ratio "β-lipoproteins" to "α-lipoproteins." This is actually the ratio of the area under the low-density peak, β-lipoproteins and chylomicrons, divided by the area under the peak representing the α-lipoproteins (12). In the past, the separations necessary

for these calculations have been obtained by ionography. A series of experiments were carried out to determine the ratio of "β-lipoproteins" to "α-lipoproteins" as obtained by paper chromatography. Chromatograms of pooled serum samples were developed with isopropyl alcohol–VOC buffer, 40:60, *v/v*, and with isopropyl alcohol–phosphate buffer, 40:60, *v/v*. The β to α lipoprotein ratio was found to lie within the same limits as determined by the ionographic procedure (12).

Results from Modifications of the Paper Chromatographic Procedure

In an attempt to increase the resolution of the paper chromatographic method, modifications of the developing solution were made. Preliminary experiments had shown that Whatman 3MM paper was superior to Whatman No. 1 for lipoprotein chromatography. The application of 40-μl samples to prewetted Whatman No. 1 paper resulted in a very large, irregular, initial spot. Also, Whatman 3MM paper possesses a greater wet strength than Whatman No. 1 paper and therefore proved more suitable for handling after the strip had been prewetted with the buffer solution.

Prewetting of the paper was found to be a necessary step in the chromatography of lipoproteins. Experiments performed on dry paper using the ultracentrifugal lipoprotein fractions, and serum, resulted in practically no movement of the fractions which are stained by Sudan Black B. Staining for protein with bromophenol blue revealed a streak of protein extending from the origin to the solvent front. In two experiments where the isopropyl alcohol content of the developing solution was reduced, some movement of the Sudan Black B staining fractions did occur on dry paper, but the extreme irregularity of the spots made interpretation impossible.

Alterations in the isopropyl alcohol content of the developing solutions did not bring about any improvement in the results compared to those obtained with the original developer, the VOC buffer–isopropyl alcohol, 60:40, *v/v*. When the concentration of the isopropyl alcohol was increased to 60% (*v/v*), it was difficult to distinguish the two principal fractions. At 80% isopropyl alcohol, the spots were very irregular. A red color appeared at the leading edge of the spots obtained from prestained serum when the isopropyl alcohol content was in excess of 60%. This red color was probably a fraction of the Sudan Black B which had been extracted from the lipoproteins. The Sudan Black B stain used in these experiments, has been shown to contain several components (13).

If secondary butyl alcohol is used instead of isopropyl alcohol, a two-phase system results with the volume ratio 40:60, secondary butyl alcohol–VOC buffer. Serum chromatograms developed for 2 hr with the lower aqueous phase resulted in no resolution of the lipoprotein components.

Alterations in the ionic strength of the VOC buffer resulted in no definite

change in the lipoprotein patterns. Buffer solutions of ionic strength 0.03, 0.015, and 0.008 were prepared by diluting the VOC buffer. The developing solutions contained the usual 40% by volume of isopropyl alcohol. On the 2-hr serum chromatograms, two lipoprotein spots were resolved. They moved the same distance as when the buffer component of the developing solution had the usual ionic strength of 0.06. The α-lipoprotein spot appeared to be irregular and poorly defined at the lower ionic strengths (0.015 and 0.008).

A series of chromatograms were developed at different pH values. Phosphate buffers with pH values of 5.8, 6.1, 6.4, 6.8, 7.3, 7.7, and 8.0 were prepared according to the directions of Kaplan (14). The chromatographic developing solutions contained 40% isopropyl alcohol and 60% of the various buffers. The prewetted serum chromatograms were developed for 2 hr. In the pH range from 5.8 to 8.0, two lipoprotein fractions appeared on all the serum chromatograms. In general, the approximate distance traveled by both lipoprotein fractions was the same as that obtained for the VOC buffer, at pH 8.6. Chromatography with a developing solution of 40% isopropyl alcohol and 60% citrate buffer, pH 3.8, resulted in no separation of the serum lipoproteins. All material stained by Sudan Black B streaked outward from the origin for about 6 cm. The spot corresponding to the α-lipoproteins as it appeared on the chromatograms for pH 5.8–8.0, was increasingly more uneven and indistinct in the more acid pH values. Increased tailing and general unevenness has been reported at acid pH values in the ionographic determination of serum proteins. This has been attributed to greater protein–paper interaction toward the acid pH values since the cellulose fibers of the paper generally bear a negative charge (10).

Centrifugally Accelerated Paper Chromatographic Separations

Figure 5*a* is a schematic representation of a centrifugally accelerated chromatogram. The part labeled *D* is shown in Fig. 5*b* after developing and staining a 40-μl sample of serum. By running simultaneously aliquots of the fractions 1, 2, and 4 of Table 1 it was evident that the farthest moving spot corresponded to the ultracentrifugally separated α-lipoproteins. Similarly, the spot closest to the origin corresponded to the low-density lipoproteins. While the spot representing the α-lipoproteins is well defined, only the leading edge of the spot representing the low-density lipoproteins could be distinguished readily, and the radial type chromatographic development resulted in a large increase in the final areas of the zones.

To eliminate the lateral spreading or "fanning out" of the migrant zones as they moved outward through the paper disk, striplike bridges or spokes were cut into the paper, as shown in Fig. 6. The "spokes" were cut radially from a point approximately 4 in. from the center to yield a spoke or strip,

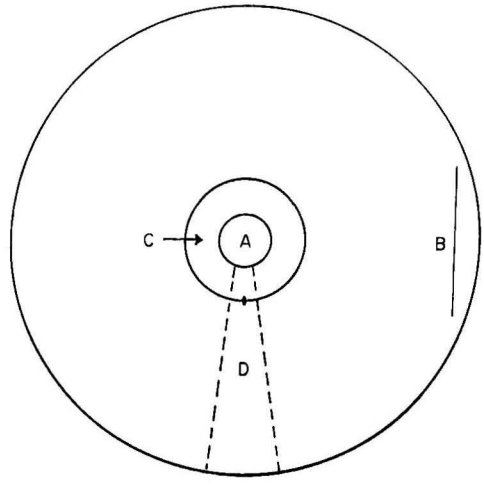

FIG. 5*a*. Schematic representation of centrifugally accelerated chromato circular hole for attachment to rotor shaft of Chromatofuge head. *B*, machine of chromatographic paper. *C*, solvent strikes paper. *D*, area cut out for Sudan Black B staining after development (see Fig. 5*b*).

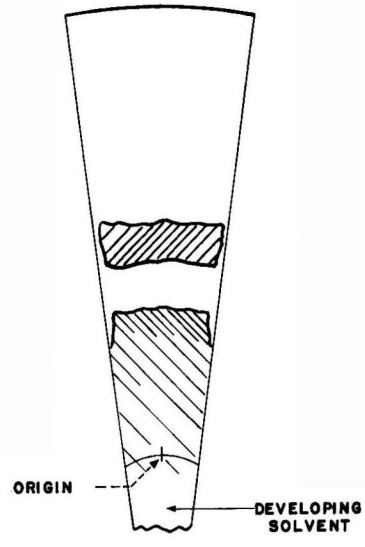

FIG. 5*b*. Centrifugally accelerated lipoprotein chromatographic diagram. Whatman 3MM paper disk prewetted with phosphate buffer. Run for 10 min at 600 rpm. Forty microliters of serum applied. Chromatogram developed for 15 min at 600 rpm; developing solvent, isopropyl alcohol–phosphate buffer, 40:60, *v/v*. Solvent flow rate, 0.92 ml/min.

4 in. in length and 0.5 in. in width, with the medial and distal end of each strip remaining attached to the paper disk.

In the "spoke technique," a 5-μl aliquot of human serum gave a good visible separation of the lipoproteins, but the size of the applied sample had to be increased to at least 10 μl for satisfactory photometric scanning of the paper spokes. A satisfactory solvent flow rate for the phosphate buffer–isopropyl alcohol developing solution was 0.25–0.35 ml/min, and a rotation of 325 rpm was used.

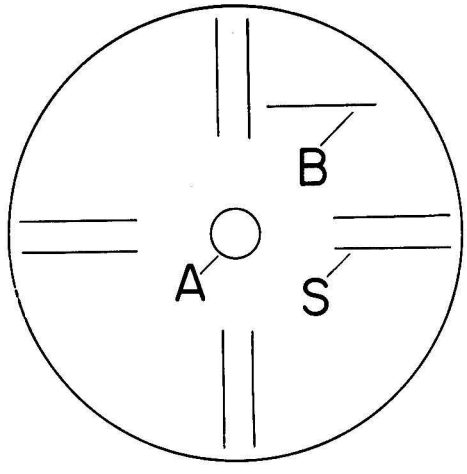

FIG. 6. Schematic representation of spokes cut into paper disk to eliminate fanning out of migrant. *A*, circular hole for attachment to rotor shaft of Chromatofuge head. *B*, machine direction of paper. *S*, paper spokes, 4 in. × 0.5 in.

In later phases of the centrifugally accelerated paper chromatographic separation of lipoproteins, the developing solution employed was ethanol (95%)–Veronal buffer (ionic strength, 0.02; pH, 8.6), 40:60, *v/v*. Currently, the authors are using as a solvent system, ethanol (95%)–phosphate buffer solution (ionic strength, 0.05; pH, 8.5–8.6), 40:60, *v/v*. As in the descending technique, it was necessary to prewet the paper spokes and the area adjacent to them with the buffer solution. Both prestaining and poststaining techniques were utilized.

ACKNOWLEDGMENTS

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SUMMARY

Employing the technique of chromatography on paper prewetted with a buffer solution (Veronal–oxalate–citrate or phosphate), two distinct lipoprotein fractions were resolved from human serum. The developing solution consisted of a mixture of the buffer solution with isopropyl alcohol or ethanol. Since the necessity of prewetting the paper negates the possibility of reporting the chromatographic properties in terms of R_f values, R_b values were introduced. They represent the ratios of the distances moved by a given substance under study divided by the distance moved by a reference substance, bromophenol blue.

The correlation between the lipoprotein fractions obtained by the paper chromatographic technique and those obtained from ultracentrifugal methods was established. On the chromatograms of the lipoproteins, the zone which moved farthest from the origin corresponded to the high-density lipoproteins, the α-lipoproteins. The α-lipoproteins as prepared by ultracentrifugal procedures separated into at least two well-defined spots. Resolution of these two α-lipoproteins by the paper chromatography of serum was not found. The spot closest to the origin corresponded to the low-density lipoproteins.

The limited studies on the effect of alterations in the composition of the developing solvent indicated that resolution of the lipoproteins is probably due in a large part to their lipid composition. Prestaining of the serum gave essentially the same type of chromatographic results as those obtained by the poststaining technique. The paper chromatographic separation of serum lipoproteins was compared with the ionographic method. The ratio of β-lipoproteins to α-lipoproteins as determined by paper chromatography agreed well with the results obtained by ionography.

Centrifugally accelerated paper chromatography was adapted to the separation of serum lipoproteins. The paper was prewetted with Veronal, Veronal–oxalate–citrate, or phosphate buffer solution and developed with mixtures of these buffers with isopropyl or ethyl alcohol. The radial type of development on the whole circular sheet caused the migrant to "fan out," yielding developed spots of an arc-type shape, which made ordinary densitometric scanning difficult. By cutting "spokes" into the paper sheet, the fanning out of the migrant zones as they moved radially outward from the point of origin was circumvented. The technique was found to be rapid and practical, and yielded chromatograms, especially of serum prestained for lipoproteins, of high quality, which were readily scannable.

REFERENCES

1. MICHALEC, C., STASTNY, M. AND NOVAKOVA, E., *Naturwissenschaften* **45**, 241 (1958)
2. MICHALEC, C., Laboratory of Proteosynthesis, Prague, Czechoslovakia; Letter dated November 5, 1958.

3. McDonald, H. J., Banaszak, L. J., and Kissane, J. Q., *Clin. Chem.* **5,** 270 (1959).
4. McDonald, H. J., Ribeiro, L. P., and Banaszak, L. J., *Anal. Chem.* **31,** 825 (1959).
5. McDonald, H. J., and Ribeiro, L. P., *Clin. Chim. Acta* **4,** 458 (1959).
6. Havel, R. J., Eder, H. A., and Bragdon, J. H., *J. Clin. Invest.* **34,** 1345 (1955).
7. Ray, B. R., Davisson, E. O., and Crespi, H. L., *J. Phys. Chem.* **58,** 841 (1954).
8. Bermes, E. W., Jr. Ph.D. dissertation, Loyola University, Chicago, 1959.
9. McDonald, H. J., Bermes, E. W., and Spitzer, R. H., *Federation Proc.* **14,** 733 (1955).
10. McDonald, H. J. "Ionography; Electrophoresis in Stabilized Media." Year Book Publishers, Chicago, 1955.
11. Pezold, F. A., deLalla, O. F., and Gofman, J. W., *Clin. Chim. Acta* **2,** 43 (1957).
12. Wilcox, A. A., Wertlake, P. T., Haley, M. I., and Peterson, J. E., *Proc. Soc. Exptl. Biol. Med.* **98,** 718 (1958).
13. Bermes, E. W., and McDonald, H. J., *Arch. Biochem. Biophys.* **70,** 49 (1957).
14. Kaplan, N. O., "Methods in Enzymology," Vol. 1, p. 143. Academic Press, New York, 1955.

ANALYTICAL BIOCHEMISTRY 1, 60–65 (1960)

A Photoelectric Method for the Measurement of Spectra of Light Sources of Rapidly Varying Intensities

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INTRODUCTION

The emission spectra of bioluminescent and chemiluminescent reactions exhibit rapidly decreasing light intensities due to the kinetics of the reactions. Heretofore the photographic technique was required to obtain a reasonably accurate spectrum of a varying intensity light source since the entire spectrum is displayed at one time and the photographic plate is essentially a light integrator. However, the assumption of reciprocity is not too well justified at high and at low light intensities and further the range of intensity that can be measured with reasonable accuracy on a single exposure seldom reaches 10:1 (1).

In this paper a simple technique is described which combines the much higher sensitivity and linearity of the electron multiplier phototube with the integrating "principle" of the photographic plate to measure the relative emission spectra of light sources independent of their time variation in intensity. This relative emission spectrum we shall designate by $f(\lambda)$.

METHOD

The experimental arrangement is shown in Fig. 1. Light from the source, S, after passing through the beam-splitting glass plate, P, is focused by the spherical mirror onto the entrance slit of a spectrometer. The phototube, PMT-1, at the exit slit receives quanta in the wavelength interval $\lambda + d\lambda$. A small fraction of the emitted light is reflected by P onto the face of the second phototube PMT-2. The light source is masked so that the image just fills the entrance slit of the spectrometer and so that its linear dimensions are smaller than those of the photocathode of PMT-2. This is a rather important point since both phototubes should "see" the same effective area of the source. (With chemiluminescent solutions the intensity usually varies over the effective surface of the solution cell due to convection when the reactants are combined.) The electrons collected at the respective anodes of the phototubes are delivered to separate capacitor current-integrating circuits so that the total charge collected by each phototube during the light flash is recorded. At a wavelength setting λ_i the charge in coulombs collected by PMT-1 is given by

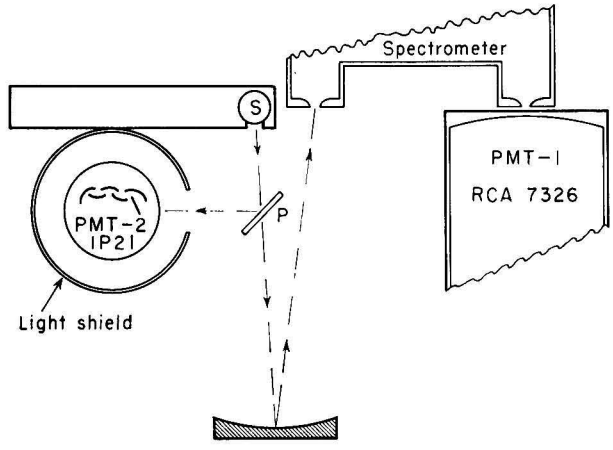

FIG. 1. Schematic drawing of the experimental method of beam-splitting in the charge normalization technique.

$$(q_1)_{\lambda_i} = \int_0^t dt I(\lambda_i,t) G_1 T(\lambda_i) \epsilon_1(\lambda_i) A_1 \times 1.60 \times 10^{-19} \tag{1}$$

where $I(\lambda_i,t)$ is the number of quanta of wavelength λ_i and at time t emitted per second by the source; G_1 is the fraction of the light focused by the mirror; $T(\lambda_i)$ is the transmission of the spectrometer which can be assumed to be flat over the wavelength interval involved; $\epsilon_1(\lambda_i)$ is the spectral efficiency of the photocathode of PMT-1 for a quantum of wavelength λ_i; and A_1 is the gain of the phototube. The total charge collected by PMT-2 is given by

$$(q_2)_i = \int_0^t \int_{\lambda_{\min}}^{\lambda_{\max}} dt\, d\lambda I(\lambda,t) R \epsilon_2(\lambda) A_2 \times 1.60 \times 10^{-19} \tag{2}$$

where R is the fraction of the light reflected by the beam-splitting plate P. Equation (2) can be simplified by separation of variables and by performing the time integration over the entire flash. Then, integrating Eq. (1) and dividing by Eq. (2), we obtain

$$\frac{(q_1)_{\lambda_i}}{(q_2)_i} = \frac{N(\lambda_i) G_1 T \epsilon_1(\lambda_i) A_1}{\int_{\lambda_{\min}}^{\lambda_{\max}} d\lambda N(\lambda) \epsilon_2(\lambda) R A_2} \tag{3}$$

where $N(\lambda_i)$ is the total number of photons emitted in the wavelength interval between λ_i and $\lambda_i + d\lambda_i$. If we now lump together all of the constants of the experiment, recognizing that $\int_{\lambda_{\min}}^{\lambda_{\max}} d\lambda N(\lambda)\epsilon_2(\lambda)$ is just the average efficiency of the phototube PMT-2 multiplied by N_0, the total number of quanta emitted by the source during the measured time interval, we arrive at the final equation

$$\frac{(q_1)_{\lambda_i}}{(q_2)_i} = K \epsilon_1(\lambda_i) \frac{N(\lambda_i)}{N_0} = K \epsilon_1(\lambda_i) f(\lambda_i). \tag{4}$$

Here the subscripts i are retained to indicate that this ratio is to be computed at each wavelength setting λ_i, and $f(\lambda_i)$ is the desired relative emission spectrum.

APPARATUS

The capacitor current integration circuits were the same as those used in previous measurements of the quantum yield of firefly bioluminescence (2). By placing the input capacitor in the negative feedback loop of a d.c. amplifier, the voltage change across it is never more than about 0.1 v so that the charging of the capacitor does not affect the phototube anode voltage to more than 0.1%. The circuit permits variation of either the voltage applied to the phototube or the size of the input capacitor used. There is a sensitive relay at the output of the VTVM portion of the d.c. amplifier which shorts out the input capacitor when the charging voltage reaches 10 v and an electrical message register then tallies the number of times this discharge has occurred. This further extends the useful range of the circuit and has the advantage that for most measurements only one capacitor need be used over all integrated intensities, thus increasing the precision. The instruments have separate settings with resistors in place of the input capacitors so that current measurements can also be made.

The spectrometer was an $f/3$ grating instrument used in first order. The dispersion was 20 A/mm at the exit slit. PMT-1 was an experimental model of the RCA 7326 phototube with an S-20 response. PMT-2 was a conventional RCA 1P-21 phototube.

SPECTRAL MEASUREMENTS

1. Chemiluminescence of Luminol (3-Aminophthalhydrazide)

One of the important criteria in the determination of the emission spectra of chemiluminescent solutions is that the concentration of the reactants be low so that the spectrum will not be distorted by self-absorption. In those instances where the quantum yield of chemiluminescence is high as in the case of the firefly (2), the problem is not so severe since relatively low concentrations of reactants will yield appreciable light. However, in the case of luminol in aqueous solution the quantum yield is low, of the order of several times 10^{-3}, and one must be careful to work with as dilute a solution as practicable. In the present case concentrations of approximately 10^{-5} M of luminol in 0.1 N sodium hydroxide were oxidized by the injection of small amounts of hydrogen peroxide and sodium hypochlorite. The light flash was just visible in a darkened room and lasted no more than 1 or 2 sec. It was necessary to repeat this procedure several times for each wavelength setting in order to obtain a reasonable precision. The control phototube PMT-2 always obtains sufficient light since the light is not dispersed before striking its photocathode. The chemiluminescence spectrum of luminol is shown in Fig. 2, together with the fluorescence spectrum of luminol at pH 7.5. The coincidence of the chemiluminescence and the fluorescence spectra would indicate that the unreacted molecule, rather than the oxidized product, is the light emitter since the final products are not fluorescent. This point has been discussed by Pringsheim (3) and by Spruit and Spruit-Van Der Burg (4). The peak positions agree almost exactly with the earlier data of Spruit and Spruit-Van Der Burg.

2. *Chemiluminescence of Hydrogen Peroxide in the Presence of Chlorine*

In the course of preliminary measurements of the quantum yield of the luminol chemiluminescence, it was found that the mixing of hydrogen peroxide and sodium hypochlorite themselves gave rise to a very small amount of light. Thus far, the following facts have been established qualitatively: (*a*) H_2O_2 + NaOCl give rise to a concentration-dependent red light-emission; (*b*) the efficiency of the reaction is increased in acid solution; (*c*) the light emission appears to be temperature dependent, becoming less intense as the temperature is increased; (*d*) the reaction H_2O_2 + Cl_2 gives rise to the same red chemiluminescence.

The rather sharp band spectrum of this red chemiluminescence is shown in Fig. 3*a*. The light consisted of a rapid but weak flash each time NaOCl

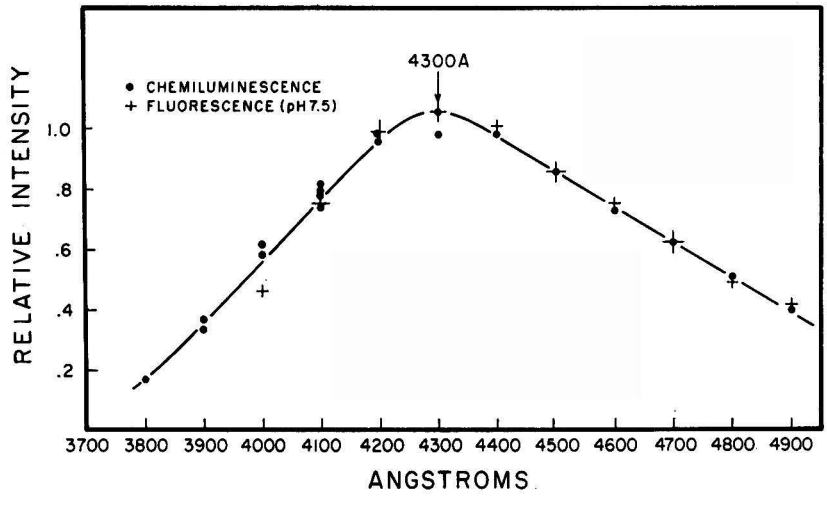

FIG. 2. Chemiluminescence and fluorescence spectrum of luminol.

was added to the H_2O_2 solution. The duration of the light flash was approximately 0.1 sec. Again it was necessary to repeat the reaction several times at each wavelength setting in order to obtain sufficient total light. It was also necessary to widen the spectrometer slits somewhat in order to increase the sensitivity. The effect of the slit width on the spectral resolution is shown in Fig. 3*b* which is a scan of the 5461A line from a low-pressure mercury discharge tube. The close agreement of the different points at each wavelength setting indicates the precision of the technique for even this extreme case of intensity variation.

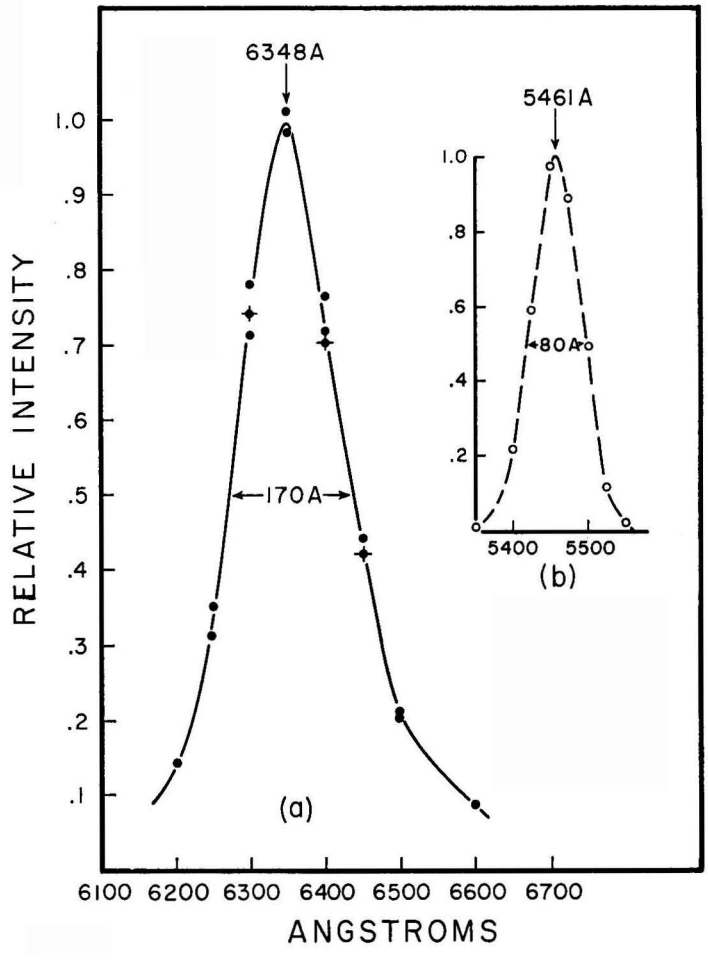

FIG. 3. (*a*) Chemiluminescence of the hydrogen peroxide–chlorine reaction; and (*b*) the effect of slit width on the resolution of the mercury 5461A line.

DISCUSSION

The charge normalization technique described above should have wide application in the measurement of spectra of intermittent light sources in chemiluminescence and in flame spectroscopy where a great deal of time and effort has been expended in devising experimental procedures to give constant light intensities. The phototube in general has higher sensitivity, greater spectral range, and gives much higher precision than the photographic plate.

It must be noted that while the application of this combination of current

integration and beam-splitting normalization for spectral measurement appears to be new, techniques based on one or the other of these principles have been used for some time. A rate-of-charge balance technique for precise measurement of currents in ionization chambers was used by Taylor (5) in 1931. More recently, the author has used this technique with ionization chambers (6) and with phototubes (2). Current integration has also become rather standard in astronomical photometry (7, 8). A variation of the normalization method was used several years ago in the gamma-gamma coincidence-counting standardization of cobalt-60, (9) as a control over possible time variations in the counting efficiencies of the different counting channels. Several recording spectrophotometers use a variation of the normalization technique by splitting a light beam at high speed alternately through the sample cell and the reference cell and electrically measuring the ratio of the two transmission intensities. The charge normalization technique is thus a logical extension of this principle.

The chemiluminescence spectrum of the hydrogen peroxide–chlorine system has not been reported previously. More detailed reports on quantum yields and spectra of chemiluminescence and fluorescence of luminol and the peroxide system will be reported in appropriate journals.

ACKNOWLEDGMENTS

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SUMMARY

A method is presented for the measurement of spectra of light sources of rapidly varying intensities using phototubes in a charge-normalization technique. The method is applied to the luminol chemiluminescence and to a newly found chemiluminescence of hydrogen peroxide and chlorine.

REFERENCES

1. GAYDON, A. G., *in* "The Spectroscopy of Flames," p. 32. John Wiley and Sons, New York, 1957.
2. SELIGER, H. H., AND MCELROY, W. D., *Arch. Biochem. Biophys.* **88,** 136 (1960).
3. PRINGSHEIM, P. *in* "Luminescence of Liquids and Solids," p. 33. Interscience Publ., New York, 1946.
4. SPRUIT, C. J. P., AND SPRUIT-VAN DER BURG, A., *in* "The Luminescence of Biological Systems" (F. H. Johnson, ed.), p. 107. Amer. Assoc. Adv. Sci., Washington, D. C. 1955.
5. TAYLOR, L. S., *Radiology* **17,** 294 (1931).
6. SELIGER, H. H., AND SCHWEBEL, A., *Nucleonics* **12,** 54 (1954).
7. GARDINER, A. J., AND JOHNSON, H. L., *Rev. Sci. Instr.* **26,** 1145 (1955).
8. WEITBRECHT, R. H., *Rev. Sci. Instr.* **28,** 883 (1957).
9. MANN, W. B., AND SELIGER, H. H., *J. Research Natl. Bur. Standards* **50,** 197 (1953).

ANALYTICAL BIOCHEMISTRY 1, 66–77 (1960)

A Fractionating Column for Analysis of Nucleic Acids[1]

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INTRODUCTION

We describe in this paper a column that is proving useful for the separation and identification of several natural and derived nucleic acids. The materials composing the column are diatomaceous earth and serum albumin, the latter made basic by esterification with methyl alcohol. These materials were used for a similar purpose but in a somewhat different way by Lerman (1).

As far as we have determined, all natural nucleic acids attach to our column material, presumably by salt linkages, from neutral solutions that are 0.4 *M* or less in sodium chloride. They can be eluted again, apparently unchanged, at higher salt concentrations that are characteristic for the nucleic acid. Our column is thus similar in principle to the histone columns described by Brown and Watson (2) and by Brown and Martin (3), but is composed of more expendable materials.

MATERIALS AND METHODS

Preparation of Nucleic Acids

We have studied only the nucleic acids of bacteriophages T2 and T4 and of *Escherichia coli*. These are prepared as follows.

The phages are collected without acid precipitation, which tends to denature some of their deoxyribonucleic acid (DNA), and are purified by filtration through Celite and fractional centrifugation (4). They are suspended at a concentration of 2×10^{12}/ml in in a solution 0.1 *M* in NaCl and 0.1 *M* in phosphate buffer of pH 7.1, chilled, and shaken for 2 min in a conical screw-capped tube with an equal volume of recently distilled, water-saturated phenol (5, 6). A mechanical shaker is used giving 240 rectilinear excursions/min, amplitude 9.2 cm, in which the tube is held parallel to the line of motion. At the DNA concentration specified, shaking for 30 min produces no appreciable breakage (recognizable as described below); at lower concentrations breakage is very rapid.

[1] Aided by a grant (C2158) from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service.

[2] Present address: Stanford Research Institute, Menlo Park, Calif.

The shaking with phenol completely dissolves the materials composing the phage particles. Brief centrifugation at about 2000 rpm suffices to separate two layers, of which the lower is discarded with a capillary pipet. The phenol extraction may be repeated, but this is unnecessary for most purposes. The dissolved phenol is removed by manual shaking with three to five changes of ethyl ether (distilled over ferrous sulfate and preserved in the cold with water and a trace of ethanol added). The DNA solution is kept cold during the extractions. Residual ether is removed as completely as desired by bubbling a stream of air through the solution. The resulting solution is clear and viscous and contains 0.4 mg DNA/ml, or virtually all of the DNA of the phage. It contains only a fraction of 1% of the protein (labeled with radiosulfur) of the starting phage, which cannot be reduced below 0.1% by repeated extraction with phenol. About 5% or less of the absorption of ultraviolet light by the solution is due to acid-soluble materials, which can be removed by dialysis. The solutions are stable in physical and chromatographic properties on storage, and can be safely transferred by pipet. When diluted, however, they should be transferred only by pouring to avoid degradation by shear (7). At 4 μg/ml, for example, reasonably careful transfer with a 10-ml serological pipet breaks about half of the DNA.

DNA labeled with radiophosphorus is prepared from labeled phages with additional precautions to avoid radiation damage (see below). At concentrations of about 10 μc/ml, very appreciable damage accumulates over a period of 2 weeks, recognizable by failure of the damaged DNA to pass our column. At much lower concentrations the labeled DNA is stable at the specific radioactivities (0.2 mc/mg P or less) appropriate to most tracer experiments.

In contrast to the simple procedure effective with the phages, bacterial nucleic acids are exceedingly difficult to extract in good yield and condition. This fact does not signify any special status of bacterial nucleic acids *in situ*, because if labeled phages are mixed with unlabeled bacteria, the labeled DNA is equally difficult to extract.

The following procedure gives virtually quantitative recovery of all the nucleic acids of *E. coli* in tracer amounts. Washed bacteria, labeled with radiophosphorus, are combined with other materials to give the following amounts per milliliter in the buffered saline mentioned above: bacteria, 5×10^8; phage T2 or T4, 10^{12}; sodium dodecyl sulfate, 10 mg. This mixture is extracted twice with phenol and then with ether as described before. The phages serve to produce a viscous extract which can be safely shaken. The extract, containing 60–85% of the labeled bacterial phosphorus, yields on chromatographic analysis DNA, soluble ribonucleic acid (RNA), and ribosomal RNA in the proportions 8:22:70, measured as radiophosphorus, and an amount of acid-soluble phosphorus about equal to that of the soluble RNA. About 80% of the extracted phosphorus passes through the column. If the detergent is omitted in this procedure, the yields of RNA are not affected but the DNA, though probably present in the extract, fails to pass the column.

Larger amounts of bacteria extracted in the manner described yield soluble RNA and DNA in the expected proportions but virtually no ribosomal RNA, which is evidently discarded with the copious precipitate of insoluble material.

Ribonucleic acids can be prepared at higher concentrations by extraction of bacteria with hot phenol (8).[3,4] We mix a suspension of washed bacteria at a concentration of 5×10^{10}/ml in buffered saline (0.02 or 0.2 *M*, pH 7.1) with an equal volume of water-saturated phenol at room temperature and then quickly place the tube containing the mixture in a water bath at 60°C, where the emulsion is stirred mechanically for 10 min.

[3] Vielmetter, W., personal communication, 1959.

[4] Cocito, C., and Hershey, A. D., work in progress, 1959.

After chilling, the suspension is centrifuged for 10 min at 10,000 rpm and the phenol layer is discarded. The remainder, consisting of a clear aqueous phase and a solid phase, is again treated with hot phenol and again centrifuged. This time only the aqueous layer is recovered. The latter is heated a third time with phenol and washed with ether to yield a solution of low viscosity containing 40% or more of the total bacterial phosphorus. Of this, 90% will pass through the column and is composed of soluble RNA and ribosomal RNA in the ratio 20:80 and an amount of acid-soluble phosphorus roughly equal to that contained in the soluble RNA. RNA is therefore extracted almost quantitatively, except for mechanical losses, and shows similar chromatographic properties to the materials extracted at low temperature. DNA is denatured and very little of it is extracted under these conditions (as shown by tests with added phage), and no alkali-stable, acid-insoluble phosphorus of bacterial origin can be recovered from the column. Evidently phenol lowers the denaturation temperature of DNA.

Preparation of the Column

Kieselguhr is purchased in the grade sold as "Hyflo Supercel" by the Johns-Manville Products Corporation, New York City. "Fraction V" bovine serum albumin powder, "for microbiological use," is purchased from the Armour Laboratories, Chicago, or from the Nutritional Biochemicals Corporation, Cleveland. The esterified albumin is prepared as follows (9).

Suspend 5 gm albumin in 500 ml absolute methyl alcohol and add 4.2 ml of 12 *N* hydrochloric acid. The protein dissolves and eventually precipitates again. Allow the mixture to stand in the dark for 3 days or more with occasional shaking. Collect the precipitate in 250-ml centrifuge bottles, and wash twice with methyl alcohol and twice with anhydrous ether in the centrifuge. Evaporate most of the ether in air and then *in vacuo* over KOH. Reduce the material to a powder and store it over KOH. Failure to remove promptly the residual acid reduces the basicity of the final product. The material is readily soluble in water and is stable either in solution or in the dry form. Reproducible preparations are easily made. The protein is used in the form of a 1% solution in water.

Washed, protein-coated kieselguhr, used for the second layer in the column, is prepared as follows. Boil (to expel air) and cool a suspension of 20 gm kieselguhr in 100 ml of 0.1 *M* buffered saline. Add 5 ml of 1% esterified albumin, stir, and add 20 ml of additional saline. Transfer the suspension to a 2-cm fractionating tube, first forming a pad of paper powder (Whatman standard) on the sintered-glass disk. The column is formed by placing 5–10-ml portions of the suspension in the tube and forcing out the excess saline under an air pressure of 3 lb/sq in. Wash the finished column, which occupies a volume of about 50 ml, with 200–300 ml of 0.4 *M* buffered saline at the pressure stated. Suspend the entire contents of the tube in 125 ml of 0.4 *M* saline and store it in the refrigerator, where it may be kept for weeks.

Buffered salt solutions are prepared to contain 0.05 *M* phosphate buffer appropriate to give a final pH of 6.7. Solutions containing sodium chloride in 0.1, 0.4, 0.6, 1.0, and 2.0 *M* concentrations are useful. Other concentrations (specified in this paper with reference to the sodium chloride only) are conveniently prepared by mixing the two nearest concentrations. The solutions are preserved by adding a few drops of chloroform to each bottle.

The column used for analysis of nucleic acids is composed of three layers in a 2-cm glass tube and is prepared as follows. Boil and cool suspensions of kieselguhr in three beakers: (**1**) 8 gm in 40 ml of 0.1 *M* NaCl; (**2**) 6 gm in 40 ml of 0.4 *M* NaCl; (**3**) 1 gm in 10 ml of 0.4 *M* NaCl. Add 2 ml of 1% esterified albumin to the first beaker, stir, and add 15 ml additional salt solution. Form the first layer of the column on paper powder with

this suspension as already described, and wash down the excess protein into the column with 0.1 *M* saline to avoid contaminating the material to be added next. Add 10 ml of protein-coated kieselguhr to the suspension in the second beaker. This mixture should not be prepared in advance because of the possibility of transfer of protein from the coated to the uncoated kieselguhr. Build up the second layer of the column with this mixture in the same manner as the first and cover the whole with the contents of the third beaker, which serves as a mechanical barrier to the working portions of the column. Promptly wash the column with 150 ml of salt solution of a concentration (to be called the starting concentration) which must be chosen to suit the occasion, as described below. The bed volume of the finished column is about 50 ml, and the liquid displacement volume is about 48 ml.

Load the washed column by passing into it under an air pressure of 3 lb/sq in. about 0.4 mg nucleic acid in 50 ml saline at the starting concentration. We have successfully used twice this amount of phage DNA and considerably larger amounts of RNA but have not determined the maximum permissible loads. Now set up a gradient of salt concentrations (see below), and collect fractions or record transmittance of ultraviolet light and radioactive emission rate of the effluent from the column as required. At the time the gradient is started, reduce the pressure to produce a flow rate of 25 ml/hr. We operate our columns at room temperature and have noticed no bad effects of temperature variation.

The columns may be successfully reused but we prefer to discard them after use.

Concentration Gradients

We produce a gradient of salt concentrations by mixing two solutions in a single mixing vessel. Under these conditions the concentration (C) of salt in the eluate is

$$C = [C_0 + C_f(e^{x/V} - 1)]e^{-x/V}$$

where C_0 = concentration in mixing vessel
C_f = concentration in reservoir
x = cumulative volume delivered less displacement volume of column (48 ml)
V = volume in mixing vessel

We find it convenient to work with the approximately constant gradients obtained when $x/V \ll 1$. Under these conditions

$$C \doteq \frac{C_0 + xC_f/V}{1 + x/V}$$

As an illustration, T2 DNA elutes in the manner shown below when $C_0 = 0.66\ M$, $C_f = 0.84\ M$, $V = 300$ ml, and one collects 3.6 ml/tube.

Principles of Operation

The following principles were worked out for phage DNA which, having an exceptionally high molecular weight, is probably more difficult to handle than most other known nucleic acids. At any rate the procedures found necessary for the one have proved satisfactory for several others.

When phage DNA is passed into a column composed of protein-coated kieselguhr only, the upper portion of the column material cakes and shrinks and liquid invariably begins to flow between the column material and the glass, often nearly to the bottom of the column. This is readily detected by adding a small amount of drawing ink to the incoming fluid. If the channeling is corrected mechanically by tamping the column during

the loading period, successful chromatograms can be obtained but the column virtually closes and the results are usually unsatisfactory. The second layer of our column minimizes these effects, presumably by preventing the DNA molecules from forming cross links from particle to particle of the column material. The second layer does not, however, prevent invisible channels from developing in the bottom layer of the column unless certain other conditions are met. These, insofar as we understand them, are as follows.

First, the middle layer of the column must stop all the DNA at the time of loading. This means that, with other variables held constant, the initial salt concentration must not be too high.

Second, we found empirically that if the initial salt concentration is too low some channeling is likely to occur. For instance, T2 DNA, which we usually pass into the column from 0.66 *M* salt solution, could at some times but not at others be run successfully under the gradient starting at 0.4 *M*.

Third, to obtain satisfactorily sharp bands the DNA must presumably elute from the middle layer at a somewhat lower salt concentration than it does from the bottom layer. This is so in fact because a full length column having the composition of our middle layer passes DNA in a very broad band eluting at a slightly lower salt concentration than the band obtained by the use of the three-layer column.

Finally, to avoid channeling in the lower layer, DNA must enter it gradually at a salt concentration very close to that at which it moves on. By good luck the second layer material we describe accomplishes these things when the proper starting concentration of salt is employed.

To determine this concentration one forms a half-column consisting of middle layer material only, loads it with DNA at a concentration of salt low enough to permit retention of the DNA, and then determines the lowest salt concentration at which the DNA just begins to elute. This may be done either under a gradient, or more reliably by passing successive 100-ml portions of salt solution, with concentration increments of 0.02 *M*, through the column. A suitable starting concentration is 0.02 or 0.04 *M* less than the initial eluting concentration found in this way. In general, a considerably lower salt concentration will work as well, except for the inconvenience that the DNA will then elute correspondingly later under the gradient. If the salt concentration from which the DNA is loaded onto the column, and the salt concentration at which the gradient is started are to be different, the above considerations apply to both of them.

In spite of these seemingly critical requirements, we rarely experience failures that cannot be accounted for by accidental violation of the stated principles.

The effects of channeling are rather surprising and are not always easy to recognize. We show in Fig. 1 an extreme case observed with DNA that is virtually homogeneous chromatographically. Results due to channeling may vary all the way from such extremes to slight irregularities in elution pattern that can be detected only by a continuous recording of the DNA concentration of the effluent. We attribute all unreproducible irregularities to channeling since we have been able to eliminate them. A valuable test of channeling can be performed by running a mixture of unlabeled DNA from T2 and labeled DNA from T4, which should elute in two bands in the absence of channeling, as illustrated below.

Still another effect of channeling should be mentioned. In a column that channels, the local flow rate and shear stress become very great and mechanical breakage of DNA results. We observed this in the one instance in which we attempted to recover valuable materials after passage through a column that had channeled.

The facts outlined above permit some inferences about the way in which the column

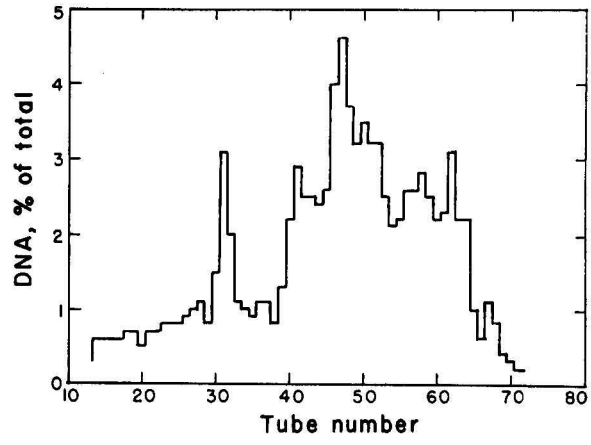

FIG. 1. Elution of T2 DNA under a gradient of increasing salt concentrations from a column composed entirely of protein-coated diatomaceous earth, showing meaningless effects of channeling within the column.

functions. We imagine that for a given species of DNA there exists a critical salt concentration at which the DNA will neither remain permanently attached to the column material nor move freely with the fluid passing through it. At this salt concentration the rate of movement of the band will be determined not only by the properties of the DNA and the amount and basicity of protein in the column material, but also by competition between like and unlike molecules in the band for attachment sites. We shall show below evidence for such competitive effects.

At a grosser level of discernment, however, one can define a salt concentration below which a given species of DNA remains attached to the column and above which it moves freely. This is shown by the fact that T2 DNA elutes at the same salt concentration under a concentration gradient or under batch elution with successively higher salt concentrations. Thus our column is operating near equilibrium and the movement of DNA through the column is not appreciably impeded by mechanical forces.

RESULTS

Phage DNA

We present below three experiments with mixtures of DNA from T2 and T4. In each case the DNA was prepared by extraction from a mixture of the two phages, one labeled with radiophosphorus, the other unlabeled. The important variables are the relative amounts of the two kinds of DNA. Fig. 2 shows a typical elution diagram for DNA from T2. It is skewed owing to self-competition among DNA molecules for attachment sites on the column, which causes the DNA to move fastest in the center of the band, sharpening its leading edge and causing its following edge to trail. (The small amount of material at the front of the band is probably broken DNA,

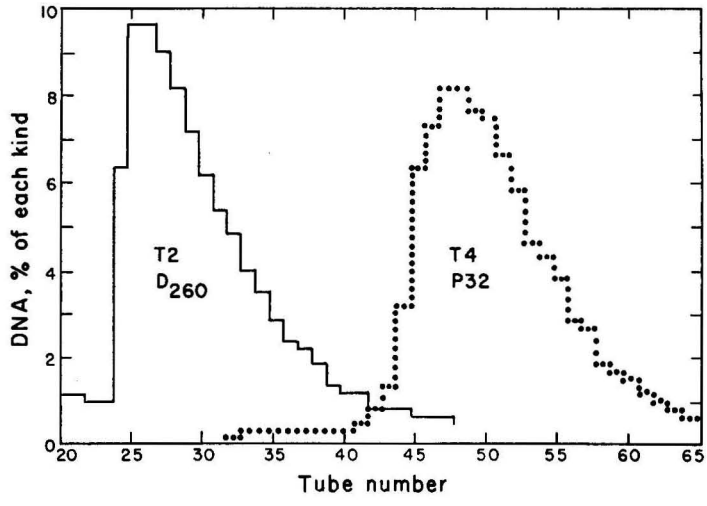

FIG. 2. Elution pattern obtained with a mixture of 0.3 mg of unlabeled T2 DNA and tracer amounts of P^{32}-labeled T4 DNA. NaCl concentration gradient 0.0012 *M* per tube (3.6 ml); concentration at T2 peak, 0.72 *M*. Tubes are numbered from the gradient front.

as shown below.) The labeled DNA from T4 shown in the same figure is well separated from that of T2, first, because T4 DNA has the greater affinity for the column material and, second, because it is present in smaller amount and moves more slowly also for this reason. Also because of the small amount, self-competition in the T4 band is minimal and a relatively symmetrical peak is produced. When run separately in similar amounts, the two kinds of DNA produce bands of identical shape. The recovery of labeled DNA from the column is 90–95% in this and similar experiments.

In Fig. 3 the effect of inverting the relative amounts of the two kinds of DNA is shown. Owing to the competitive effects already discussed, T2 DNA emerges as an exceedingly sharp band at the leading edge of a normal-appearing T4 band.

In Fig. 4 is illustrated the results obtained with an equal mixture of two kinds of DNA. They form what may appear to be a single band, but the distribution of radioactivity shows that the two kinds of DNA are fairly well separated. Since the total amount of DNA here is small, none of the effects of competition are evident. It should be added that mixtures of labeled and unlabeled DNA of the same kind show excellent coincidence of optical density and radioactivity, or only small differences that are negligible compared with the effects illustrated.

These and analogous findings with other materials permit a useful generalization: To achieve maximum separation of two dissimilar nucleic acids,

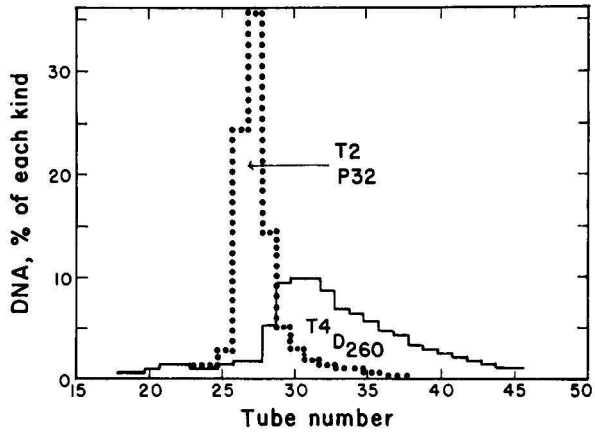

FIG. 3. Elution pattern obtained with a mixture of 0.3 mg of unlabeled T4 DNA and tracer amounts of P^{32}-labeled T2 DNA. Gradient as in Fig. 2.

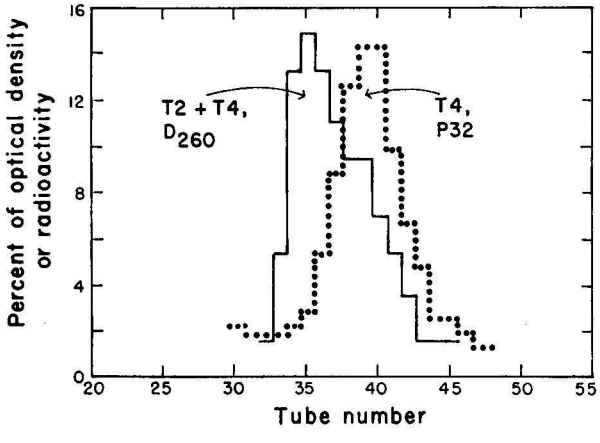

FIG. 4. Elution pattern obtained with a mixture of 0.03 mg each of DNA from T2 and T4, that from T4 only labeled with P^{32}. Gradient as in Fig. 2.

to demonstrate the absence of a common component for example, a mixture containing a large amount of the one that moves faster and a small amount of the one that moves more slowly should be used. It should be noted, also, that the information obtained by the use of differentially labeled mixtures could scarcely be got in any other way.

We are unable to account for the difference in behavior of the DNA's of T2 and T4 unless it is directly related to their slight difference in glucose content (10). Circumstantial support for this hypothesis is gained from our

findings, made in collaboration with Dr. G. Streisinger, that a T2-T4 hybrid having the glucose content of T4 contains DNA chromatographically identical with that of T4, and that a hybrid of intermediate glucose content yields DNA eluting from our column at a salt concentration intermediate between those characteristic of the DNA's of the two parental phages. DNA's from several mutants of T2 or T4 do not differ from those of the parental phages. Likewise, the similar but genetically different lines known as T2H and T2L contain indistinguishable DNA's, as do T4B and T4D.

Protein Content of Phage DNA

As already mentioned, our preparations of phage DNA contain variable traces of acid-insoluble sulfur depending on the care with which they are prepared and on the cleanliness of the starting phage (phenol does not extract previously coagulated protein). In one instance, a single treatment with phenol of a sample of phage representing 5×10^6 counts/min due to radiosulfur, yielded a preparation of DNA containing 5×10^4 counts/min of acid-insoluble radiosulfur. When this sample of DNA was passed through the column, no significant amount of protein radiosulfur eluted with the DNA. (The samples were freed from salt by precipitation with trichloroacetic acid and carrier serum albumin for radioassay.) The few counts recorded in excess of background, if taken literally, would correspond to 0.002% of the radiosulfur of the starting phage in the entire sample of DNA. Evidently the sulfur-containing phage proteins have nothing to do with the behavior of DNA on our column and are efficiently separated from the DNA by it. Column fractionation cannot be used as a primary step in purification, however, because the DNA in crude preparations prepared by osmotic shock will not pass through the column.

Mechanical Breakage

Owing to its high (but still unknown) molecular weight, DNA from T2 and T4 is extremely susceptible to breakage by hydrodynamic shear (7). Our column proves to be useful in measuring such breakage.

As illustration, we present the experiment shown in Fig. 5. Labeled DNA from T2, shaken for 5 min at a concentration of 2.5 μg/ml in 0.2 *M* buffered saline under the conditions described above for preparation of DNA, was mixed with a large excess of carrier DNA and passed through a column. The labeled DNA eluted in two bands, one corresponding to the carrier DNA and one eluting at a lower salt concentration. Material taken from either band and rerun with additional carrier eluted at the position corresponding to that from which it was taken. Labeled DNA diluted in the same manner but not shaken, and then mixed with carrier DNA, did not separate from the latter (Fig. 5*B*). Likewise DNA shaken at a slightly

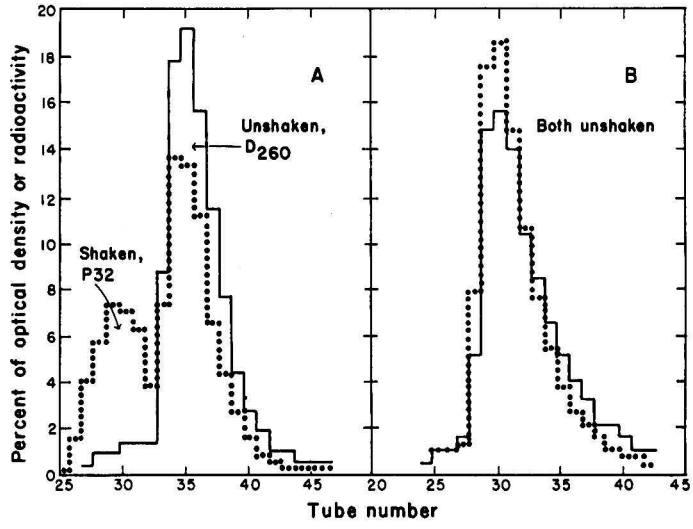

FIG. 5. Breakage of T2 DNA by shaking. *A*. Elution pattern of a mixture of tracer amounts of labeled DNA, previously shaken for 5 min at 2.5 μg/ml in buffered saline, and 0.3 mg of unlabeled DNA. *B*. An otherwise identical unshaken control.

higher concentration (5 μg/ml) was not altered. As will be shown in detail elsewhere, the component produced by shaking consists of broken molecules, and the susceptibility to breakage is strongly concentration-dependent, being greater the lower the concentration.

Evidently the protective effect of high concentrations explains why our standard DNA preparations are not already broken in the manner shown. In point of fact, such breakage may account for the small and variable amount of DNA in all our preparations that elutes ahead of the main band (e.g., Fig. 2). It should be added that the effects of shaking are much more severe in the presence of phenol or chloroform than in their absence: Whereas a concentration of 3 or 4 μg/ml is sufficient to protect against breakage when the DNA is shaken in saline under our conditions, a concentration greater than 40 μg/ml is necessary in the presence of an equal volume of a phenol phase.

The obvious questions, whether our DNA has been broken already during extraction under optimal conditions and what, in fact, is its molecular weight, are under investigation. At this time we conclude only that our column can detect the effects of breakage.

Denaturation

Heat-denatured DNA from T2 fails completely to pass through our column under the usual salt gradient, or at higher salt concentrations up to

4.0 *M*. For example, labeled DNA from T2 diluted to 1.3 μg/ml in 0.01 *M* saline, heated for 10 min at 90°C, and mixed after cooling with carrier DNA was completely retained (86%) by the column material. The carrier DNA eluted normally.

DNA subjected to radiation damage (presumably the effect of free radicals produced in the surrounding fluid under our conditions) is also retained by the column. For example, a purified phage stock labeled with P^{32} at a specific radioactivity of 1.0 mc/mg P and stored for 35 days at an initial concentration of 1.8 μc/ml showed a surviving phage titer of 50%. DNA prepared at this time and passed through a column was retained to the extent of 67% by the column. Admixed carrier DNA, prepared from a stock more than 1 year old, passed through normally, as had DNA prepared earlier from the radioactive stock. Virtually the same quantitative relation between phage survival and retention of DNA was observed with other preparations. Evidently the killing of a phage particle under these conditions is accompanied by the "denaturation" of a large portion of its DNA.

A similar retention of part of the DNA by the column was seen with phage preparations in which acid precipitation had been used as one step in purification (4), a procedure which we have accordingly abandoned.

These experiments do not distinguish between the effects of intramolecular denaturation and possible intermolecular bonds subsequently formed, although the low concentration of DNA employed in the heating experiments was designed to minimize aggregation. At any rate it appears that passage through our column effectively separates out denatured DNA.

Bacterial Nucleic Acids

For the purpose of the following notes, "soluble RNA" means RNA that does not sediment with ribosomes in suitable bacterial extracts (11) and is completely soluble in *M* NaCl, and "ribosomal" RNA means RNA that does sediment with ribosomes and, after phenol extraction, is insoluble in *M* NaCl.[3]

A mixture of nucleic acids from *E. coli* and from phage T2 prepared in one of the ways already described can be separated as follows:[4] The mixture is passed into a column in 0.66 *M* sodium chloride and the column is washed with the same solvent. The effluent contains low molecular weight materials and soluble RNA. Under a gradient of increasing salt concentration, bacterial DNA will now elute at about 0.70 *M*, phage DNA at about 0.75 *M*, and microsomal RNA at about 0.9 *M*, without admixture. Soluble RNA may be separated from the first effluent by passing it into a second column in 0.4 *M* NaCl, from which the RNA will elute at about 0.5 *M*.

It should be possible to separate all four nucleic acids in a single run

starting with low salt concentrations, but in our trials, with mixtures containing appreciable amounts of phage DNA, channeling invariably occurred. In the absence of DNA, the two kinds of RNA could be adsorbed to a column and separately eluted without difficulty, however.

ACKNOWLEDGMENT

We were greatly assisted by the advice of Dr. Gebhard Koch both concerning the preparation of nucleic acids and the design of our column.

SUMMARY

A readily prepared fractionating column is described that separates several ribonucleic and deoxyribonucleic acids from each other, including the closely similar nucleic acids of T2 and T4 bacteriophages. The column also distinguishes DNA that has been denatured by heat and DNA that has been broken by hydrodynamic shear from the starting material.

Some notes are recorded also concerning the preparation of nucleic acids from bacteria and phage by the phenol method.

REFERENCES

1. LERMAN, L. S., *Biochim. et Biophys. Acta* **18,** 132 (1955).
2. BROWN, G. L., AND WATSON, M., *Nature* **172,** 339 (1953).
3. BROWN, G. L., AND MARTIN, A. V., *Nature* **176,** 971 (1955).
4. HERRIOTT, R. M., AND BARLOW, J. L., *J. Gen. Physiol.* **36,** 17 (1952).
5. GIERER, A., AND SCHRAMM, G., *Nature* **177,** 702 (1956).
6. KIRBY, K. S., *Biochem. J.* **66,** 495 (1957).
7. DAVISON, P. F., *Proc. Natl. Acad. Sci. U. S.* **45,** 1560 (1959).
8. WECKER, E., *Virology* **7,** 241 (1959).
9. FRAENKEL-CONRAT, H., AND OLCOTT, H. S., *J. Biol. Chem.* **161,** 259 (1945).
10. JESAITIS, M. A., *J. Exptl. Med.* **106,** 233 (1957).
11. TISSIERES, A., AND WATSON, J. D., *Nature* **182,** 778 (1958).

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Drift of Drops in Density Gradient Columns

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INTRODUCTION

The fall of a drop of an aqueous test solution in a density gradient column composed of mixtures of water-immiscible organic solvents may be described by two stages. In the first stage, the rate of fall *decreases* gradually, reflecting the approach of the drop to the level of its own density. In the second stage, the rate of fall is *constant,* reflecting a progressive increase in density of the drop. The fall of a drop in the second stage may be referred to as its drift.

A study of factors affecting drift and position of drops in density gradient columns is presented in this paper. The factors studied include size of drop, concentration and nature of solute in drop, nature of solvents used for preparing the density gradient columns, presaturation of components of the columns with water or different salt solutions, and presaturation of the aqueous test samples with mixtures of components of the columns. Also dealt with is a new method for obtaining linearity of gradient. The apparatus and procedures used are based to a large degree on those of Linderstrøm-Lang and co-workers (1–3).

PROCEDURES

Aqueous Solutions of Standard Density

Aqueous solutions of standard density were prepared with sodium chloride instead of potassium chloride (1) because of a projected measurement of partial specific volumes of certain substances in aqueous media containing high concentrations of sodium chloride, and the supposition that it might prove advantageous to have the same salt in standards as in unknowns. The compositions of sodium chloride solutions of standard density at 25°C were determined by interpolation of data supplied by the *International Critical Tables* (4). The solutions were prepared in 100-ml volumetric flasks. Weighings were made with calibrated weights to five places beyond the decimal, and corrections were made for buoyancy. Densities of resulting solutions were then calculated to five places beyond the decimal. The solutions were stored at 4°C under a thin layer of kerosene.

Preparation of Density Gradient Columns

The design of the gradient column tube was that of Hvidt *et al.* (3). The outside diameter of the lower bulb was 5.7 cm; that of the upper bulb, 4.8 cm. The outside diameter of the cylindrical section between the bulbs was 2.8 cm; its length, 20 cm.

Density gradient columns were prepared with the aid of the apparatus shown in Fig. 1. The procedure was based on principles used in continuous gradient chromatography (5). First, a 500-ml Erlenmeyer flask, *C*, used as a mixing vessel, and its outlet tube, *D*, were filled to within 12 ml of the top with the heavier of two solvent mixtures. Next, a further quantity of the heavier mixture, corresponding to the capacity of the lower bulb plus one-sixth of the cylindrical portion of the gradient density column tube, *E*, was introduced into the separatory funnel, *A*, used as a reservoir. The mixture was then allowed to flow into the lower bulb of the gradient

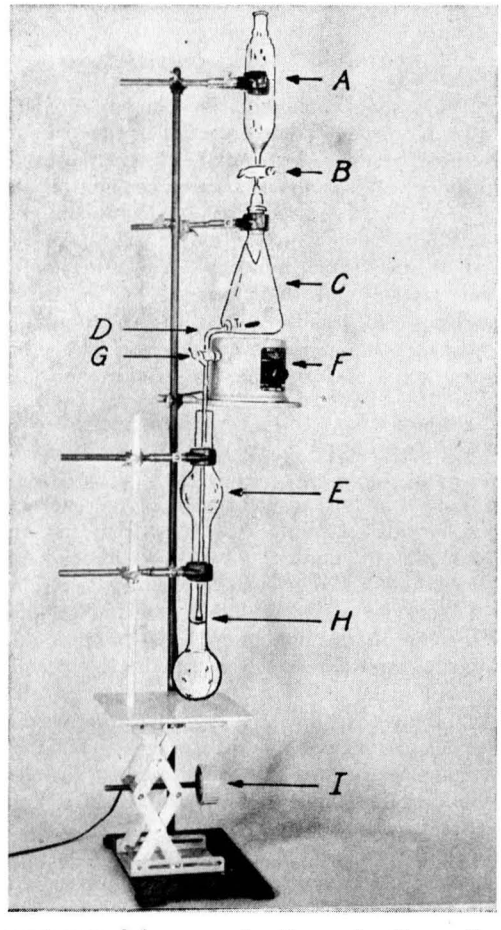

FIG. 1. Apparatus used for preparing linear density gradient columns.

column tube until the level in the separatory funnel came to the stopcock, *B*. At this time a quantity of the lighter solvent mixture, corresponding to two-thirds the capacity of the cylindrical portion of the gradient column tube, was introduced into the reservoir and was allowed to pass slowly into the mixing vessel where it was vigorously stirred with the aid of a magnetic stirrer, *F*, and thence into the gradient column tube. By gradually lowering the jack, *I*, during this process, the level of a sintered-glass plate, *H*, used as a diffuser at the bottom of the outlet tube, was maintained just below the level of the liquid within the gradient column tube. After the reservoir had emptied, flow of liquid from the mixing vessel into the gradient column tube was continued until the level in the tube had risen to within 3 or 4 cm of the top of the stem above the upper bulb. Flow was controlled in the various steps by manipulation of stopcocks *B* and *G*.

The density gradient column with contents was finally placed in a vibration-free bath at 25°C, regulated to ±0.01°C. As will be pointed out later, however, the accuracy of measurements could very likely be improved by closer regulation.

Preparation of Solvent Mixtures

Merck reagent-grade bromobenzene and an odorless preparation of kerosine, Deo-Base, marketed by Howe and French, were the principal solvents used to prepare the density gradient columns. Two mixtures were prepared in each instance. The heavier mixture possessed a density at the upper limit of the range of density desired. The density of the lighter mixture was predetermined as follows: The characteristics of gradients obtainable with the aid of the apparatus shown in Fig. 1 were first determined by substituting aqueous salt solution for the heavier density mixture in the mixing vessel, and water for the lighter density mixture in the reservoir, and measuring conductivities of successive 10-ml aliquots of product emerging from the outlet tube of the mixing vessel. The proper density of the lighter solvent mixture could then be calculated with the aid of the expression,

$$\frac{\Delta k/\text{ml}}{k_2 - k_1} = \frac{\Delta \rho/\text{ml}}{\rho_2 - \rho_1}$$

where Δk/ml represented the slope of the line relating conductivity to volume in milliliters of mixture of salt and water emerging from the outlet tube; k_2, the original conductivity of the salt solution; k_1, the original conductivity of the water; $\Delta\rho$, the desired slope of the density gradient, calculated on a milliliter basis; ρ_2, the known density of the heavier solvent mixture; and ρ_1, the unknown density of the desired lighter solvent. In the present work the range of density used within the columns was 0.02 and the volume of the cylindrical portion of the tube which included this range was 55 ml.

The proportions of bromobenzene and of kerosine which would yield the desired densities for the heavier and lighter mixtures were estimated on the basis of the assumption that no volume changes occurred on mixing. The densities of the mixtures which were obtained were checked with the aid of a Westphal balance, and further adjustments were made if necessary.

Presaturation of components of the column with water or salt solutions was accomplished by agitating the components with the appropriate saturating medium at room temperature (about 25°C) on a tumbling machine, allowing the aqueous and solvent components to clarify, and finally separating the resulting layers. In

tests with dried solvent mixtures, the above washing procedure was first carried out with water and was then followed by drying of the solvent layers over calcium chloride.

Use of Density Gradient Columns

Drops of aqueous test solutions were introduced into the column with the aid of a micrometer syringe equipped with a 2-in. No. 25 needle. The procedure was to insert the tip of the needle below the surface of the column, force out the desired size of drop, and release the drop by withdrawing the needle through the solvent-air meniscus. Before drops were introduced into the column, however, several were first forced out under kerosine in a waste container. If this procedure was not followed, the first drops introduced into the column exhibited higher densities than subsequent drops, due, apparently, to evaporation of test samples at the tip of the syringe needle. For convenience, positions of drops in the column were generally measured at their lower edges as recommended by previous investigators (1–3). Readings were made to ± 0.0001 cm with the aid of a special cathetometer made by Ole Dich, Copenhagen. Drops of aqueous samples after measurement were removed from the column by guiding them with a thin glass rod to the wall of the tube where they readily adhered.

RESULTS

Linearity of Column

The linearity of density gradient columns prepared by the procedure described above is shown by the results in Fig. 2. In this test the component mixtures from which the column was prepared were presaturated with water. Their densities were 0.923 and 1.051, respectively. The drops of solutions of standard density which were used were 1 μl in volume and readings of their heights in the column were made 20 min after entry. The fact that the points shown in Fig. 2 lie fairly close to a straight line can be taken as justification for the assumption of exact linearity in tests where the densities of drops of unknown samples, located between drops of known samples, are to be determined. It was found that linearity was obtained much more dependably by the procedure described herein than by procedures previously described (1, 3, 7).

Characterization of Fall of Drops

The change in rate of fall of a drop having a volume of 1 μl and a density of 1.04295 is shown in Fig. 3. The two stages which characterize the fall are designated on this figure. The linear nature of the second stage of fall (the drift) was confirmed in numerous additional tests, for periods of observation up to 90 min.

Effect of Drop Size

The effect of drop size upon drift and position of drops is shown in

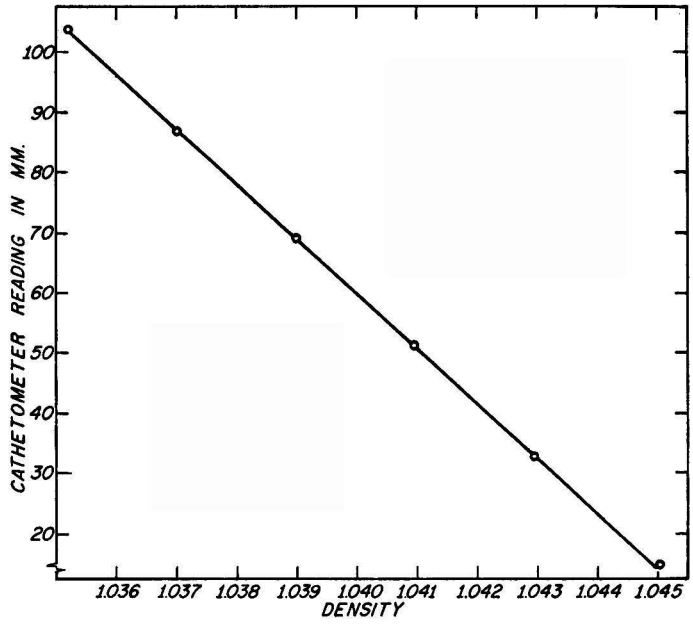

FIG. 2. Linearity of height of drop in column *vs.* density of drop.

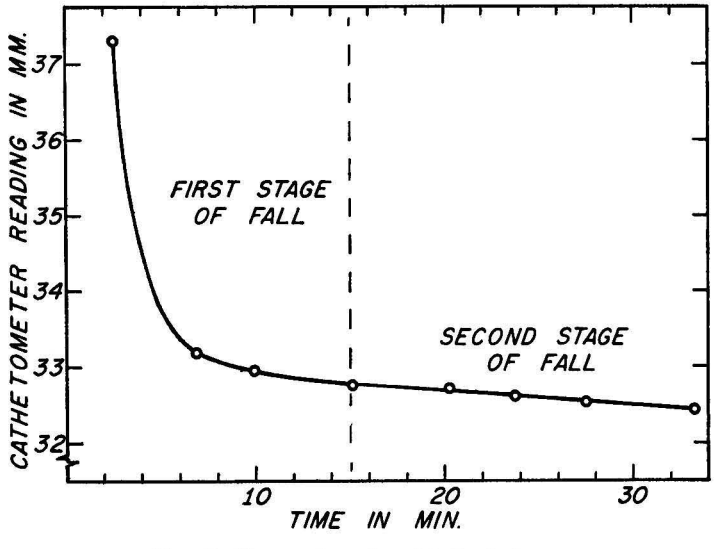

FIG. 3. Change in rate of fall of drops.

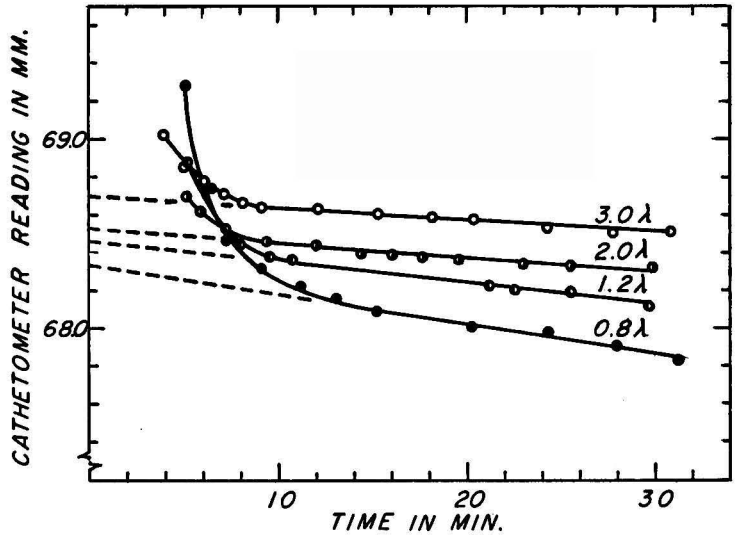

FIG. 4. Effect of size of drop on drift.

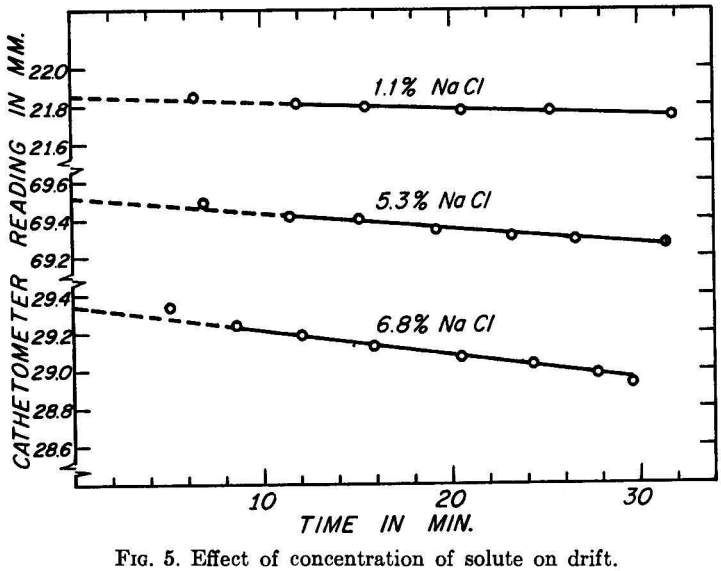

FIG. 5. Effect of concentration of solute on drift.

Fig. 4. The density of the drops used was 1.03905. To permit extrapolation of the data, positions of the drops in these tests were measured at their centers instead of their bottom edges since the centers are more exactly related to the density of the column. The results show that the

drift decreases with drop size and that the height of drops in the column increases with drop size. Extrapolation of the drift data to zero time corrects only partially for the differences observed with drops of different size. The findings were closely substantiated in additional tests. From practical considerations, a drop size of 1 μl appeared best for most work since, on the one hand, it avoids the greater drift encountered with smaller drops, and, on the other hand, avoids crowding within the column which is encountered with larger drops. This size was used in all further studies to be described.

Effect of Concentration of Solute

Results presented in Fig. 5 show that drift rate increases with concentration of solute in the aqueous test samples. When the data of Fig. 5 are used to plot drift rate against concentration of solute and the relation is assumed to be linear, the rate at infinite dilution is about half that at the 1.1% concentration.

Effect of Nature of Solute

Drops of aqueous solutions containing sucrose or salts other than sodium chloride also exhibited drift. Tests were carried out with drops of solutions of the different solutes at equal density (approximately 1.036), introduced into a column which had been presaturated with sodium chloride solution. When the drift rates which were observed were related to that shown by sodium chloride solution at the same density, the results shown in Table 1 were obtained. The reason for the apparent differences observed with different solutes is not known.

TABLE 1
RELATIVE RATES OF DRIFT OF DROPS CONTAINING DIFFERENT SOLUTES

Solute	Relative rate
NaCl	100
KBr	124
KI	68
Na_2SO_4	56
KCl	44
Sucrose	44

Nature of Organic Solvents Used in Column

When columns were made up of mixtures of bromobenzene and xylene, the drift rate was three times that observed with comparable columns made up of bromobenzene and kerosine. Effects of drop size on drift rate

were found to be analogous in the two different solvent systems. Because of the high drift rate encountered with the bromobenzene–xylene mixture, it was not used further.

Effect of Presaturation of Column

When components of the column were saturated with water, then dried over calcium chloride, the drift rate was more than twice as great as when the components were saturated with water but not dried. Drift rate observed when the components were saturated with sodium chloride solution of density of 1.05 was essentially the same as that observed when water alone was used. Attempts to decrease drift by increasing the period of agitating components of the column with water from a few minutes to overnight were without effect, suggesting that equilibrium conditions were reached. When the components of the column were saturated with potassium bromide solution of density 1.03, however, the drift rate was strikingly reduced and amounted to only about one-fourth that observed when water alone was used.

The importance of close regulation of temperature became apparent after drift was reduced by presaturation of components of columns with the potassium bromide solution. A slow oscillation of drops could then be observed which coincided with the period of the temperature-regulating mechanism. Previous investigators (3) obtained excellent reproducibility of drop position by regulation of temperature to $\pm 0.001°C$.

Effect of Presaturation of Aqueous Test Solutions with Components of the Columns

In numerous tests, using drops of different sizes, it was found that presaturation of aqueous test samples with mixtures of solvents such as were used in typical columns had no significant effect on the drift rate.

DISCUSSION

The great convenience as well as the extraordinarily high accuracy of the density gradient column method has been brought out by its original authors (1–3). Drift as a source of error, suggested by the present work, has not heretofore been revealed as a factor of major consequence, however, due to the fact that previous studies were concerned primarily with aqueous test solutions of low solute concentrations, whose drift can now be expected to have been minimal. Drift is considerable at higher solute concentrations and is magnified by the use of drops of small size.

The most likely explanation of drift is the transfer of water from drops to surrounding solvent, which has been referred to as evaporation (6). This is supported by the fact, which can be deduced from Fig. 4,

that drift rate increases as the ratio of surface area to unit volume increases. The present work further indicates that preferential transfer of bromobenzene from solvent mixture to drops of aqueous test samples does not occur to any detectable degree and cannot, therefore, account for the observed phenomenon.

The effectiveness of potassium bromide solution for presaturation of the components of columns as a means of reducing drift has been described previously (1), but the rationale behind the procedure does not appear to have been made clear until the present work. The mechanism whereby the bromide solution should be more effective than the corresponding chloride solution is not known. The fact that a solution of a salt containing the bromine atom is effective for saturation of a solvent mixture of which one of the components also contains the bromine atom may be significant. Tests with columns containing chlorobenzene or carbon tetrachloride as one of the components might yield information on this question.

When presaturation cannot be resorted to, and where aqueous solutions of high density are to be tested, drift should be investigated and, if necessary, allowances made for it. Examples of circumstances where presaturation is undesirable are those involving the measurement of the density of water containing oxygen-18 or deuterium (3, 7). An example of a circumstance where samples of high density are to be tested is the use of the gradient density column method for the measurement of partial specific volume (6, 8) in the special case where solubility or other considerations necessitate the use of media containing high concentrations of salt. Allowance for drift could be accomplished by close regulation of time of reading of position of drop after entry or by extrapolation of observed data to zero time. The present data do not reveal which procedure might be preferable.

ACKNOWLEDGMENT

Kermit Lowry, Jr., Marguerite Donlon and Earl Weiss contributed to this work in its preliminary stages.

SUMMARY

The rate of drift of drops of sodium chloride solution in density gradient columns composed of mixtures of bromobenzene and kerosine increases with concentration of solute and decreases with drop size. Size also affects position of drop, larger drops tending to come to rest at higher levels than smaller drops. Drift is greatest in columns whose components are dried over calcium chloride, is diminished in columns whose components are presaturated with water or sodium chloride solution, and

is nearly eliminated in columns whose components are presaturated with potassium bromide solution. It is not affected significantly by presaturation of aqueous test samples with the mixtures of solvents used in the columns. Drift is also observed with test samples containing solutes other than sodium chloride. Further, it is greater in columns composed of bromobenzene and xylene than in those composed of bromobenzene and kerosine. The significance of these findings as they may affect error of measurement of density is pointed out.

A new method for obtaining linearity in density gradient columns also is described.

REFERENCES

1. LINDERSTRØM-LANG, K., AND LANZ, H., JR., *Compt. rend. trav. lab. Carlsberg, Sér. chim.* **21,** 315 (1935–8).
2. LINDERSTRØM-LANG, K., JACOBSEN, O., AND JOHANSEN, G., *Compt. rend. trav. lab. Carlsberg, Sér. chim.* **23,** 17 (1938–41).
3. HVIDT, A., JOHANSEN, G., LINDERSTRØM-LANG, K., AND VASLOW, F., *Compt. rend. trav. lab. Carlsberg, Sér. chim.* **29,** 129 (1954).
4. "International Critical Tables," Vol. III, p. 79. McGraw-Hill Book Company, New York, 1928.
5. ALM, R. S., WILLIAMS, R. J. P., AND TISELIUS, A., *Acta Chem. Scand.* **6,** 826 (1952).
6. SCHACHMAN, H. K., *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. IV, p. 32. Academic Press, New York, 1957.
7. ANFINSEN, C., *in* "Preparation and Measurement of Isotopic Tracers." J. W. Edwards, Ann Arbor, Mich., 1946.
8. HARRINGTON, W. F., AND SCHELLMAN, J. A., *Compt. rend. trav. lab. Carlsberg, Sér. chim.* **30,** 21 (1956).

ANALYTICAL BIOCHEMISTRY 1, 88–95 (1960)

SHORT COMMUNICATIONS

The Hydroxyproline Content of Porcine Gelatin

A slightly low hydroxyproline content may be expected with certain gelatins that contain a small percentage of a coagulable mucoprotein complex (1, 2) and give rise to substantial humin formation when hydrolyzed by strong acids at 100°C. Acid-processed pig skin gelatins however have generally been regarded as very pure since they normally give colorless solutions, free from haze, and show insignificant amounts of mucoprotein complex under the conditions of Sheppard, Hudson, and Houck (2) and no humin on hydrolysis. Nonetheless, impurities might be expected in pig skin gelatins since Courts (3) has shown that acid processing is not efficient in removing non-collagenous (and hence non-hydroxyproline-containing) material from gelatin.

The only reported amino acid analysis of a porcine gelatin was by Eastoe (4) who found the hydroxyproline content to be 13.5% for an acid-processed pig skin gelatin. No great significance has been attached to this single result. However, the hydroxyproline value for gelatins from bovine collagens is generally much higher, and Eastoe (4) reports the value of 14.4% for a purified bovine hide gelatin. This value appears to represent the highest hydroxyproline content so far reported for gelatins. If pure gelatin is assumed to have a constant hydroxyproline content of at least 14.4%, the value of 13.5% found in the acid-processed pig skin gelatin would indicate the presence of a very substantial amount of impurity.

In order to elucidate this question, we have determined the hydroxyproline contents of five commercial acid-processed pig skin gelatins and of two specially prepared porcine gelatins, from bone by an acid process, and from skin by an alkaline process.

Details of the gelatins examined and the results of tests are shown in Table 1.

Acid-Processed Pig Skin. The normal commercial practice is to equi-

libriate the skin to a pH of about 4 using hydrochloric acid and to extract the gelatin at this pH with water at 50–70°C.

Alkali-Processed Bovine. These gelatins were extracted at a pH near neutrality from hides that had had a prolonged soak in cold, saturated lime water, pH 12.6.

Acid-Processed Bone. Fresh compact bone, taken from the femur shaft, was disintegrated and defatted by repeated washing with cold trichloroethylene. Demineralization was carried out in 5% (*w/w*) HCl in 1 day at 18°. A small proportion of solubilized collagen was removed by extraction in 0.15 *M* citrate buffer, pH 3.7 for 24 hr at 18°. The residue was washed, suspended in water, and adjusted to pH 2.5 with sulfur dioxide. Extraction of gelatin was made at 60° for 2 hr.

TABLE 1
HYDROXYPROLINE CONTENTS OF PORCINE AND BOVINE GELATINS

Sample no.	Source and process	Isoionic pH	Viscosity[b] (cs. at 40°C)	Rigidity[a] (dynes/cm²) at 10°C	Hydroxyproline % of gelatin[b]
97	Pig skin, acid commercial	8.54	5.5	60,000	13.2 (1)[c]
136	Pig skin, acid commercial	8.12	10.7	67,000	13.3 (1)
177	Pig skin, acid commercial	8.41	5.4	65,000	13.1 (3)
149	Pig skin, acid commercial	8.83	11.0	90,000	12.6 (8)
215	Pig skin, acid commercial	7.09	3.4	31,000	13.3 (2)
H.92	Pig bone, acid laboratory	8.40	9.2	90,000	13.6 (3)
J.73	Pig skin, alkali laboratory	4.80	12.6	82,000	14.2 (5)
127	Ox skin, alkali commercial	4.94	8.7	68,000	14.3 (3)
141	Ox skin, alkali commercial	4.98	7.0	67,000	14.1 (1)

[a] Measured on 5.65% gelatin.
[b] Mean values calculated on moisture and ash free gelatin.
[c] Figures in parentheses show the number of determinations made. All values were within ±2% of the mean.

Alkali-Processed Skin. Dried and finely subdivided pig skin was defatted in chloroform–methanol (2:1, *v/v*) and washed in ether. The pretreatment using NaOH in the presence of Na_2SO_4 was carried out as described by Courts (3). Exhaustive treatment (24 hr) at 18°C with citrate buffer removed a soluble fraction. The properties of this fraction will be reported elsewhere. The residue was washed to pH 7 and then heated in water at 60°C for 1 hr to yield gelatin.

The hydroxyproline contents of the gelatins were determined by the method of Neuman and Logan (5) as modified by Leach (6).

The hydroxyproline contents of all acid-processed materials, whether from skin or bone, were as low as or lower than the result of 13.5% reported

by Eastoe (4). The similarity in the contents of hydroxyproline of the gelations from skin and bone is also in line with Eastoe's work on bovine collagen. The low values for porcine materials, however, would appear to result from the manner of processing rather than a species difference since the alkali-processed pig skin gelatin had a hydroxyproline content similar to that of "pure" bovine materials.

Bowes (7) found that the hydroxyproline content of bovine skin increased from 13.1 to 13.9% as a result of soaking at pH 12.6 in sodium hydroxide. This suggested that a large proportion of non-collagen impurities (materials not containing hydroxyproline) was removed into the alkali. At least some of these might be expected to remain and be extracted with the gelatin under the conditions of the acid processing. Courts (8) has shown that acid-processed gelatins, including porcine samples, contain 1–2% mucoprotein, in comparison with alkali-processed gelatins which contain virtually none. Courts (3) has also shown that the alkali processing of connective tissue accounts for the removal of mucoprotein.

Thus, qualitatively, low hydroxyproline values for acid-processed porcine gelatins can readily be accounted for by the presence of non-collagen impurities not removed by that process. Some of the present results however can only be accounted for on this hypothesis by the presence of more than 10% of impurity. This is of similar order to the total proportion removed from skin by Bowes (7). It is however difficult to conceive that so large a proportion of impurity can be present in pig skin gelatins which are apparently pure and form clear solutions over a wide pH range. The chemical composition of the impurity would clearly be very different from that of the coagulable impurities detected by Leach (1) in bovine gelatin which were precipitated at pH 4. Here the level of impurity was always less than 1%.

The alternative conclusion is that the hydroxyproline contents of "pure" gelatins are much more variable than has previously been assumed. It is apparent that a detailed survey of hydroxyproline values is necessary before this method can be used as a valid criterion of purity for mammalian collagens and gelatins. This work is now being undertaken in these laboratories.

ACKNOWLEDGMENT

We gratefully acknowledge the assistance of Mr. W. B. Langston.

REFERENCES

1. LEACH, A. A., *Biochem. J.* **74,** 61 (1960).
2. SHEPPARD, S. E., HUDSON, J. H., AND HOUCK, R. C., *J. Am. Chem. Soc.* **53,** 760 (1931).
3. COURTS, A., *Biochem. J.* **74,** 238 (1960).
4. EASTOE, J. E., *Biochem. J.* **61,** 589 (1955).

5. NEUMAN, R. E., AND LOGAN, M. A., *J. Biol. Chem.* **184,** 299 (1950).
6. LEACH, A. A., *Biochem. J.* **74,** 70 (1960).
7. BOWES, J. H., *J. Soc. Leather Trades' Chemists* **43,** 203 (1959).
8. COURTS, A., *Biochem. J.* **73,** 600 (1959).

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An Improved Zaffaroni-type Solvent System for Paper Chromatography[1]

During studies on the isolation of 17-hydroxydeoxycorticosterone (Substance S) from adrenal venous blood there arose a need for a Zaffaroni-type solvent system that would aid in separating this substance from contaminating materials. The system toluene–propylene glycol is suitable for preliminary separation of Substance S from adrenal or urinary extracts (3), but separation of corticosterone from Substance S is not achieved in this system. Some of the systems of Bush (1) and of Eberlein and Bongiovanni (2) will achieve this separation, but the capacity of these systems is relatively low.

Paper chromatographic solvent systems generally used in the Zaffaroni-type procedure contain stationary phases of a relatively high boiling point such as propylene glycol. The components used in the Bush type systems are all lower boiling solvents, and chromatographic development requires a more rigid temperature control. It would be of advantage if the salient features of these two types of systems could be combined. The paper chromatographic solvent system methylcyclohexane–dimethylformamide described here resulted from a search for solvents for the stationary phase of lower boiling point which might be used in the manner described by Zaffaroni. The boiling point of dimethylformamide is 152–154°C in contrast to that of propylene glycol which is 189°C. This system has been used successfully to separate Substance S from corticosterone in adrenal venous blood (3).

Table 1 lists the relative mobilities of a number of naturally occurring steroids in the new system. Mobilities in toluene–propylene glycol and

[1] This work was supported in part by Research Grants A-1509 and CY-3644 from the National Institutes of Health and the John G. Clark Memorial Fund.

TABLE 1

MOBILITIES OF STEROIDS IN PAPER CHROMATOGRAMS

Steroid[a]	MC:DMF[b]	T.PG[b]	MC:PG[b]
Cortisone	0.16	0.041	—
H_4B	0.23	0.066	0.01
H_4A	0.25	0.14	0.02
H_2E	0.28	0.069	—
Corticosterone	0.59	0.20	0.015
11-Dehydrocorticosterone	0.60	0.50	0.075
H_4S	0.62	0.041	—
17-Hydroxydeoxycorticosterone	1.0	0.15	0.02
11β-Hydroxyandrostenedione	1.6	0.5	0.09
17-Hydroxyprogesterone	2.7	—	0.31
Deoxycorticosterone	2.4	1.0	1.0
Progesterone	10.2	—	6.3

[a] The designation H_2 refers to the steroid with the 4-double bond reduced. H_4 refers to the Ring A reduced form.

[b] MC:DMF, methylcyclohexane–dimethylformamide.
T:PG, toluene–propylene glycol.
MC:PG, methylcyclohexane–propylene glycol.

methylcyclohexane–propylene glycol are also given for comparison. The compounds are arranged according to decreasing oxygen content; for compounds with the same number of oxygens, the arrangement is in the order of decreasing polarity. The data in this table were obtained using the procedure described below.

The solvents used were of reagent grade, and it has not been found necessary to redistill them. A mixture of methylcyclohexane and dimethylformamide was shaken in a separatory funnel at room temperature. The top phase was used for the mobile phase and enough was poured in a chromatography jar lined with paper to keep it saturated. The chromatographic paper strips (standard length 57 cm) were drawn through a trough containing the dimethylformamide and blotted between filter paper. The steroids or extracts were immediately applied to the starting line, and the strip was again saturated only below the starting line and blotted lightly before being transferred to the jar. Mobile phase was added to the troughs immediately after the strips were placed in the jar, and descending development was carried out. Development is carried out for the predetermined length of time or until the required amount of effluent has been collected. For separation of Substance S from corticosterone, the volume of effluent collected was 15 ml per ½ in. width of paper; this took about 4 days. For 11-deoxycorticosterone, development time was 18 hr or the effluent volume was 0.5 ml. After development of the chromatogram the strips were dried

in air, preferably in a dark room, and the steroids were detected by ultraviolet illumination or appropriate color tests.

A new Zaffaroni-type solvent system for paper chromatography of steroids is described. The system methylcyclohexane–dimethylformamide has a relatively high capacity and provides for separating steroids not otherwise resolved by other Zaffaroni-type systems.

REFERENCES

1. BUSH, I. E., *Biochem. J.* **50,** 370 (1952).
2. EBERLEIN, W. R., AND BONGIOVANNI, A. M., *Arch. Biochem. Biophys.* **59,** 90 (1955).
3. TOUCHSTONE, J. C., COOPER, D. Y., AND BLAKEMORE, W. S., *J. Clin. Endocrinol. and Metabolism* **19,** 812 (1959).

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A Micro Calcium Method Accurate in the Presence of Magnesium and Phosphate

Yalman *et al.* (1) in the macroanalysis of ashed urine and bone observed that, in titrating calcium with ethylenediamine tetraacetate (EDTA), interference by phosphate and magnesium could be avoided if the titration were run as a back titration with calcium following the addition of excess EDTA. These results were in agreement with extensive unpublished experiments on serum in our laboratory which showed that reliable results could be obtained on a *micro* scale with the EDTA titration for calcium but that, if magnesium and phosphate were present, it was necessary to carry out the titration as a back titration.

The micro method is given below. The method of ashing is semiautomatic and enables the ashing and titration to be carried out in the same tube.

Serum, 0.500 ml, in a 100 × 13 mm Pyrex test tube is dried at 110° and, after the addition of 0.20 ml of a mixture of equal parts of concentrated nitric acid and of 70% perchloric acid, the tube is placed for 5 min at a depth of 20 mm; for 15 min at a depth of 40 mm; and for 30 min to within a couple of millimeters of its full length in aluminum blocks

heated at 180° C and provided with suitable holes and plugs for the adjustment of the depth of insertion of a dozen tubes. The resultant ash is dry and readily soluble. To the ash are added: (*a*) 0.5 ml water, (*b*) 2 drops of a 1:5 fresh aqueous dilution of stock dye (stock: 2% calcein in 0.25 *N* NaOH), (*c*) 0.4 ml of 2 *N* NaOH (CO_2-free) and, after mixing, (*d*) about 0.5 ml (measured with 0.1% accuracy with a syringe pipet of 0.004 *M* EDTA freshly diluted with water from stock and standardized with each set of titrations (stock: 0.04 *M* EDTA made by dissolving 14.9 gm disodium dihydrogen Versenate and 4.5 ml of 18 *N* NaOH in 1000 ml water). The mixture is titrated, against a white porcelain background under illumination from a "daylight" bulb, with 0.100 *M* calcium chloride from a microburet with stirring by filtered air.

All water used is distilled water, treated in a mixed-bed deionizer, and all glassware, after cleaning with dichromate and dilute HCl, is cleaned with this water. The calcein is sold by the Frederick Smith Chemical Co., Columbus, Ohio. The microburet used was a mercury-containing buret with a total expressed volume of 0.1 ml manufactured by the Manostat Corporation. The Krogh-Keyes syringe pipet of 1.0 ml total capacity that was used is manufactured by Alfred Bicknell Associates, Cambridge, Mass.

To test the method, serum was decalcified by oxalate precipitation and found, by the above method, to contain less than 0.0002 mg calcium/ml. Aliquots of 0.500 ml of the decalcified serum were ashed by the above method, and to the ash were added known amounts of calcium. Fifty-two analyses on four successive days with amounts of added calcium ranging from 19 to 77 μg per analysis gave the results shown in Table 1. Similar results were obtained with decalcified and ashed rat tissue.

TABLE 1
RESULTS OF ANALYSES

Number of analyses	Calcium added	Calcium found mean	Mean error
	μg	μg	μg
12	19.2	19.3	0.2
12	38.4	38.1	0.5
12	63.4	63.9	0.6
16	76.8	77.0	0.4

ACKNOWLEDGMENT

The technical assistance of Miss Janet Woodson and of Miss Frances Harnach is gratefully acknowledged. Part of the expenses of this investigation were paid by a grant from the U. S. Public Health Service.

REFERENCES

1. YALMAN, R. G., BRUEGEMANN, W., BAKER, P. T., GARN, S. M., *Anal. Chem.* **31,** 1230 (1959).

Department of Biochemistry and Zoller Clinic THOMAS B. COOLIDGE
University of Chicago
Chicago, Illinois
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Cytology and Evolution

By E. N. WILLMER

Reader in Histology, Clare College, University of Cambridge

April 1960, 440 pp., illus., $10.00

From a description of the main classes of cells which develop in simple tissue cultures, the author proceeds in search of the origin and significance of these classes both in embryology and in phylogeny. Clues based on phylogenetic evidence lead to an investigation of certain amoebae whose structure and behaviour are directly dependent upon fairly simple and universal factors in their environment. These same environmental factors are then considered as possible influences in establishing the basic pattern for a simple colonial metazoan organism, and thus probably for the embryonic forms of the majority of invertebrates and, possibly, of vertebrates also. Early differentiation of cells into a few main types is shown to influence the cellular organization of other body tissues. A tentative genealogical tree of the families of cells of an organism is proposed and considered as a key to the manner in which the cells differentiate and to the manner in which they behave in tissue cultures.

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ANALYTICAL BIOCHEMISTRY 2, 97–102 (1960)

A System for Fractionating by Density Gradient Procedures [1]

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Received March 29, 1960

INTRODUCTION

During studies on the sedimentation of botulinum toxin (1) using density gradients it became apparent that, for certain applications at least, the conventional methods others employed for fractionation were deficient. In the system where individual layers are removed through the side of the plastic tube by syringe and needle, it is necessary for the layers to be visible. When visibility of layers is not imparted by virtue of color, light-scattering, or abrupt refractive-index changes, this method is not applicable. In the commonly employed system of allowing the material to drip from a small hole in the bottom of the tube, it is difficult to obtain a suitable and consistent flow rate. This fractionation procedure has been improved, or made more generally applicable, by the development of a system which facilitated the insertion of a cannula into the bottom of a tube without leakage and provided a regulated flow rate so that the contents of the tube could be divided into any preselected number of fractions. This method and the results of some tests in which it was used are described in this report.

MATERIALS AND METHODS

Construction

The system consisted essentially of a metal tube to hold a plastic centrifuge tube, a mechanism for inserting a cannula into the bottom of the tube, and an electrolytic device (3) to control the flow rate. Only dimensions applicable to a ½ × 2-in. plastic tube are given.

[1] This work was sponsored by the Bureau of Medicine and Surgery, United States Navy, and the Office of Naval Research under a contract with the Regents of the University of California. Opinions contained in this report are not to be construed as reflecting the views of the Naval Service.

1. The metal tube, 14–15 mm i.d., was about 65 mm long and the cap was about 12 mm high. Slots were cut in the sides (Fig. 1) to permit observation and manipulation of the plastic tube. The cap and upper portion of the tube were threaded, 12 per inch. The cap had a 9-mm hole in its center to allow passage of the hub of a hypodermic needle, while the tube had a hole, about 3 mm diameter in the bottom to permit the sampling cannula to protrude.

2. The sampling cannula was made from a 25-mm section of 18-gage hypodermic needle. An external thread was formed on the lower portion of the hypodermic needle by soldering an open coil of 22-gage wire about 5 mm from the end of the needle. A stylet was bent around to engage this wire on the needle as shown in the insert of Fig. 1 so that rotation of the stylet caused it to be drawn into the needle. With the stylet in position the tip of the needle and stylet were ground as a unit to produce what might best be described as a trocar and cannula. The use of such a conically pointed trocar minimized tearing of the plastic which has been observed when the usual beveled needle was used. In addition, the cannula makes it possible to obtain sharper separation of layers than with the beveled hypodermic needle. A needle ground to a double point, as described by Britten and Roberts (2), is also superior for separating layers, but the plastic tube was more difficultly pierced with such a needle than with either a single-pointed needle or the trocar and cannula described above.

A disk, about 7 mm diameter and 1 mm thick, was soldered about 10 mm from the sharpened end of the cannula. In use the disk rested on the bottom of the holder, thus positioning the cannula.

The cannula with the trocar was pushed into section of a No. 0 rubber stopper 8–9 mm thick with the trocar point just flush with the upper surface of the rubber. This arrangement causes the rubber to be pressed firmly against the bottom of the plastic tube before it is pierced. Application of a spot of grease to the stopper around the cannula effectively prevented leakage during the fractionation.

3. The upper end of the plastic tube was fitted with a No. 00 rubber stopper which was trimmed so that it would not touch the contents of the tube. A 20-gage needle was inserted through this stopper for connection with the electrolytic device.

4. The construction of the electrolytic gas generator and manometer is shown in Fig. 1. The electrodes were made of 24-gage platinum wire, and the generator was filled to within 1 cm of the top with dilute sulfuric acid.

5. A power supply consisting of a 25-v transformer rheostat or variable transformer, a selenium rectifier, and a 0–50 ma meter was used

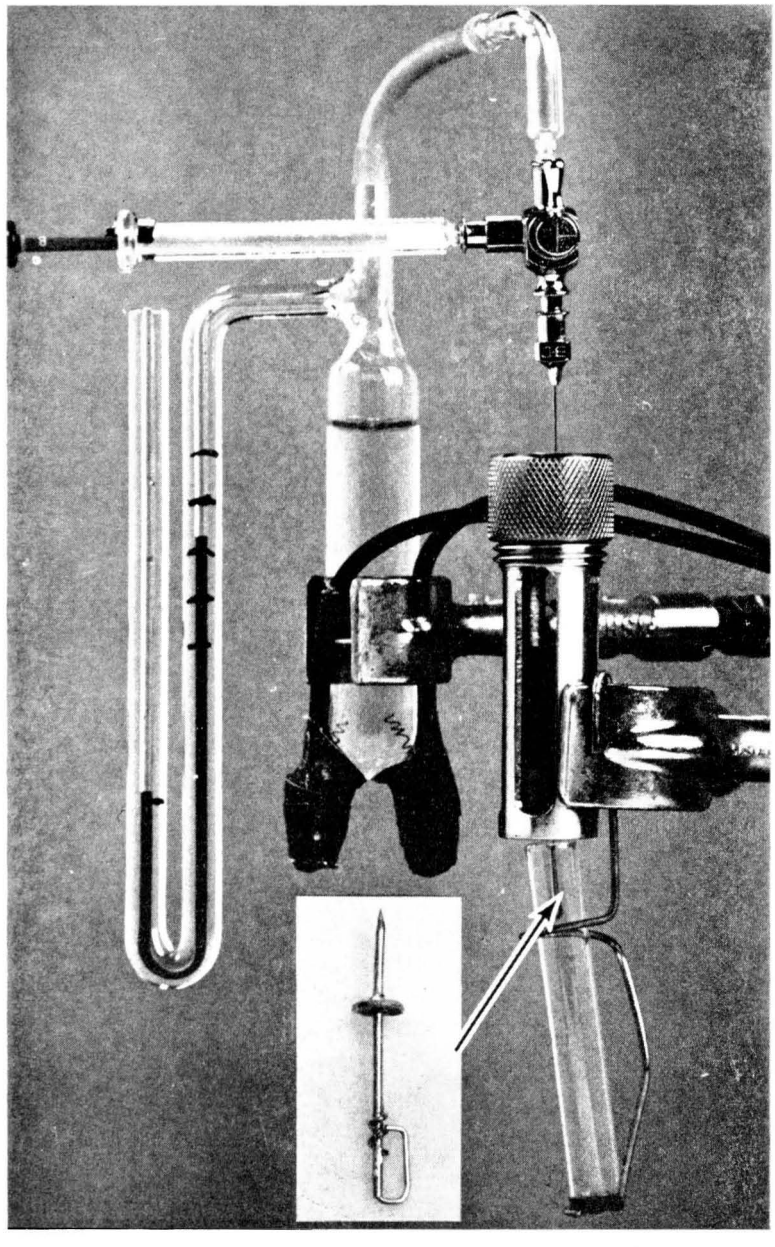

FIG. 1. An apparatus for fractionating the contents of a plastic centrifuge tube. The power supply, 0–50 ma range, is not shown.

to supply the current for electrolysis. Other aspects of the construction will be brought out in a description of a typical fractionation procedure. A syringe drive or other means of introducing air or oil at a regulated rate could, of course, be used instead of the electrolytic device.

Operation

1. The holder, with the trocar and cannula assembly in place, was clamped in a vertical position as shown in Fig. 1.

2. The No. 00 stopper with the hypodermic needle was carefully inserted in the plastic tube before placing it in the holder, and the screw cap was slightly tightened to retain the tube.

3. After the 3-way valve was attached to the needle in the stopper, the gas generator was attached to the upper opening of the valve and a ¼-ml syringe was attached to the other opening as shown in Fig. 1.

4. A vacuum was applied with the syringe to balance the hydrostatic head in the tube being fractionated, and the valve was turned to connect the gas generator with the tube to be fractionated.

5. The cap of the holder was then tightened so that the plastic tube was punctured by the trocar, but only the tip of the cannula, less than 0.1 mm, was pushed through the bottom of the plastic tube. The applied vacuum was insufficient if a drop of the liquid flowed as the trocar was removed. Too much vacuum, of course, would cause a bubble of air to enter and seriously disrupt the boundaries but, fortunately, due to the high surface tension of aqueous systems, there is considerable latitude between too little and too much vacuum.

6. Fractionation of glycerol gradients varying from 0 to 60% was carried out at a constant flow rate, and fractions were obtained at fixed intervals. The collection tubes were held against the delivery tip and changed manually. The volumes obtained by this method were more uniform than those obtained by allowing drops to form and fall by gravity. Automatic fraction collection, using a drop counter, can be utilized in those instances in which variation of drop size is acceptable. Obviously flow rates can be adjusted by changing the electrolyzing current to suit the response time of the counter or to any slower rate to minimize mixing of the layers.

RESULTS

The fractionation procedure was evaluated by incorporating methylene blue in alternate layers of decreasing glycerol concentrations. These layers in the ½ × 2-in. plastic centrifuge tubes represented bands as would be obtained by ultracentrifugation in density gradients. Immediately after filling, the tubes were fractionated as described above.

Results comparable to those shown in Fig. 2 were obtained when fractions were collected at 1-min intervals using a current of 10 ma. As shown in Fig. 2, the volumes of the first fractions were nearly identical with the volumes of the last fractions despite the marked differences in density, viscosity, and surface tension.

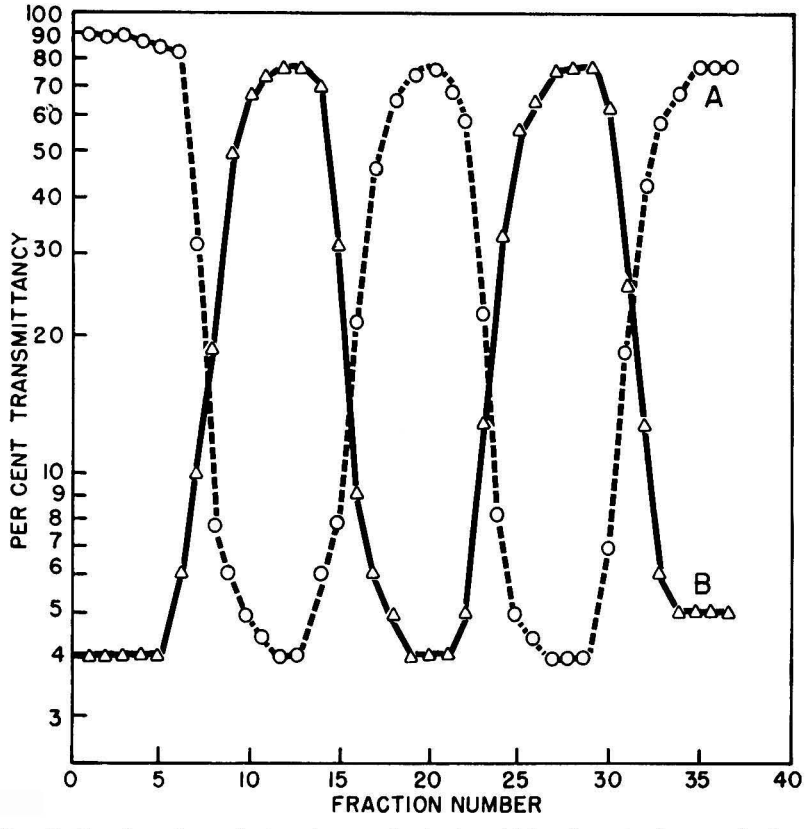

FIG. 2. Fractionation of stepwise gradients in which alternate layers of glycerol contained methylene blue. Five layers, each 0.95 ml, were prepared in ½ × 2-in. plastic tubes using 0, 5, 10, 20, and 40% glycerol. The 2nd and 4th layers in tube A and the 1st, 3rd and 5th layers in tube B contained 0.05% methylene blue. The electrolysis current was set at 41 ma, and fractions were collected at 15-sec intervals to obtain approximately 0.12-ml fractions. Each fraction was diluted to 5 ml and read at 650 mμ in 18-mm test tubes.

Most of the deviation of the curves from a square-wave pattern was caused by the mixing which was observed at the time of preparing the layers rather than by carry-over or mixing on sampling. Nevertheless, the positions of the boundaries were easily definable on the basis of the

colorimetric measurements either in terms of fraction number, as was done in Fig. 2, or in terms of distance from the meniscus. Unfortunately, the exact position of a component in the cell was not determined easily because a finite, but variable, amount of material at the bottom was not drained. It was observed that the bottom of the plastic tube was indented slightly as the needle was inserted. While the tube was more easily punctured by a smaller needle, it was found that use of a 22-gage needle resulted in an appreciable back pressure with a current of 10 ma. This, in turn, resulted in a marked change in flow rate of the liquid as fractionation proceeded. However, needles or cannulas smaller than 18 gage may be satisfactory for solutions less viscous than glycerol, such as the popular cesium chloride solutions.

SUMMARY

A system is described which has been found useful for density gradient fractionation. It consists of a screw-capped holder with a trocar and cannula in the bottom to puncture the plastic tube and an electrolytic gas generator to control the outflow rate. The size of the fractions was uniform and independent of density or surface tension of the fluid.

REFERENCES

1. Britten, R. J., and Roberts, R. B., *Science* **131,** 32 (1960).
2. Heckly, R. J., Hildebrand, G. J., and Lamanna, C., *J. Exptl. Med.* **111,** 745 (1960).
3. Heckly, R. J., *Science* **127,** 233 (1958).

ANALYTICAL BIOCHEMISTRY 2, 103–106 (1960)

An Ultramicro Method for the Determination of Total Serum Cholesterol[1]

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From the Department of Foods and Nutrition, The Pennsylvania State University, University Park, Pennsylvania

Received March 31, 1960

INTRODUCTION

The determination of total cholesterol in blood serum has assumed great importance in recent years due to the possible relationship of the cholesterol level to various disorders. Many methods for the determination of total serum cholesterol have been developed including those of Schoenheimer and Sperry (1), Sperry and Webb (2), Abell *et al.* (3), and Pearson, Stern, and McGavack (4). The direct method of Zlatkis, Zak, and Boyle (5) is a simple one and a more stable color is developed than in previous methods. However, this method requires 0.1 ml of serum which is too large a sample for many procedures.

The present method is an ultramicro adaptation of the method of Zlatkis, Zak, and Boyle (5) for the determination of total cholesterol in serum using a 5-μl sample. This size sample can be collected in a capillary tube from the tip of the tail of a rat, one tube supplying triplicate samples of serum. The method described is simple, accurate, and rapid and permits quantitative recovery of added cholesterol.

METHOD

A calibration curve using standard solutions of cholesterol was prepared as follows:

A stock standard solution containing 400 mg% cholesterol was prepared by dissolving the weighed dry cholesterol in glacial acetic acid. Aliquots of the stock standard were diluted to obtain standards containing 300, 200, 100, and 50 mg% cholesterol.

1. 5.1 μl of the 50 mg% standard was pipetted[4] in triplicate into

[1] Home Economics Research Publication No. 172.

[2] Submitted to the Graduate School of The Pennsylvania State University in partial fulfillment of the requirements for the Master of Science degree.

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70 × 7 mm test tubes. This was repeated with the 100, 200, 300, and 400 mg% solutions in four other sets of tubes.

2. 299.4 μl of glacial acetic acid was added to each tube and mixed.

3. 200.4 μl was taken from the mixture and pipetted into clean tubes.

4. 200.4 μl of the sulfuric acid–ferric chloride reagent (1 ml of 10% ferric chloride solution diluted to 100 ml with concentrated sulfuric acid) was added to each tube by allowing the reagent to flow down the side of the tube forming two layers. The tubes were shaken vigorously using an electric buzzer. A brown color forms immediately followed by a change to rose and then to violet depending on the concentration of cholesterol. The absorption was measured after 30 min at 560 mμ in the Beckman DU Spectrophotometer fitted with a micro attachment and 2.5-mm quartz cuvettes.

5. The readings of the standards were corrected for reagent blanks and a calibration curve of the net readings of the standards was plotted against the equivalent concentrations. From the formula:

$$K = \frac{\text{net reading of standard}}{\text{equivalent mg\% of cholesterol}}$$

a K value for each standard was calculated and an average K computed.

The procedure employed for analyzing the serum and a commercial cholesterol control[5] differed from the analysis of the standards only in centrifugation between steps 2 and 3 in order to precipitate the protein and obtain a clear supernatant. Total cholesterol in the serum was calculated as follows:

$$\text{Total cholesterol} = \frac{\text{net reading of sample}}{\text{average } K}$$

As a further test of the accuracy of the method, recoveries were made of known quantities of cholesterol added to the glacial acetic acid which was used to separate the serum components (step 2). Four solutions were prepared containing 6.25, 3.13, 1.56, and 0.78 mg% of cholesterol. An average K value for these solutions was calculated and referred to as the recovery K.

To calculate the cholesterol recovery, the average optical density of the plain serum analyzed in the same run was subtracted from the optical density of the serum plus the additional cholesterol. This figure,

[4] Constriction pipets were used, and the indicated volumes are based on calibrations in this laboratory.

[5] Choles-Trol, Dade Reagents.

which corresponds to the optical density of the added cholesterol, was divided by the recovery K. Thus, the per cent of recovery was obtained:

$$\frac{\text{OD of added cholesterol}}{\text{recovery } K} = \text{mg\% recovered}$$

$$\% \text{ recovery} = \frac{\text{mg\% recovered} \times 100}{\text{mg\% added}}$$

RESULTS AND DISCUSSION

Calibration curves obtained with cholesterol standards indicated that optical density was proportional to concentration between 50 and 400 mg%. The average K value of the standards was 0.0022 with a standard error of ± 0.000019.

The commercial cholesterol control had a cholesterol concentration of 200 mg% according to the manufacturer's analysis. Forty-three sets of triplicate samples of this control were analyzed giving a range of values from 193 to 205 mg%. The average concentration of cholesterol in these samples was 199.58 mg% with a standard error of ± 0.58.

Fifty triplicate samples of blood serum from one normal rat were analyzed. During each run, triplicate commercial cholesterol control samples were included for the purpose of providing a control for each series. The cholesterol concentration of the serum ranged from 54.1 to 59.1 mg%, averaging 56.1 mg% with a standard error of ± 0.05.

Table 1 gives the results of the recovery test at each of the four levels of added cholesterol. In all, 30 sets of triplicate samples were analyzed. The average recovery ranged from 98.7 to 101.6%.

TABLE I

RECOVERY OF CHOLESTEROL ADDED TO RAT SERUM SAMPLES

No. of triplicate samples	Cholesterol added (mg%)	Average cholesterol recovery	
		mg%	%
7	0.78	0.77 ± 0.01[a]	98.7
7	1.56	1.59 ± 0.01	101.6
8	3.13	3.16 ± 0.05	100.6
8	6.25	6.28 ± 0.04	100.4

[a] Standard error of the mean.

The reproducibility of the calibration curves, the values for the cholesterol control, and the serum samples, as well as the accurate recoveries of added cholesterol, are evidence of the reliability of this ultramicro adaptation of the Zlatkis, Zak, and Boyle method for the determination of total cholesterol in blood serum.

SUMMARY AND CONCLUSIONS

An ultramicro adaptation of the Zlatkis, Zak, and Boyle method for the determination of total serum cholesterol utilizing a 5-μl sample has been described. The data demonstrate that this adaptation provides an accurate, rapid, and practical method for the determination of total serum cholesterol.

REFERENCES

1. Schoenheimer, R., and Sperry, W. M., *J. Biol. Chem.* **106,** 745 (1934).
2. Sperry, W. M., and Webb, M., *J. Biol. Chem.* **187,** 97 (1950).
3. Abell, L. L., Levy, B. B., Brodie, B. B., and Kendall, F. E., *J. Biol. Chem.* **195,** 357 (1952).
4. Pearson, S., Stern, S., and McGavack, T. H., *J. Clin. Endocrinol. and Metabolism* **12,** 1245 (1952).
5. Zlatkis, A., Zak, B., and Boyle, A. J., *J. Lab. Clin. Med.* **41,** 486 (1953).

ANALYTICAL BIOCHEMISTRY 2, 107–126 (1960)

Purification and Measurement of Microgram Amounts of Radioactive Nucleic Acids and Proteins from Animal Cells in Tissue Culture

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Received April 11, 1960

INTRODUCTION

The use of suitable isotopic precursors in growth media of controlled composition makes possible studies of animal cells in tissue culture which would, had conventional colorimetric analyses been used, have required the use of many expensive and inherently variable replica cultures. These advantages are offset, however, by the laborious purifications (usually of a chromatographic nature) which are necessary to achieve radiochemical purity of the cell fractions examined, as well as by the further time consumed during the plating of the samples prior to measurement of their radioactivity.

In this paper very rapid and uncomplicated analytical procedures are described for isolating and measuring the radioactivity of microgram amounts of protein and nucleic acids from animal cells which have incorporated either adenine-8-C^{14}, glycine-1-C^{14}, or phosphate-P^{32} during growth in tissue culture. Although different methods had to be developed to deal with the specific pattern of incorporation of each of the precursors, the following basic similarities of design are present in all of the methods:

(*a*) Almost invisible amounts of proteins and nucleic acids are quantitatively recovered by filtration through membrane filters. These filters have previously been applied to the study of bacterial metabolism, particularly in the separation of acid-soluble pools from acid-precipitable materials (1, 2).

(*b*) The separation of ribonucleic acid (RNA) from deoxyribonucleic acid (DNA) and the nucleic acids from the proteins follows, in principle, the classical Schmidt-Thannhauser procedure (3) and its modification by Schneider (4). However, certain steps of these analyses have been greatly abbreviated and others modified to make them compatible with the use of the filters.

(*c*) Activated charcoal is used to selectively adsorb nucleotides and nucleic acid bases from solutions often highly contaminated by extraneous activity. Although charcoal has had a similar application numerous times in nucleic acid research (5, 6), efforts had usually been made to elute the adsorbed radioactive materials from the charcoal before measuring them. Here, on the other hand, it has been shown possible, using filtration again, to measure directly and reproducibly the radioactivity bound onto the charcoal, thus eliminating any need for elution.

In the description which follows of the techniques used to analyze variously labeled cells, no attempt will be made at first to validate the conditions employed or the designations given to the fractions which were isolated. Rather, the control experiments which were performed will be presented in a single section and will be discussed in relation to those particular procedures which, as indicated above, are shared by several techniques.

MATERIALS AND METHODS

1. Biological

Tissue Culture Techniques. A subline of Earle's L cell (7) was grown as a suspension of single cells at 37°C in a rapidly rotated roller tube (8) containing 20–45 ml medium. Two media were used; a complete medium composed of 2 parts normal or dialyzed horse serum plus 8 parts C.M.R.L. 1066 (9), and a minimal medium consisting of 1.5 parts dialyzed horse serum and 8.5 parts 1066 from which the deoxynucleosides, coenzymes, glycine, glutathione, and serine had been omitted. In the former medium the cell generation time was 24 hr; in the latter, 36 hr. The cell concentration ranged from 1 to 4×10^5 cells/ml (20–80 μg dry weight/ml).

Sampling and Storage of Labeled Cells. After cells had incorporated radioactive precursors from the media, they were sampled in aliquots containing up to 10^6 cells (the maximum number the filters used in these analyses could collect before they became clogged). No lower limit was found to the number of cells which could be successfully analyzed, save that imposed by the level of radioactivity in the cells.

The samples containing the cells were diluted at least fivefold in buffered saline, then centrifuged at 2°C for 10 min at $1500 \times g$. After the supernatant fluid was removed by means of a Pasteur pipet attached to a vacuum line, the open sample tubes were stored in an insulated cabinet containing solid CO_2 at —78°C. No change in radioactivity of the nucleic acids or proteins was detected in samples stored in this way for even one month.

2. Chemical

Radioactive Chemicals. Carrier-free orthophosphate-P^{32} was obtained from Atomic Energy of Canada Ltd. and was autoclaved in 1 *N* HCl prior to use, to hydrolyze any pyrophosphates or peroxides present. Adenine-8-C^{14} sulfate, glycine-1-C^{14}, and L-lysine-C^{14} (uniformly labeled) were obtained at a specific activity of 1–5 mc/mole from Nuclear Chicago Corp. Tritiated thymidine (360 mc/mole was obtained from Schwarz Laboratories Inc.

Measurement of Radioactivity. Radioactivity was measured using a D-47 gas-flow counter (Nuclear Chicago) with a 1 mg/sq cm Mylar end window. Counting efficiency of infinitely thin C^{14} samples was 30% of the theoretical maximum; that of P^{32} was 95%. With the end window removed, tritium could be counted with a maximum efficiency of 5%. By immersing a thin-walled Geiger tube (D-52, Nuclear Chicago Corp.) into liquids containing P^{32}, their activities could be measured with 10% efficiency.

Filtration Equipment. In all of the routine procedures described, radioactive proteins, nucleic acids, and charcoal-adsorbed nucleic acid derivatives were ultimately collected by filtration on the surface of membrane filters. "Hydrosol Assay" Millipore filters, (25 mm in diameter, having a usable surface area of 2.26 sq cm and an average pore size of 0.45 μ and glass microanalysis filter holders, were both obtained from the Millipore Filter Corp., Bedford, Mass. To measure their radioactivity, filters carrying radioactive proteins and/or nucleic acids were mounted on 3-cm diameter stainless steel planchets with rubber cement.

Use of Charcoal. To adsorb radioactive nucleotides and bases, Norit A, an activated vegetable charcoal, was used. It was first treated with 6 *N* HCl under vacuum to remove adsorbed gases (10), and after extensive washing was resuspended at a concentration of 10 mg/ml in 0.1 *M* HCl to form a stock suspension. Whenever aliquots of this stock were combined with radioactive solutions, vigorous aeration was employed to keep the charcoal in suspension, any number of samples being supplied with a constant stream of air from a manifold constructed in the following way: A length of rubber tubing was closed at one end and attached to a compressed air line at the other. An adjustable clamp was placed on the tube close to the air inlet, and a number of fine-bore hypodermic needles were inserted into the longer, low-pressure segment of the tube. A constant flow of air could then be led from each of these needles to the sample tubes via the plastic tubing and attached needle adapter of a disposable blood transfusion kit.

After adsorption of radioactivity, the charcoal was collected by filtration of the suspension through a membrane filter. Its radioactivity was

then counted by mounting the filter bearing the resulting charcoal pad on a steel planchet to which had been added 0.5 ml of a 2% aqueous polyvinylpyrrolidone solution, the latter material serving, as it dried, both to stick the filter to the planchet and to keep the pad from disintegrating.

Use of Nucleases. In tests which will be described of the validity of the procedures used to analyze P^{32}-labeled cells, the following enzymes were used: Crystalline ribonuclease (RNAse) and deoxyribonuclease (DNAse) were obtained from Worthington Biochemicals Corp. Crude Russel's viper venom was obtained from Ross Allen's Reptile Institute, Silver Springs, Fla., and used as a source of phosphodiesterase. The latter enzyme was purified free of 5′-nucleotidase activity by the method of Hurst and Butler (11).

When cells were to be treated with RNAse or DNAse prior to their fractionation, they were first suspended at a concentration of 10^6 cells/ml in 0.05 *M* potassium phosphate buffer, pH 7.5, or 0.02 *M* magnesium sulfate, pH 6.5, respectively [the conditions required for optimal activity of the corresponding enzymes (12)] and were then disrupted in a 250-w 10-kc Raytheon sonic disintegrator. When small volumes of suspension were used, they could be handled most conveniently by placing them in short lengths of 5-mm diameter dialysis tubing which were in turn submerged in cold buffer in the chamber of the disintegrator during the 10 min it took the instrument, running at full power, to rupture or totally disintegrate the cells. The sonicates were then incubated with 0.5 mg enzyme for 4 hr at 37°C, conditions found to result in a maximum hydrolysis of the nucleic acid concerned.

When DNA in isolated cell fractions was to be degraded to its constituent nucleotides, it was first digested with DNAse (0.5 mg/ml) for 4 hr at 37°C in 0.02 *M* $MgSO_4$, pH 6.5, the digest was brought to pH 9.2 with 0.25 *M* sodium glycinate, and sufficient phosphodiesterase was added to cause complete hydrolysis to mononucleotides by the end of another 4 hr of digestion at 37°C.

ANALYTICAL PROCEDURES

Analysis of Adenine-8-C^{14} Labeled Cells

A (1) *Total Acid-Insoluble Nucleic Acids (DNA plus RNA).* A frozen sample was thawed in ice water, 5 ml of ice-cold 0.3 *N* perchloric acid (PCA) was added, and the contents of the tube were actively stirred up by scraping with a nichrome microspatula. After 15 min at 0°C, the suspension was sucked through a membrane filter, which was then washed with additional cold 0.3 *N* PCA. Excess acid was removed by

pulling air through briefly, and the filter, which at that stage carried the total DNA and RNA activity, was counted.

A (2) *Acid-Soluble Purines.* The filtrate from the above steps contained radioactivity from the acid-soluble purine pool of the cells and from any medium carried over in the original sample. To adsorb this radioactivity, 1 ml of the stock charcoal suspension was added to a volume of filtrate not exceeding 15 ml. The charcoal, stirred by aeration, was kept at 0°C for 10 min, and then was filtered, washed thoroughly with water, and counted.

A (3) *The Separation of RNA from DNA Radioactivity.* Two-tenths of a milliliter of 1 *N* NaOH was added directly to a second frozen sample, which was then incubated 1 hr at 37°C to render RNA acid soluble. The tube was cooled in an ice bath and acidified with 5 ml of cold 0.2 *N* PCA to precipitate DNA. Ten minutes later, the suspension was filtered through a membrane filter which was washed with 5 ml of 0.2 *N* PCA and sucked dry of adherent acid. The radioactivity of the filter was due solely to DNA. The radioactivity of RNA was calculated by subtracting the DNA activity [step A (3)] from the total acid-insoluble activity [step A (1)].

Analysis of Cells Labeled with Glycine-1-C^{14}

G (1) *Isolation of Protein Activity.* One sample was first extracted using procedures described in sec. A (1), and the filtrate was kept for measurement of the acid-soluble purines. The filter, instead of being counted, was placed in a 25-mm diameter vial containing 10 ml of 1.6 *N* PCA (14% by volume of 70% PCA) and was heated at 60°C for 9 min to release the purines from the precipitated nucleic acids. When the acid had cooled, the filter was removed (care being taken to avoid tearing or perforating the filter), replaced on the glass holder, and, after the strong acid had been filtered through it to recover any protein which might have come off the filter during the hydrolysis, it was washed with additional 0.3 *N* PCA. The radioactivity remaining on the filter was due solely to proteins, while the strong acid filtrate was kept for measurement of the total nucleic acid radioactivity.

G (2) *Isolation of DNA Activity.* The procedure described in sec. A (3) was applied to a second sample, resulting in a filter carrying radioactivity from protein and DNA but not from RNA. The DNA activity was then released by a hot acid hydrolysis and refiltration identical to that described in sec. G (1). In this case, however, only the acid filtrate (containing the DNA purines) was kept.

G (3) *Purification and Adsorption of Purine Activity from Filtrates.* Radioactive non-purine contaminants present initially in the three types

of acid filtrates had to be converted to forms not adsorbable by charcoal. This was done without loss of purine activity, by adding PCA to the filtrates to a final concentration of 1.6 *N*, then autoclaving them at 132°C (2.6 atm steam) for 4 hr. The filtrates were afterward treated with charcoal using procedure A (2). Activities of the acid-soluble pool, total nucleic acid purines, and DNA purines were thus obtained directly; the RNA purine activity was determined by subtracting DNA purine from total nucleic acid purine activities.

Analysis of P^{32}-Labeled Cells

P (1) *Isolation of Acid-soluble Pool.* Frozen samples were melted in an ice bath, extracted for 10 min with 5 ml of 0.3 *N* PCA containing 1 mg of bovine serum albumin as carrier, and centrifuged at 2°C for 10 min at 1500 × g. The supernatants were decanted and treated 10 min at 0°C with 20 mg charcoal to which the nucleotides became adsorbed; after which the charcoal was filtered off, washed with water and counted.

P (2) *Isolation of DNA Activity.* The pellet from the previous centrifugation was dissolved in 0.2 ml of 1 *N* NaOH and heated in a boiling water bath for 10 min to render RNA acid soluble. Six milliliters of a lipid solvent composed of 4 vol $CHCl_3$ and 6 vol of absolute ethanol was added, and the mixture was heated for 15 min at 60°C, resulting in the precipitation, among other things, of DNA and the ribonucleotides. On cooling, the mixture was filtered quickly through a dry membrane filter. The filter was washed with an additional 5 ml of fresh solvent, and the filtrates were discarded. Five milliliters of 0.1 *N* PCA was subsequently added on top of the filter and permitted to remain there 10 sec before suction was applied to ensure complete precipitation of DNA on the one hand and the solution of the ribonucleotides on the other. The original sample tube, after being scraped vigorously with a spatula to remove any residue sticking to the walls, was washed out with additional acid. All radioactivity remaining on the acid-washed filter was then due to DNA.

P (3) *Isolation of RNA Activity.* To the acid filtrate from the previous step, 20 mg charcoal was added, and the suspension was aerated *vigorously* at 0°C for 15 min. The charcoal, which was then filtered off and washed well with water before counting, had radioactivity due to ribonucleotides.

Self-Absorption Corrections

In these analyses the radioactivity of cell fractions deposited as thin layers on filters is compared with those adsorbed onto much thicker pads of charcoal. Corrections for the different degrees of self-absorption

expected under these two conditions, especially for C^{14}, depended on the following considerations.

When cells containing P^{32} or C^{14} were deposited directly onto filters, no self-absorption was detected even up to the clogging limit of the filters, 1.2×10^6 cells. At this limit the thickness of the sample was only 0.1 mg/sq cm, well within the region of infinite thinness for C^{14}, let alone P^{32}.

In determining the self-absorption of radioactivity in the charcoal, use was made of the fact that cells grown in the presence of adenine-8-C^{14} incorporated activity only into their nucleic acid purines. This activity could then be measured either directly on a filter as in method A (1) or after adsorption to a charcoal pad, as in methods G (1–3). Such comparisons, which were made whenever a fresh lot of charcoal was prepared, showed that an average pad thickness of 5.5 mg/sq cm for charcoal, (as compared with 5.6 mg/sq cm for $BaCO_3$-C^{14}) (13) reduced the activity of adsorbed C^{14}-labeled material by one-half, and that the 10 mg charcoal normally used reduced activity by 45%.

No significant self-absorption of P^{32} radioactivity could be detected when labeled nucleotides were adsorbed onto 20 mg charcoal.

RESULTS AND DISCUSSION OF CONTROL EXPERIMENTS

1. Magnitude of Losses Occurring on Filtration

Losses from the Total Acid-Precipitable Fraction. Since the membrane filter has been frequently used in the analysis of bacteria (1, 2), it was expected that the filter would also recover quantitatively from the animal cell all its acid-precipitable material. The recovery of total acid-insoluble material which was in fact obtainable was tested using cells which had incorporated activity into both proteins and nucleic acids during growth in a minimal medium containing glycine-1-C^{14}.

One sample of cells was extracted with cold acid and centrifuged [as described in procedure P (1)]. The supernatant from these cells contained no activity which could be retained by the membrane filter. The centrifuged cells were dissolved in 0.3 *N* NH_4OH and plated directly on a planchet bearing a lens tissue disk. The other sample was extracted and filtered by procedure A (1). The activity of this extracted and filtered sample was, in fact, 2.5% higher than that of the other sample, after corrections had been made for self adsorption losses. Close agreement was also obtained between the charcoal-adsorbable activities of the two acid extracts (the supernatant and the filtrate), indicating a complete removal from the filtered cells of their acid soluble pool.

Losses of DNA. It was possible that even if losses had occurred during

precipitation and filtration, the presence of radioactive contaminants might have masked them. However, no such problem could arise if radioactive thymidine, a precursor of DNA alone, were used. Accordingly, to test for possible losses of DNA thymidine activity, cells were grown for one generation in a *minimal* medium containing 2 μg of tritiated thymidine/ml (specific activity-1 μc/μg). An activity of 1200 counts/min/10^5 cells was incorporated into the DNA over this period. To estimate the proportion of DNA activity passing *through* the filter under the conditions routinely employed, the acid and solvent filtrates obtained on treating the cells by methods A (3) and P (2) were concentrated on a flash evaporator, and much of the PCA was removed by neutralization with KOH. The concentrates were divided; one half was combined with a 0.3 *N* NH_4OH solution in which the DNA activity retained by the filter had been dissolved and was then evaporated down on a steel planchet bearing a lens paper disk, while the other half was plated directly. On comparing in this way the total activity and the losses respectively under identical conditions of self-absorption, it was shown that the acid filtrate from procedure A (3) contained 0.5% of the total DNA activity, while the acid and solvent filtrates of P (2) had 3% and 2%, respectively, of the original label. Losses of DNA to the acid filtrate increased with increasing acid concentrations, amounting, for example, to 15% when 0.3 *N* PCA was used in procedure A (3) with either thymidine- or adenine-labeled cells. The losses of DNA reported by Hanawalt (2), who used membrane filters in a somewhat similar way to analyze P^{32}-labeled *Escherichia coli* cells, might have resulted from his use of these higher concentrations of acid.

Surprisingly, when adenine-labeled cells were passed through procedure P (2), up to 15% of their DNA activity was in fact found in the solvent filtrates and was shown to result there from depurination during lipid extraction. This might have resulted from a lowering of pH on reaction of NaOH with $CHCl_3$ (to form HCl and tetrachloroethylene), for at 60°C. the alkali was totally consumed by this reaction within 4 min. While these losses do not affect the recovery of DNA phosphorus, they do render the DNA obtained in this way unsuitable for further treatments, such as base analyses, which may require undegraded material.

Losses of P^{32}-Labeled Ribonucleotides. The possibility of loss of P^{32}-labeled ribonucleotides either to the lipid solvent or to the DNA residue (as a result of incomplete extraction by acid in this latter case) was tested by hydrolyzing 250 μg yeast RNA in alkali, extracting it with the lipid solvent, as in step P (2), and comparing the optical density at 260 mμ of the resulting acid extract with that of an equal volume of acid to which the alkaline ribonucleotides had been added directly. The two

optical densities agreed within 5%. Since the radioactivity of the ribonucleotide-containing fraction isolated from P^{32}-labeled cells was the same whether 250 μg of RNA carrier had been added or not, it was concluded that no losses of ribonucleotides occurred during the fractionation of cells by methods P (1–3).

2. Completeness of Adsorption onto Charcoal

Quantitative adsorption of purine bases and nucleotides under the conditions used was demonstrated by spectrophotometric measurements of solutions of these compounds before and after their adsorption on charcoal. These studies confirm those of Tsuboi and Price (6). The addition of similar amounts of these compounds to filtrates containing very low concentrations of their radioactive counterparts (derived from the analyses of labeled cells) did not result in an uptake of activity exceeding that from carrier-free filtrates.

Since traces of $CHCl_3$ inevitably carried over into the acid filtrate during step P (2) will poison the adsorption capacity of charcoal, vigorous aeration is particularly important during step P (3) in order that all of the solvent may be blown off. A similar reduction in adsorption capacity occurs in the presence of 5% trichloroacetic acid and therefore renders this precipitating agent unsuitable for use in these techniques.

3. Losses Occurring during Hydrolysis

Alkaline Hydrolysis. The mild alkaline hydrolysis of C^{14}-labeled cells was patterned after that used by Scott, Fraccastoro, and Taft (14). It was shown, in confirmation of their work, that the release of nucleic acid activity from adenine-labeled cells reached a maximum after 45 min of hydrolysis, the remaining acid-insoluble activity being then attributable to DNA.

It has already been shown with tritiated thymidine that losses of DNA on vigorous hydrolysis with alkali are minor. Hurst (15) has found a similar stability of DNA to hot alkali. As for the lability of RNA phosphorus, it was shown by direct estimations of inorganic phosphate that less than 3% of a mixture of purified 2′- and 3′-ribonucleotides exposed to 1 *N* NaOH for 10 min was dephosphorylated. (To perform the phosphate analyses, using the method of Fiske and SubbaRow (16), excess nucleotides had first to be removed by adsorbing them on charcoal to prevent their being precipitated by ammonium molybdate.)

Acid Hydrolysis. Table 1 shows that the conditions of acid hydrolysis of purines used in procedure G (1) [which were also adapted from those used by Scott, Fraccastoro, and Taft (14)] resulted in a 99% liberation of activity from cells labeled by adenine-8-C^{14}, quite in keeping with the

incorporation of this activity into nucleic acids by acid-labile purine bonds. Prolonging hydrolysis, while not significantly improving the yield of purines in adenine-labeled cells, did, however, cause hydrolysis of protein, since under these conditions there was a decrease in the acid-precipitable radioactivity of cells which had incorporated lysine-C^{14} exclusively into their proteins. This activity which was lost was not

TABLE 1
LIBERATION OF RADIOACTIVITY FROM C^{14}-LABELED CELLS EXPOSED TO HOT ACID[a]

Duration of exposure to 1.6 *N* PCA at 60°C (min)	Radioactivity (counts/min/10^5 cells)							
	Adenine-8-C^{14}-labeled cells		Cells labeled by glycine-1-C^{14} during growth in				Lysine-C^{14}-labeled cells	
			minimal medium		*complete* medium			
	res	*filt*	*res*	*filt*	*res*	*filt*	*res*	*filt*
0	2840	—	7210	—	3172	—	1461	—
4	350	2450	5613	1534	3208	15	1392	3
9	30	2818	5011	2097	3059	31	1510	5
15	9	2870	4803	2045	2898	27	1267	9

[a] Replicate samples, each containing 10^5 cells labeled in designated media, were treated by procedure G (1) modified to permit varying exposure of the cells to hot acid. The term *res* denotes the activity recovered on the filter; *filt* denotes the activity rendered filterable which could also be adsorbed on charcoal after purification by procedure G (3).

appreciably adsorbed by charcoal, in keeping with charcoal's specificity for aromatic and heterocyclic compounds. A fraction of the activity of cells labeled with glycine in a complete medium—an activity present only in the proteins since exogenous purines in this medium suppressed incorporation into nucleic acids—was also lost from these cells when hydrolysis exceeded 9 min.

Since 9 min hydrolysis liberated a maximum amount of *adsorbable* activity from cells which had incorporated glycine radioactivity into both proteins and nucleic acids during growth in a minimal medium, it was concluded that the conditions specified in G (2) result in a separation of nucleic acid from protein activity, with minimum losses to the latter.

4. Radiochemical Purity of Fractions Isolated

Contamination by Unincorporated Precursors. No appreciable retention of activity was observed when 0.1 *N* PCA solutions containing orthophosphate-P^{32} glycine-1-C^{14}, adenine-8-C^{14} were passed through membrane filters, provided they were then washed with 10 ml of cold, nonradioactive acid. Similar results were obtained when 10^6 *nonradioac-*

tive cells were included in the radioactive acid samples, thus ruling out nonspecific adsorption of activity to cells.

Charcoal, on the other hand, adsorbed 0.05% of carrier-free P^{32} orthophosphate and 0.5% of glycine-1-C^{14} (specific activity-3mc/mmole) from cold solutions of these isotopes in 0.1 *N* PCA. However, almost all of the unincorporated label was ordinarily removed during the centrifugation of cells preceding storage, so that these retentions seldom amounted to an appreciable fraction of the incorporated activity that was to be measured.

Purity of Adenine-8-C^{14}-Labeled Fractions. The assumption that adenine activity was incorporated solely into nucleic acids was justified by the finding (see Table 1) that this activity was completely released during an acid treatment which had little effect on proteins, the only other fraction into which adenine activity might have been assumed to enter. Contamination of the DNA fraction with unhydrolyzed RNA or of the RNA fraction with DNA has already been ruled out by demonstrations of completeness of alkaline hydrolysis and absence of DNA losses, respectively.

Purity of Glycine-1-C^{14}-Labeled Fractions. When lipid was extracted from glycine-labeled cells using the conditions described by Schmidt and Thannhauser (3), the defatted residue of proteins and nucleic acids, upon further analysis by procedures G (1–3) showed the same distribution of activity as an undefatted sample. Glycine, therefore, was assumed to be incorporated only into the proteins and nucleic acids.

Preliminary chromatographic studies of the unautoclaved acid-soluble and DNA-containing filtrates showed them to contain *charcoal-adsorbable* non-purine activity which was often far in excess of the purine activity. On the assumption that these contaminants were peptides that occurred either naturally in the acid-soluble pool or were liberated from proteins during exposure to alkali in procedure G (2), conditions of hot acid hydrolysis were sought which would degrade these peptides to non-adsorbable fragments without affecting the recovery of purine activity.

In studying the progress of degradation of these contaminants, use was made again of the 10-fold suppression by exogeneous purines of incorporation of glycine into purines (see condition II, Table 2). The radioactivity of the nucleic acid fractions could, under these conditions of labeling, be considered almost entirely due to non-purine contaminants. Similarly, use was made of the fact that any decrease in the activity recoverable from *adenine*-labeled cells, where all activity is authentically in the nucleic acids, could be taken to reflect destruction of purines.

It may be seen from Table 2 that the purine fractions of glycine-

TABLE 2

FRACTIONATION OF ADENINE-8-C^{14} AND GLYCINE-1-C^{14}-LABELED CELLS; THE EFFECT OF HOT ACID ON THE PURITY AND RECOVERABILITY OF PURINE RADIOACTIVITY[a]

Labeled precursor employed	Fraction isolated	Activity before purification (c.p.m./10^5 cells)	Charcoal-adsorbable activity (c.p.m./10^5 cells) Filtrates containing purines and 1.6 *N* PCA heated in autoclave				
			1 hr	2 hr	4 hr	7 hr	10 hr
I: Adenine-8-C^{14} (3 mc/mmole) 5×10^{-2} μc/ml in complete medium	Cold acid-soluble purines	807	821	842	786	697	603
	Total nucleic acid purines	6430	6190	6238	5963	5014	4622
	DNA purines	1991	2113	1947	1851	1481	1393
	RNA purines	4582	4487	4621	4289	3673	3001
II: Glycine-1-C^{14} (2 mc/mmole) (.1 μc/ml in minimal medium plus 20 μg/ml nonradioactive adenine.	Cold acid-soluble purines	1110	372	127	30	21	19
	Total cold acid-insoluble activity	6472	—	—	—	—	—
	Protein	6310	—	—	—	—	—
	Total nucleic acid purines	104	113	101	97	83	67
	DNA purines	1431	274	59	39	35	21
	RNA purines	1009	261	81	64	52	41
III: Glycine-1-C^{14} (2 mc/mmole) 0.1 μc/ml in minimal medium	Cold acid-soluble purines	1305	471	195	153	137	101
	Total cold acid-insoluble activity	5491	—	—	—	—	—
	Protein	3797	—	—	—	—	—
	Total nucleic acid purines	1511	1530	1484	1434	1199	1052
	DNA purines	1949	637	501	437	380	292
	RNA purines	2019	1304	1215	1085	844	735

[a] Cells which were grown for two generations in specified media had incorporated activity from medium I, exclusively into nucleic acid purines; from II, almost exclusively into proteins; from III, into both nucleic acids, purines, and proteins. All cells were extracted by procedure P (1), to remove acid-soluble pools, then separated into the fractions listed, by procedures G (1) and (2), the cold acid filtrate from G (2) being retained for the measurement of RNA. Progressive destruction of contaminants in the acid filtrates, and later of the purines themselves, was followed by varying the duration of heating in step G (3).

labeled cells showed, after 4 hr in the autoclave, losses of adsorbable activity as high as 98%, while adenine-labeled fractions under these conditions lost no more than 7%. With further heating equal proportions of activity were lost from both adenine- and glycine-labeled fractions, suggesting strongly that the same fraction, namely the purines, was being destroyed in both cases. Therefore, the adsorbable activity of the glycine-labeled purine fraction was at that point truly purine activity. The total nucleic acid purines derived by procedure G (1) from glycine-labeled cells were independently demonstrated to be free of non-purine-adsorbable material by chromatography of this fraction (without first autoclaving it) on Dowex 50 cation-exchange resin using the HCl elution system of Cohn (17).

A final indication of the destruction of impurities and recovery of purine activity is the close agreement between the total cold acid-insoluble activity and the sum of protein plus the total nucleic acid activity. (The sum of the RNA and the DNA measurements could be substituted equally well for the latter quantity.) Although little purine activity was lost, chemical changes did occur in at least one of the purine bases, adenine, as evinced by changes in its ultraviolet absorption spectrum as heating progressed. Absorption spectra measured on the same solution of adenine, after it had been 1, 2, and 4 hr in the autoclave, passed through an isosbestic point at 275 mμ, indicating, therefore, that a single, as yet unidentified, ultraviolet-absorbing compound probably resulted from the acid treatment. Nevertheless, all ultraviolet-absorbing material was removed by charcoal, in keeping with the over-all conservation of purine activity.

Purity of P^{32}-Labeled Fractions. The frequently used method of Schmidt and Thannhauser (3) for analyzing P^{32}-labeled cells was shown by Davidson and Smellie (18) to yield an RNA fraction contaminated by low-molecular-weight non-nucleotide materials which incorporated label rapidly, relative to the nucleotides. Since charcoal was known to adsorb heterocyclic and aromatic compounds (such as nucleotides) selectively, its use was expected to minimize these contaminants. It was decided to test this selectivity most critically by using cells which had been exposed to phosphate-P^{32} for only a short time in order that the activity of any such metabolically active contaminants as might have been present would be accentuated. The distribution of activity in such cells is presented in Table 3 together with the results obtained on digestion of the same cells with nucleases (using the procedures described under *Materials and Methods*) prior to their fractionation.

The charcoal-adsorbable activity of the acid-soluble pool was accepted as being entirely due to nucleotides in light of the demonstration

TABLE 3

THE FRACTIONATION OF P^{32}-LABELED CELLS BY METHODS P (1), (2), AND (3) COMPARED TO THAT OBTAINED USING THE SCHMIDT-THANNHAUSER METHOD; THE EFFECT OF PRIOR DIGESTION OF CELLS WITH NUCLEASES ON THE ACTIVITY OF NUCLEIC ACID FRACTIONS AS A MEASURE OF THEIR PURITY[a]

Nominal radioactive component in fraction (ignoring contaminants)	Isolation procedure			
	Methods P (1), (2) and (3)		Schmidt-Thannhauser method	
		(c.p.m./10^6 cells)		(c.p.m./10^6 cells)
Acid-soluble Nucleotides	Activity adsorbed on charcoal	19,100		—
Total activity precipitable with cold PCA	Activity collected on filter	15,800	Activity plated directly on planchet	16,100
"Lipids"	Activity in $CHCl_3$–ethanol measured with immersion counter	1,810	Activity in pooled solvents (ethanol–ether) measured with immersion counter	6,450
RNA	Total activity in acid filtrate before adsorption on charcoal (measured with immersion counter)	11,900	Total activity measured with immersion counter	7,600
	Activity adsorbed onto charcoal	4,680	Activity adsorbed onto charcoal	4,850
	Cells pretreated with RNAse; activity remaining adsorbed on charcoal	374		—
DNA	Activity on filter [P(2)]	1,605	Activity plated on planchet directly	1,570
	Activity remaining after cells were pretreated with DNAse	54		

[a] Cells, grown for 5 hr in a minimal medium containing 5 mc/mmole of P^{32}-orthophosphate, were analyzed either directly or after sonic disintegration and digestion with nucleases.

by Tsuboi and Price (6) that charcoal selectively adsorbed nucleotide phosphorus from the acid-soluble pools of rat tissues. The relatively high activity of the pools described in Table 3 is related to the brief labeling period, since, in uniformly labeled cells, this activity was usually no greater than the sum of activities incorporated into RNA and DNA.

The low activity of the lipid fraction obtained in step P (2) probably reflected an extensive hydrolysis of phospholipids during the hot alkali digestion since the "missing" activity appeared instead in the acid filtrate of P (3).

The presence of contaminants in the Schmidt-Thannhauser "RNA" fraction is well demonstrated by the difference between its activity measured first directly then after adsorption on charcoal. The latter activity agreed with that obtained by procedure P (3). No unequivocal estimate of the purity of the activity adsorbed by charcoal from the RNA fraction could be made from the effect of predigesting the cells with RNAse since the undigested residue (8% of the total activity) might well have represented the acid-precipitable "RNAse resistant core" (19) of the original RNA. On the other hand, the almost complete elimination of activity from the DNA fraction on predigestion of the cells with DNAse implies that this fraction *is* radiochemically pure.

Since Davidson and Smellie (18) had provided a precedent for the possible existence of low-molecular-weight contaminants which might have been bound to the nucleic acids and then released during their hydrolysis, a more direct analysis of the purity of the RNA and DNA fractions was made by the ion-exchange chromatography of these fractions on Dowex 1 anion-exchange resin. Before the ribonucleotide fraction isolated in step P (3) could be chromatographed, most of the acid in the filtrate was removed as an insoluble perchlorate by the addition of KOH, and most of the remaining salt was precipitated by evaporation of the neutral solution on a flash evaporator at 40°C. The deoxyribonucleotides in the DNAse–diesterase digest, on the other hand, were adsorbed directly to the column. Since the amounts of labeled nucleotides involved gave immeasurably small optical densities to the eluates, the appropriate nucleotides were added as carrier. After the ribonucleotides had been adsorbed, the column was washed with 500 ml of 0.02 *M* ammonium formate and eluted using the formate system of Cohn (17). The deoxyribonucleotides were washed on the column with 500 ml of 0.02 *M* ammonium acetate and then eluted with the acetate system of Hurst *et al.* (20). Further details are specified in Figs. 1 and 2.

The eluates were collected in 10-ml fractions, and after their optical densities had been measured at 260 mμ, they were chilled and combined with enough PCA to lower their pH to 0.75. Their charcoal-adsorbable

activity was then isolated by procedure P (3), and after the charcoal had been filtered off, the nonadsorbable activity remaining was measured using an immersion counter.

Figure 1 shows the existence of two as yet unidentified radioactive peaks which together comprise 9.2% of the total charcoal-adsorbable

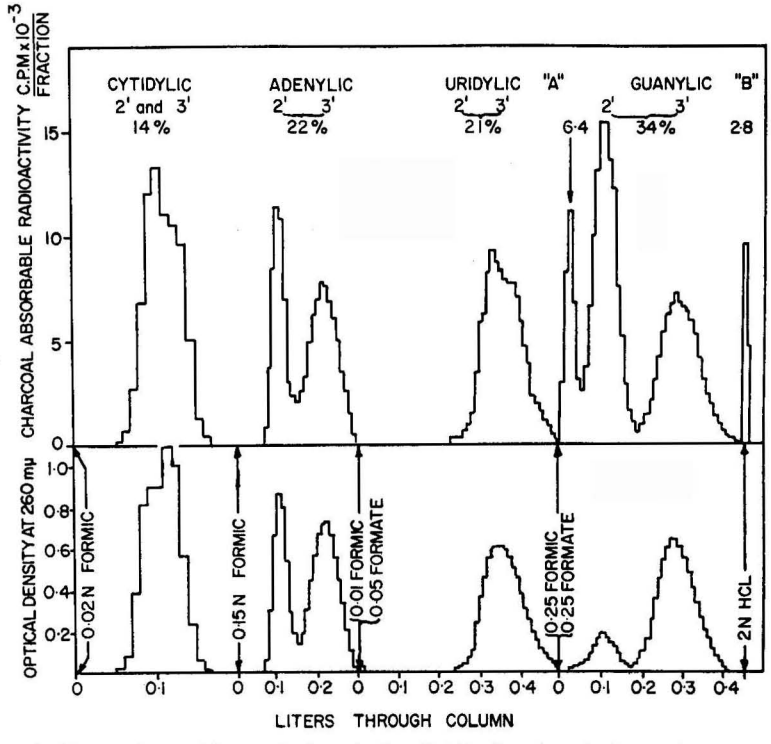

FIG. 1. Chromatographic analysis of the RNA fraction isolated by procedure P (3) from 10^6 cells labeled 5 hr in a complete medium containing phosphate-P^{32}, 15 mc/mmole. Nonradioactive carrier: 2.5 mg each of the four ribonucleotides cited. Exchanger: Dowex 1-X8 formate, 200–400 mesh; 10 cm × 0.80 sq cm. Influent: Formic acid, sodium formate, and HCl as shown; flow rate: 0.75 ml/min. Only charcoal-adsorbable activities are recorded. Negligible activity remained on the column after elution with 2 *N* HCl.

activity of the RNA fraction. In further experiments it was shown that no ultraviolet absorbing material appeared in the region of peak A when the carrier nucleotides were also exposed to 1 *N* NaOH at 100°C for 10 min, thus ruling out the possibility that this peak represented, say, the deaminated purine nucleotides. In view of the capacity of charcoal to adsorb a wide variety of ultraviolet-absorbing compounds, it is possible

that peak A is identified with the unidentified ultraviolet-absorbing radioactive component, also designated as "compound A," which Davidson and Smellie (18) found on electrophoresis of P^{32}-labeled RNA hydrolyzates. In both cases, these compounds contained 6% as much phosphorus as the four nucleotides taken together.

Incidentally, no particular significance could be attached to the great discrepancy between the specific activities of the two isomers of guanylic acid since the optical density referred to the carrier added while the radioactivity referred to the traces of cellular ribonucleotides.

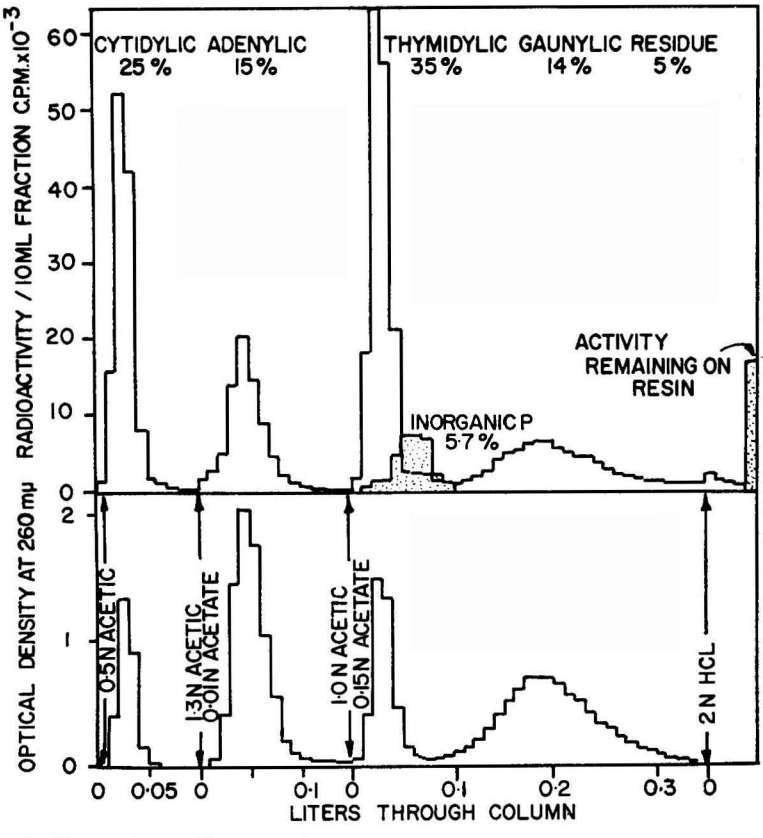

FIG. 2. Chromatographic analysis of a DNAse–phosphodiesterase digest of the DNA fraction isolated by procedure P (2) from 5×10^6 cells labeled 5 hr in a complete medium containing phosphate-P^{32}, 15 mc/mmole. Nonradioactive carrier: 1.5 mg each of the four monodeoxyribonucleotides. Exchanger: Dowex 1-X8 acetate, 200–400 mesh; 10 cm × 0.08 sq cm. Influent: Acetic acid, sodium acetate, and HCl as shown; flow rate: 1.0 ml/min. Both charcoal-adsorbable and nonadsorbable activities were measured; the latter, after correction for self-absorption, being represented by shaded areas.

Figure 2 shows that the activity of the DNAse–phosphodiesterase digest of the DNA fraction was resolved by chromatography into charcoal-adsorbable mononucleotides comprising 90% of the total activity, a nonadsorbable peak identified by its total precipitability with the magnesia reagent (21) as inorganic phosphate, and a fraction which remained uneluted on the column. The inorganic phosphate almost certainly resulted from the dephosphorylation of mononucleotides by traces of 5′-nucleotidase in the phosphodiesterase. This nucleotidase activity could be readily demonstrated by following the disappearance with time of charcoal-adsorbable activity in a solution containing purified radioactive deoxyribonucleotides and diesterase. Indeed it may be taken as a further proof of the purity of the DNA fraction that *crude* snake venom (containing relatively high levels of nucleotidase), when used in place of the purified diesterase caused a liberation of inorganic phosphate from 99% of that portion of the total DNA fraction (95% of the total) which could be rendered acid soluble by enzymic digestion. Therefore, at most 5% of the DNA activity is due to contaminants.

Reproducibility of Results

Although no detailed statistical analysis was made of the reproducibility of these procedures, it may be stated that the maximum difference between results obtained from replicate samples (usually three or more in number for routine analyses) rarely exceeded 10% and was usually around half this value. Greater variation than 10% could almost always be related to a loss of cells on incautious removal of supernatants following centrifugation or with insufficient scraping of residues from the bottom of the sample tubes. It was particularly important, for example, to ensure that all of the residue remaining after the lipid extraction of P^{32}-labeled cells was either dissolved or resuspended in cold acid.

CONCLUSIONS

In this laboratory, the methods which have been described have been used not only to analyze the fate of precursors incorporated over a relatively short period of time, but also, where conditions of uniform labeling have been achieved, to permit a measure of the net synthesis of proteins and nucleic acids. Since these techniques are very rapid, an experienced worker may perform, for example, as many as 60 separate P^{32} fractionations in an 8-hr day.

These methods have been used so far only in the analysis of the labeling of mouse fibroblasts (L cells) in tissue culture. Nevertheless the absence, to date, of any qualitative differences in the metabolism of the

precursors with which this paper deals among the mammalian cell strains commonly grown in tissue culture, ensures that these techniques will be applicable without further modification to the analysis of these cells. In more distantly related materials, particularly where there is some likelihood that the C^{14} precursors may be catabolized to smaller fragments, radioactivity may be incorporated into cell components in addition to those found labeled in the mammalian cells. In such cases it is best to use an operational definition of the isolated fractions (such as their acid lability or solubility, their charcoal adsorbability, etc.) until their true identity can be independently established.

It should be noted, however, that these methods do *not* permit the analysis of any fewer cells than could be handled by conventional techniques employing suitable amounts of carrier materials. What they do permit is the elimination of the repeated centrifugations, prolonged hydrolyses, careful pipettings, and quantitative transfers which are essential to these conventional methods of analysis. In addition, they permit measurement of radioactivity under optimal conditions without requiring the evaporation of extracts and the self-absorption losses this often entails. Finally, the purity of the fractions obtained, especially from P^{32}-labeled cells, exceeds considerably that achieved by other non-chromatographic methods.

SUMMARY

Rapid procedures are presented for analyzing microgram quantities of animal cells which have incorporated either radioactive adenine, glycine, or phosphate during growth in tissue culture. After proteins, ribonucleic acid and deoxyribonucleic acid are separated using abbreviated versions of the Schmidt-Thannhauser and Schneider methods, the acid-precipitable fractions are collected on membrane filters. The acid-soluble nucleic acid derivatives are adsorbed selectively on charcoal, and this in turn is collected on a filter. Both types of filter are then counted directly. The radioactivity bound by charcoal was shown to be relatively free of the non-nucleic acid impurities, which commonly interfere with conventional analytical procedures.

ACKNOWLEDGMENTS

The author is grateful to Prof. Louis Siminovitch of this department for the facilities he has provided for this research and the constant interest he has taken in it. Prof. C. W. Helleiner, also of this department, donated the purified diesterase used in this work and provided much useful advice as well. This research was aided by grants from the National Cancer Institute of Canada and the Public Health Service of the National Institutes of Health of the United States.

REFERENCES

1. ROBERTS, R. B., COWIE, D. B., BOLTON, E. T., ABELSON, P. H., AND BRITTEN, R. J., *Carnegie Inst. Wash. Publ. No.* **607** (1955).
2. HANAWALT, D., *Science* **130,** 386 (1959).
3. SCHMIDT, G., AND THANNHAUSER, S. J., *J. Biol. Chem.* **161,** 83 (1945).
4. SCHNEIDER, W. C., *J. Biol. Chem.* **161,** 293 (1945).
5. THRELFALL, C. J., *Biochem. J.* **65,** 694 (1957).
6. TSUBOI, K. K., AND PRICE, T. D., *Arch. Biochem. Biophys.* **81,** 223 (1959).
7. SANFORD, K. K., EARLE, W. R., AND LIKELY, G. D., *J. Natl. Cancer Inst.* **9,** 229 (1948).
8. GRAHAM, A. F., AND SIMINOVITCH, L., *Proc. Soc. Exptl. Biol. Med.* **89,** 326 (1955).
9. HEALY, G. M., FISCHER, D. C., AND PARKER, R. C., *Proc. Soc. Exptl. Biol. Med.* **89,** 71 (1955).
10. STADTMAN, E. R., AND KORNBERG, A., *J. Biol. Chem.* **203,** 47 (1953).
11. HURST, R. O., AND BUTLER, G. C., *J. Biol. Chem.* **193,** 91 (1951).
12. SCHMIDT, G., *in* "The Nucleic Acids," (E. Chargaff and J. N. Davidson, eds.), Vol. I, p. 555. Academic Press, New York, 1955.
13. ARANOFF, S., "Techniques of Radio-biochemistry," p. 57. The Iowa State College Press, Ames, Iowa, 1956.
14. SCOTT, J. F., FRACCASTORO, A. P., AND TAFT, E. B., *J. Histochem. Cytochem.* **4,** 1 (1956).
15. HURST, R. O., AND KUKSIS, A., *Can. J. Biochem. Physiol.* **36,** 919 (1958).
16. FISKE, C. H., AND SUBBAROW, Y., *J. Biol. Chem.* **66,** 375 (1925).
17. COHN, W. E., *in* "The Nucleic Acids" (E. Chargaff and J. N. Davidson, eds.), Vol. I, p. 211. Academic Press, New York, 1955.
18. DAVIDSON, J. N., AND SMELLIE, R. M. S., *Biochem. J.* **52,** 599 (1952).
19. KUNITZ, M., *J. Gen. Physiol.* **24,** 15 (1940).
20. HURST, R. O., MARKO, A. M., AND BUTLER, G. C., *J. Biol. Chem.* **204,** 847 (1953).
21. LELOIR, L. F., AND CARDINI, C. E., *in* "Methods in Enzymology" (S. P. Colwick and N. O. Kaplan, eds.), Vol. III, p. 850. Academic Press, New York, 1957.

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Measurement of Carboxymethylcellulase Activity

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INTRODUCTION

The method outlined by Reese[1] (10) for measuring cellulase activity, involving action of the enzyme on carboxymethylcellulose followed by determination of the reducing power of the resulting sugars with the aid of the dinitrosalicylic acid reagent of Sumner and Sisler (11), appears to be one of the most useful of many reported. Carboxymethylcellulose is favored over native cellulose as a substrate because of the great advantage of its solubility in water, while dinitrosalicylic acid reagent is favored over other reagents for determination of reducing sugar because of its great convenience. To avoid ambiguity, however, with regard to the presumptive relationship of enzymes acting on carboxymethylcellulose to those acting on native cellulose, the former will be designated carboxymethylcellulase, as suggested by Festenstein (1).

Through experience with the method, technical details have been worked out to produce a simplified procedure which will be described in the present paper. The use of iodine–potassium iodide (4, 7) for determination of the reducing sugar produced in the test is also described. Comparisons are made of results obtained with the two different sugar reagents and with carboxymethylcellulases from different sources.

SOURCES OF CARBOXYMETHYLCELLULASE

Samples of carboxymethylcellulase studied were a lyophilized preparation from *Myrothecium verrucaria* QM 460 (6), cellulolytic culture filtrates from *Trichoderma viride* QM 6a, *Penicillium pusillum* QM 137g, and *Pestalotiopsis westerdijkii* QM 381 and an acetone precipitate of a filtrate from Basidiomycete QM 806. The filtrates and the acetone precipitate were prepared and placed at our disposal by Dr. E. T. Reese and Dr. Mary Mandels of this laboratory.

[1] Reese, E. T., private communication.

REAGENTS

Buffered Carboxymethylcellulose Substrate

Forty-four and eight-tenths grams of Hercules 50T carboxymethylcellulose (CMC) are dissolved in 2800 ml water at 90°C. This is best accomplished with the aid of a 1-gal Waring blendor which effectively disperses gelatinous lumps of the CMC. The mixture is cooled, 800 ml of 0.5 *M* citrate buffer (35 gm citric acid monohydrate and 98 gm sodium citrate dihydrate per liter), 0.4 gm Merthiolate, and 0.4 gm glucose are added and the solution is diluted to 4 l. The reagent is stored at 4°C. The final solution thus prepared contains 1.00% CMC based on a 12% moisture content found for the original CMC powder. The citrate concentration is 0.1 *M*; the pH, 5.0; the glucose concentration, 0.1 mg/ml. This level of glucose (0.1 mg/ml) provides a safe excess over the level required to compensate for the destruction of reducing sugar in the color test (5), yet does not contribute to too high a blank reading. The Merthiolate serves as a preservative.

Dinitrosalicylic Acid Reagent

Modified dinitrosalicylic acid reagent containing Rochelle salts (5) is prepared by dissolving a mixture of 40 gm dinitrosalicylic acid, 8 gm phenol, 2 gm sodium sulfite, and 800 gm Rochelle salts in 2 l of 2% sodium hydroxide and diluting to 4 l.

Iodine–Potassium Iodide Reagents

Two-hundredths normal iodine–10% potassium iodide, 0.75 *M* sodium carbonate, and 1.2 *N* phosphoric acid reagents are prepared as described elsewhere (7).

PROCEDURES

Procedure Using Dinitrosalicylic Acid as Sugar Reagent

(*a*) One-milliliter aliquots of cold (4°C) CMC substrate are added to 1-ml aliquots of enzyme samples and of glucose standards in 14-mm o.d. colorimeter tubes. The samples are mixed by inverting 10 times. (*b*) The mixtures are incubated at 50°C for a desired period of time, usually 20 min, and are then cooled in running tap water. (*c*) Three-milliliter aliquots of dinitrosalicylic acid reagent are added to each digest and the reactants are mixed by inverting five times. (*d*) The mixtures are then heated in a boiling water bath for 15 min, followed by cooling in running tap water adjusted to ambient temperature. (*e*) Readings of absorbance are finally made in a Beckman model DU spectrophotometer

at a wavelength of 640 mμ and a slit width of 0.2 mm. Shorter wavelengths will be found suitable where readings are made in colorimeters using color filters.

Procedure Using Iodine–Potassium Iodide as Sugar Reagent

Steps (*a*) and (*b*) are those described in the preceding section. (*c*) Following the procedure for aldoses (7), 1 ml of 0.75 *M* sodium carbonate and 2 ml of 0.02 *N* iodine–potassium iodide are next added to each tube, and the contents of the tubes are mixed by twirling. (*d*) The tubes are allowed to stand at 25°C for 1 hr. (*e*) Each mixture is acidified with 5 ml of 1.2 *N* phosphoric acid and is carefully stirred with a glass rod. (*f*) Absorbances are measured in a Beckman model DU spectrophotometer at a wavelength of 480 mμ and a slit width of 0.2 mm.

RESULTS

Definition of Unit of Carboxymethylcellulase Activity

For convenience the unit of carboxymethylcellulase activity may be defined as that activity shown by an amount of enzyme in 1 ml of solution which will produce an amount of reducing sugar equivalent to 1.0 mg glucose when incubated for 20 min at 50°C with 1 ml of 1% carboxymethylcellulose dissolved in 0.1 *M* citrate buffer at pH 5, as determined with the dinitrosalicylic acid reagent, or 0.5 mg glucose, as determined with the iodine–potassium iodide reagent. This unit closely approximates the Cx unit of Reese and co-workers (2, 10). The reason for the different amounts of glucose corresponding to 1 unit of activity when the different sugar reagents are used will become clear from results given in the next section.

Dilution Curves for Carboxymethylcellulase from Myrothecium verrucaria

Data obtained for amounts of reducing sugar formed when various dilutions of a solution of carboxymethylcellulase from *Myrothecium verrucaria* are incubated with carboxymethylcellulose for widely varying periods of time (20 min and 24 hr) are shown graphically in Fig. 1. The amounts of reducing sugar indicated by the dinitrosalicylic acid reagent (upper curves) are about twice those indicated by the iodine–potassium iodide reagent (lower curves). This can be explained, at least to a large degree, by the fact that with the dinitrosalicylic acid reagent oligosaccharides from cellulose yield on an equimolecular basis much more color than does glucose,[2] while with the iodine–potassium iodide

[2] Miller, G. L., and Slater, R. W., unpublished work.

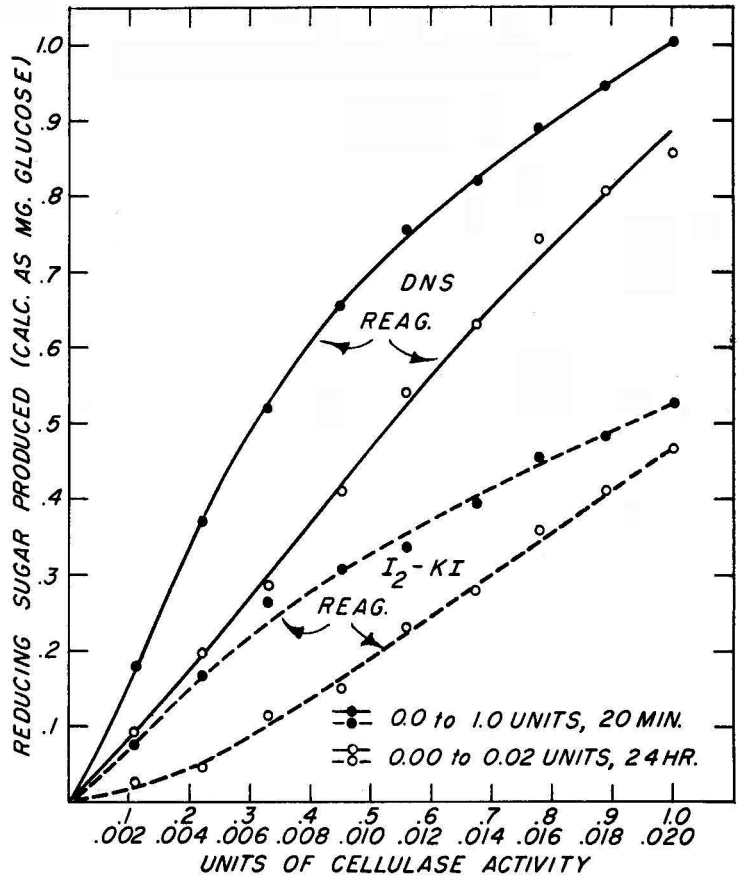

FIG. 1. Dilution curves for carboxymethylcellulase from *Myrothecium verrucaria*, obtained with different ranges of enzyme activity and different periods of incubation and with different sugar reagents.

reagent the degrees of reaction of oligosaccharides and glucose are the same (7).

Comparison of Dilution Curves for Carboxymethylcellulases from Different Fungi

Dilution curves of carboxymethylcellulases from *Myrothecium verrucaria* and *Trichoderma viride*, measured under conditions of 20 min incubation, but covering 10 times the range of activity given in Fig. 1, are shown in Fig. 2. It is clear that the dilution curves for the different cellulases are different from one another and cannot be superimposed. Curves obtained for carboxymethylcellulases from other fungi, not shown

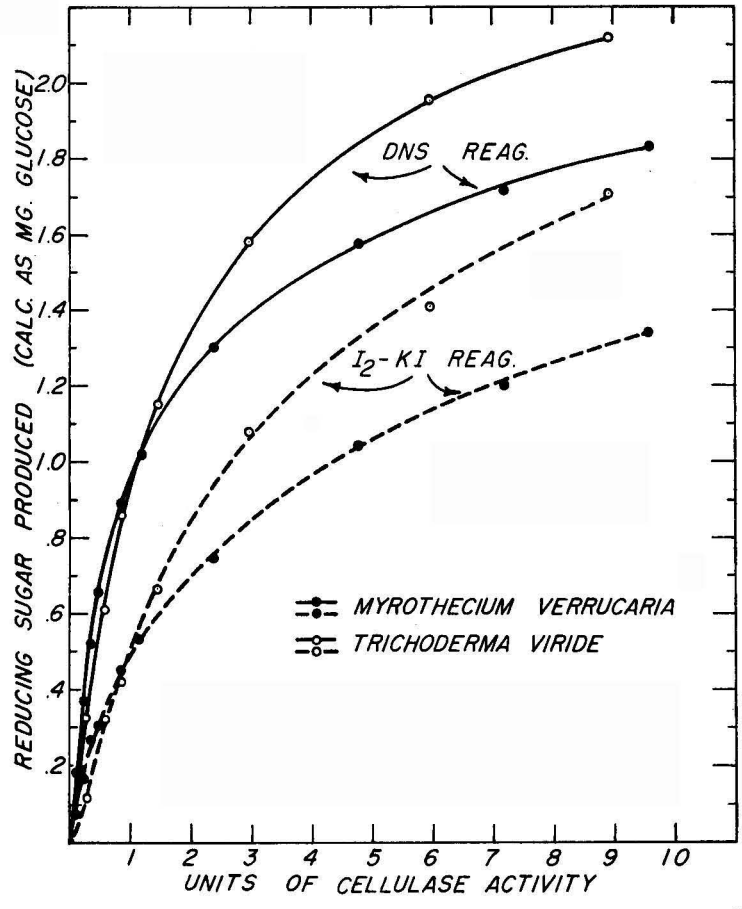

FIG. 2. Comparison of dilution curves for carboxymethylcellulases from different fungi, obtained over a wide range of enzyme activity and with different sugar reagents.

on the figure, also were found to be distinctive. Further, the differences in the dilution curves for different carboxymethylcellulases were found to be reproducible.

DISCUSSION

Within certain limitations the procedures described in this paper will be found to be highly useful. For many purposes dilution curves of the types shown in Figs. 1 and 2 can be used as standards of reference for estimations of activities of unknowns, although where this is done the enzyme used for the dilution curve and that contained in the unknown should correspond to one another. The wide ranges of activities which

can be measured is clearly apparent from the data. When relative values of activity suffice, as in measurements of zone-electrophoretic fractions, values of absorbance may be used as first approximations of enzyme activity, with excellent reproducibility (8). The dinitrosalicylic acid reagent is more convenient for measuring reducing sugar than the iodine-potassium iodide reagent. However, the latter should prove to be the more reliable in kinetic studies because of its greater specificity for aldehyde groupings.

It can be shown for the present data that a constant product of enzyme activity and time fails to yield a constant amount of reducing sugar, as required by first-order reaction kinetics (9). Further, the dilution curves of the different enzymes reveal, to varying degrees, anomalous *S* shapes. These deviations may be due to the operation of complicating factors such as inhomogeneity of substrate (degree of substitution, particle size), multiplicity of enzyme components (2, 3, 6, 8), and presence or absence of activators or inhibitors. It may be presumed that other cellulase methods described in the past are subject to similar deviations. Methods for controlling such factors are not available at present. Caution is particularly advisable in making comparisons of activities of enzymes from different sources. Empirical applications of the described procedures should, however, aid in resolving the apparent complexities of cellulase systems.

SUMMARY

Methods are described for measurement of carboxymethylcellulase activity involving action of the enzyme on carboxymethylcellulose followed by determination of reducing power of resulting sugars with the aid of dinitrosalicylic acid or iodine–potassium iodide. Results obtained with the different reagents and with carboxymethylcellulases from different sources are compared. The applicability of the methods is discussed.

REFERENCES

1. Festenstein, G. N., *Biochem. J.* **65,** part **2, 23,** (1957).
2. Gilligan, W., and Reese, E. T., *Can. J. Microbiol.* **1,** 90 (1954).
3. Hash, J. H., and King, K. W., *J. Biol. Chem.* **232,** 381 (1958).
4. Jermyn, M. A., *Australian J. Sci. Research, Ser. B,* **5,** 409 (1952).
5. Miller, G. L., *Anal. Chem.* **31,** 426 (1959).
6. Miller, G. L., and Blum, R., *J. Biol. Chem.* **218,** 131 (1956).
7. Miller, G. L., and Burton, A. L., *Anal. Chem.* **31,** 1790 (1959).
8. Miller, G. L., Blum, R., and Hamilton, N. F., *J. Chromatog.* **3,** 576 (1960).
9. Moelwyn-Hughes, E. A., *in* "The Enzymes" (Sumner, J. B., and Myrbäck, K., eds.), Vol. 1, Part 1, p. 28. Academic Press, New York, 1950.
10. Reese, E. T., Siu, R. G. H., and Levinson, H. S., *J. Bacteriol.* **59,** 485 (1950).
11. Sumner, J. B., and Sisler, E. B., *Arch. Biochem.* **4,** 333 (1944).

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Micro Column Chromatographic Method for Analysis of Oligosaccharides

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INTRODUCTION

The successful resolution of malto- and cellodextrins of different degrees of polymerization by ethanol–water gradient elution from chromatographic columns composed of stearic acid-treated mixtures of charcoal and Celite (1, 2) suggested the development of a micro analytical method based on these principles. The method, described below, was worked out with the aid of cellodextrins of degree of polymerization of 2–7 and of selected oligosaccharides of other series. It provides means for qualitative identification of individual oligosaccharides and quantitative measurement of their concentrations. One of its more important uses might be in kinetic studies on the breakdown, or formation, of different polysaccharides.

EXPERIMENTAL

Adsorbent

Forty grams of Darco G-60[1] and 40 gm of Celite 545[2] were mixed with 320 ml of 2½% stearic acid (technical) in absolute ethanol for ½ hr, followed by filtering with suction. The adsorbent mixture was then suspended in 320 ml of 50% ethanol saturated with stearic acid, refiltered, resuspended in 200 ml of water, and refiltered again. The final product was dried to a powder in a vacuum desiccator over calcium chloride.

Test Materials

The cellodextrins used were prepared from acid hydrolyzates of cellulose (2). Individual cellodextrins were dissolved in water to give concentrations of 0.1%. In special experiments 1% concentrations also

[1] An activated carbon preparation manufactured by Atlas Powder Company, Wilmington, Del.

[2] A siliceous filter-aid manufactured by Johns-Manville Co., New York, N. Y.

were used. Solutions of mixtures of cellodextrins were made up to contain 0.1% glucose, 0.1% cellobiose, and 0.1% concentrations of each of other homologs of the series up to and including celloheptaose.

Laminaribiose, laminaritriose, laminaritetraose, isomaltose, and isomaltotriose, obtained from enzymic hydrolyzates of laminarin and dextran, were supplied by Dr. E. T. Reese. A preparation of maltose which was tested was obtained from Central Scientific Company, New York.

Preparation and Use of Column

Five hundred milligrams of dry adsorbent mixture, prepared as described above, was placed in a fritted-glass micro filter tube which was 5.5 cm long, 1.0 cm inside diameter, and medium–coarse porosity. To wet

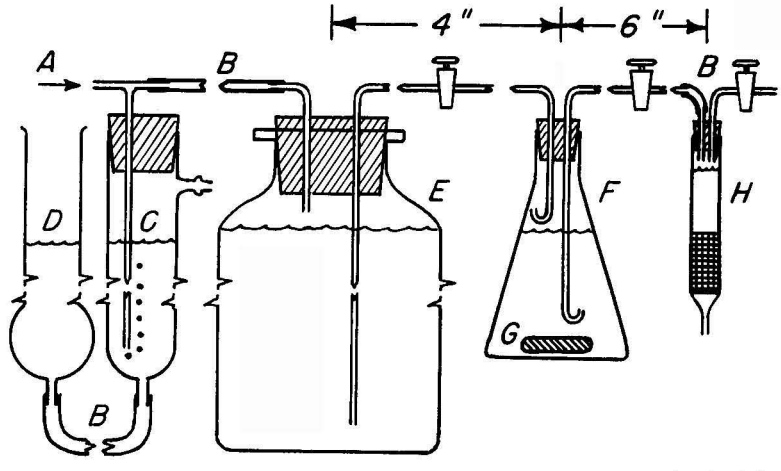

FIG. 1. Micro column chromatography apparatus. *A*, compressed air inlet. *B*, flexible rubber tubing connections. *C*, modified side-arm test tube containing water, used as a manostat. *D*, drying tube used as leveling bulb for manostat. *E*, 500-ml bottle used as reservoir for absolute ethanol. *F*, 50-ml Erlenmeyer flask used as mixing vessel. *G*, Teflon-covered magnetic bar operated by magnetic stirrer, not shown. *H*, elongated fritted-glass micro filter tube, used to contain column of adsorbent. The stoppers are rubber.

the adsorbent, it was suspended in 2–3 ml of 20% ethanol. The liquid was pushed through the adsorbent by blowing through a rubber tube connected to the top of the filter tube, and ethanol which remained on the adsorbent was removed by washing the column five times with 0.5-ml portions of water.

A 0.2-ml sample of individual mono- or oligosaccharide, or of mixture

of oligosaccharides, was introduced into the absorbent, followed by 0.1 ml water. Two milliliters of water was then layered above the adsorbent, prior to commencement of the elution. For the elution, the top of the column was connected to a 50-ml Erlenmeyer flask used as a mixing vessel, as shown in the diagram of the apparatus in Fig. 1. The mixing vessel, charged with 50 ml water and operated with the aid of a magnetic stirrer, was fed from a reservoir containing absolute ethanol. Air pressure, equal to a few centimeters of water, was applied to the system with the aid of a manostat. For convenience in starting and stopping the elution process and in charging the various components of the system, stopcocks were placed in the line between the column and the mixing vessel, between the mixing vessel and the reservoir, and on a vent at the top of the filter tube. The apertures of inlet and outlet tubes to the mixing vessel were each bent upward to prevent bubbles from entering the tubes. The aperture of the inlet tube was open to an air space above the surface of the liquid in the vessel.

The eluate from the column was collected in 13 × 100 mm tubes with a drop-counting type of fraction collector, each tube receiving 10 drops. The pressure was adjusted to produce an initial rate of elution of one drop per 60 sec, and was not further adjusted. About 16 hr was required for elution of the most slowly eluted oligosaccharide tested, namely, celloheptaose.

Analysis for Carbohydrate

The entire eluate obtained for each fraction was used in the analyses for carbohydrate content. The fractions were each mixed with 3-ml of 0.2% orcinol in 70% sulfuric acid (v/v), heated for 20 min at 100°, and cooled to room temperature. Absorbances of the colored solutions which resulted were measured at a wavelength of 550 mμ with the aid of an automatic recording colorimeter (3). The values of absorbance which were obtained were compared with those of similarly treated glucose standards consisting of duplicate samples of 0.0, 0.05, 0.10, and 0.15 mg glucose, each in 0.5 ml water. The amounts of carbohydrate in each eluate could then be calculated in terms of glucose, when desired.

An important prerequisite for good results was the strict exclusion of dirt and dust from fraction collector tubes and from reagent.

RESULTS AND DISCUSSION

Characterization of Elution

Because of surface tension effects, sizes of drops of eluate diminished as the concentration of ethanol increased. The magnitude of this effect

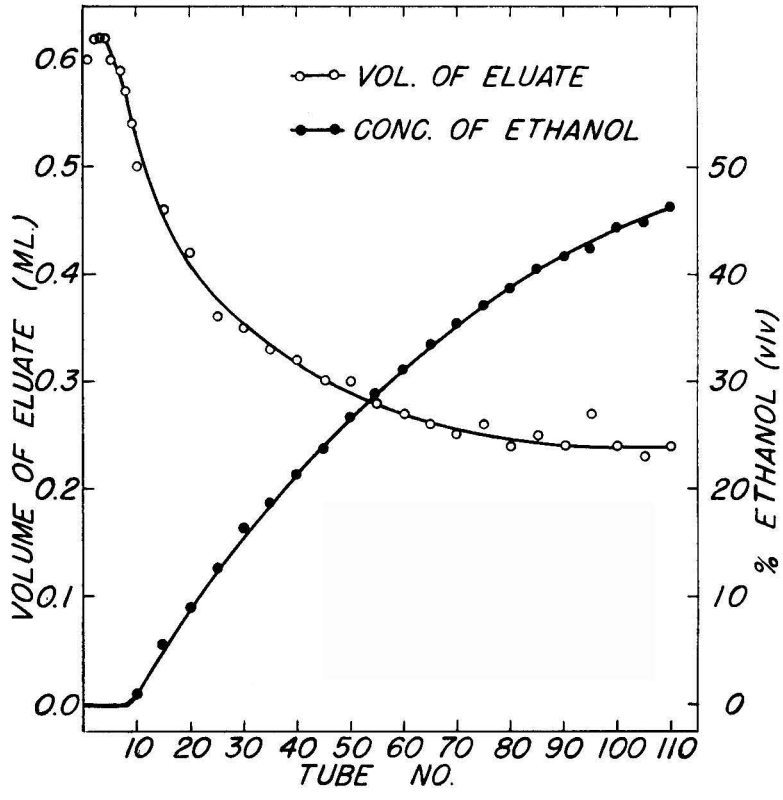

FIG. 2. Relation of volume of eluate and concentration of ethanol to tube number of eluate.

is shown in Fig. 2 by the relation of volume of eluate and of concentration of ethanol to tube number of eluate. Concentration of ethanol was measured refractometrically.

Qualitative Aspects of Method

Distribution patterns for individual cellodextrins and for a mixture of cellodextrins, in which values of absorbance are related to tube numbers of eluates, are shown in Fig. 3. The degrees of polymerization of the different components are indicated alongside the corresponding peaks in the patterns. It may be seen that individual oligosaccharides separated at the same tube numbers when tested in the presence of homologs as in their absence. In replicate tests it was found that positions of peak maxima seldom varied more than ± 1 tube. The concentrations of ethanol at which the different components were eluted from the ad-

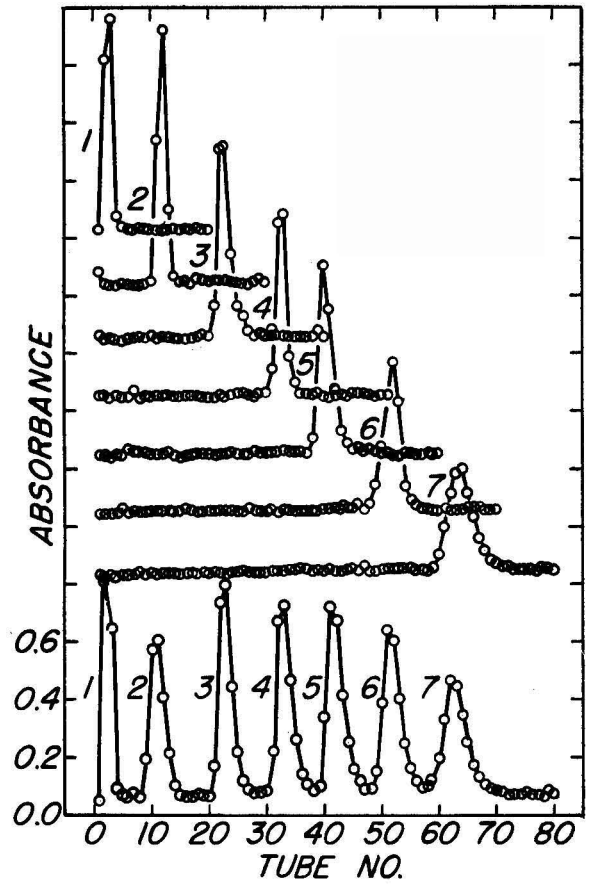

FIG. 3. Distribution patterns for individual cellodextrins and for a mixture of cellodextrins.

sorbent may be found by reference to Fig. 2. The slight "tailing" which was observed with each component could not be avoided.

Distribution patterns for laminaribiose, -triose, and -tetraose, not shown, revealed peaks at tube nos. 14, 27, and 40, respectively. Patterns for isomaltose and isomaltotriose revealed peaks at tube nos. 5 and 12, respectively. The commercial sample of maltose showed one major and two adjacent minor peaks; assuming the minor peaks to represent glucose and maltotriose, the peaks of the di-, and trisaccharide of this series appeared at tube nos. 8 and 15, respectively. It is clear from these results that resolution of homologs can be effected in series other than that of the cellodextrins and that corresponding isomers from different series may be distinguished from one another by differences in the tube

numbers at which they appear. The findings also suggest the possible use of the method for sugars differing in ways other than in degree of polymerization or in mode of linkage of monomeric units; for example, in length of carbon chain, in presence or absence of reducing groups, in nature of glycosidic substituent, in occurrence of aldose versus ketose groups, etc.

Quantitative Aspects of Method

The recoveries of glucose and of the different cellodextrins from the chromatographic column were found to be fairly quantitative when

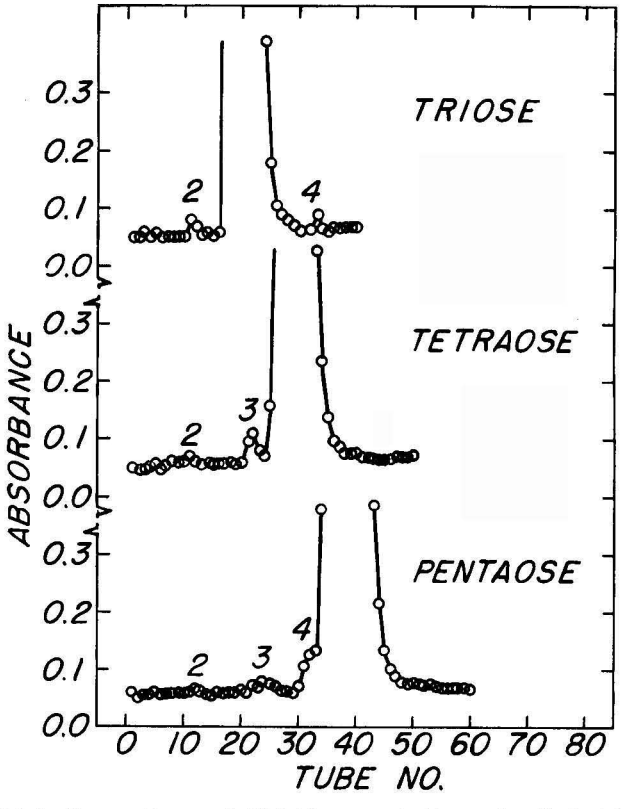

FIG. 4. Distribution patterns of 10-fold concentrations of cellodextrins, showing presence of homologs as contaminants.

appropriate corrections were applied to allow for differences in volumes of different eluate fractions, effect of ethanol on development of color, and uptake of water by the oligosaccharides as a result of hydrolysis in the color test. The best method of handling quantitative aspects of the

method, however, was by determination of empirical correction factors from data obtained by measurement of apparent recoveries of the different sugars, in terms of glucose. The factor found for glucose was 1.11; that for cellobiose, 0.94; those for cellotriose to -heptaose, 0.77. To calculate the true value for the concentration of cellotriose, for example, its apparent concentration in terms of glucose should be multiplied by 0.77. Results obtained by this procedure were accurate to $\pm$ 5%.

When cellotriose and -pentaose were added as artificial contaminants to cellotetraose in proportions of 2% of the total, they were readily detected on chromatographic analysis under the above conditions. Since homologs were not revealed as contaminants in the distribution patterns shown in Fig. 3, it could be concluded that contamination by any individual homolog was probably lower than 2%. More precise evaluation of contamination was possible, however, by analyzing the cellodextrins at 10-fold concentrations. Results obtained for cellotriose, -tetraose, and -pentaose under these conditions are shown in Fig. 4. It was calculated from the data that total contaminations with homologs was of the order of 1%, the principal contaminant in each instance being the next lower homolog. The more common occurrence of lower, rather than higher, homologs is explained by the nature of the tailing which occurred in the original chromatographic fractionation of the oligosaccharides (2). Had higher homologs been present in the different oligosaccharides, however, they would have been detected since it was found that cellopentaose added to cellotetraose in as low a proportion as 0.2% of the total sample could readily be detected. Similar analyses for cellohexaose and -heptaose, not shown, were somewhat less satisfactory. Excessive spread of the cellohexaose peak appeared to obscure any -pentaose present, while the low solubility of the -heptaose handicapped the significance of its analysis.

Variables in the Method

When charcoal–Celite mixtures were used which were untreated with stearic acid, the separation of oligosaccharide components from the column was retarded and the resolution was poor, confirming the original observations of Alm (1) in this respect.

The use of large test samples, or of more rapid elution rates, decreased the resolution of components, but did not change materially the numbers of the tubes at which the different peaks appeared. The use of steeper ethanol–water gradients or of shorter columns also decreased resolution, but at the same time accelerated the separation of components from the column and thereby affected the positions at which peaks appeared.

Reuse of columns led to tailing, doubling of peaks, and eventually to

slower rates of separation of components from the column. For best results, therefore, columns should not be reused.

Alteration of certain variables may be advantageous under special circumstances. For example, more rapid elution rates or steeper gradients may be used where only the qualitative aspects of the test are of importance. On the other hand, slower elution rates or shallower gradients may be used where greater separations of components are desired and where the longer period of time required for the test is not a serious drawback.

Different batches of adsorbent may show somewhat different chromatographic properties. To allow for this, a calibration could be carried out for a given batch of adsorbent, using a slowly eluted material such as cellohexaose as a reference material. The tube numbers at which other oligosaccharides appear may then be expected to be proportional to that at which the cellohexaose appears.

SUMMARY

A micro column chromatographic method for qualitative identification and quantitative measurement of oligosaccharides from cellulose and other series of oligosaccharides is presented. Analyses are made with 0.2-mg quantities of test material. Contaminants in the form of homologs are estimated with the use of 2-mg quantities.

REFERENCES

1. ALM, R. S. *Acta Chem. Scand.* **6,** 1186 (1952).
2. MILLER, G. L., DEAN, J., AND BLUM, R., *Arch. Biochem. Biophys.* In press (1960).
3. MILLER, G. L., AND CUMMINGS, C. L., *Anal. Chem.* **31,** 481 (1959).

ANALYTICAL BIOCHEMISTRY 2, 141–150 (1960)

Chromatographic Separation of Enzymes for Analytical Purposes[1]

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INTRODUCTION

In our studies on the enzymes of hexose metabolism, we needed quantitative analyses of some of the enzymes in crude tissue samples, and the measurements were frequently difficult because of the presence of competing activities. An exploration of the use of the cellulose derivatives developed by Peterson and Sober (1) was made. Chromatography with these derivatives is now a common tool, but in order to obtain the reproducibility and speed required for our purposes several departures from the usual practice were necessary. The quality of the results that have been obtained suggests that the new technique may have more general value, and it is described in the following.

METHODS

1. General Description

The sample of soluble proteins is passed through a column of DEAE-cellulose[2] and then through a column of CM-cellulose, the DEAE-cellulose having been charged as the Mg $EDTA^{2-}$ salt, and the CM-cellulose as the NH_4^+ salt. The columns are separated when the sample has passed through them, and they are individually eluted with a solution of $(NH_4)_2Mg$ EDTA. The elution is made with a concentration gradient that accelerates to a linear rise in concentration. This gradient is established by using two mixing flasks in series with a reservoir of the salt solution.

[1] This investigation was supported by research grant No. RG-5431 from the National Institutes of Health, U. S. Public Health Service.

[2] The following abbreviations are used: DEAE-cellulose for diethylaminoethylcellulose; CM-cellulose for carboxymethylcellulose; EDTA for ethylenedinitrilotetraacetate.

2. *Apparatus*

The arrangement used for eluting a column is shown in Fig. 1. The reservoir *A*, air inlet *B*, two-neck mixing flasks *E*, and Luer adapters *D* were especially made by the Kontes Glass Company, Vineland, New Jersey. Similar reservoirs with air inlets were used for adding sample

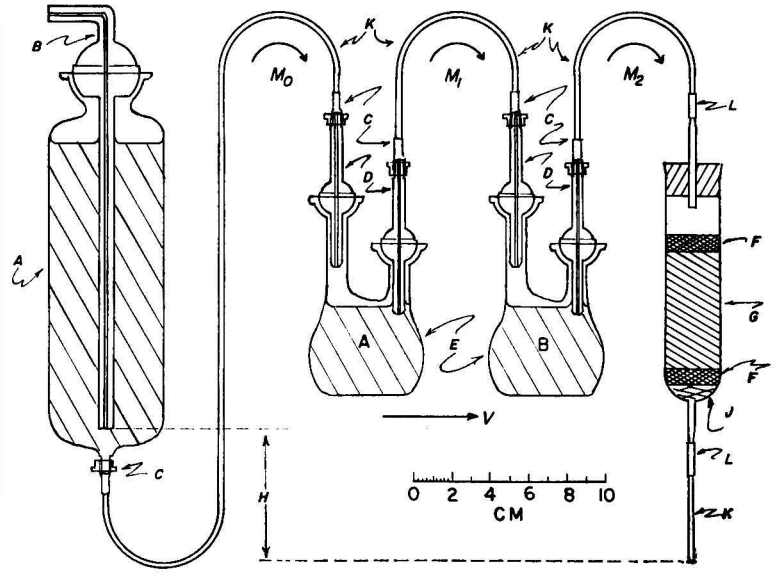

FIG. 1. Apparatus assembled for gradient elution. *A:* Reservoir fitted with 35/20 ball joint and Luer taper, filled with eluant of concentration M_0. *B:* Air inlet to maintain constant hydrostatic head. *C:* Polyethylene fittings for Luer joints, made by flattening a warmed piece of 3/16 in. tubing to fit snugly over a short piece of Clay-Adams, Inc. No. PE320 tubing. *D:* Luer adapters for 18/9 ball joints. *E:* Two-neck mixing flasks *A* and *B*, magnetically stirred and filled initially with V_A and V_B ml of concentrations M_A and M_B, respectively, which become M_1 and M_2 after V ml have passed through. *F:* Glass beads, 0.5 mm diameter, from Arthur S. LaPine and Co. *G:* Column packing of DEAE- or CM-cellulose. *H:* Hydrostatic head. *J:* Borosilicatae glass wool. *K:* Clay-Adams, Inc. No. PE260 polyethylene tubing. *L:* Clay-Adams, Inc. No. PE320 polyethylene tubing.

solutions and for charging columns. The glass columns were made in the shape shown. Connectors were made from polyethylene tubing. The metal adapters available commercially for connecting polyethylene tubing to Luer fittings are corroded by the eluant used in these studies, but they have been convenient with other eluants. All of the equipment was rinsed with water redistilled from all-glass apparatus before use.

3. Preparation of the Columns

Two departures from the usual techniques have been made to get quick fractionation. The ratio of the length to diameter of the column packing was made small, and the column was packed loosely. Under these circumstances, especial care is necessary in obtaining uniform packing.

The column size is chosen to give the desired flow rate with a head of 5–10 cm, measured to the bottom of the outlet from the column. Flow rates of 1–3 ml/min were obtained with column packings varying from 23 to 33 mm diameter with 40–60 mm length. Adjustments of size were made from the approximation that the flow varies with $(\text{diameter})^2$ and $(\text{length})^{-1}$. Thus, if the bed volume was changed by a factor n, the diameter was changed by $n^{1/4}$ and the length by $n^{1/2}$, if constant flow rate was required.

From 0.2 to 0.3 gm of DEAE- or CM-cellulose/ml of desired bed volume was made into a light slurry with water. The amount of material necessary to achieve a column of satisfactory flow rate after discarding fines was determined by experience with each lot. The slurry was brought to a boil *in vacuo* to eliminate trapped air.

The CM-cellulose slurry was charged by mixing with 50 ml of 0.02 *M* NH_4Cl–NH_3, pH 7.6, per gram. The pH was returned to 7.6 with 1 *M* NH_3 at the glass electrode. Fines and smaller fibers were discarded by decantation. The remaining compacted mass was drained and mixed with 10–20 vol water. The smaller particles were again discarded by decantation, the remainder drained and mixed with 20 ml of 0.01 *M* $(NH_4)_2Mg$ EDTA–NH_3, pH 7.6, per initial gram of CM-cellulose. If necessary, alternating washes and buffer were repeated until the pH of the slurry remained over 7.55.

The DEAE-cellulose slurry was charged in a similar manner, using 50 ml of 0.01 *M* $(NH_4)_2Mg$ EDTA–NH_3, pH 7.6, per gram of cellulose, alternated with water rinses until the pH of the slurry dropped below 7.65.

The glass columns, fitted with clamped polyethylene tubing on their outlets, were first plugged with borosilicate glass wool, and partially filled with water. Glass beads, 0.5 mm diameter, were added to form a bed up to the bottom of the straight-walled portion of the column. The outlet tubing was raised until its open end was about 1 cm below the surface of the beads. The water level was allowed to drop almost to the beads, and then the cellulose slurry was added in portions. Each portion was added along a glass rod that was being rotated around the walls of the column, or with a broad-tipped pipet moving over the sur-

face as the slurry was delivered. If the surface of the beads was disturbed, the column was repacked. After enough cellulose to make about 8 mm of bed had been added, the column was drained dropwise until most of the cellulose was compacted. Then the open end of the outlet tubing was raised, more slurry was added and gently stirred to mix it uniformly with the uncompacted material in the column. This process was repeated until the column was filled to the desired level. All the lengths of packing cited were measured at this point in the procedure. The water level was cautiously dropped to the top of the bed, and when no free-standing liquid remained, the column was slowly drained further until the bed had shrunk 1–2 mm. Additional buffer was added to a depth of about 1 cm. A bed of glass beads, 0.5 mm diameter, was added to the top of the column with great care to avoid disturbing the cellulose.

Care was exercised at all times after the columns were packed to avoid drainage under hydrostatic heads of more than a few centimeters. The compaction that would otherwise result not only lessened the flow rate, but usually made the packing less uniform, resulting in smearing of the fractions. Low flow rates indicated a need for discard of more of the small fibers from the packing, not a need for more hydrostatic pressure.

When the columns were packed, they were rinsed with 2–3 bed volumes of 0.0012 *M* $(NH_4)_2$Mg EDTA–NH_3, made by diluting the buffer used in charging the DEAE-cellulose column. The columns were placed in the cold overnight or longer. The flow rate was tested before use by rinsing with more buffer. The DEAE- and CM-cellulose preparations were obtained from Distillation Products, Inc., and Bio-Rad Laboratories, Inc., respectively.

4. Addition of Samples

The samples used in this laboratory were the supernatant obtained by centrifugation (40 min at 74,000 × *g*) of homogenates made with 4 ml of 0.25 *M* sucrose/gm tissue. The supernatants were adjusted to pH 7.6 with 1 *M* imidazole and diluted with 6.67 vol water. The diluted sample was passed through the columns, using a reservoir at a constant pressure head of approximately 10 cm. Collection of fractions was begun immediately, and the eluant was tested for sucrose by the anthrone method (2) to determine the holdup volume of the columns. When the last of the sample had entered the top (DEAE-cellulose) column, the mixing flask arrangement shown in Fig. 1 was connected, and the elution was begun. When a volume sufficient to clear the sample solution from the DEAE-cellulose column had passed in, the columns were discon-

nected, and the bottom CM-cellulose column was attached to similar apparatus on a second fraction collector.

5. Gradient Elution

A concentration gradient was established for eluting the columns by filling the mixing flasks with known volumes of water, 115 ml in each being customary. A solution of 0.52 *M* $(NH_4)_2Mg$ EDTA–NH_3, pH 7.6 measured at 0.2 *M* and 25°, was placed in the reservoir for most of the studies reported here. The shape of the resultant concentration gradient is shown in Fig. 2. This technique has been used in our laboratory for a

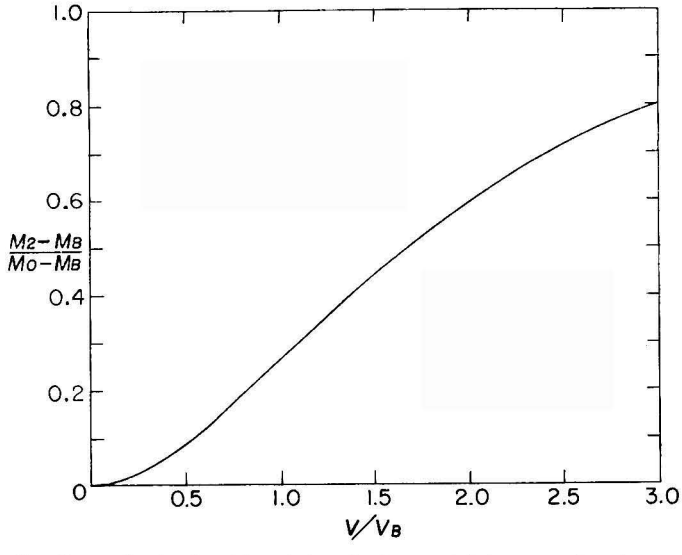

FIG. 2. Gradient obtained with mixing flasks containing equal volumes at equal initial concentrations. The fraction of the possible rise in concentration is shown as a function of the number of mixing-flask volumes of eluant delivered. See text for symbols.

variety of concentration gradients other than that shown, and more general methods of calculation are described in an appendix to this report. The technique has also been used in other laboratories at this Medical School for the separation of a glyceraldehyde phosphate dehydrogenase from *Alcaligenes faecalis* (3), the separation of lysozyme from lung tissue (4), and the separation of catalase from human erythrocytes.[3]

[3] Thorup, O. A., Jr., personal communication.

6. Preparation of Ammonium Magnesium EDTA

The preparation of this compound is complicated only by impurities in commercially available forms of EDTA. The free acid, in our experience, must be purified via the disodium salt for chromatographic purposes, and it is simpler to start with the latter compound, obtainable in adequate quality from Distillation Products, Inc. The disodium salt was suspended in 9 ml water/gm and heated to 95–100°. The resultant solution was acidified with 2.1–2.2 equiv of 12 *M* HCl per mole of salt, added with brisk stirring. Upon standing, crystallization of the acid began, and, after cooling to room temperature, the crystals were collected by filtration and washed with water until a solution of the crystals in nitric acid gave a negative test for chloride.

After drying *in vacuo*, the anhydrous acid was suspended in 1 l water per mole, and one mole of MgO was stirred in. After cessation of heat evolution, 2.5 moles of 15 *M* NH_3 per mole of acid used was cautiously stirred in. After cooling, any solid, which indicates poor quality in the reagents, was discarded by filtration, and 4.5 vol of absolute ethanol was added to the filtrate, with stirring. The crystalline chelate was collected by filtration and washed with ethanol. Acetone was added to the combined filtrate and washings to collect more of the solid. The crops were dried separately. In earlier preparations, quality was gaged by the ammonia and magnesium content. Later, nonchelated EDTA was estimated by the ultraviolet absorption at pH 9.0 and total EDTA by absorption after addition of excess cupric ions at pH 5.5 (5). With either criterion the first crop of crystals usually had a negligible amount of nonchelated EDTA and assayed as $(NH_4)_2Mg$ EDTA $\cdot 3H_2O$ with 80–83% yield. The second crop, approximately 10% yield, frequently required reprecipitation with the addition of more ammonia.

RESULTS

Analyses of typical enzymes in a sample of rat liver are shown in Fig. 3. Collection of the fractions shown was complete in less than 4 hr after the rat was killed. As markers, the position of catalase, which is superimposed on the 6-phosphogluconic dehydrogenase, and of an uncharacterized, sharply eluted hemochromogen that is coincident with the peak of the aldolase from the CM-cellulose column were useful. Catalase was located qualitatively by adding approximately 50 μl of each fraction to 3 ml of 1% hydrogen peroxide and noting the appearance of bubbles. In practice, the position of each enzyme may be predicted within one fraction for each run under the conditions shown, and this reproducibility has greatly aided the use of the columns for analytical purposes, since only those fractions in which the enzyme to be assayed

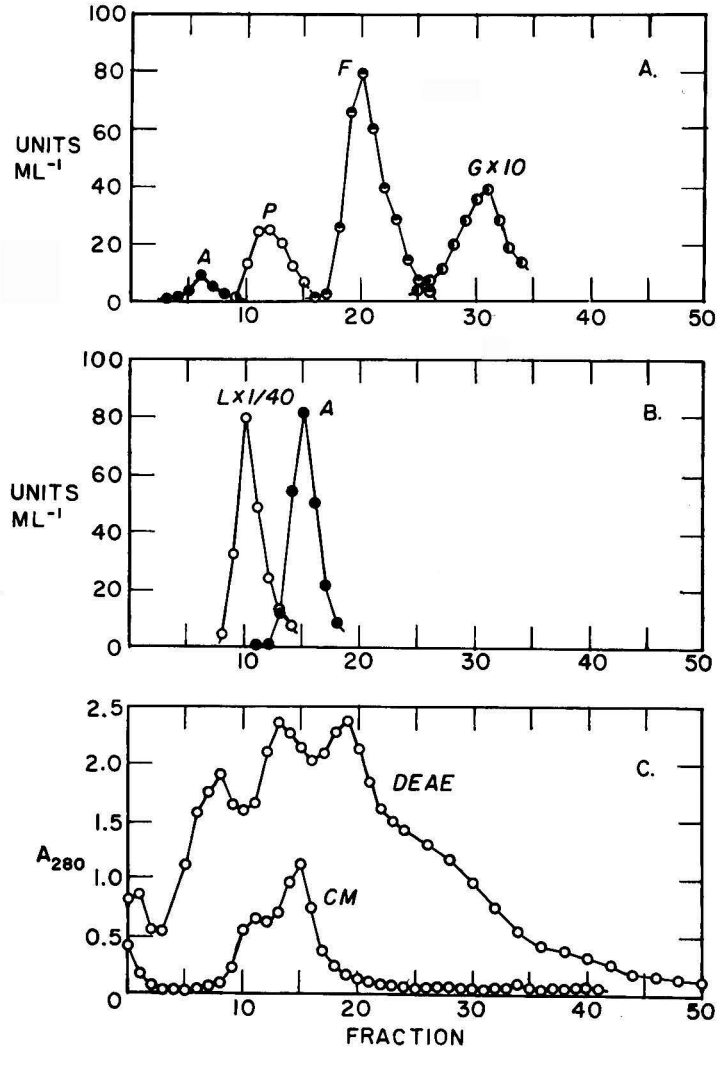

FIG. 3. Typical elution pattern of soluble cytoplasmic proteins from rat liver.

A. Eluate from DEAE-cellulose column. *A* represents minor aldolase fraction; *P*, 6-phosphogluconic dehydrogenase; *F*, fructosediphosphatase; *G*, glucose-6-phosphate dehydrogenase.

B. Eluate from CM-cellulose column. *L* represents lactic dehydrogenase; *A*, the major aldolase component.

C. Absorbance at 280 mμ of eluates from DEAE- and CM-cellulose columns.

Units are micromoles of product formed/hr. Fractions of 3.5 ml volume are numbered after sample has passed through column in question.

is predicted to occur need be measured. It may also be noted in passing that no evidence has been found for the occurrence of more than one molecular species of any of the enzymes tested, except for aldolase, when the samples are protected from denaturation.

If analyses of the type shown in Fig. 3 are to be of value, the samples must be reasonably stable. To determine if this was the case, repeated measurements were made with the same fractions, which were kept at 2–4°. Glucose-6-phosphate dehydrogenase showed no significant change after 13 days. 6-Phosphogluconic dehydrogenase was unchanged after 2 days, but most of the fractions had slightly less activity after 10 days, the maximum decrease being 8%. The aldolase activity eluted from CM-cellulose was mostly unchanged the next day, with a maximum decrease of 3½%, but all fractions were lower after 12 days, the maximum loss being 28%. The aldolase activity from DEAE-cellulose showed no significant change after 12 days. Fructosediphosphatase activity was uniformly higher after 4 days, with a maximum increase of 15%, suggesting an activation similar to that previously reported (6). The activity after 9 days was slightly lower, but still above the initial values. Lactic dehydrogenase activity was decreased as much as 35% after 13 days. It is not known how much of the decrease in activity in any case resulted from the repetitious handling of the fractions without especial precautions against surface denaturation. However, it is apparent that instability of samples is not an important problem with the above enzymes, with the possible exception of lactic dehydrogenase, which has not been studied intensively.

A more detailed report of results of assays made by this technique for these and other enzymes from differing sources will be made elsewhere.

APPENDIX

1. Calculation of Concentration Gradients

The following symbols are used:

V = volume of fluid passed through the system,
V_A, V_B = respective volumes of solutions in flasks A and B,
M_0 = molarity of solution in reservoir,
M_1 = molarity of solution in flask A after passage of V ml,
M_2 = molarity of solution delivered to column from flask B, and
M_A, M_B = respective molarities of solutions initially present in flasks A and B.

The behavior of the system is defined by the equation:

$$dV = \frac{V_B dM_2}{M_1 - M_2} = \frac{V_A dM_1}{M_0 - M_1} \tag{1}$$

In the general case with mixing flasks of different volumes, the fraction of the possible rise in concentration that has been attained after passage of V ml is given by the expression:

$$\frac{M_2 - M_B}{M_0 - M_B} = 1 - \frac{e^{-\frac{V}{V_B}}\left[\frac{V_A}{V_B}\frac{(M_A - M_B)}{(M_0 - M_B)} - 1\right] + e^{-\frac{V}{V_A}}\left[\frac{V_A}{V_B}\left(1 - \frac{M_A - M_B}{M_0 - M_B}\right)\right]}{\frac{V_A}{V_B} - 1} \quad (2)$$

Analysis of the solutions of this equation have revealed no gradients with advantages over those obtained with the technically simpler arrangement of two flasks with identical volumes. A solution of this special case has already been published (7) and is:

$$\frac{M_2 - M_B}{M_0 - M_B} = 1 - e^{-\frac{V}{V_A}}\left[1 + \frac{V}{V_A}\left(1 - \frac{M_A - M_B}{M_0 - M_B}\right)\right] \quad (3)$$

In the present study, it has been advantageous to have the initial increase in gradient illustrated in Fig. 2, and this is obtained by having the solution in both mixing flasks of the same initial concentration. For other analyses in this laboratory, we have desired a linear gradient, and the means of obtaining this are discussed in the next two sections.

2. Obtaining a Linear Gradient

If $(M_A - M_B)$ is greater than 0.3 $(M_0 - M_B)$, the concentration gradient will be essentially linear until $(M_2 - M_B)$ rises over 0.55 $(M_0 - M_B)$. Greatest economy of reagents is obtained by starting with $(M_A - M_B) = 0.3(M_0 - M_B)$, and this is the routine condition used in this laboratory. Calculated values for the resultant gradient are given in Table 1.

TABLE 1
CALCULATED VALUES FOR A LINEAR CONCENTRATION GRADIENT[a]

V/V_B	0.1	0.2	0.4	0.6	0.8	1.0	1.2	1.6	2.0	2.4	3.0
$\frac{M_2 - M_B}{M_0 - M_B}$	0.032	0.066	0.143	0.220	0.300	0.374	0.446	0.572	0.676	0.757	0.845

[a] The fraction of the total possible rise in eluant molarity is listed as a function of the mixing-flask volumes that have been delivered. See text for symbols used. $(M_A - M_B)$ is $0.3(M_0 - M_B)$.

3. Extending a Linear Gradient

A linear gradient may be extended indefinitely by appropriate changes in the reservoir and mixing flask concentrations. A change that has been

most useful because it does not require discard of any solutions is made by beginning with a volume in the reservoir equal to $1.51V_A$, and with $(M_A - M_B) = 0.3(M_0 - M_B)$. When the reservoir has completely drained, $(M_2 - M_B)$ will be $0.545(M_0 - M_B)$, and $(M_1 - M_B)$ will be $0.845(M_0 - M_B)$. If the reservoir is now refilled with a solution of a concentration $0.545(M_0 - M_B)$ above its original value, it may be seen that the original *differences* in concentration are restored, and the linear rise will continue for another 1.51 mixing flask volumes. Using the apparatus described, a deviation of less than 2% from the predicted value has been found after two such changes in reservoir concentration.

Similarly, a gradient with an initial acceleration, as shown in Fig. 2, may have its linear portion extended. In this case, the linear portion begins with $V/V_A = 0.428$, and $(M_2 - M_B) = 0.068\ (M_0 - M_B)$. When $V/V_A = 1.94$, $(M_2 - M_B) = 0.576(M_0 - M_B)$, and at this point raising the concentration in the reservoir by $0.508(M_0 - M_B)$ will continue the linear gradient for 1.51 mixing flask volumes.

SUMMARY

1. The apparatus and necessary calculations for conveniently obtaining linear and accelerating concentration gradients with two mixing flasks connected in series are described.

2. Procedures for charging and packing columns of cellulose ion-exchange derivatives so as to obtain rapid flow rates and reproducible results in the analysis of enzymes from crude tissue samples are given. These procedures use diethylaminoethylcellulose columns charged with magnesium ethylenedinitrilotetraacetate ions, and carboxymethylcellulose columns charged with ammonium ions.

3. The preparation of diammonium magnesium ethylenedinitrilotetraacetate is described.

4. Illustrative results with commonly measured enzymes are given, with evidence that the resultant fractions are stable enough to permit accurate analysis of the activities with the expectation that the values represent the state of the original sample.

REFERENCES

1. PETERSON, E. A., AND SOBER, H. A., *J. Am. Chem. Soc.* **78,** 751 (1956).
2. MOKRASCH, L. C., *J. Biol. Chem.* **208,** 55 (1954).
3. BRENNEMAN, F. N., AND VOLK, W. A., *J. Biol. Chem.* **234,** 2443 (1959).
4. OSIMA, S., MYRVIK, Q. N., AND LEAKE, E. S., *Bacteriol. Proc.* 113 (1960).
5. MARTELL, A. E., AND CALVIN, M., "Chemistry of the Metal Chelate Compounds," p. 223. Prentice-Hall, New York, 1952.
6. POGELL, B. M., AND MCGILVERY, R. W., *J. Biol. Chem.* **197,** 293 (1952).
7. SCHWAB, H., RIEMAN, W., AND VAUGHAN, P. A., *Anal. Chem.* **29,** 1357 (1957).

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A Planchet-to-Planchet Microstill[1]

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INTRODUCTION

The still illustrated in Figs. 1 and 2 was devised to permit the separation of small amounts of acetate and other similarly volatile acids labeled with carbon-14 from a small volume of solution also containing labeled glucose and lactate. The separation was followed by measurement of the volatile acids by standard counting procedures. The still could presumably be used for other kinds of separation by distillation. It has an advantage over other arrangements for distillation of radioactive compounds in that the volatile materials are distilled directly from one planchet to another, thus aiding efficient recovery of small samples and simplifying counting of distillate and residue at each stage of a succession of redistillations. Moreover, only two simple parts of the still (a collar and a spacer) become contaminated in use, and these can be quickly washed and dried; the enclosure is completed by the donor and recipient planchets which are not reused, except in successive distillations within a given analysis.

APPARATUS AND PROCEDURES

The still was assembled from materials already available in the laboratory with the exception of the Lucite spacer, which was simple to make. Mounting the container of freezing solution on an old microscope stand was a considerable convenience, but other arrangements could have been devised.

For each distillation, a small volume (e.g., 10 μl) of a suitable trapping agent (e.g., NaOH for volatile acids) was placed on the recipient planchet, which was then inverted on the Lucite spacer. The donor planchet, usually carrying 0.1 ml of solution, was placed on the stove,

[1] This work was supported by a research grant (B 702) from the Institute of Neurological Diseases and Blindness of the National Institutes of Health, U. S. Public Health Service.

and the collar, spacer, and recipient planchet were quickly put in place. The brass rod from the freezing mixture was brought against the recipient planchet, a drop of alcohol was added to ensure good thermal contact, and 15 min was allowed for distillation. The recipient planchet was then flipped over on the stove, revealing a snowlike condensate

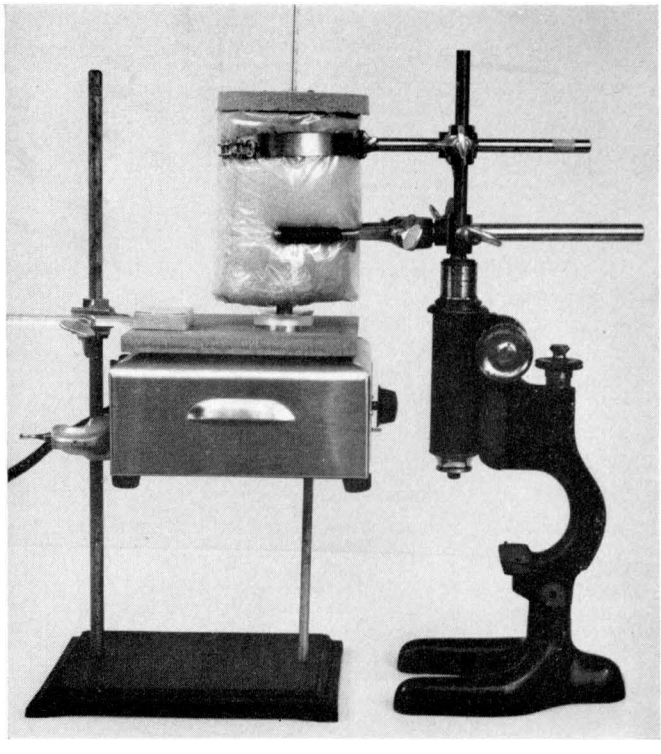

FIG. 1. General arrangement of apparatus. Surface temperature of the thermostated electric stove (Temco type 1900) was measured by a thermometer which projects to the left, the bulb of the thermometer being covered by a small box lined with insulating material. An old microscope stand supported a container of dry ice and acetone. The container was a tinned food can, 3 in. in diameter by 4 in. high, with a brass rod 7/16 in. in diameter soldered through the bottom. The can was covered with insulating material which was protected from condensation of moisture by a polyethylene food bag.

which immediately melted and was stirred with a glass rod to ensure good mixing with the trapping agent. The two planchets were left on the stove until dry.

The procedure followed in a typical application is illustrated in Fig. 3, which is largely self-explanatory. This procedure was used in an

analysis of metabolic products of sympathetic ganglia excised from rats. The experimental sample placed on planchet 1 was 25 μl of a bicarbonate-buffered physiological solution from a microincubation vessel in which the solution had been exposed to the tissue for 1 hr, plus 60 μl of 0.01 *N* NaOH used to wash out the vessel. Asterisks indicate radioactive compounds, labeled with carbon-14, which were expected to be

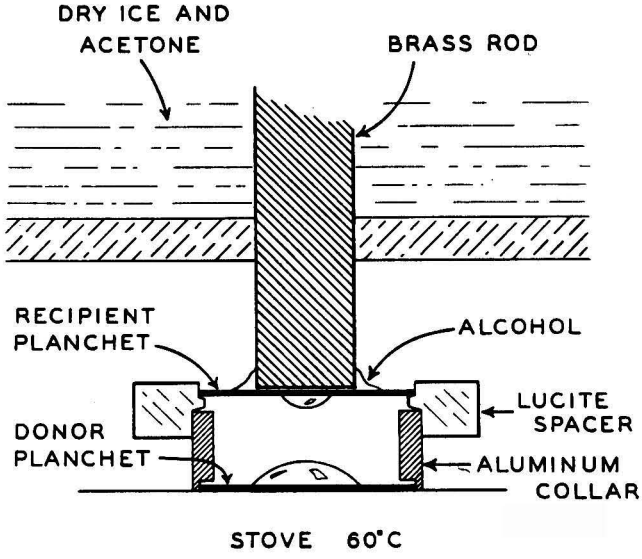

FIG. 2. Details of the still, approximately to scale. The planchets were 1 in. in diameter (Tracerlab E-24A, stainless steel). The aluminum collar was an inverted planchet holder (Tracerlab E-19). The spacer was bored from a square of Lucite, ¼ in. thick.

present in the experimental samples. Labeled glucose had been provided as substrate; labeled lactate and CO_2 were known to be produced by metabolism; the production of labeled acetate or some acid of comparable volatility was suspected and was being investigated.

As indicated in Fig. 3, labeled CO_2 was removed before distillation. This was done by placing planchet 1 for ½ hr in a container through which 100% CO_2 was passed. Acid was used as trapping agent on planchet 2 under the assumption that some volatile alkali might possibly be distilled as the sample became alkaline due to loss of CO_2 the first time it was warmed and dried.

TESTS WITH KNOWN MATERIALS

To test the effectiveness of the distillation procedures, known labeled samples of the materials expected to be present were added individually

to control samples of bathing fluid or to water containing 1 μmole NaOH (to simulate the excess nonvolatile base of the usual samples). Results of the tests are shown in Table 1 and Fig. 4.

A period of ½ hr was chosen for the initial wash in 100% CO_2 because it was found that the evolution of labeled CO_2 essentially stopped after

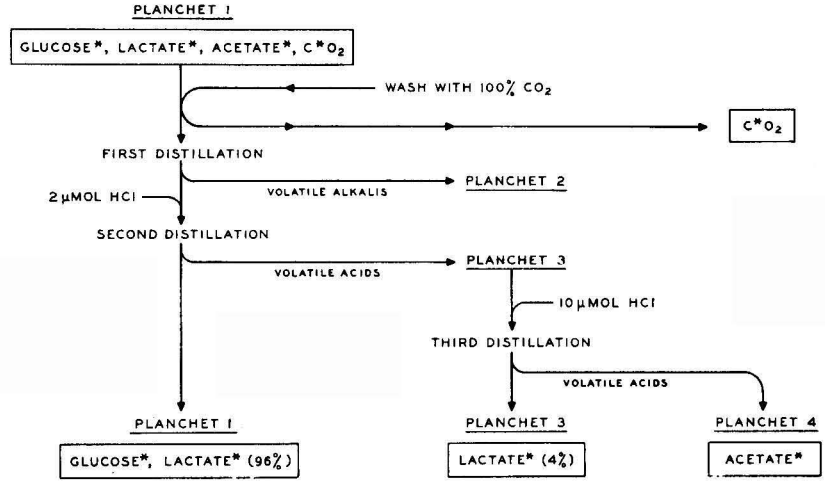

FIG. 3. An example of application of the still. Asterisks indicate substances labeled with carbon-14. Washing with 100% CO_2 was done by placing planchet 1, carrying the original liquid sample, in a small container through which 100% CO_2 flowed for ½ hr at a rate of 2–2.5 ml/min (see Fig. 4). In the first distillation, 1 μmole HCl was placed on planchet 2 to trap possible volatile alkalis; in the second, 5 μmoles NaOH was placed on planchet 3 to trap volatile acids; in the third, 10 μmoles NaOH was put on planchet 4. If all of the CO_2 were lost in the first distillation, the original sample would be left with 1 μmole of free base. Acids added to donor planchets before the second and third distillations were in 0.1 ml water. For further explanations, see text.

20 min (Fig. 4). The completeness of removal of labeled CO_2 was further attested by its absence from planchet 1 after the first distillation (Table 1, tests 1 and 2).

Lactate mostly remained on planchet 1 during the first and second distillations (Table 1, tests 3, 4 and 5). However, a few counts appeared on planchet 3 in the one test (test 5) where this was examined. A similar number of counts was again transferred in this test when the lactate on planchet 1 was redissolved in water and the distillation repeated onto another planchet 3 (not shown in Table 1). Although it is possible that the counts transferred were due to a labeled impurity, the possibility was not excluded that some of the lactate itself might have been trans-

ferred, especially since others have reported that a small percentage of lactate is lost under some conditions of distillation (1). Therefore, the third distillation was introduced as a precautionary although unproven attempt at further separation of lactate from acetate.

TABLE 1
TESTS OF STILL[a]

Test No.	Labeled material placed on planchet 1	Counts/min after wash with 100% CO_2 and first distillation			Counts/min after second distillation		Counts/min after third distillation	
		A In gas[b]	B Planchet 1	C Planchet 2[c]	D Planchet 1[d]	E Planchet 3	F Planchet 3	G Planchet 4
1	Carbonate	275	3					
2		3,500	2	0				
3	Lactate		183		130			
4			2,080		1,790			
5			720	5	670	31		
6	Acetate	−2	161					
7			1,460	8	14	1,190		
8							15	1,220
9					37	1,190	4	1,090
10	Glucose				13,800	47	5	22
11				1	14,800	61	7	48
12				1	14,100	17	7	9

[a] Counts were made to a minimum of 5000 counts or 10 min. in a counter with a background of about 1 count/min. Background has been subtracted from the tabulated figures. Large differences between counts in successive tests with a given material are due to the use of different amounts of material. Counts are not corrected for self-absorption, since the amounts of material were small (less than 0.6 mg of solids on any planchet), and high accuracy was not needed to show that acetate was adequately separated from the other substances.

[b] The 100% CO_2 used to wash out the labeled CO_2 was lead to a cell under a Geiger counter (Fig. 4). Total counts recorded in the gas phase were converted to equivalent counts per minute on a planchet after appropriate calibration experiments. Figures given in Column A are thus comparable with the data in the other columns.

[c] In tests 1–9, alkali was used as trapping agent on planchet 2 instead of the usual acid trap, since the known labeled materials present were acids.

[d] Counts from glucose samples in column D were attenuated with an absorber and by increased distance to prevent overloading the counting system. The tabulated data has been corrected for this attentuation to permit comparison with the other results.

Acetate, the material of primary interest in the present application was not lost during the wash with CO_2 or during the first distillation and was transferred satisfactorily in the second and third distillations (tests 6, 7, 8, and 9). Efficiencies of the transfers cannot be calculated

accurately from the counting rates given in Table 1, since there was some self-absorption even with the small samples used and the total dry mass of the sample was not the same on all planchets. However, it is obvious that percentage recoveries were high.

When labeled glucose was added in an amount equal to that used in the actual experiments (tests 10, 11, and 12), a few counts (less than 0.5%) were carried over to planchet 3 and many of these were further transferred to planchet 4. It is presumed that these counts represented

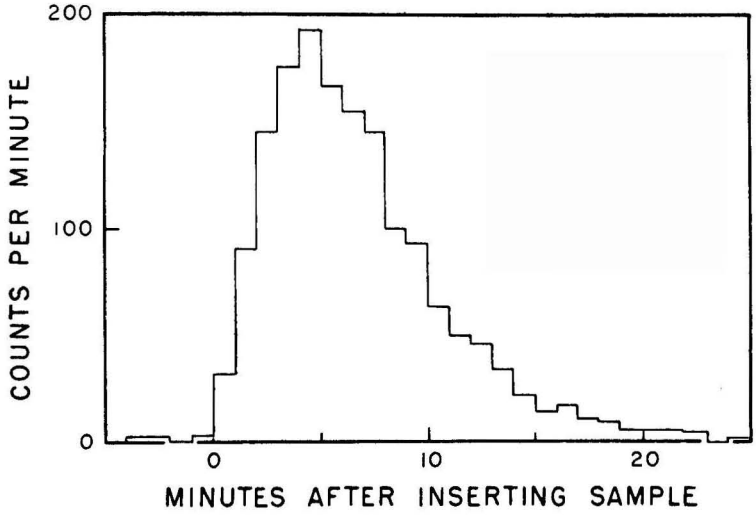

FIG. 4. Clearing of CO_2 labeled with carbon-14 by washing a sample with 100% CO_2. Sample consisted of 30 μl of a physiological solution plus 60 μl of 0.01 N NaOH. Sample was placed on a planchet in a small vessel through which 100% CO_2 passed at about 2 ml/min at room temperature. Counts were recorded by passing the gas leaving the vessel through a chamber under a Geiger counter.

either minor labeled contaminants in the glucose or products of microorganisms to which it had been exposed. In this connection it is relevant that the glucose solutions used in tests 10, 11, and 12 had been kept 1 hr at 37° in a nonsterile incubation vessel before transfer to planchet 1. These counts were allowed for, without further identification, by always running control samples of bathing fluid through the same procedures of incubation, washing, and distilling as those employed for the experimental samples. Production of volatile acid by the tissue was then calculated from the difference between experimental and control samples. Such control procedures permitted the microstill to be used for the application for which it was intended without further refinement.

EXPERIMENTAL RESULTS

Results of three experiments on superior cervical ganglia excised from rats are given in Table 2. Details of excising and weighing the ganglia, preparing the bathing solutions, the form of the incubation vessels, and provision for oxygenation have been described elsewhere (2, 3, 4). These details will not be repeated here where the purpose is to illustrate an application of the microstill. In the average of three

TABLE 2

OUTPUT OF VOLATILE ACIDS BY A SYMPATHETIC GANGLION

Experiment Weight of tissue (μg)	1/15/60 156	1/22/60 178	1/30/60 132
Counts/min on planchet 4 (av. with no. of samples in parentheses)			
Controls (in incubation vessel without tissue for 1 hr)	9.0(2)	46.2(4)	23.2(5)
Experimental (exposed to tissue for 1 hr)	14.8(3)	61.0(6)	35.7(6)
Increment	5.8	14.8	12.5
Volatile acid produced from glucose[a]			
μμmole C/hr	293	747	631
μmole C/gm dry weight/hr	1.9	4.2	4.8
μmoles as acetate/gm dry weight/hr	1.0	2.1	2.4

[a] Specific activity of the glucose used as substrate = 19,800 counts/min./μmole C, when dried on a planchet with 10 μmoles NaOH to simulate conditions on planchet 4

experiments presented in Table 2, the rate at which the tissue caused labeled carbon to appear on planchet 4 was 3.6 μmole C/gm dry weight/hr. Since this is only about 1% of the rate at which the tissue takes up carbon as glucose (2), it is evident that very little of the consumed glucose was metabolized to acetate. Moreover, since the experiments were undertaken to test a hypothesis that a considerably larger fraction might be metabolized to acetate, the hypothesis was considered definitively refuted, and further refinements of method were not attempted.

REFERENCES

1. BERNSTEIN, I. A., AND WOOD, H. G., *in* "Methods in Enzymology" (S. D. Colowick and N. O. Kaplan, eds.), Vol. IV, p. 563, Academic Press, New York, 1957.
2. HOROWICZ, P., AND LARRABEE, M. G., *J. Neurochem.* **2**, 102 (1958).
3. LARRABEE, M. G., *Methods in Med. Research* **9**, in press (1961).
4. LARRABEE, M. G., HOROWICZ, P., STEKIEL, W., AND DOLIVO, M., *in* "Metabolism of the Nervous System" (D. Richter, ed.), p. 208. Pergamon Press, New York, 1957.

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Effect of Formation of Insoluble Polysilver Salts on Argentometric Titration of Mercaptoacids

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INTRODUCTION

The two most widely used volumetric reagents for the determination of the thiol group are standard solutions of iodine and of silver nitrate. The iodimetric titration, first employed by Klason and Carlson (1), is based on the oxidation of the thiol to the corresponding disulfide. The titration with silver involves formation of non-ionized silver mercaptide (2). In 1946, Kolthoff and Harris introduced amperometric techniques in titration of thiols with silver nitrate in ammonia buffers and were able to perform titrations in the milligram range with excellent accuracy (3). In a later publication, they demonstrated that titration with silver nitrate in an ammoniacal medium is applicable, in general, to all monofunctional mercaptans, that is to all mercaptans containing the thiol group as the only functional group (4). They found, however, that the course of reaction of such mercaptans with iodine depended on whether the thiol group was primary, secondary, or tertiary.

Because of its simplicity, good accuracy at low levels, and apparent selectivity toward the thiol group, the method of Kolthoff and Harris seemed well suited for biochemical work. Benesch and Benesch adapted it, in 1948, to the determination of thiol groups in materials of biological importance (5). Further in 1955, Benesch, Lardy, and Benesch introduced tris(hydroxymethyl)aminomethane (Tris) as the complexing agent for silver in place of ammonia (6). This enabled them to carry out determinations in neutral media. Because of the susceptibility of thiols to air oxidation in alkaline solutions, the new complexing agent offered an important advantage.

Nevertheless, the selectivity of ammoniacal silver toward thiol groups, in the presence of other functional groups which are present in materials of biological interest, was questioned by Cecil in 1950 (7). Kolthoff and Stricks observed that when cysteine is titrated amperometrically with silver nitrate in ammonia buffers, an excess of silver ammine is tied with silver cysteinate to form a higher complex (8). The

complexing of silver was quite pronounced when the concentration of titrated cysteine exceeded 5×10^{-4} *M*. Cecil and McPhee examined conditions for the potentiometric titration of certain thiols with silver nitrate (9). The pH range had to be controlled carefully; otherwise, high results were obtained, and were attributed to the formation of higher complexes between the silver mercaptide and additional silver reagent.

In 1958, Burton published a critical review of argentometric methods as applied to cysteine, glutathione, and other substances (10). In numerous cases, the observed consumption of silver was markedly higher than can be accounted for by the stoichiometric relation

$$RS^- + Ag^+ \rightarrow RSAg$$

The errors occurred regardless of whether Tris or ammonia was used to complex silver. In view of these findings, Burton concluded that amperometric titration of thiols with silver is not universally applicable.

This paper presents evidence that formation of salts of the general formula AgSRCOOAg is responsible for high results observed when argentometric titration is applied to substances possessing both thiol and carboxylic acid functions. Since many biologically important thiols belong to this class of compounds, high results reported by Cecil, Burton, and others (11) may be caused by formation of analogous complexes.

EXPERIMENTAL

Four mercaptoacids were analyzed by selected volumetric methods. The compounds were: mercaptoacetic acid, β-mercaptopropionic acid, mercaptosuccinic acid, and α,α'-dimercaptoadipic acid. These substances were titrated by several independent methods: iodimetric, alkalimetric, and argentometric. In addition, the following experiments were performed. A solution of the mercaptoacid was introduced into a moderate excess of ammoniacal silver. Under these conditions, three of the four mercaptoacids (dimercaptoadipic acid was an exception) formed precipitates. From a determination of the amount of residual silver in the solution by back titration with potassium thiocyanate, the silver consumed in forming the precipitate could be calculated. The composition of the precipitate was ascertained by the determinations of silver, sulfur, and sulfhydryl content, and by infrared spectroscopy.

MATERIALS

Mercaptoacetic acid (72% solution in water) was distilled at 107°C, at a pressure of 16 mm Hg; lit., 107–108°/16 mm (12*a*). Since highly concentrated solutions of this acid contain some thiolactone, the concentrate, prior to use, was diluted with water and allowed to stand over-

night to open the thiolactone ring. It was established separately that only negligible amounts of disulfide are formed under these conditions. Dithiodiacetic acid was recrystallized from ethyl acetate–benzene, m.p. 102–103°C; lit., 108–109°C (12*b*). β-Mercaptopropionic acid was distilled at 106°C, at a pressure of 9 mm Hg; lit., 114–115.5°C at 13 mm (12*c*). Mercaptosuccinic acid was recrystallized from water, m.p. 152–153°C; lit., 151°C (12*d*). α,α′-Dimercaptoadipic acid was recrystallized from ethyl acetate–benzene, m.p. 186–188°C; lit., 188°C (13).

METHODS

Volumetric determinations were performed, in most cases, on 0.1 *M* aqueous solutions of the mercaptoacids. An exception was made in the case of dimercaptoadipic acid because of its limited solubility in water. For this reason, solid dimercaptoadipic acid was weighed out for each individual titration. To facilitate comparison with other mercaptoacids, the results were recalculated to refer to a hypothetical 0.1 *M* solution (20.82 g/l) of dimercaptoadipic acid.

Amperometric titrations were performed using conditions and apparatus recommended by Kolthoff and Harris (3). The 0.1 *M* stock solutions were diluted 10- or 20-fold. Aliquots of the dilute solutions were introduced into the titration vessel and were titrated with 0.01 *M* silver nitrate solution. No potential was applied between the rotating platinum electrode (r.p.e.) and the mercuric iodide reference cell except in the titration of mercaptopropionic acid. In the latter case, in the absence of applied potential, an anodic current was observed which was possibly caused by the oxidation of the sulfhydryl group at the r.p.e. This anodic process obscured the end point. To minimize this undesirable effect, titrations of mercaptopropionic acid were conducted at the potential of −0.1 v applied to the r.p.e. Under these conditions, the anodic current was absent and the end point was detectable with good precision.

The results of the amperometric determinations were calculated back to refer to the concentrations of the original 0.1 *M* stock solutions.

Iodimetric titrations of the thiol groups were performed in weakly acid solutions (pH = 3–4) using 0.1 *N* iodine solution and starch indicator. These conditions had to be modified in the cases of mercaptopropionic and mercaptosuccinic acids. The former showed a tendency to become oxidized beyond the disulfide stage while the latter, a secondary mercaptan, could possibly enter into a side reaction with iodine and form a sulfenyl iodide (4). To suppress these undesirable reactions, titrations of these two mercaptoacids were performed in strongly acid media (pH = 1) and in the presence of 2–3 gm potassium iodide. The latter was introduced into the solution immediately prior to titration.

Alkalimetric titration of the carboxylic groups were performed with

0.1 N sodium hydroxide. The end point was detected potentiometrically with a Beckman model G pH meter.

The behavior of mercaptoacids and their quantitative analysis in the presence of an excess of silver diammine complex was examined in the following manner. Fifty milliliters of standard 0.1 M silver nitrate solution, 1 gm ammonium nitrate, and 2 ml of conc. ammonia were introduced into a 100-ml volumetric flask. Then, an aliquot of the solution of the mercaptoacid, containing 0.5–2.0 mmoles of the latter, was introduced under continuous swirling. The swirling was continued for about one more minute. Water was added to bring the volume to the 100-ml mark, and the contents were mixed thoroughly. The amount of silver remaining in the solution was determined in the following manner. The suspension of the precipitated material was filtered, and the filtrate was collected in a dry container. An aliquot of the filtrate (25 ml) was acidified with 25 ml of 1 N nitric acid and titrated with standard 0.1 N potassium thiocyanate solution to the ferric alum indicator end point according to the usual Volhard procedure. This procedure gave quantitative results when applied to solutions of known concentration of mercaptoacetic acid if the content of the latter was calculated from the stoichiometry of the equation

$$HSCH_2COOH + 2Ag^+ \rightarrow Ag_2SC_2H_2O_2 + 2H^+$$

The same procedure was applied to solutions of mercaptopropionic acid with the following minor changes. Only 1 ml of conc. ammonia was used. The precipitate began to form after several minutes. The suspension was allowed to stand for 2 more hours and shaken occasionally to make the precipitation complete. The concentration of mercaptopropionic acid was calculated assuming that 2 moles of silver were equivalent to 1 mole of the mercaptoacid.

The composition of the disilver salt of mercaptoacetic acid obtained above was verified by the following procedure. The fresh precipitate was collected on a sintered-glass filter, washed with water containing 10% methanol, and dried to constant weight over phosphorous pentoxide. To establish the thiol content, the precipitate was dissolved in dilute acetic acid, an excess of potassium iodide was added to precipitate the silver ion, and the resulting mixture was titrated immediately with 0.1 N iodine solution. Another portion of the precipitate was dissolved in dilute nitric acid and titrated for silver ion by the Volhard method using standard 0.1 N potassium thiocyanate. The sulfur content was obtained by destructive oxidation in a Parr bomb by the usual sodium peroxide treatment. The oxidation products were leached out with hot water, and the insoluble silver residue was filtered off and washed. The filtrate was combined with the washings and acidified with dilute hydrochloric acid

TABLE 1

TITRATION OF MERCAPTOACIDS BY VARIOUS PROCEDURES: COMPARISON OF RESULTS

Mercaptoacid titrated	Concentration found, moles/l[a]			
	Method			
	Iodimetric	Alkalimetric	Amperometric	Precipitation and back-titration
Mercaptoacetic	0.0997–0.1001 (3)	0.1038–0.1038 (2)	0.1200–0.1300 (2)	0.0996–0.1013 (4)[b]
Mercaptopropionic	0.0861–0.0871 (4)	0.0855–0.0860 (4)	0.0888–0.0920 (4)	0.0850–0.0858 (3)[c]
Mercaptosuccinic	0.0994–0.0999 (4)	0.0994–0.1000 (3)	0.1020–0.1080 (4)	—[d]
Dimercaptoadipic	0.0967–0.0973 (4)	0.0980–0.0981 (4)	0.1150–0.1220 (4)	—[e]

[a] Range of observed concentrations is given. Figures in parentheses denote number of determinations.

[b] The amounts of mercaptoacetic acid taken were in the range 0.5–2.0 mmole. In this range, the result of a determination did not depend on the size of the sample taken for analysis.

[c] The amounts of mercaptopropionic acid taken for analysis were in the range 0.42–1.26 mmole.

[d] Results were not satisfactory from a quantitative view. From the determination of the silver ion remaining in the solution, it was established that the precipitate contained about 1.8–2.0 moles of silver per mole of mercaptosuccinic acid.

[e] No precipitate was formed between the mercaptoacid and an excess of ammoniacal silver.

to a pH of about 3. The sulfate content was then determined in the usual manner by precipitation with barium chloride.

The following procedure was used to see whether the precipitate contained ammonia, either present as a silver diammine ion or as an ammonium ion. The precipitate was freshly prepared by introducing mercaptoacetic acid into an excess of ammoniacal silver according to the method already outlined. The precipitate was collected, without washing, on filter paper, and blotted to remove most of the mother liquor. The blotted precipitate was then divided into two portions. Portion 1 was weighed and introduced into a Kjeldahl distillation apparatus to determine the total ammonia content of the moist precipitate. Portion 2 was weighed and dried to determine the amount of mother liquor contained in the precipitate. Ammonia bound by the precipitate was then calculated as a difference between the total ammonia and the known ammonia content contributed by the mother liquor. In a separate experiment, it was established that the presence of silver ion does not interfere with the quantitative recovery of ammonia in the Kjeldahl distillation.

RESULTS

The various analytical determinations are assembled in Tables 1–3.

TABLE 2

DETERMINATION OF MERCAPTOACETIC ACID (1.000 MMOLE) BY PRECIPITATION AND BACK-TITRATION WITH THIOCYANATE IN THE PRESENCE OF VARIABLE AMOUNTS OF DITHIODIACETIC ACID

Dithiodiacetic acid introduced (mmoles × 100)	Mercaptan found (mmoles)
0.00	1.0005
1.00	1.0015
3.00	0.9955
5.00	1.0015
	Average 0.9998

DISCUSSION

Mercaptoacetic acid was studied in more detail than the other compounds since it is believed that it is representative of the behavior of mercaptoacids. As can be seen from Table 1, the results of the amperometric determinations were high in each case and out of line with the results of the other procedures. The magnitude of this discrepancy was significant and not explainable in terms of the uncertainty associated with the detection of the end point. In contrast, excellent agreement was obtained between the iodimetric method and the method based on precipitation followed by back-titration with thiocyanate. The alkalimetric titrations gave somewhat higher results, but the discrepancy is very

TABLE 3
COMPOSITION OF THE PRECIPITATE FORMED BETWEEN MERCAPTOACETIC ACID AND AN EXCESS OF AMMONIACAL SILVER

Type of analysis	Per cent found	Per cent calculated for $Ag_2C_2H_2O_2S$
Silver	70.09	70.54
Sulfur	10.17	10.48
($^-SCH_2COO^-$)	29.77[a]	29.44
NH_3[b]	0.2	0.0[c]

[a] Determined by the iodimetric titration of the thiol group.

[b] Determined on a freshly prepared precipitate which was not washed. Excess moisture was removed by blotting (see *Methods*). The figure was corrected for the liquid content of the precipitate (under 5%) and the ammonia content of the mother liquor.

[c] Ammonia content of the hypothetical compound $AgSCH_2COOAg(NH_3)_2$ is 10.0%.

likely caused by the presence of small amounts of disulfide or perhaps other impurity of an acidic character.

In the course of an amperometric titration, the initial color of the precipitate was yellow. Upon addition of more silver, the color gradually faded until it appeared white. A similar process took place when the mercaptoacetic acid solution was introduced into an excess of ammoniacal silver. In the first few seconds, a yellow color was observed. Agitation of the suspension for about one more minute brought about a sudden change of color from yellow to white. It would seem that the yellow color is characteristic of silver mercaptide of mercaptoacetic acid, while the disilver salt is, apparently, white. With the back-titration method, it was our experience that if, for any reason (such as insufficient swirling), the precipitate failed to change from yellow to white, the disilver salt was not formed quantitatively and, as a result, the stoichiometry corresponded to a precipitate containing a mixture of mono- and disilver salts.

The precipitate formed in the precipitation and back titration procedure is stoichiometrically the disilver mercaptoacetate, $AgSCH_2COOAg$. This conclusion was confirmed by the direct analysis of the precipitate, as shown in Table 3. Infrared spectra of the precipitate also substantiate this view. The presence of a band at 6.35 μ rather than at 5.9 μ indicates that the carboxylic group is in the ionic form (14*a*). For example, absorption of the carboxylic group in its non-ionized form in acetic acid occurs at 5.80 μ, while the carboxylate ion in silver acetate was found to absorb at 6.30 μ. Furthermore, the absorption band at 3.9 μ, characteristic of the —SH group, is missing, as would be expected if a mercaptide had formed (14*b*). The amount of ammonia held by freshly formed precipitate, as seen from Table 3, is insignificant. Consequently, silver diammine or ammonium ion cannot be the counterions of the mercaptide

or carboxylate groups, and hence silver ion is the only remaining candidate for salt formation. The view that a definite compound has been formed is further strengthened by the fact that the composition of the precipitate remained constant over a fourfold change in the amount of the mercaptoacid (see Table 1, footnote *b*). Had some other phenomenon been responsible for the consumption of silver (such as adsorption or coprecipitation), the amount of silver tied with the precipitate could be expected to depend on the ratio of concentrations of the silver diammine ion and mercaptoacetic acid.

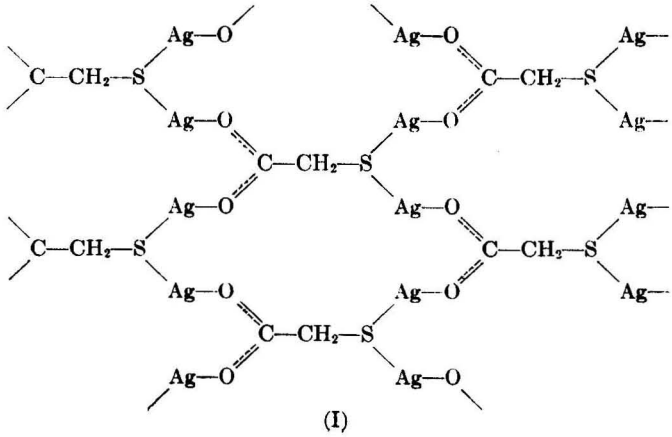
It appears that the carboxyl group of a mercaptoacid is endowed with special properties as regards combination with silver so that it is not at all comparable to other carboxylates.

The difference in behavior of mercaptoacetic acid and its disulfide suggests a plausible structure for the disilver salt of the former. We recall first that the silver ion has a coordination number of 2, but that the bonds tend to be colinear (15). The stability of the group

$$Ag^{+} \ldots {}^{-}O\underset{O}{C}-$$

is evidently not very strong for an ordinary carboxylate, such as that in dithiodiacetic acid, for, as the data in Table 2 show, the latter does not remove silver from the silver diammine ion. Furthermore, when silver ion was introduced into an ammoniacal solution of dithiodiacetic acid under conditions which were employed for the amperometric determination of mercaptoacids, there was not indication that the silver diammine ion formed a complex with dithiodiacetic acid.

In a mercaptoacid, however, double linkages of the following type become possible:



(I)

The likelihood of the silver ion being removed from solution is now much greater because both mercaptide and carboxylate groups are involved in bonding. Furthermore, conditions for the buildup of an infinite lattice present themselves, and hence the extra stabilization due to crystallization becomes available. Bonds such as pictured in (I) are not possible in an ordinary carboxylic acid nor in the disulfide, dithiodiacetic acid. In the former no sulfur is present; in the latter an—SS—is available but the affinity of the disulfide group toward metal ions is exceedingly weak.

Although complete analytical data for the precipitate of disilver salt were obtained only for mercaptoacetic acid, the behavior of the other mercaptoacids fits the scheme pictured in (I). Amperometric titrations invariably give high results, indicative of an uptake of the silver ion beyond that equivalent to —SH group alone, and under proper conditions a precipitate will form containing a high content of silver.

It is of interest to note that an attempt to prepare a trisilver salt of mercaptosuccinate, which has one sulfhydryl and two carboxylic groups, was unsuccessful, although a disilver salt is apparently obtained (Table 1, footnote *d*). Evidently, the second carboxyl does not have sufficient affinity to hold a third silver. Such behavior is fully in accord with the structure pictured in (I), since the second carboxyl does not have a matching mercaptan group to supply the additional affinity needed to hold the additional silver ion.

Many biological substances containing sulfhydryl groups also contain carboxylate ions. It seems likely that in these cases, excess silver ion may also be taken up if the carboxylate group is suitably placed with respect to the mercaptan. If these groups are attached to a macromolecule, lattice formation would not follow, since the groups holding silver would not be free to approach each other. Nevertheless, the amount of silver ion bound could exceed the equivalent amount of mercaptan and hence lead to the high results which have been observed in some amperometric titrations.

SUMMARY

High results observed when mercaptoacids are titrated in ammoniacal media with silver nitrate are caused by the formation of insoluble precipitates containing silver in the forms of both the mercaptide and the carboxylate salt. In the presence of a moderate excess of ammoniacal silver, mercaptoacetic acid or β-mercaptopropionic acid can be precipitated quantitatively as a disilver salt. The readiness with which silver combines with the carboxylate ion in compounds of this type is postulated to be favored by the crystal structure of the resulting compound.

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REFERENCES

1. KLASON, P., AND CARLSON, T., *Ber.* **39,** 738 (1906).
2. TAMELE, M. W., AND RYLAND, L. B., *Ind. Eng. Chem. Anal. Ed.* **8,** 16 (1936).
3. KOLTHOFF, I. M., AND HARRIS, W. E., *Ind. Eng. Chem. Anal. Ed.* **18,** 161 (1946).
4. KOLTHOFF, I. M., AND HARRIS, W. E., *Anal. Chem.* **21,** 963 (1949).
5. BENESCH, R., AND BENESCH, R. E., *Arch. Biochem.* **19,** 35 (1948).
6. BENESCH, R. E., LARDY, H., AND BENESCH, R., *J. Biol. Chem.* **216,** 663 (1955).
7. CECIL, R., *Biochem. J.* **47,** 572 (1950).
8. KOLTHOFF, I. M., AND STRICKS, W., *J. Am. Chem. Soc.* **72,** 1952 (1950).
9. CECIL, R., AND MCPHEE, J. R., *Biochem. J.* **59,** 234 (1955).
10. BURTON, H., *Biochim. et Biophys. Acta* **29,** 193 (1958).
11. SLUYTERMAN, L. A., *Biochim. et Biophys. Acta* **25,** 402 (1957).
12. "Dictionary of Organic Compounds" (I. Heilbron and H. M. Bunbury, eds.), Oxford Univ. Press, New York, N. Y., 1953. (*a*) Vol. IV, p. 495; (*b*) Vol. II, p. 144; (*c*) Vol. IV, p. 496; (*d*) Vol. IV, p. 497.
13. HREDYA, A., *Ber.* **71B,** 289 (1938).
14. BELLAMY, L. J., "The Infrared Spectra of Complex Molecules," J. Wiley & Sons, New York, N. Y., 1958. (*a*) pp. 174–6; (*b*) p. 352.
15. WELLS, A. F., Structural Inorganic Chemistry," pp. 504–6. Oxford Univ. Press, London, 1945.

ANALYTICAL BIOCHEMISTRY 2, 168–177 (1960)

A Spectrophotometric Method for the Assay of Bacterial Mucopolysaccharases[1]

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INTRODUCTION

Acid mucopolysaccharides have, in recent years, been found to assume important physiological and structural roles in the proper functioning of living organisms. A number of enzymes capable of degrading these substances have been obtained from mammalian and microbial sources. Enzymes from microorganisms that can depolymerize these materials have been reported to produce disaccharides that are unsaturated α, β to the uronic acid carboxyl group (1, 2). Such a structure (i.e., α, β-unsaturated carboxylic acids) has a marked absorption in the ultraviolet region: a property that can be utilized to advantage for a spectrophotometric method for measuring the appearance or disappearance of these substances during the course of enzymic reactions.

Rapid and convenient tests will be described in this paper for the bacterial enzymes catalyzing the degradation of hyaluronate and chondroitin sulfate. This method can also be adapted for studying the enzymic utilization of the unsaturated uronides. A similar method has been described for the assay of fumaric and *cis*-aconitic acids (3).

Previous studies on the purification and properties of chondroitinase from *Proteus vulgaris* by Dodgson and Lloyd (4) and by Martinez, Wolfe, and Nakada (5) originally reported on the production of saturated disaccharides, but more recent work by Nakada and Wolfe (to be published elsewhere) showed that their original report was in error.

[1] This investigation was supported in part by a grant from the U. S. Public Health Service (A-1645).

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These authors now have evidence that the mechanism of action of *Proteus* chondroitinase was similar to the mechanism of action of bacterial hyaluronidases in that unsaturated disaccharides are produced by enzymic degradation of chondroitin sulfate.

MATERIAL AND METHODS

Potassium chondroitin sulfate-C was obtained by the method of Einbinder and Schubert (6) from beef trachea and hyaluronic acid was purchased from Sigma Chemical Company. *Proteus vulgaris* chondroitinase was prepared as previously described (7), and a generous gift of streptococcal hyaluronidase was obtained from Lederle Laboratories Division of American Cyanamid Company. The histidine decarboxylase from *Clostridium welchii* (Worthington Biochemicals) was found to have hyaluronidase activity. The method of Reissig, Strominger, and Leloir (8) was used for acetylhexosamine determinations, and the Dische (9) procedure was used for uronic acid assays.

ASSAY PROCEDURE

(a) Bacterial Chondroitinase (pH 7.9 Method)

Reagents. Chondroitin sulfate, potassium salt, 6 mg/ml adjusted to pH 7.9; tris(hydroxymethyl)aminomethane-HCl, 0.25 *M* pH 7.9; enzyme solution.

To quartz cuvettes (1-cm light path) add 0.1 ml chondroitin sulfate, 0.7 ml buffer, and water to a total volume of 2 ml including enzyme. The blank can contain all components of the system except either enzyme or chondroitin sulfate. When using crude enzymes, it is advisable to have both an enzyme and a substrate blanks in order to obtain more accurate zero-time optical densities. Add enzyme and read at 232 mμ at intervals of about 1 min for several minutes. Owing to the high extinction coefficient of the unsaturated disaccharide, higher wavelengths (up to 255 mμ) can be used to decrease the sensitivity of the method for studying the reaction at higher substrate or enzyme concentrations.

(b) Bacterial Chondroitinase (Acid Method)

Reagents. Chondroitin sulfate, K salt, pH 7.9; tris(hydroxymethyl)-aminomethane-HCl buffer, 0.25 *M* (pH 7.9); enzyme solution; KCl-HCl buffer pH 1.8; 95% ethanol made approximately 0.1 *N* with HCl.

To a test tube add 0.1 to 1 ml chondroitin sulfate, K salt; tris(hydroxymethyl)aminomethane-HCl 0.3 ml, enzyme, and water to a total volume of 2 ml. Remove 0.1-ml samples at appropriate time intervals and pipet into 1.9 ml of KCl-HCl buffer or ethanol-HCl. If proteins

precipitate, remove by centrifugation and read the solution at 232 mμ. Zero-time readings can be used as the blank. Under these conditions, the absorption at 232 mμ was stable for at least a week. This method is especially useful for following the course of large-scale experiments or for studying the enzymic degradation at high substrate levels or with crude or whole cell preparations.

(c) Bacterial Hyaluronidases

The procedures are essentially the same as outlined above for chondroitinase except that 0.25 *M* Na acetate buffer pH 6.0 replaces tris-(hydroxymethyl)aminomethane-HCl, and 0.2 ml hyaluronic acid (3 mg/ml) replaces the chondroitin sulfate K salt. With dilute enzyme solutions, up to 0.5% gum arabic can be added to help stabilize the enzyme.

RESULTS AND DISCUSSION

Most of the work to be reported on this method was based on the use of the enzyme chondroitinase, isolated and partially purified from *Proteus vulgaris 31 M*, but can be adapted for use with any of the unsaturated disaccharide-producing bacterial mucopolysaccharases with minor modifications in variables such as pH and concentration.

The disaccharide product of the reaction was isolated on charcoal–Celite columns (7) and crystallized and recrystallized as the pyridine salt. The pyridine was removed by passage through Dowex 50. This material was assayed and showed the theoretical amount of acetylgalactosamine and 96% of the theoretical uronic acid. While there is presumptive evidence for the product having a structure which can be tentatively formulated as an *N*-acetylchondrosin sulfate that is unsaturated α, β to the carboxyl group of the uronic acid moiety, formal proof for the structure is lacking, so the analytical values presented were based on *N*-acetylgalactosamine and uronic acid assays of the product. The ultraviolet absorption spectra of the products of chondroitinase action on chondroitin sulfate and hyaluronate are recorded in Fig. 1. The product of pneumococcal hyaluronidase action on hyaluronate was kindly supplied by Dr. Karl Meyer. For comparison the absorption spectrum of undegraded chondroitin sulfate has been included. At pH 1.8 a maximum of 232 mμ and a minimum of 218 mμ were found, but at pH 7.9 no such peak was observed. An equivalent quantity of undegraded chondroitin sulfate had a low absorption in the vicinity of 232 mμ at either pH. The unsaturated disaccharide from enzymic hyaluronate degradation showed an absorption spectra essentially identical

to those shown for the product of chondroitin sulfate depolymerization. Synthetic *N*-acetylchondrosin, *N*-acetylgalactosamine, glucuronic acid, or the product of bovine testicular hyaluronidase action on chondroitin sulfate or hyaluronate had no appreciable absorption at this wavelength.

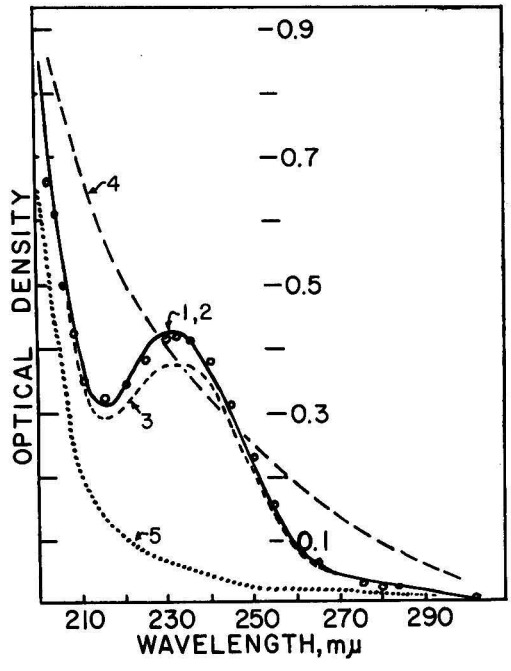

FIG. 1. Ultraviolet absorption spectra of the products of chondroitin sulfate and hyaluronate degradation by bacterial enzymes. Instrument: Cary model 14 automatic recording spectrophotometer. Curve 1 (solid line) and curve 3 show the absorption spectra at pH 1.8 of the unsaturated disaccharides obtained from *P. vulgaris* chondroitinase action on chondroitin sulfate and hyaluronate. Curve 2 (open circles, not connected) represents the absorption spectrum at pH 1.8 of the product of pneumococcal hyaluronidase action on hyaluronate (kindly supplied by Dr. Karl Meyer). Curve 4 shows the absorption spectrum of the unsaturated *N*-acetylchondrosin sulfate at pH 7.9, and curve 5 the absorption spectrum of undegraded potassium chondroitin sulfate (pH 1.8). The spectrum of potassium chondroitin sulfate does not change appreciably with pH.

Figure 2 shows the linear relationship between optical density at 232 mμ with increasing amounts of disaccharide product. Note that at pH 7.9, the product has less absorption than at pH 1.8. Though not shown, the pH 6.0 values were essentially identical to those found at pH 7.9. The higher pH was found to be more convenient in that the reaction could be followed continuously at the pH at which chondroitinase

action was maximal. The lower pH lacks convenience but was found to be more reproducible since the product has an absorption peak in acid solution, thus allowing for minor variations in wavelength without sacrificing accuracy. These standard curves were used to calculate the amount of unsaturated disaccharide produced in the experiments to be reported later in this paper.

That this method can be used for the quantitative determination of bacterial chondroitinase or hyaluronidase is shown in Fig. 3. The increments in optical density at 232 mμ are proportional to the amount of

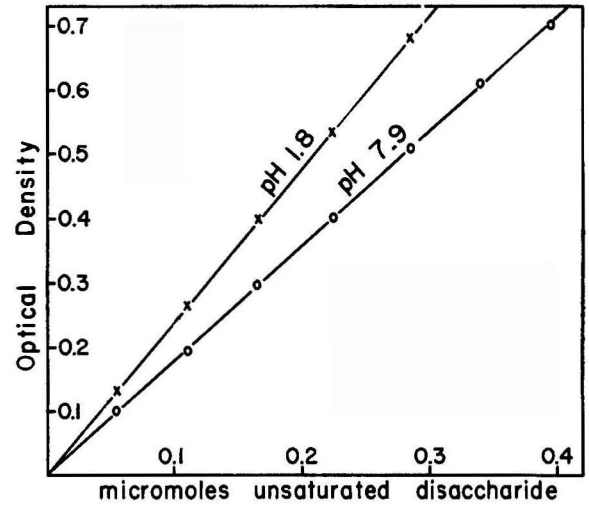

FIG. 2. Calibration curves for the unsaturated disaccharide at pH 1.8 and 7.9.

enzyme added, and this was found for crude or purified chondroitinase as well as for streptococcal hyaluronidase. A unit of enzyme activity has been defined as that amount of enzyme necessary to produce 0.01 μmole of unsaturated disaccharide per minute under standard assay conditions and specific activity as units/mg protein. Units defined in this manner are adaptable to changes in procedure.

An example of the acid procedure is shown in Table 1. In this case, a large-scale experiment using a relatively high concentration of chondroitin sulfate was followed with concurrent assays of uronic acid, *N*-acetylhexosamine release and unsaturated disaccharide production. At the indicated times, 0.1-ml aliquots were removed for the three assays. A reasonably close parallel was found between the amount of acetylhexosamine groups released and the amount of unsaturated disaccharide formed. The uronic acid values remained fairly constant through-

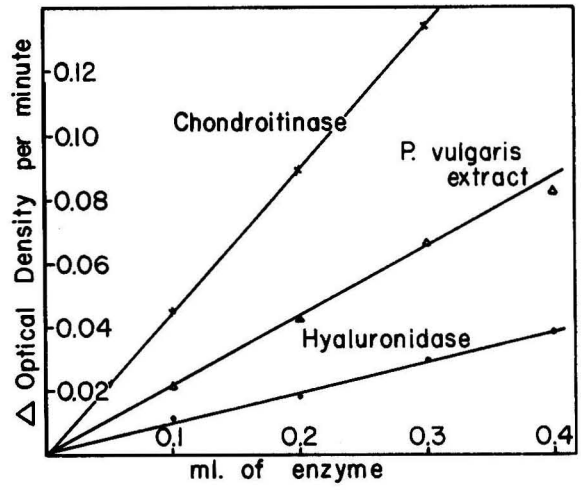

FIG. 3. Quantitative determination of the *Proteus* chondroitinase and streptococcal hyaluronidase. Relation of enzyme concentration to reaction velocity. *Proteus* chondroitinase concentration (100 μg/ml). *Proteus* crude extract (5 mg/ml). Streptococcal hyaluronidase concentration (10 mg/ml). The reactions were run using the standard conditions used for enzyme assay. (See *Methods*.)

TABLE 1

ENZYMIC DEPOLYMERIZATION OF CHONDROITIN SULFATE ASSAYED BY ACID METHOD AND *N*-ACETYLGALACTOSAMINE RELEASE[a]

Time (min)	Micromoles/0.1 ml		
	Glucuronic acid	*N*-Acetylgalactosamine	Unsaturated disaccharide
0	0.140	0.0	0.0
5	0.141	0.031	0.023
10	0.141	0.049	0.048
15	0.138	0.073	0.085
20	0.144	0.113	0.108
25	0.143	0.117	0.118
30	0.141	0.121	0.126
40	0.142	0.129	0.135
50	0.141	0.142	0.142
60	0.141	0.145	0.144
70	0.141	0.143	0.144

[a] Reaction vessel contained 5 mg chondroitin sulfate, K salt; 75 μmoles tris(hydroxymethyl)aminomethane-HCl, pH 7.9; enzyme (60 μg); and water to total volume 6 ml. One-tenth milliliter samples were removed at time intervals indicated for analysis for glucuronic acid, acetylhexosamine, and unsaturation. The unsaturation read in KCl-HCl buffer pH 1.8 (1.9 ml + 0.1 ml sample). Incubation at room temperature (approximately 26°). Theory: 0.153 μmole disaccharide per 0.1-ml sample.

out the run. The final amounts of uronic acid, acetylgalactosamine, and unsaturated disaccharide closely approached the theoretical value, which was based on the weight of chondroitin sulfate added.

TABLE 2
ENZYMIC DEPOLYMERIZATION OF CHONDROITIN SULFATE FOLLOWED BY DIRECT SPECTROPHOTOMETIC ASSAY[a]

Time (min)	Optical density (232 mμ) at indicated times					
	Enzyme	CSA	Boiled enzyme	Boiled enzyme + CSA	Enzyme + CSA	Enzyme + CSA
0	0	0.046	0.038	0.088	0.030	0.030
1	0	0.046	0.037	0.088	0.119	0.208
5	0	0.046	0.038	0.089	0.479	0.534
10	0	0.045	0.038	0.088	0.652	0.687
15	0	0.046	0.038	0.088	0.682	0.721
20	0	0.046	0.038	0.089	0.713	0.751
30	0	0.046	0.038	0.080	0.732	0.775
40	0	0.048	0.038	0.088	0.750	0.800
50	0	0.050	0.037	0.088	0.774	0.822
60	0	0.046	0.038	0.087	0.795	0.850
90	0	0.046	0.038	0.088	0.870	0.920
120	0	0.046	0.038	0.089	0.870	0.920
Disaccharide (μmole)	0	—	—	—	0.497[b]	0.516[b]
Glucuronic acid (μmole)	0	0.507	0	0.495	0.475	0.515
Galactosamine released (μmole)	0	0	0	0	0.506	0.545
Theory (μmole) (based on amount of CSA added, 0.3 mg. including 5.5% water)	0	0	0	0.520	0.520	0.520

[a] Each Beckman quartz cuvette contained the indicated components in the following quantities: 0.3 mg chondroitin sulfate K salt, *Proteus vulgaris* chondroitinase 30 μg, tris(hydroxymethyl)aminomethane-HCl (pH 7.9) 75 μmoles, and water to total volume of 2 ml. Reaction was followed at 232 mμ until a plateau had been reached. Samples were then removed for glucuronic acid and *N*-acetylgalactosamine determinations.

[b] Corrected for decrease in chondroitin sulfate content.

In Table 2 are shown the results of an experiment run at pH 7.9 in Beckman cuvettes. No enzyme or boiled enzyme runs show the expected results. In the presence of enzyme and substrate, the increase in optical density due to enzyme action was followed as a function of time. At the end of the experiment, the cuvette contents were assayed

for uronic acid and *N*-acetylgalactosamine. Again, a fair correlation was found between the amount of disaccharide product and *N*-acetylgalactosamine groups released, and these values closely approximated the theoretical values calculated from the amount of chondroitin sulfuric acid added.

Since chondroitin sulfate itself has some absorption at 232 mμ (Fig. 1) a correction can be made to account for the disappearance of the chondroitin sulfate during the course of enzymic reaction. At either pH 7.9 or 1.9 chondroitin sulfate was found to have about 5.5% of the absorbency of the unsaturated product; so for better accuracy, the change in optical density due to enzyme action can be increased by 5.5% to account for the disappearance of chondroitin sulfate.

The kind and concentration of salts influence the rate of chondroitinase action, but it has been found that tris(hydroxymethyl)-aminomethane-HCl can maintain the pH and exert a maximum activating effect over a wide range of concentrations. Ordinarily, the salt activation effect reached a maximum as the concentration was increased, then became quite inhibitory at higher concentrations. Since the velocity of chondroitinase action is extremely sensitive to ionic strength, one should be cautious regarding salt concentration when assaying unknown solutions of enzyme or substrate. An extensive study of the ion effects using the spectrophotometric assay is to be published elsewhere. Most of these studies were carried out on the Cary automatic recording spectrophotometer. A typical experiment utilizing this instrument is shown in Fig. 4. The linear portion of this curve was used to calculate reaction velocity. The initial slower reaction rate was probably due to the addition of cold enzyme to the reaction mixture. When the enzyme was added at ambient temperatures, no such lag period was noted.

Direct rate studies of some enzymes of bacterial origin that were tested for their ability to degrade hyaluronic acid are shown in Table 3. Chondroitinase from *P. vulgaris* was extremely active. Streptococcal hyaluronidase could be easily measured, and *C. welchii* enzyme, though not particularly active, could be detected with no difficulty. The latter preparation was an aqueous extract of *C. welchii* histidine decarboxylase. Note that direct measurements could be carried out even though the enzyme was crude and the protein concentration was relatively high. A larger slit width was required, but no other precaution was necessary. *Lactobacillus pentosum* was typical of a number of bacterial extracts that were tested and found to be devoid of hyaluronidase activity.

The drawbacks of this method lie in the fact that some materials absorb strongly in the vicinity of 232 mμ. For example, nitrate, iodide, chlorate, trichloracetate, maleate, and high concentrations of gum arabic

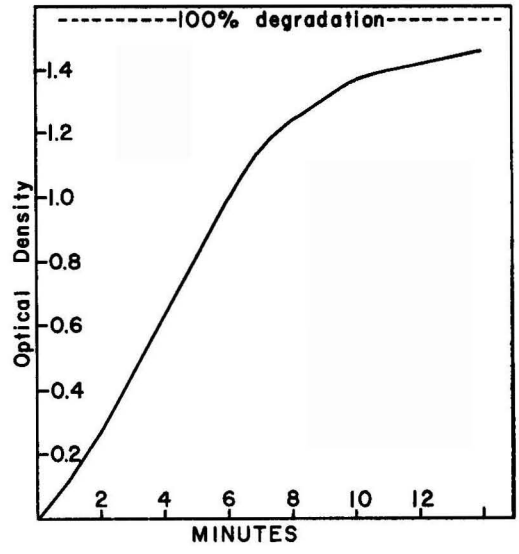

FIG. 4. Depolymerization of chondroitin sulfate by *P. vulgaris* chondroitinase. The reaction mixture contained 0.6 mg potassium chondroitin sulfate, 0.7 ml tris-(hydroxymethyl)aminomethane-HCl buffer (pH 7.9, 0.25 *M*), 0.05 ml chondroitinase (40 μg), and water to a total volume of 2 ml (in a 1-cm light-path quartz cuvette). The extent and rate of reaction was measured at 232 mμ in a Cary model 14 automatic recording spectrophotometer at a chart speed of 1 in./min.

TABLE 3

ASSAY OF DIFFERENT PREPARATIONS FOR HYALURONIDASE ACTIVITY[a]

C. welchii extract		*P. vulgaris* chondroitinase		Streptococcal hyaluronidase		*L. pentosum* extract	
Time (min)	O.D. at 232 mμ	Time (min)	O.D. at 232 mμ	Time (min)	O.D. at 232 mμ	Time (min)	O.D. at 232 mμ
0	0.100	1	0.125	0.5	0.340	0	0.076
20	0.120	5	0.510	3	0.423	20	0.076
40	0.139	10	0.700	6	0.535	60	0.078
60	0.155	15	0.995	10	0.679	90	0.076
80	0.175	20	1.22	14	0.810	120	0.075
100	0.196	25	1.50	18	0.985	240	0.076
120	0.215	30	1.71	22	1.12	(20 hr.)	0.079
				25	1.24		

[a] Each cuvette contained 0.6 mg hyaluronate, 20 μmoles Na acetate buffer pH 6.0, enzyme, and water to a total volume of 2 ml. Enzyme was added at zero time in the following amounts: *P. vulgaris* chondroitinase (0.04 mg protein), *C. welchii* extract (4 mg protein), streptococcal hyaluronidase (4 mg protein), and *L. pentosum* extract (4 mg protein). Reaction followed in Beckman DU at 232 mμ.

could not be used, but most of the common inorganic and organic salts associated with living systems were tested with no difficulty.

SUMMARY

A simple spectrophotometric method for the assay of the bacterial mucopolysaccharases, chondroitinase, and hyaluronidase has been described.

REFERENCES

1. LINKER, A., MEYER, K., AND HOFFMAN, P., *J. Biol. Chem.* **219,** 13 (1956).
2. HOFFMAN, P., LINKER, A., SAMPSON, P., MEYER, K., AND KORN, E. D., *Biochim. et Biophys. Acta* **25,** 658 (1957).
3. RACKER, E., *Biochim. et Biophys. Acta* **4,** 20 (1950).
4. DODGSON, K. S., AND LLOYD, A. G., *Biochem. J.* **68,** 88 (1958).
5. MARTINEZ, R. J., WOLFE, J. B., AND NAKADA, H. I., *J. Biol. Chem.* **234,** 2236 (1959).
6. EINBINDER, J., AND SCHUBERT, M., *J. Biol. Chem.* **185,** 725 (1950).
7. MARTINEZ, R. J., WOLFE, J. B., AND NAKADA, H. I., *J. Bacteriol.* **78,** 217 (1959).
8. REISSIG, J. L., STROMINGER, J. L., AND LELOIR, L. F., *J. Biol. Chem.* **217,** 959 (1955).
9. DISCHE, Z., *J. Biol. Chem.* **183,** 489 (1950).

ANALYTICAL BIOCHEMISTRY 2, 178–179 (1960)

SHORT COMMUNICATION

Sudan Black B in Ethyl Acetate-Propylene Glycol for Prestaining Lipoproteins[1]

McDonald and Ribeiro (1) have reported the preparation and utilization of a 0.5 gm per cent solution of Sudan Black B in ethylene and propylene glycols in the prestaining of serum lipoproteins for electrophoresis in various stabilized media. This technique has been widely used in the ionographic study of lipoproteins; lately, however, a new solubilizing procedure has been developed which greatly increases the concentration of Sudan Black B and results in a more intensely stained lipoprotein pattern on the resultant ionograms or paper chromatograms (2).

The lipid-staining reagent is now prepared by adding 0.1 gm of Sudan Black B (Hartman-Leddon Co., Philadelphia, Pa.) to 1 ml of analytical grade ethyl acetate, at room temperature, thoroughly agitating until the dye is dissolved, diluting the solution with 9 ml of propylene glycol and stirring, until homogeneous, with a glass rod. The reagent is stoppered and stored in the dark until ready for use.

For prestaining purposes the Sudan Black B reagent is added to human serum, at room temperature, with a swirling action, in the ratio of 1:5, v/v, respectively. The serum–dye mixture is allowed to stand for 15 min and is then centrifuged to remove excess dye, which precipitates as fine particles during the initial mixing of the dye reagent and the serum.

An acetylated Sudan Black B solution, prepared in an analogous manner for purposes of comparison, was found to offer no detectable advantage in stain intensity or color stability.

The incorporation of ethyl acetate into the dye solution did not produce any detectable deleterious effects upon the serum lipoproteins, and typical lipoprotein patterns were achieved after chromatography by the centrifugally accelerated method.

[1] This work was supported in part by a grant-in-aid of research from the Chicago Heart Association.

REFERENCES

1. McDonald, H. J., and Ribeiro, L. P., *Clin. Chim. Acta* **4**, 458 (1959).
2. McDonald, H. J., Banaszak, L. J., and Kissane, J. Q., *Anal. Biochem.* **1**, 44 (1960).

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Reader in Histology, Clare College, University of Cambridge

April 1960, 440 pp., illus., $10.00

From a description of the main classes of cells which develop in simple tissue cultures, the author proceeds in search of the origin and significance of these classes both in embryology and in phylogeny. Clues based on phylogenetic evidence lead to an investigation of certain amoebae whose structure and behaviour are directly dependent upon fairly simple and universal factors in their environment. These same environmental factors are then considered as possible influences in establishing the basic pattern for a simple colonial metazoan organism, and thus probably for the embryonic forms of the majority of invertebrates and, possibly, of vertebrates also. Early differentiation of cells into a few main types is shown to influence the cellular organization of other body tissues. A tentative genealogical tree of the families of cells of an organism is proposed and considered as a key to the manner in which the cells differentiate and to the manner in which they behave in tissue cultures.

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ANALYTICAL BIOCHEMISTRY, 1, 181–186 (1960)

The Quinoline Pathway of Tryptophan Oxidation in *Pseudomonas*: Synthesis of 7,8-Dihydroxykynurenic Acid and Kynurenic Acid-C^{14} *

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Received June 6, 1960

INTRODUCTION

Recent work from two laboratories (1–4) has shown that the quinoline pathway of tryptophan oxidation in *Pseudomonas*[3] proceeds from kynurenic acid through 7,8-dihydroxykynurenic acid and leads eventually to the formation of L-glutamic acid.

Kynurenic acid labeled with C^{14} has proved useful in these investigations, and we wish to report here its synthesis as well as that of 7,8-dihydroxykynurenic acid, the last intermediate in this pathway prior to rupture of the quinoline ring system. Our synthesis of C^{14}-labeled kynurenic acid, obtained enzymically from tryptophan, is diagrammed in the scheme shown. The outlines of a somewhat different method have been published (4). DL-Tryptophan-C^{14} was converted, by the use of a partially purified tryptophan pyrrolase preparation together with rat liver formamidase, to L-kynurenine.[4] L-Amino acid oxidase was then used to convert L-kynurenine to *o*-aminobenzoylpyruvic acid which undergoes spontaneous cyclization to kynurenic acid. It was essential that the tryptophan pyrrolase preparation be relatively free of kynureninase activity, since, although kynureninase is typically present in those strains of *Pseudomonas* which degrade tryptophan via the aromatic

* This investigation was supported by U. S. Atomic Energy Commission Contract No. AT(30-1)-901 with the New England Deaconess Hospital.

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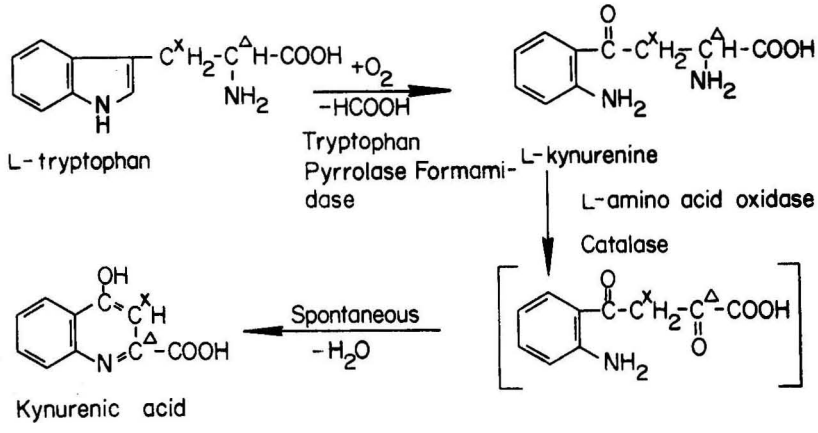
[2] Postdoctoral Research Fellow of the National Cancer Institute, U. S. Public Health Service.

[3] We have used strain RS-1: We are grateful to Prof. R. Y. Stanier for supplying us with a culture of this organism (5).

[4] Hayaishi has published a macro procedure for the conversion of tryptophan to L-kynurenine (6).

pathway, it is present in small quantity in quinoline pathway organisms as well (7).

We have briefly reported the synthesis of 7,8-dihydroxykynurenic acid (1); the details of this synthesis are reported here. 2,3-Dimethoxybenzoic acid was converted to 2,3-dimethoxyaniline via the Schmidt



The synthesis of kynurenic acid-C^{14} from tryptophan-C^{14}.

reaction. The aniline was condensed and cyclized with diethyloxalacetate in a modified Conrad-Limpach synthesis (8) to yield ethyl 7,8-dimethoxy-4-hydroxyquinoline-2-carboxylate which gave the desired compound after treatment with 48% HBr.

EXPERIMENTAL[5]

Kynurenic Acid-C^{14}. Preparation of Partially Purified Tryptophan Pyrrolase

Step I was identical to that described by Tanaka and Knox (9). The crude supernatant fraction obtained from 10 gm of tryptophan-grown *Pseudomonas* cells was centrifuged for 1 hr at 30,000 $\times$ *g* and the precipitate discarded. Sufficient saturated ammonium sulfate, pH 7.2, was added to the clear supernatant material to reach 40% saturation. The precipitate was collected by centrifugation and dissolved in 30 ml of 0.1 *M* phosphate buffer, pH 7.2. This fraction was then brought to 34% saturation with saturated ammonium sulfate; the precipitate was centrifuged and finally redissolved in 6 ml. of 0.1 *M* phosphate buffer, pH 7.2. The solution was dialyzed overnight against 2 l of 0.02 *M* phosphate

[5] All melting points are corrected. Analyses were performed by Dr. Carol K. Fitz, Needham Heights, Mass.

buffer pH 7.2, containing 10 mg disodium ethylenediaminetetraacetic acid. Tryptophan pyrrolase and kynureninase activity were assayed spectrophotometrically (9) by measuring either the appearance or disappearance of L-kynurenine at 365 mμ. The activity of a typical preparation of this kind (expressed as micromoles L-kynurenine formed or degraded/ml enzyme/min) was: tryptophan pyrrolase, 41.2, and kynureninase, 0.085.

The Conversion of DL-*Tryptophan to Kynurenic Acid +* D-*Tryptophan*

Kynurenic acid-2-C^{14} was prepared from DL-α-tryptophan-C^{14} (Tracerlab, Inc.) and kynurenic acid-3-C^{14} from DL-β-tryptophan-C^{14} (New England Nuclear Corp.). Five milligrams of DL-tryptophan-C^{14} (24.5 μmoles) dissolved in 2 ml water (2.98×10^7 counts/min) was placed in the main compartment of a Warburg flask, and 1.0 ml of partially purified tryptophan pyrrolase was added to the side arms. KOH was present in the center well. The control flask contained no tryptophan. After thermal equilibration, the enzyme preparation was tipped in. Within 30 min the theoretical quantity of oxygen had been consumed for the conversion of 12.25 μmoles L-tryptophan to formyl-L-kynurenine. The conversion to kynurenic acid was effected by the addition of 0.2 ml of a purified rat liver formamidase preparation (10), 1 mg of crystalline catalase (Worthington), and 100 mg of snake venom (*Agkistrodon piscivorus,* Ross Allen's Reptile Institute) to each flask. The oxygen uptake was again followed until, after 60 min, the theoretical quantity was reached for the conversion to kynurenic acid.

Separation of Kynurenic Acid and D-*Tryptophan*

The reaction mixture from the last step was deproteinized by heating at 100°C for 5 min. The precipitated proteins were removed by centrifugation, and the precipitate was washed with 8 ml of water. The washings were added to the initial supernatant material. This material was then passed through a column (4 × 1 cm) of Amberlite IRA-400 in the chloride form. D-Tryptophan was quantitatively removed from the column by washing first with 135 ml water and finally with 100 ml of 0.02 *N* HCl. Kynurenic acid was then eluted by washing the column with 40 ml of 88% formic acid. The recovery of radioactive material was 95% of theoretical: water + HCl washes gave 1.42×10^7 counts/min; the formic acid eluate gave 1.41×10^7 counts/min.

Isolation of Kynurenic Acid

Unlabeled kynurenic acid, 290 mg, was dissolved in 40 ml of hot formic acid and added to the formic acid eluate from the column. The

formic acid was then removed by distillation *in vacuo*, the residue dissolved in 40 ml of 1 *M* acetate buffer, pH 6.5, and the solution treated with Norit and filtered. Kynurenic acid was precipitated by the addition of 10 ml of conc. HCl. The precipitate was collected by filtration and washed with cold 1 *N* HCl, water, and finally with acetone. Crystallization from 130 ml of 40% acetic acid yielded 232 mg kynurenic acid, specific activity 6110 counts/min/μmole. Recrystallization from water yielded 195 mg kynurenic acid monohydrate, specific activity unchanged.

7,8-Dihydroxykynurenic Acid (4,7,8-Trihydroxyquinoline-2-carboxylic Acid). 2,3-Dimethoxyaniline

2,3-Dimethoxybenzoic acid, 54.5 gm (0.3 mole) (Aldrich Chemical Co.), was dissolved in 1 l of dry chloroform, 100 ml of conc. sulfuric acid was added, and the mixture was heated to 45° with good stirring. Sodium azide, 39 gm (0.6 mole), was added in small portions at such a rate that the temperature was maintained between 50 and 55°C. After completing the addition of sodium azide, the reaction mixture was cooled, water was added, and the aqueous and chloroform layers were separated. The cooled aqueous layer was made basic and the aniline extracted with ether. Evaporation of the ether yielded 33 gm (73.5%) of crude material which was purified by distillation *in vacuo*. The *N*-acetyl derivative was prepared by reaction with acetic anhydride and gave m.p. 82–83°C. Vermeulen reports 84°C (11).

Ethyl 7,8-Dimethoxy-4-hydroxyquinoline-2-carboxylate

2,3-Dimethoxyaniline, 29.1 gm (0.155 mole), and 23.7 gm (0.155 mole) of freshly prepared diethyloxalacetate (12) were mixed in a beaker, five drops of conc. HCl was added, and the mixture was allowed to stand for 4 days over conc. sulfuric acid in a desiccator. The crude condensation product was rapidly added to 200 ml of boiling Dowtherm A, (Dow Chem. Co.), and the solution was refluxed for 10 min. After cooling, and the slow addition of 1 l of hexane, the mixture deposited 15 gm of yellow crystals together with a quantity of tar. The tar was dissolved in 95% ethanol, and the solution was treated with charcoal and filtered. Water was added to the hot solution until a faint cloudiness appeared. After standing overnight at 5°C, this mixture yielded an additional 14 gm of crude ester, giving a total crude yield of 67%. Recrystallization from a water-ethanol mixture gave very pale yellow crystals, m.p. 149–151°C.

Anal. Calcd. for $C_{14}H_{15}NO_5$: C, 60.6; H, 5.4; N, 5.1; found: C, 60.3; H, 5.4; N, 5.1.

4,7,8-Trihydroxyquinoline-2-carboxylic Acid

Crude ethyl 7,8-dimethoxy-4-hydroxyquinoline-2-carboxylate, 28 gm, was refluxed for 6 hr in 100 ml of 48% aqueous HBr. The hydrolyzate was cooled, diluted with water, and filtered to yield 14.5 gm (65%) of the crude product. The compound was purified by recrystallization from dilute HCl. The material did not form a discrete hydrate at this stage. After drying in an Abderhalden apparatus for 1 hr at 100°C over P_2O_5 (0.1 mm Hg), the compound had the following properties: yellow needles, m.p. greater than 300°C, dec. Its ultraviolet spectrum showed three maxima in 2 *N* HCl: $\epsilon_{385\ m\mu}$ 2100, $\epsilon_{325\ m\mu}$ 4600, $\epsilon_{265\ m\mu}$ 35,000.

Anal. Calcd. for $C_{10}H_7NO_5 \cdot 1/2H_2O$: C, 52.2; H, 3.5; N, 6.1; found C, 52.5; H, 3.9; N, 6.3.

Chromatography of the product in the *n*-butanol–ethanol–water system of Pridham (13), using molybdate-treated paper, showed in addition to the major spot, $R_f = 0.04$, a diffuse yellow spot of higher R_f, apparently indicating incomplete cleavage of the methoyl ether linkages.[6] Accordingly, the crude material was further treated as follows: 2.5-gm lots were refluxed with 60 ml of 48% aqueous HBr for 6 hr. Upon cooling, the mass of orange needles was filtered (sintered glass), pressed dry, and recrystallized by dissolution in 70 ml of boiling 48% aqueous HBr. The hot solution was rapidly filtered through sintered glass, reheated to dissolve the precipitate, and then slowly cooled. The crystals of *4,7,8-trihydroxyquinoline-2-carboxylic acid hydrobromide monohydrate* were filtered on sintered glass, washed with cold 48% aqueous HBr, and finally dried thoroughly at 0.1 mm Hg over KOH for 2 hr. Chromatography in the system mentioned above gave one spot, which reacted as a molybdate complex, reddish brown, $R_f = 0.04$. Long orange needles, m.p. 290–291° (fast heating in capillary), 301–302° (slow heating in capillary).

Anal. Calcd. for $C_{10}H_7NO_5 \cdot HBr \cdot H_2O$: C, 37.5; H, 3.15; N, 4.38; Br, 25.0; found: C, 37.3, 37.3; H, 3.1, 3.2; N, 4.2, 4.3; Br, 25.1, 25.2. Its ultraviolet spectrum showed three maxima in 3 *N* HCl: $\epsilon_{385\ m\mu}$ 2060, $\epsilon_{325\ m\mu}$ 4790, $\epsilon_{265.5\ m\mu}$ 33,800.

SUMMARY

The enzymic synthesis of kynurenic acids-2- and 3-C^{14} from the correspondingly labeled tryptophans is described. The method utilized successive treatment with tryptophan pyrrolase, formamidase, and L-amino acid oxidase. 7,8-Dihydroxykynurenic acid has been synthesized by

[6] Difficulty has been reported in achieving complete ether fission in the synthesis of xanthurenic acid (14, 15).

application of the Conrad-Limpach method to 2,3-dimethoxyaniline and diethyloxalacetate. These compounds are of importance in the elucidation of the pathway of tryptophan metabolism by certain bacteria of the *Pseudomonas* species group.

REFERENCES

1. BEHRMAN, E. J., AND TANAKA, T., *Biochem. Biophys. Research Communs.* **1,** 257 (1959).
2. BEHRMAN, E. J., AND TANAKA, T., *Federation Proc.* **18,** 189 (1959).
3. HAYAISHI, O., KUNO, S., TASHIRO, M., TANIUCHI, H., YAMADA, H., HORIBATA, K., SAKAN, T., SENO, S., AND TOKUYAMA, T., *Federation Proc.* **19,** 5 (1960).
4. HAYAISHI, O., TANIUCHI, H., TASHIRO, M., YAMADA, H., AND KUNO, S., *J. Am. Chem. Soc.* **81,** 3483 (1959).
5. STANIER, R. Y., HAYAISHI, O., AND TSUCHIDA, M., *J. Bacteriol.* **62,** 355 (1951).
6. HAYAISHI, O., *in* "Biochemical Preparations," (E. E. Snell, ed.), Vol. 3, p. 108. Wiley & Sons, New York, 1953.
7. MILLER, I. L., TSUCHIDA, M., AND ADELBERG, E. A., *J. Biol. Chem.* **203,** 205 (1953).
8. SURREY, A. R., AND HAMMER, H. F., *J. Am. Chem. Soc.* **68,** 113 (1946).
9. TANAKA, T., AND KNOX, W. E., *J. Biol. Chem.* **234,** 1162 (1959).
10. KNOX, W. E., *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. 2, p. 246. Academic Press, New York, 1955.
11. VERMEULEN, H., *Rec. trav. chim.* **48,** 969 (1929).
12. BESSMAN, S. P., *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. 3, p. 418. Academic Press, New York, 1957.
13. PRIDHAM, J. B., *J. Chromatography,* **2,** 605 (1959).
14. MEBANE, A. D., AND OROSHNIK, W., *J. Am. Chem. Soc.* **73,** 3520 (1951).
15. FURST, A., AND OLSEN, C. J., *J. Org. Chem.* **16,** 412 (1951).

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A Modified Procedure for the Automatic Analysis of Amino Acids

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The analysis of amino acids by ion-exchange chromatography can now be done routinely with a great deal of precision owing to the development of automatic analytical procedures. The most widely used method was devised by Spackman, Stein, and Moore (1); it is also the basis for several commercial instruments. A 150-cm column and a 15-cm column are used to analyze a protein hydrolyzate in a single day. Two samples of about 1–2 mg each are optimal, although good analyses can be obtained on less than 0.5 mg. If the sample is more complex than a protein hydrolyzate, such as a blood plasma, an additional procedure which provides greater resolution can be employed. It requires a third column (50 cm) and 2 days for a complete analysis. We have been using alternate manual procedures designed primarily for the analysis of collagens and gelatins (2), but also suitable for other biological samples (3). It was our purpose to adapt these methods to the automatic system of Spackman *et al.* (1) and to employ a single column and a continuous gradient. This was made practicable by the use of a highly cross-linked resin (Dowex 50X12) and the development of a gradient-producing device (Varigrad) by Peterson and Sober (4) which is well suited to the present problem. This communication describes the final analytical system now in routine use in our laboratory (5–9). It permits a complete analysis of a protein hydrolyzate in 24 hr using a single sample and requiring only 1 hr of operator time for the preparation of the instrument for an analysis. Considerably less glassware and mechanical equipment is required for this procedure than the one developed by Spackman *et al.* resulting in a lower construction cost and simplifying the maintenance.

MATERIALS

A schematic diagram of the amino acid analyzer is shown in Fig. 1. The mechanical and electronic portions of the instrument are similar to

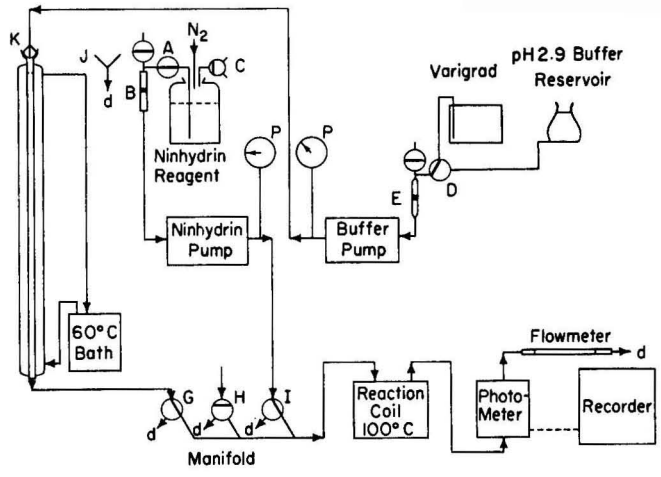


FIG. 1. A schematic diagram of the components of an automatic system for the analysis of amino acids. See text for details.

those devised by Spackman *et al.* (1), unless otherwise noted. Reference should be made to their publication for details not included here.

Pumps

We employ plunger type pumps (Minipump MMI-B-29; Milton Roy Company, 1300 E. Mermaid Lane, Philadelphia 18, Pa.) with column valve and 1/8-in. plunger constructed of Hastelloy C and purchased without motors. They are fitted with Bodine motors, 41 in.-lb at 9.6 rpm (Type NSI-12Rb, Bodine Electric Company, Chicago, Ill.). This is one-third the speed at which these pumps are usually operated. At the slower speed they employ a larger proportion of their total capacity (about 30%) and thus are less susceptible to variable output resulting from air bubbles or solid particles trapped in the liquid end. Their operation is not otherwise affected. The Milton Roy Company has recently developed a modified pump designed especially for chromatographic work which would probably serve the present application.

Both pumps are operated at 30 ml/hr. At the combined flow rate of 60 ml/hr, the resistance of the reaction coil (see below) develops about 18 lb/ sq in. at the manifold making a device to provide an artificial back pressure for the ninhydrin pump unnecessary. The buffer pump operates against 30–50 lb/sq in. with the added resistance of the ion-exchange column.

Ninhydrin Reservoir

An amber 1- or 2-gal bottle is used for the ninhydrin reagent. The oil reservoirs (1), used to maintain a nitrogen atmosphere over the nin-

hydrin reagent, can be replaced by nitrogen supplied directly from a tank through a suitable regulator at 1 lb/sq in.[1] Solid particles or gas bubbles are prevented from reaching the pump by a trap (*B*, Fig. 1). Details of its construction are shown in Fig. 2. The trap should be

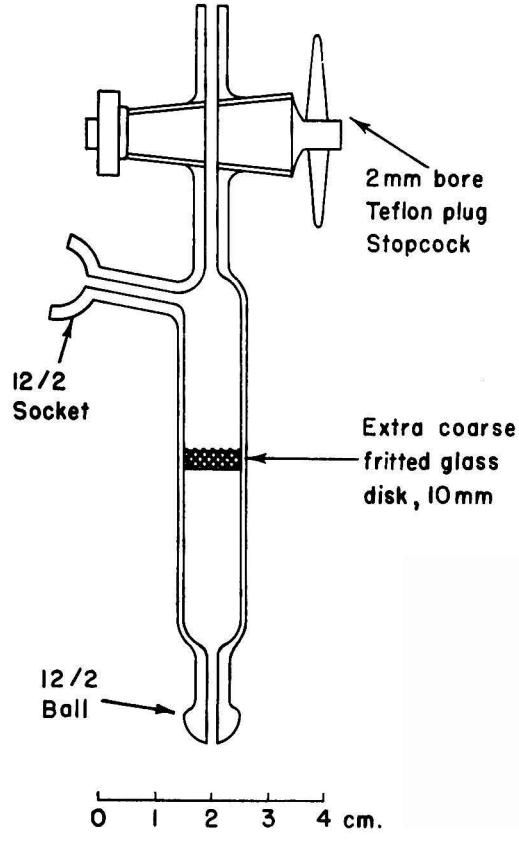


FIG. 2. Details of the construction of the bubble trap. See *B* and *E*, Fig. 1, and text.

examined every few days since it may gradually fill with gas released as solution passes through the filter disk. If it is partially empty, or after cleaning, it can be refilled by closing stopcock *A*, evacuating the trap through its stopcock, and filling with reagent through stopcock *A*. It should be removed and thoroughly cleaned about once a month.

Every 2 or 3 months it is advisable to dismantle and clean the ninhydrin reservoir and associated glassware. The necessity for this is indicated by the appearance of sediment in the reservoir and the trap. If this is not done, the trap becomes easily clogged and the pump oper-

[1] Dr. S. Moore, personal communication.

ates erratically. This is seen on the charts, particularly the 440-mμ trace, as an irregular base line. The blank values may also increase from day to day as sediment forms.

Gradient Device (Varigrad) and Buffer Reservoir

The buffer pump is fed during an analytical run from a 1-l nine-chambered Varigrad, similar to those described by Peterson and Sober (4).[2] The outlet from the Varigrad leads to a three-way Y stopcock (*D*, Fig. 1) and then to a trap (*E*, Fig. 1, and Fig. 2) identical to the one in the ninhydrin line. The other arm of the three-way stopcock leads to a reservoir (pH 2.91 buffer) which consists of a 250-ml flask with a side arm sealed near the bottom.

A deaerator [see (1)] is not employed in the line to the buffer pump. If the buffer is stored or equilibrated at room temperature before use, it does not contain enough dissolved air to form bubbles in either the column or the reaction coil. The trap (*E*, Fig. 1) will collect small bubbles which may inadvertently enter or form in the lines. It should be cleaned and filled as described above for the trap in the ninhydrin line.

Ion-Exchange Column

The jacketed column is identical in construction to those previously described (10). It is 140 cm from the disk to the 18/9 socket at the top of the column and is 9 mm inside diameter. Since the diameter may vary slightly, it is necessary to insure that the volume which the resin is to occupy is known and can be reproduced. To do this, the column is closed at the bottom, filled with 85 ml water, and marked at this level. This is 133 cm from the disk if the diameter is exactly 9 mm. The column is maintained at 60°C by water circulated from a water bath (No. 4-96, American Instrument Co., Silver Spring, Md.). The water level must be checked every few days since loss by evaporation is rapid at this temperature.

A small funnel (*J*, Fig. 1), with tubing leading to a drain, is mounted near the column to hold the ball joint (*K*, Fig. 1) while the sample is being added to the resin.

Manifold

The manifold is constructed in a similar manner to that previously described (1). Since only one column is used, only two three-way stopcocks are necessary although a third is convenient if an additional column is contemplated or for the introduction of a wash into the

[2] Equivalent items are now available commercially from Technicon Instruments Corp., Chauncy, N. Y., and Phoenix Instrument Company, Philadelphia, Pa.

reaction coil. The drain tubing from the stopcock controlling the flow of ninhydrin reagent (*I*, Fig. 1) is led to a drain higher than the ninhydrin reservoir to prevent siphoning.

Reaction Bath

The heating coil where the ninhydrin reagent and column effluent react is constructed of 0.7-mm bore Teflon tubing as previously described (1) but with a total volume of 15 ml (approximately 130 ft). It is contained in a 1-l resin reaction flask with heating mantle and a standard top section. This size coil keeps the effluent and ninhydrin reagent at 100° for the required 15 min.

Automatic Stopcock

Although stopcock *D* (Fig. 1) can be operated manually, a source of operator error is omitted if it is done automatically. A suitable type has been described (1) which is operated by a timer. However, the time at which the change is made is quite critical since the Varigrad must be emptied to complete the elution of arginine but air must not be drawn into the pump. Therefore, it is better if the stopcock is activated by the interruption of a microcurrent between two platinum electrodes in the line between the Varigrad and stopcock *D*. When the Varigrad is empty, air is drawn into the line, the circuit is broken, and a relay operates a solenoid which turns the three-way stopcock 120° directing buffer from the pH 2.91 buffer reservoir to the pump. In constructing such a stopcock, it must be possible for it to be turned manually to any of its three positions during the preparation of the instrument for an analysis.[3]

Photometer and Recorder

We have used the same type recorder and have constructed a photometer identical to that described by Spackman *et al.* (1). The absorption cell has been redesigned to provide a slightly longer light path (3 mm) and to minimize mixing (see Fig. 3). Flow through the cell is upward so that the gas bubbles that may inadvertently enter the stream are not trapped in the cell.

Resin

We originally employed Dowex 50X12, 20–30 μ spheres obtained by regrading —400 mesh resin by the hydraulic fractionation procedure of Hamilton (11). However, the yield is very small (about 5%) and the

[3] A device of this type has been designed by the Instrument Engineering and Development Branch of the National Institutes of Health. Plans are available on request.

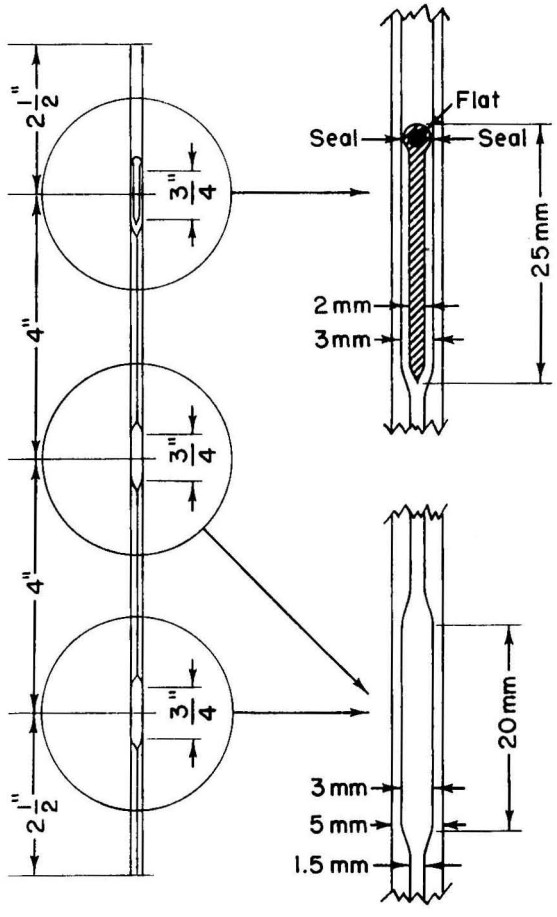


FIG. 3. Details of the construction of the photometer cell. See text.

source is uncertain.[4] We now use a ground Dowex 50 resin, AG 50X12, 32–25 μ (Bio-Rad Laboratories, 32nd and Griffin Ave., Richmond, Calif.), which has similar properties, although the two forms are not directly interchangeable. This size is small enough for optimal sharpness of the amino acid peaks at an elution rate of 30 ml/hr and can be operated at a moderate pressure (about 25 lb/sq in. for the column alone).

[4] Dowex 50 has been largely superseded by Dowex 50W which is manufactured by a different procedure to yield a lighter colored resin. Although amino acids chromatograph well on Dowex 50W, the adsorption characteristics are different, making it unsuitable for the present procedure. For example, threonine and serine do not separate and tyrosine and phenylalanine are found in the region occupied by leucine and isoleucine.

To be certain that the column will perform properly, it may be necessary to wash and regrade the resin, one half pound at a time, as follows.[5] Wash successively on a funnel (medium porosity sintered-glass disk) with 2 l of 4 *N* HCl, 2 l water, 4 l of 2 *N* NaOH, and 2 l water. Fractionate the resin in the Na form as described by Hamilton (11), using a 3-l separatory funnel and distilled water at room temperature. Discard all material that comes over at a flow rate of 140 ml/min (less than an average diameter of about 25 μ), maintaining this rate for 6–8 hr. Then collect the resin that comes over at a flow rate of 250 ml/min[6] (an average diameter of about 32 μ).

The column is poured in sections as described (10). If the resin has been graded properly, the boundaries between the sections will not be visible. The column should originally be filled 5 or 6 cm above the 85-ml mark and washed with 0.8 *M* sodium citrate and then with pH 2.91 buffer before being used. During this wash and the first few runs, the column will shrink and can then be adjusted to the final height by the removal or addition of resin.

Ninhydrin Reagent

The reagent employed in our procedure is identical to the one used by Spackman *et al.* (1) except that some of the Methyl Cellosolve is replaced by water and the concentration of $SnCl_2$ is reduced slightly. It is prepared as described (1) in 3-l quantities from 1875 ml Methyl Cellosolve, 375 ml water, 750 ml of 4 *N* acetate buffer (12), 60 gm ninhydrin, and 1.00 gm $SnCl_2 \cdot 2H_2O$. This quantity is sufficient for four complete analyses. Larger quantities can be prepared if used in less than 2 weeks. In this case a 2-gal reservoir is required.

Buffer

Only one buffer, pH 2.91, is required. It is prepared from 440 gm sodium citrate $\cdot 2H_2O$, 1460 gm citric acid $\cdot H_2O$, water to make a total volume of 18 l, and 1 ml *n*-caprylic acid. It is stored at room temperature. Thiodiglycol (5 ml/l) and BRIJ 35 detergent solution (2 ml/l) are added to 1-l quantities before use (10). If it is determined that the ammonia content of the buffer is too high (see *Discussion*), it is reduced as follows: A column is prepared from 2-l (about 5 lb) of Dowex 50X8, 100–200 or 200–400 mesh resin (or its equivalent). It is washed

[5] We have tested three different lots of resin supplied by Bio-Rad Laboratories. It was necessary to regrade the first two lots; the third and most recent preparation could be used directly.

[6] These flow rates are faster than would be required for Dowex 50X8 in the same size range (11) because of the higher density of Dowex 50X12.

with 4 l of 2 *N* NaOH and then with 2 l of water. The buffer is introduced and collection is begun when the pH of the effluent is the same as the original buffer. It is conveniently collected in a large flask under reduced pressure to maintain an adequate flow rate which may be as high as several liters per hour. A column this size is sufficient for 18 l of buffer. It must then be regenerated with NaOH and washed with water before additional buffer is treated.

Sodium Citrate Solution

Sodium citrate, 0.8 *M*, is prepared in 4 l quantities. It is stable indefinitely at room temperature without a preservative and is used without further additions.

PROCEDURE

At the conclusion of a run, as indicated by the completion of the recording of the arginine peak on the chart, the following procedure is started. The early afternoon is most convenient, but it may be started at any time desired. If the instrument was not in use the previous day, steps 1, 2, and 4 are omitted.

1. Turn off the ninhydrin pump and turn stopcock I at the manifold to drain. The pressure on the ninhydrin line will immediately fall to zero.
2. Turn off the recorder and remove the used portion of the chart.
3. Prepare the Varigrad: Fill the open channels between the chambers with water, close the stopcocks, and pour off the excess water. Fill the chambers as described in Table 1, set on the support, place the cover in position, and level.

TABLE 1

PREPARATION OF THE VARIGRAD[a] FOR CHROMATOGRAPHY[b]

Chamber	pH 2.91 citrate (ml)	0.8 *M* sodium citrate (ml)	water (ml)
1	75	0	0
2	75	0	0
3	75	0	0
4	70	5	0
5	50	24	0
6	40	19	15
7	30	10	34
8	5	52	15
9	0	71	0

[a] Peterson and Sober (4).

[b] These volumes can be measured with a volumetric cylinder to the nearest milliliter The small changes in total volume compensate approximately for density difference.

4. Turn off the buffer pump no sooner than 35 min after the ninhydrin pump (to insure that the reaction coil is cleared of ninhydrin reagent) and turn stopcock *G* on the manifold to drain.

5. When the pressure on the buffer line falls below 10 lb, open the top of the column, wipe the ball joint, and place it in the waste funnel (*J*, Fig. 1).

6. Disconnect the line to the Varigrad and lower it below the pH 2.91 reservoir. Turn stopcock *D* to join this line and the reservoir and allow it to backwash and fill with pH 2.91 buffer (to remove solution remaining from the end of the previous run), using suction to start the flow if necessary. Turn the stopcock back to its original position, connecting the pH 2.91 reservoir to the pump, and turn on the buffer pump (to clear the line between pump and column of solution released from the pressure gage). Reattach the line to the Varigrad.

7. Remove the buffer from above the resin with a suction line and add the sample. Apply 3–5 lb/in. air pressure to the column and open stopcock *G* to drain. Watch carefully as the sample drains into the column to insure that air is not forced into the column. Follow the sample with a small wash of about 0.5 ml of pH 2.91 buffer.

8. When the wash is in, close stopcock *G*, remove the air line, and fill the space above the column with pH 2.91 buffer.

9. Place the ball joint (*K*, Fig. 1) in place on the column and clamp with two #18 clamps. As the pressure reading on the gage rises, start the stirrers and open the stopcocks on the Varigrad.

10. When the buffer pressure has reached the value which experience has shown to be near the equilibrium value (about 30 lb/sq in.), open stopcock *G* to the reaction coil and turn on the recorder. Turn stopcock *D* to join the Varigrad to the buffer pump. When it is certain that the buffer pressure is at equilibrium (allow about 5 min), measure and record the flow rate. (As experience is gained, this measurement can be omitted.) The flow rate should be 30 ml/hr.

11. Close stopcock *I* and turn on the ninhydrin pump. When the pressure is 8 lb/sq in., which is about one half the equilibrium value, turn stopcock *I* to send ninhydrin reagent to the reaction coil.

12. If stopcock *D* is to be operated automatically, activate this device.

13. About 20 min later, after the mixture of ninhydrin and buffer has reached the photometer as evidenced by an increase in the base line on the recorder to a stable line, set the base lines with the controls on the photometer and check and record the flow rate. It should now be 60 ml/hr. Record the pressures indicated on the gages. The buffer pressure is now about 10 lb/sq in. above the value observed when buffer alone was flowing through the reaction coil and will climb an additional 10

lb/sq in. during the run as the viscosity of the eluting solution increases.

14. The next morning, about 21 hr after the run was started, the Varigrad will be empty except for a few milliliters which tend to remain behind in the higher numbered chambers. At this time turn stopcock *D* (if it is manually operated) to supply pH 2.91 buffer to the pump. All of the amino acids are now through the column except arginine. The instrument is allowed to continue in operation without attention about 2 hr longer to complete the analysis of arginine and regenerate the column before the next run.

15. Repeat the above procedure for the next run. If another run is not to be made immediately, turn off the ninhydrin pump and recorder (steps 1 and 2) and 35 min later turn off the buffer pump (step 4). It is best to leave on all other parts of the instrument unless it is not to be used for more than several days.

RESULTS AND DISCUSSION

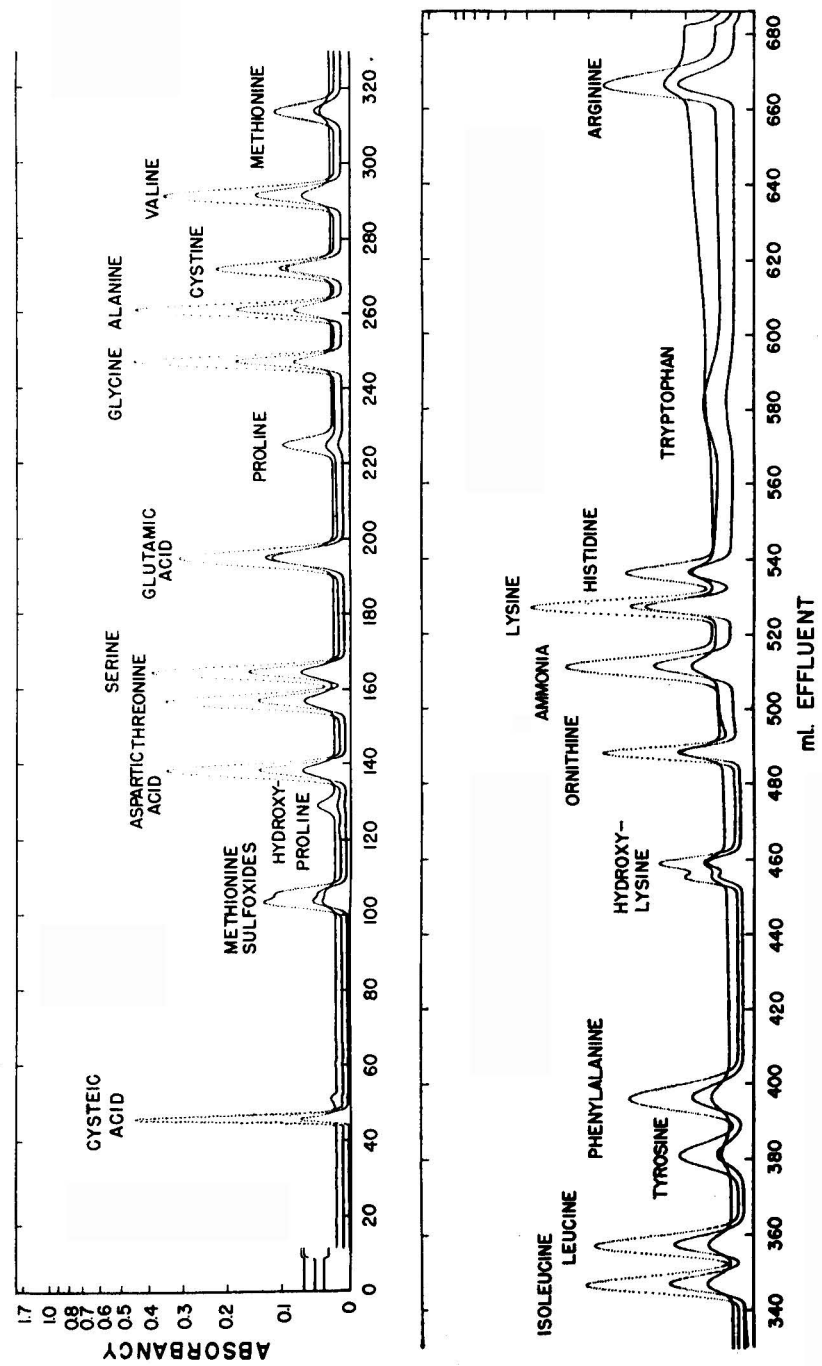
Figure 4 shows an analysis of a standard sample containing 0.5 or 0.25 μmoles of all the amino acids usually encountered in protein hydrolyzates including hydroxyproline and hydroxylysine. Resolution of all the components is complete except for several slight overlaps which, however, do not prevent the precise calculation of the individual components by the usual procedures (1). For convenience in measuring blank values, the base lines are set about 0.005 absorbancy unit apart on the chart. The color values obtained with mixtures of known amino acids are in general similar to those reported by Spackman *et al.* (1). These values will, of course, depend on the exact size of the photometer cells and to a certain extent on the procedures used for elution and color development. For precise work they must be determined independently for each instrument and analytical scheme.

We have found that the precision of the method is at least as good as described by Spackman *et al.* (1). From a total of ten analyses of three different standard solutions at levels of 0.05–4 μmoles analyzed at intervals over several months, coefficients of variation between 1 and 3% were calculated for all of the amino acids appearing in Fig. 4. Hydroxyproline showed a larger variation owing to its poor color yield when present in amounts less than about 1 μmole. It was found that amounts as small as 0.01 μmole of most of the amino acids could be analyzed with a precision of about 10%. As noted (1), this high sensitivity is possible as a result of the extremely stable base line that can be obtained. Under ideal conditions (replicate analyses of more than 0.1 μmole of each amino acid done on successive days) the precision was generally less than 1%.

The use of a Varigrad to produce a continuous gradient permits considerable flexibility. The general principles have been discussed by Peterson and Sober (4). In the present application the nine chambers are filled with buffer mixtures which increase in pH and ionic strength from chamber 1 through 9. As buffer is drawn from the first chamber by the pump, the levels in all the chambers fall together since they are connected in series. Efficient mixing is provided by motor-driven stirrers. It can be seen that each chamber contributes to the composition of the effluent but that the maximal effect of any one chamber will be limited to one part of the chromatogram which is determined by the position of that chamber relative to the others. With this arrangement changes can often be made in portions of the eluant to effect specific separations with little or no effect on the rest of the chromatogram, or part of the chromatogram can be compressed to allow any portion of interest to be chromatographed under conditions which would provide greater resolution. The elution scheme devised for the present problem (Table 1) is essentially empirical and was chosen partly for its simplicity. The use of a buffer, 0.8 *M* citrate, and water allows a wide selection of gradients to be constructed.

There are several parts of the chromatogram where separations are dependent on the exact conditions. The pH of the starting buffer can be lowered to move aspartic acid further behind hydroxyproline or raised to move them closer together. The position of cystine will depend primarily on the amount of 0.8 *M* sodium citrate in chamber 4. The amount can be increased to move it closer to alanine or decreased to move it closer to valine. Lysine must be placed precisely between ammonia and histidine to be separated from both. It can be slowed relative to its neighbors by increasing the proportion of water at the expense of 0.8 *M* citrate in chamber 7 or speeded by the opposite procedure. If the arginine peak is not completely eluted before the appearance of pH 2.91 buffer following the completion of the gradient and it is certain that the Varigrad was emptied, it is an indication that the column contains too much resin. It should be shortened 1 to 2 cm at a time until the arginine is completely eluted.

The analysis of many of the components in physiological fluids is also possible using the present system. The positions of some of the compounds which might be encountered have been determined for the gradient in Table 1. Taurine and urea follow cysteic acid as in other systems (1). Asparagine and glutamine precede and overlap threonine. Citrulline appears between glutamic acid and proline, well separated from both. Glucosamine forms a shoulder on the trailing edge of the glycine peak. Galactosamine follows and overlaps cystine. α-Aminobutyric acid pre-


ABSORBANCY
1.7
1.0
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320
CYSTEIC ACID
METHIONINE SULFOXIDES
HYDROXY-PROLINE
ASPARTIC ACID
THREONINE
SERINE
GLUTAMIC ACID
PROLINE
GLYCINE
ALANINE
CYSTINE
VALINE
METHIONINE
ISOLEUCINE
LEUCINE
PHENYLALANINE
TYROSINE
HYDROXY-LYSINE
ORNITHINE
AMMONIA
LYSINE
HISTIDINE
TRYPTOPHAN
ARGININE
340 360 380 400 420 440 460 480 500 520 540 560 580 600 620 640 660 680
mL. EFFLUENT

cedes valine and is usually well separated from cystine. γ-Aminobutyric acid appears between the hydroxylysines and ornithine.

The major difference between the present procedure and that developed by Spackman *et al.* (1) is the use of a single column and a continuous gradient to obtain a complete analysis from a single run. No separate washing step is required since the gradient removes all the amino acids. Regeneration also occurs as part of the analytical operation. In this manner an analysis is completed and the same column is ready for the next run every 24 hr. An experienced technician needs only an hour a day at the instrument (exclusive of maintenance) and an approximately equal time for the necessary calculations.

It is necessary to use more ninhydrin reagent in the procedure described here than was used by Spackman *et al.* (1) owing to the lower pH and higher ionic strength of the starting buffer. At the slower flow rate for the ninhydrin reagent of 15 ml/hr (1), the color yields of the amino acids appearing early in the chromatogram are much reduced. It is also necessary to change the composition of the ninhydrin reagent by the replacement of part of the Methyl Cellosolve with water. Otherwise crystallization may occur in the line where reagent and effluent mix.

The citrate buffer always contains traces of ammonia present as impurities in the reagents. This results in an ammonia plateau which normally appears in the effluent as a sharp rise in blank just before the ammonia in the sample. If this is small, it does not affect the calculations even though the base line may not be horizontal. If it is very large, or if peaks fall where the blank is changing rapidly, it is necessary to treat each batch of buffer when it is prepared to remove ammonia (see the experimental section). When this is done, the ammonia plateau is small (Fig. 4).

The use of a 12% cross-linked resin has several advantages over the 8% cross-linked resin which has been more commonly used. The higher

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FIG. 4. Chromatographic analysis of a known mixture of amino acids by the procedure described in the text. Five-tenths micromole of each of the amino acids was present, except 0.25 μmole cysteic acid, methionine sulfoxide (sum of isomers), cystine, methionine, tyrosine, hydroxylysine (sum of diastereoisomers), ornithine, histidine, and tryptophan. After addition of the sample to the column (0 ml effluent) the recorder is allowed to print at any convenient position. Shortly after the appearance of ninhydrin in the effluent as indicated by the change in the blank values (at about 10 ml effluent), the base lines are set as follows: 440-mμ trace at 0.008, 570-mμ trace (3-mm cell) at 0.004, and 570-mμ trace (1-mm cell) at 0.000 absorbancy unit. The blank values increase gradually during the run owing to the changing composition of the effluent. The completion of the gradient is indicated by the precipitate fall in the blank values as the starting buffer enters the stream (at about 685 ml effluent) following the arginine peak.

cross-linking produces a denser resin which is less subject to volume changes with changes in ionic strength. The sodium-ion concentration in the present procedure varies from 0.25 to 2.4 N without a significant change in the column volume. The 12% cross-linked resin also has about 25% greater exchange capacity on a volume basis and therefore makes a more efficient column. It has a disadvantage in that equilibrium between solute and exchange sites is slower than with 8% cross-linked resin. For this reason, the particle size must be less than 32 μ and a higher temperature (60°C) is required for optimal resolution.

Existing amino acid analyzers of the type developed by Spackman *et al.* (1) can be easily adapted to the column and gradient described here. An additional column, 140 cm long, must be provided. It could be substituted for one of the original columns or attached directly to the manifold if a spare position is available. It must be jacketed at 60° either by changing the temperature of the existing water bath or supplying another. The Varigrad[2] (4) and buffer reservoir should be connected to one of the buffer pumps with a line containing a trap (Fig. 1) which by-passes the deaerators.[7] If the connection is made through a three-way stopcock, both the original and the new arrangement can be retained and selected as desired. The composition of the ninhydrin must be changed as described and its flow rate doubled. To maintain optimal color yield under these conditions, the size of the reaction coil should be increased to 15 ml and the photometer cells should be changed to provide a longer light path. The latter modification is not critical since the loss of sensitivity resulting from the larger reaction volume is only 25%. An instrument modified in this fashion can still be used with the original columns and elution schemes since the changes in composition and flow rate of the ninhydrin reagent are compatible. It would merely be necessary to redetermine the color factors.

SUMMARY

A simplified scheme for the automatic analysis of amino acids has been developed. It employs a single column, 133 × 0.9 cm, of Dowex 50X12, 25–32 μ (ground), which is eluted with a continuous gradient. The gradient is produced by a nine-chambered device (Varigrad) which allows a wide variety of elution schemes to be constructed. The procedure described allows the complete analysis of a protein hydrolyzate, including collagen, in 24 hr using a single sample. It is also suitable for many other biological amino acid mixtures.

[7] Some of the commercial instruments most recently supplied have bubble traps and no deaerators.

REFERENCES

1. SPACKMAN, D. H., STEIN, W. H., AND MOORE, S., *Anal. Chem.* **30,** 1190 (1958).
2. PIEZ, K. A., AND GROSS, J., *Biochim. et Biophys. Acta* **34,** 24 (1959).
3. PIEZ, K. A., AND EAGLE, H., *J. Biol. Chem.* **231,** 533 (1958).
4. PETERSON, E. A., AND SOBER, H. A., *Anal. Chem.* **31,** 857 (1959).
5. PIEZ, K. A., AND GROSS, J., *J. Biol. Chem.* **235,** 995 (1960).
6. EAGLE, H., OYAMA, V. I., AND PIEZ, K. A., *J. Biol. Chem.* **235,** 1719 (1960).
7. PIEZ, K. A., WEISS, E., AND LEWIS, M. S., *J. Biol. Chem.* **235,** 1987 (1960).
8. PIEZ, K. A., OYAMA, V. I., LEVINTOW, L., AND EAGLE, H., *Nature* **188,** 59 (1960)
9. FOLK, J. E., PIEZ, K. A., CARROLL, W. R., AND GLADNER, J. A., *J. Biol. Chem.* **235,** 1987 (1960).
10. MOORE, S., SPACKMAN, D. H., AND STEIN, W. H., *Anal. Chem.* **30,** 1185 (1958).
11. HAMILTON, P. B., *Anal. Chem.* **30,** 914 (1958).
12. MOORE, S., AND STEIN, W. H., *J. Biol. Chem.* **176,** 907 (1948).

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A New Exchanger for the Chromatographic Fractionation of Nucleic Acids[1]

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Fractionations of the nucleic acids have been accomplished by selective elution from various adsorbents and exchanger materials such as calcium phosphate (1), Dowex 2 (2), a modified cellulose exchanger Ecteola (3), histone-coated kieselguhr (4), methylated bovine serum albumin, and Celite (5, 6), and by the dissociation of denatured nucleoprotein complexes (7, 8). Of these materials the one which has seemingly proved to be the most useful is Ecteola, a triethanolamine derivative of cellulose (9). It has been used for the fractionation of both deoxyribonucleic acid (DNA) (3), and ribonucleic acid (RNA) (10, 11). It is reported, however, that as much as 3 weeks may be required to complete a fractionation (12). Attempts to shorten this time by increasing the elution rate resulted in a reduction of the resolving power of the exchanger and caused the peaks to overlap (12).

A survey of commercially available exchanger materials was made in an attempt to find a material which might offer advantages not exhibited by existing exchangers. One such product that has been investigated in this laboratory is a cationic starch called Cato-2 (formerly known as Cato-8, National Starch Products, Inc., 750 Third Ave., New York 17, N. Y.). This report describes the technique used for fractionating nucleic acids on columns of Cato-2 and presents results to substantiate the effectiveness of these fractionations.

METHODS

Cato-2 is manufactured under U. S. Patent No. 2,813,093 and is described therein as an ungelatinized tertiary amino alkyl ether of starch.

[1] This work was supported by funds from the National Cancer Institute, National Institutes of Health, Public Health Service (Grant C-2896); initial pilot studies were aided by an allocation from an American Cancer Society Institutional Grant to Stanford University.

It behaves as an anion exchanger. The sample of Cato-2 used in the experiments to be reported here (Lot EFR 239) has a nitrogen content of 0.23% and a capacity for DNA of about 3 mg/gm of large mesh exchanger (see below). The capacity for soluble RNA is about three times that found for DNA.

Cato-2 is supplied as a fine powder and as such is not suitable for use in columns because of impaired flow rates. To make it suitable for column use it must first be converted to a larger mesh size. One hundred grams of Cato-2 was suspended in 1 l water and allowed to settle for about 1 hr. The supernatant was decanted and the procedure repeated. The starch was then mixed with approximately an equal volume of water and filtered in a Büchner funnel (11 cm diameter). When the excess moisture disappeared from the top of the cake, it was lightly scored with a spatula. The vacuum was immediately released when the cake cracked in half on the scored line. The cake was then put bottom side down on an enamel pan and placed in a 165° oven for 3 hr, then removed and allowed to cool.

The pure white, nongelatinized top portion of the cake was crumbled off and sorted to remove traces of gelatinized material, lightly ground in a mortar, and then screened through perforated stainless steel screens. Good flow rates were obtained with material which passed a screen having $\frac{1}{32}$-in. holes, but most of the work reported here was done with material which passed through a screen having $\frac{1}{16}$-in. holes but not through a screen having $\frac{1}{32}$-in. holes.

For the fractionation of 1–2 mg nucleic acid, columns 0.8 cm in diameter have been used, containing about 10 cc of packed Cato-2 (about 2 gm of dry Cato-2). To assure equilibration with NaCl, 100 ml of 1 *M* NaCl was run through the columns at a flow rate of 0.4 ml/min. The excess 1 *M* NaCl was then washed out with about 40 ml of 0.01 *M* NaCl at the same flow rate. The excess Cato-2 was washed out to a predetermined volume and a glass wool plug was introduced to prevent resuspension of the Cato-2. Up to 2 mg nucleic acid in 5 ml of 0.01 *M* NaCl was allowed to percolate through the column at a flow rate of 0.2 ml/min. When the sample had passed into the column bed, washing was continued with 15 ml of 0.01 *M* NaCl. For the elution of the nucleic acids, the flow rate was increased to 0.4 ml/min and 2.0-ml samples were collected.

For linear gradient elution (13) four schedules have been used: (A) a linear gradient from 0.0 to 1.0 *M* NaCl; 50 fractions were collected; (B) a linear gradient from 0.0 to 1.0 *M* NH_4OH in 1.0 *M* NaCl; 50 fractions were collected; (C) 12 fractions were eluted with 1.0 *M* NaCl; (D) elution with 0.1 *M* NaOH in 1.0 *M* NaCl. Cato-2 swells in NaOH so

that the flow of liquid through a column stops. In order to elute with 0.1 *M* NaOH in 1.0 *M* NaCl, the Cato-2 was extruded from the columns into a centrifuge tube. The starch was gently shaken in about 50 ml of this reagent for 15 min, then centrifuged and the supernatant transferred to a 50-ml volumetric flask, diluted to volume with reagent, and assayed.

After elution with 0.1 *M* NaOH, the Cato-2 was discarded because of the large percentage of mechanical breakage of the Cato-2 particles during this extraction procedure. When the strongest eluting reagent used was 1 *M* NH_4OH, as when fractionating soluble RNA, the columns were reused after complete regeneration with NaCl.

When the nucleic acid sample contained as much as 80% DNA as in the samples of total tissue nucleic acids from mouse thymus (see below), the recoveries from the columns were better than 96% when uncorrected for column blank. There was some non-nucleic acid material that adsorbed at 260 mμ which was eluted from blank columns (mostly in area B). The average column blank per fraction was 0.006 optical density units. When a correction was made for this column blank, the recovery of nucleic acids (when mostly DNA) was about 85%. When the nucleic acid samples contained only RNA, however, the corrected recoveries averaged 99%.

RESULTS

Reproducibility

A sample of mouse thymus nucleic acids representing 87% of the total tissue nucleic acid content and consisting of a mixture of DNA and RNA in the ratio of 4:1 was prepared by a modification (14) of the detergent method of Crestfield, Smith, and Allen (15). Duplicate aliquots of the same sample of nucleic acid were submitted to linear gradient elution from two different columns of Cato-2 (Fig. 1). Although the samples were run on columns of different length so that some of the peaks in the shorter columns come off a tube or two sooner, the almost exact superimpossibility of the two diagrams is obvious. Furthermore, when duplicate aliquots of the same sample of mouse thymus RNA prepared by extraction with phenol (16) were submitted to separate columns and fractionated by the technique of discontinuous elution (see below), the amount of material eluted in the various peaks differed by less than 3% between the two columns (14).

Rechromatography

Although the method gives reproducible results for duplicate columns, it is also of importance to determine whether the emergence of material

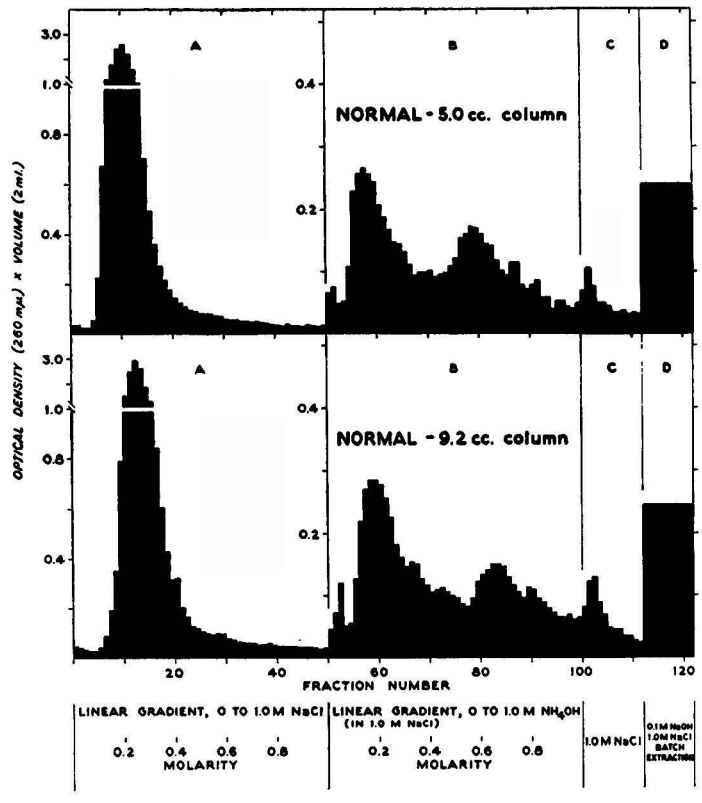

FIG. 1. Chromatography of duplicate samples of the same preparation of mouse thymus nucleic acids (DNA and RNA) representing 87% of the total tissue nucleic acids, on Cato-2 columns (0.8 cm diameter) of different length. Linear gradient elution was used at a flow rate of 0.4 ml; 2-ml samples were collected.

in any one peak along the elution diagram is a reproducible characteristic of that particular material on the column or is a secondary consequence of the over-all interaction between the exchanger and the various nucleic acid species present. If the former is true, then a peak should emerge from the column under the same conditions whether it has been adsorbed separately or as part of a mixture of nucleic acids. To test this, a sample of commercial DNA (Worthington Biochemical Corp., Freehold, N. J.) was fractionated on a column of Cato-2 (Fig. 2). The nucleic acid from the shaded area was recovered by alcohol precipitation and then resubmitted to a second column giving the results in the black areas. Ninety per cent of the resubmitted nucleic acid was again eluted from the column under the same conditions as before. Probability curves constructed for a theoretical resubmission experiment have the same

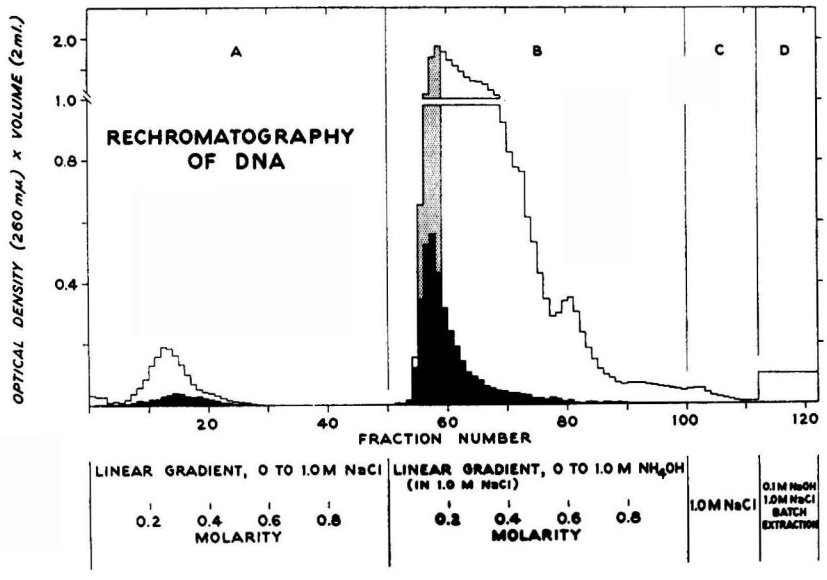

FIG. 2. Rechromatography of commercial calf thymus DNA on Cato-2 columns (0.8 × 18.3 cm): (*a*) original pattern given by solid line; (*b*) fraction to be resubmitted after recovery by alcohol precipitation indicated by shaded area; (*c*) results of resubmission experiment given in solid black areas. Linear gradient elution was used at a flow rate of 0.4 ml; 2-ml samples were collected.

shape as the curve obtained in area B. The degree to which a given peak will mimic its original behavior when resubmitted to columns of Cato-2 is therefore considered highly satisfactory.

Effect of Molecular Size

Figure 3 shows the results of one experiment to investigate the importance of molecular weight in determining the chromatographic behavior of the nucleic acids. The dark curve is the elution profile for a commercial sample of DNA (Worthington Biochemical Corp., Freehold, N. J.). The shaded area is the elution pattern for a deoxyribonuclease (Worthington Biochemical Corp., Freehold, N. J.) digest of a sample of this same DNA. It is apparent that molecular size does play a role in the fractionation obtained on columns of Cato-2. In other experiments it was found that adenine ribonucleotide was eluted in the same area as the digest of DNA, while uracil did not adhere to the column and was completely removed in the 0.01 *M* NaCl washing step. Since the largest polynucleotide in a deoxyribonuclease digest is probably an octanucleotide (17), either the column is not very selective for materials with molecular weights below about 3000 or else the conditions of elution do not make use of its selectivity (see below).

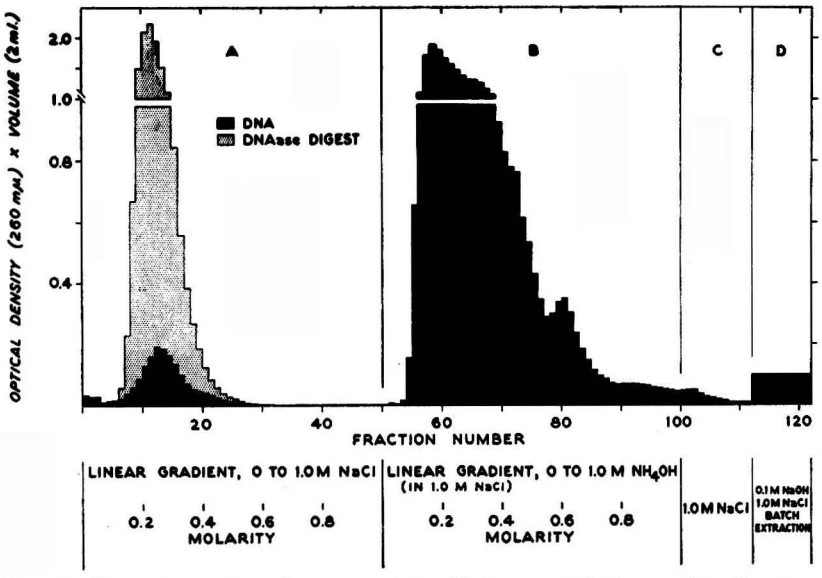

FIG. 3. Chromatography of commercial calf thymus DNA on Cato-2 columns (0.8 × 18.3 cm) before and after deoxyribonuclease digestion; using linear, gradient elution at a flow rate of 0.4 ml/min; 2-ml samples were collected.

Fractionation by Discontinuous Elution

The above result with the deoxyribonuclease digest was at first disappointing because the digest was eluted under the same conditions as a large part of the total mouse thymus nucleic acids (compare with Fig. 1). Quite different results were obtained, however, when the various samples were eluted by the technique of discontinuous elution instead of linear gradient elution. The method used for discontinuous elution does not differ from that already outlined for linear gradient elution as regards the preparation of the column, elution flow rates, etc., except that the eluting solutions were changed manually when the optical density at 260 mμ fell to a value below 0.040, and usually below a value of 0.020. To cover that portion of the diagram designated as area A in the linear gradient method, five discontinuous elutions have been used; followed then by the batch extraction of the Cato-2 as described in elution schedule D. The fractionations obtained by continuous elution and by discontinuous elution may be compared in Fig. 1 and Fig. 4, respectively.

With discontinuous elution adenylic acid could be eluted with 0.05 *M* salt, and deoxyribonuclease digests of DNA and ribonuclease digests of RNA could be eluted with 0.10 *M* salt. Less than 10% of the total tissue nucleic acids of mouse thymus was eluted with 0.10 *M* salt. These results with discontinuous elution indicate that the columns do distinguish be-

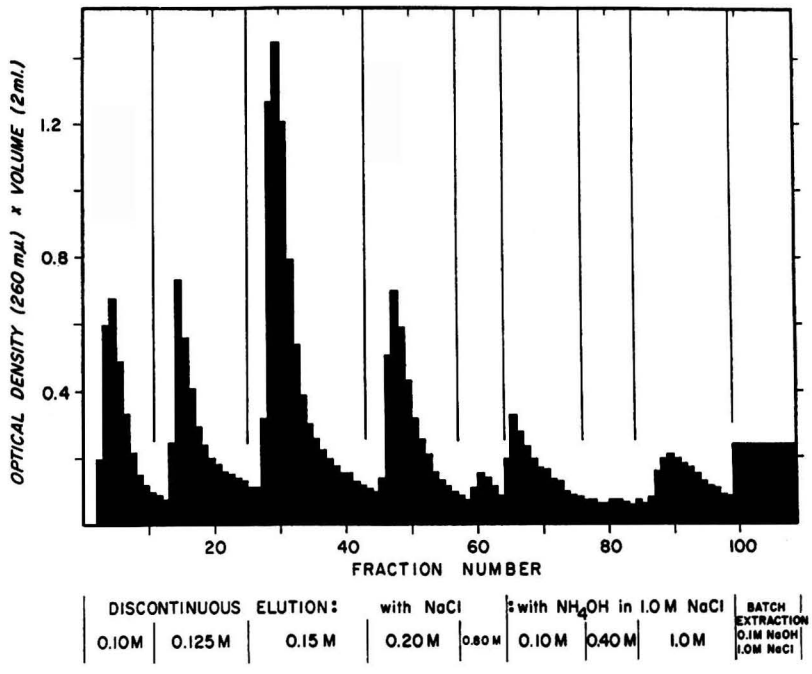

FIG. 4. Chromatography of mouse thymus nucleic acids (DNA and RNA), representing 92% of the total tissue nucleic acids, on a Cato-2 column (0.8 × 18.3 cm) using discontinuous elution at a flow rate of 0.4 ml/min; 2-ml samples were collected.

tween lower molecular weight polynucleotides. In an effort to obtain similar results with gradient elution, the rate of change of the gradient was decreased. Even a 20-fold decrease in the rate of change of the gradient still did not bring about fractionation in the neutral salt area.

Separation of Biologically Active Nucleic Acids

Nucleic acids recovered from the peaks obtained by the method of discontinuous elution have demonstrated different biological activities. Soluble RNA from guinea-pig liver has been fractionated into samples which show a differential ability to incorporate specific amino acids (18). The RNA was fractionated by an elution schedule similar to that given in Fig. 4. It was found that the RNA which combines with tyrosine under the influence of an enzyme also found in the soluble fraction was eluted primarily in the 0.125 *M* NaCl peak, while the RNA which combines with leucine was found primarily in the 0.20 *M* NaCl peak. The ratio of the specific activities of leucine-RNA to tyrosine-RNA in the unfractionated material was 4:1. This ratio changed to 1:1 in the 0.125 *M* peak and was 21:1 in the 0.20 *M* NaCl peak. Assays for the incorpora-

tion of lysine, isoleucine, and threonine showed that some fractionation of RNA's specific for these amino acids was also achieved. We thus have an example of nucleic acids which differ not only chromatographically but also on the basis of a functional parameter.

Separation of Soluble and Microsomal RNA

It was reported that at least a partial separation of transfer RNA and microsomal RNA was obtained on columns of Cato-2 since 85–90% of the former was eluted with neutral 1.0 *M* NaCl while about 65% of the microsomal RNA remained adsorbed under these conditions and was eluted with alkaline salt solutions (18). We now find that when using washed mouse liver microsomes as the source of the RNA, better than 80% of the RNA is not eluted with neutral salt but can be eluted with alkaline salt solutions (19). The 20% of the RNA that is eluted by neutral salt does not come off the column until a concentration of 0.2 *M* is used, while about 50% of the soluble RNA is eluted from the columns with 0.15 *M* salt. It is remarkable that the washed microsomes of the type used for the isolation of the above RNA are still capable of incorporating amino acids into protein without the addition of soluble RNA. Furthermore the fraction of RNA isolated from washed microsomes that is eluted from the columns by neutral salt is capable of accepting amino acids in the presence of purified pH 5 enzymes. These results either point up the extreme heterogeneity of amino acid acceptor RNA's, or as preliminary experiments suggest only a portion of the amino acid acceptor RNA isolated from tissue supernatants obtained by spinning for 60 min at 105,000 $\times$ *g* is involved in microsomal protein synthesis; the remainder is probably involved in other pathways of amino acid metabolism.[2] Work to clarify this point is in progress.

Variation in Base Composition of Chromatographic Fractions

The various peaks obtained by discontinuous elution exhibit differences in chemical composition. Calf thymus RNA was prepared by extraction with 90% phenol (16). Forty-nine milligrams of RNA in 13 ml of 0.01 *M* NaCl were adsorbed on a column of $\frac{1}{32}$-in. mesh Cato-2 (2.1 cm $\times$ 32 cm) at a flow rate of about 1 ml/min. Twelve-milliliter samples were collected. Elution was performed at a flow rate of about 2 ml/min and was continued until the optical density at 260 mμ fell below a value of 0.020 before changing to the next eluting solution. The six fractions thus obtained were recovered by alcohol precipitation.

Aliquots of the recovered material were then subjected to alkaline hydrolysis (0.3 *M* KOH at 37° for 18 hr), and the nucleotides were

[2] See Bates, H. M., and Lipman, F., *J. Biol. Chem.* **235**, 22P (1960).

separated by the technique of paper electrophoresis in 0.4 *M* formate buffer at pH 3.2. Areas containing the nucleotides were located by ultraviolet photography (20). These areas were cut from the papers and the nucleotides were extracted with 0.01 *M* HCl. Concentrations were determined with a Beckman DU spectrophotometer using the following millimolar extinction coefficients at 260 mμ: adenylic acid, 14.2; guanylic acid, 11.8; cytidylic acid, 6.8; and uridylic acid, 10.0. The elution conditions and the nucleotide composition of the various fractions are given in Table 1.

TABLE 1
COMPOSITION OF CALF THYMUS RIBONUCLEIC ACID FRACTIONS

Chromatographic fraction	Per cent of total RNA	Nucleotide composition (moles/100 moles)				$\frac{G}{A}$	$\frac{C}{U}$	$\frac{A+G}{C+U}$
		C[a]	A	G	U			
Unfractionated RNA		32.1[b]	18.1	31.6	18.2	1.75	1.76	0.99
0.125 *M* NaCl	4.1	32.9	16.8	32.1	18.2	1.91	1.81	0.96
0.150 *M* NaCl	6.4	31.9	18.1	31.4	18.5	1.74	1.72	0.98
0.200 *M* NaCl	24.7	31.5	18.5	31.4	18.6	1.70	1.70	1.00
0.800 *M* NaCl	12.5	31.3	18.7	31.6	18.5	1.69	1.69	1.01
1.0 *M* NaCl + 1.0 *M* NH_4OH	51.5	31.7	18.7	30.3	19.2	1.62	1.65	0.96
1.0 *M* NaCl + 0.1 *M* NaOH	0.8	—	—	—	—	—	—	—
	Recovery[c]	98%	102%	97%	103%			

[a] C, A, G, and U represent the nucleotides: cytidylic acid, adenylic acid, guanylic acid, and uridylic acid, respectively.

[b] Average deviation is 2%.

[c] Nucleotide composition of calf thymus RNA calculated as the sum of the increments contributed by each fraction and expressed as the per cent of the values found for the unfractionated sample.

The various RNA fractions show a progressive increase in adenine content relative to the guanine content as the eluting solutions necessary for their removal from the column become more concentrated. A similar progressive change has been obtained in the base composition of DNA fractions (4, 7). A chemical explanation for this phenomenon has been published (4, 10).

The various RNA fractions show a purine to pyrimidine ratio near unity. This is of special significance in the fractions eluted by neutral salt since the major portion of these fractions is composed of RNA from the soluble fraction of the cells (18, 19). The relatively smaller size of this class of RNA has implied the lack of a requirement for base pairing as is found in DNA and more recently in microsomal RNA (21). It

would now appear that there may be base pairing even in these smaller ribonucleic acids.

DISCUSSION

It is unreasonable to assume that one exchanger material would meet the requirements for the complete fractionation of anything so complex as the total nucleic acids of a tissue. An exchanger exhibiting weak basic properties is required for the efficient release of the larger molecular weight nucleic acids, and somewhat more basic exchangers are to be preferred for the fractionation of lower molecular weight polynucleotides; nevertheless, the selection of an exchanger material for a particular separation is largely empirical. Discussions of some of the problems involved in the fractionation of the nucleic acids with special emphasis on the influence of the chemical and physical heterogeneity of the nucleic acids (4, 10) and the heterogeneity of the exchanger materials themselves have been published (10).

Although Cato-2 was not developed commercially for chromatographic use, it has proved to be very useful in this capacity. A simple pretreatment of Cato-2 converts it to a mesh size more useful for column work. It exhibits characteristics that are to be desired in any exchanger material: (*a*) duplicate columns yield duplicate results; (*b*) isolated peaks rechromatograph as they did originally; (*c*) it distinguishes between samples which differ chemically and/or by molecular weight; and (*d*) it apparently does not alter the sample during its passage through the column since fractionated soluble RNA retains its biological activity as a binding agent for specific amino acids. Cato-2 offers an important technical advantage in that fractionation remains sensitive at much faster flow rates than those employed with other exchangers. This allows the completion of an experiment in days instead of weeks.

Preliminary accounts of the application of this method to the study of the nucleic acid changes in isologous tumor and x-irradiated tissues have appeared (22, 23). This work will be reported in more detail elsewhere.

SUMMARY

1. A new exchanger material, Cato-2 (a cationic starch), has been found to be suitable for the chromatographic fractionation of the nucleic acids. Duplicate columns yield duplicate results and isolated peaks rechromatograph as they did originally.

2. Much faster flow rates than those usually employed with other exchangers can be used with Cato-2 without loss of resolution. This allows the completion of an experiment in days instead of weeks.

3. That the fractions obtained under these conditions represent real and significant differences in the nucleic acids present is documented by the fact that the fractions differ not only in chemical composition but also in biological activity.

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REFERENCES

1. MAIN, R. K., AND COLE, L. J., *Arch. Biochem. Biophys.* **68,** 186 (1957).
2. MIURA, K., AND SUZUKI, K., *Biochim. et Biophys. Acta* **22,** 565 (1956).
3. BENDICH, A., PAHL, H. B., AND BEISER, S. M., *Cold Spring Harbor Symposia Quant. Biol.* **21,** 31 (1956).
4. BROWN, G. L., AND WATSON, M., *Nature* **172,** 339 (1953).
5. LERMAN, L. S., *Biochim. et Biophys. Acta* **18,** 132 (1955).
6. MANDELL, J. D., AND HERSHEY, A. D., *Anal. Biochem.* **1,** 66 (1960).
7. CHARGAFF, E., CRAMPTON, C. F., AND LIPSHITZ, R., *Nature* **172,** 289 (1953).
8. LUCY, J. A., AND BUTLER, J. A. V., *Nature* **174,** 32 (1954).
9. PETERSON, E. A., AND SOBER, H. A., *J. Am. Chem. Soc.* **78,** 751 (1956).
10. BRADLEY, D. F., AND RICH, A., *J. Am. Chem. Soc.* **78,** 5898 (1956).
11. GOLDTHWAIT, D. A., *J. Biol. Chem.* **234,** 3245 (1959).
12. BENDICH, A., PAHL, H. B., KORNGOLD, G. C., ROSENKRANZ, H. S., AND FRESCO, J. R., *J. Am. Chem. Soc.* **80,** 3949 (1958).
13. CHERKIN, A., MARTINEZ, F. E., AND DUNN, M. S., *J. Am. Chem. Soc.* **75,** 1244 (1953).
14. SMITH, K. C., AND KAPLAN, H. S., in preparation.
15. CRESTFIELD, A. M., SMITH, K. C., AND ALLEN, F. W., *J. Biol. Chem.* **216,** 185 (1955).
16. KIRBY, K. S., *Biochem. J.* **64,** 405 (1956).
17. SINSHEIMER, R. L., *J. Biol. Chem.* **208,** 445 (1954).
18. SMITH, K. C., CORDES, E., AND SCHWEET, R. S., *Biochim. et Biophys. Acta* **33,** 286 (1959).
19. SMITH, K. C., AND DOELL, R. G., *Federation Proc.* **19,** 318 (1960).
20. SMITH, K. C., AND ALLEN, F. W., *J. Am. Chem. Soc.* **75,** 2131 (1953).
21. DOTY, P., BOEDTKER, H., FRESCO, J. R., HASELKORN, R., AND LITT, M., *Proc. Natl. Acad. Sci. U.S.* **45,** 482 (1959).
22. SMITH, K. C., AND KAPLAN, H. S., *Federation Proc.* **17,** 458 (1958).
23. SMITH, K. C., AND KAPLAN, H. S., *Federation Proc.* **18,** 507 (1959).

ANALYTICAL BIOCHEMISTRY, 1, 213–217 (1960)

The Determination of Uric Acid in Biological Fluids: I. A Modification of the Method of Bergmann and Dikstein

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A reliable method for the determination of uric acid by means of specific precipitation with acidified mercuric acetate, re-solution in acidified sodium chloride, and measurement of the optical density at 290 mμ has been published by Bergmann and Dikstein (1). The reaction contrasts with those of various colorimetric procedures (2–6) in being stoichiometric, although Henry *et al.* (7) report results which follow Beer's law. The enzymic method of Kalckar (8) proved unsuitable in our hands for use with cockroach feces in which we were especially interested. Brown's method (5) also gave irregular results. The method of Bergmann and Dikstein in its published form was also found unsuitable, however, for determining low concentrations of uric acid as found in cockroach excreta. For example, our solutions usually contained 4 μg/ml, or less. The final concentration by Bergmann and Dikstein's procedure would be 0.2 μg/ml, or less, and gives too low an absorbance for accuracy. It was necessary to modify their method, and we have further simplified it so as to make a single procedure applicable to various biological fluids. Our procedure has been proved by the recovery of added uric acid from various biological solutions, including extract of roach feces, and especially by its favorable comparison with results obtained by the enzymic method of Kalckar with human urine and blood plasma.

MATERIALS AND PROCEDURE

The following reagents are employed: Solution 1, 2.6 *N* perchloric acid; Solution 2, 0.2 *M* sodium tetraborate (borax) in 0.9 *N* NaOH; Solution 3, 0.13 *M* mercuric acetate in 6.5% acetic acid; Solution 4, 0.5 *M* sodium chloride in 1% acetic acid.

The procedure is as follows: To a conical-bottom 40-ml centrifuge tube containing 4.0 ml of the solution to be tested, which should preferably contain between 2 and 15 μg uric acid/ml, add 0.5 ml of Solution 1 dropwise while shaking. After 10 min, centrifuge for 10 min at 3000 rpm (Model 2 International centrifuge) to pack the protein precipitate. Decant into another centrifuge tube, draining thoroughly, and add 1.0 ml of Solution 2 to the supernatant dropwise while shaking. Then add dropwise and very slowly, while swirling continuously and vigorously, 1.0 ml of Solution 3. Place the tube with contents in a boiling water bath for 15 min and shake gently once or twice to prevent formation of a dried ring. Chill in an ice bath. Centrifuge. Decant thoroughly and discard the supernatant. Dissolve, or disperse by shaking, the precipitate with the aid of 4.0 ml of Solution 4, and clarify the mixture by centrifuging for 5 min. Decant into an ultraviolet absorption cell and read the optical density at 290 mμ against Solution 4. Divide the observed optical density by the optical density, 0.065, of 1 μg/ml uric acid in Solution 4 to obtain micrograms uric acid/ml sample. In protein-free samples, precipitation with mercuric acetate in acetic acid (Solution 3) may be performed directly after first adding 1.5 ml water to the 4.0 ml sample.

The extracts of feces from the cockroach, *Periplaneta americana,* were made with 0.003 *M* LiOH by homogenization in a Virtis "45" followed by centrifugation at 3000 rpm. The urine was diluted 1:100 and the heparinized plasma 1:10 in water. Uric acid was added either as an aqueous dilution of Folin's standard uric acid solution (3) or as a solution in 0.008 *M* borax for the series of enzyme oxidations. Twenty, 10, or 5 μg uric acid was added in 0.5 ml borax solution to 4.0 ml sample. Five-tenths milliliter borax was added to the control tubes.

For measurements of uric acid by Kalckar's procedure, uricase was used in borate buffer at pH 8.8 and incubated at room temperature or at 40°C. Four different samples of enzyme were used, all of which were obtained from the Sigma Chemical Co., Saint Louis.

RESULTS AND DISCUSSION

Table 1 compares our procedure with that of Bergmann and Dikstein's. Our procedure is uniform and permits the use of small original samples of low concentration of uric acid and better control of the pH of the reaction. The pH of the mixture from which the uric acid is precipitated varies from 3.2 for extracts to about 4.5 for plasma; below 2.8 some uric acid is lost, while above pH 4.8 there is an excessive precipitation of substances which results in high absorbance, especially with

TABLE 1

AUTHORS' PROCEDURE

Sample			Soln. 1		Soln. 2	Soln. 3		Soln. 4	
Urine / 100 or 4.0 ml	or Plasma / 10	or Fecal Ext.	0.5 ml	Centrifuge 10 min	1.0 ml	1.0 ml	Place in boiling water bath 15 min; chill; centrifuge 10 min pH 3.2–4.5	4.0 ml	Centrifuge 5 min

BERGMANN AND DICKSTEIN'S PROCEDURE

Sample					
Urine / 5 3.0 ml	3 ml 6% $HClO_4$; filter	Dilute filtrate 5× with 3% borax	Add 4 ml to 1 ml of precipitant in boiling water bath dropwise during 4 min while shaking. Continue heating 10 min. Chill. Centrifuge. Pipet off. pH 4.2	Wash ppt. with 5 ml satd. uric acid soln. Centrifuge. Pipet off	Dissolve in 8 ml NaCl–acetic acid soln. Filter
Plasma / 5 10.0 ml	10.0 ml $HClO_4$. Heat 5 min at 60°. Chill; filter	To 10 ml add 2.5 ml NaOH–borax solution	To 10 ml add 2.0 ml of precipitant. Boil 1 min over flame. Heat 5 min in boiling water bath. Centrifuge. Decant. Pipet off. pH 7.0		Dissolve in 8 ml NaCl–acetic acid soln. Filter

extracts of cockroach feces. The use of reaction mixtures at a pH below 4.8 eliminates the need for washing.

The efficiency of the method as measured by the recovery of uric acid added in the amount of 5 μg/ml to test solutions of urine, plasma, and extracts of cockroach feces is shown in Table 2. Equally good recovery

TABLE 2

DETERMINATION BY DIFFERENT METHODS OF URIC ACID IN, AND RECOVERY OF URIC ACID ADDED TO HUMAN URINE, BLOOD PLASMA, AND EXTRACT OF COCKROACH FECES

	Authors' method		Bergmann and Dikstein's method		Kalckar's method	
	μg/ml	% recovery[a]	μg/ml	% recovery[a]	μg/ml	% recovery[a]
Urine (7 samples)		94.6 ± 3.1[b]				
Plasma (6 samples)		93.6 ± 3.2				
Fecal exts. (16 samples)		95.1 ± 3.4				
Urine sample 1	591	99.4	592		593	99.6
Urine sample 2	730	102.0	716	98.5	726	98.5
Urine sample 3	1320				1350	
Urine sample 4	1100				1050	
Plasma sample 1	68	95.6			69	97.0
Plasma sample 2	51.6				52.6	

[a] Five micrograms uric acid added per milliliter of test solution before treatment with $HClO_4$.

[b] Standard deviation.

is obtained when 2.5 1.25 μg/ml is added. Comparison of Kalckar's method with that of the authors with urine and blood plasma, and with Bergmann and Dikstein's with urine gave results which are in good agreement, as shown in Table 2, and permits the conclusion that the method proposed does indeed measure the amount of uric acid in solution.

SUMMARY

A simplified, generally applicable modification of Bergmann and Dikstein's method for uric acid analysis is proposed which has been verified by means of Kalckar's uricase method when applied to urine and blood plasma.

Recovery of 95.1 ± 3.4% has been obtained with 5 μg uric acid/ml added to an extract of cockroach feces containing 3 μg uric acid/ml, and equally good results with the addition of 2.5 or 1.25 μg. Similar recoveries were obtained from urine and plasma.

REFERENCES

1. BERGMANN, F., AND DIKSTEIN, S., *J. Biol. Chem.* **211,** 149 (1954).
2. STEEL, A. E., *Biochem. J.* **68,** 306 (1958).
3. FOLIN, O., *J. Biol. Chem.* **106,** 311 (1934).
4. KERN, K., AND STRANSKY, E., *Biochem. Z.* **290,** 419 (1937).
5. BROWN, H., *J. Biol. Chem.* **158,** 601 (1945).
6. BORSOOK, H., *J. Biol. Chem.* **110,** 481 (1955).
7. HENRY, R. J., SOBEL, C., AND KIM, J., *Am. J. Clin. Pathol.* **28,** 152 (1957).
8. KALCKAR, H. J., *J. Biol. Chem.* **167,** 429 (1947).

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Ultraviolet Fluorescence of Sulfosalicylic Acid for the Detection and Differentiation of Ketoses and Aldoses

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Dip or spray methods utilizing aniline oxalate–permanganate (1), cysteine–carbazole (2), 2,4-dinitrophenylhydrazine (3), resorcinol (4–6), napthoresorcinol (7, 4), orcinol (8, 5, 9), vanillin (10), *p*-anisidine hydrochloride (5), urea hydrochloride (5), anthraquinone (5), and 2,5-diphenyl-3-*p*-styrylphenyltetrazolium chloride (11) have been used for the differentiation of ketoses from other sugars on paper chromatograms. Some of the methods depend upon color differences while others are sensitive for ketoses only, aldoses giving no more than traces of color on the paper chromatograms.

A procedure was developed earlier (12) for the nonspecific detection of organic compounds on paper chromatograms. This procedure utilized the ultraviolet fluorescence of quinine sulfate or sulfosalicylic acid as a background for spot detection. When the developed chromatograms were dipped in a solution of sulfosalicylic acid in acetone, dried at room temperature, and examined under ultraviolet light, ketoses, aldoses, sugar alcohols, amino acids, *O*-methyl sugars, saponins, and sapogenins were detected as light fluorescent spots against an intense gray fluorescent background. However, on the basis of this test alone none of the organic compounds could be differentiated.

This paper reports a new simple and sensitive adaptation of the ultraviolet fluorescence procedure which is useful for the detection and differentiation of ketoses from aldoses and other organic compounds. This adaptation is unique in that following the preliminary nonselective detection of sugars and other organic compounds, one may selectively detect the ketoses only, merely by heating the paper.

MATERIALS AND METHODS

The aldoses and ketoses used for this procedure were chromatographed on the papers by an appropriate solvent system. When the developed and

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

dried chromatograms were dipped in a freshly prepared solution of 1.0% sulfosalicylic in acetone, dried at room temperature, and viewed in the dark under ultraviolet light of 2537 A wavelength, sugars and other organic compounds were nonselectively detected (12). The area of each of the spots which are visible should be lightly marked on the paper in pencil at this time. At this point in the procedure two choices are available. First, it is possible to develop a tan color from ketose spots only by heating the paper for 5 min in a forced air oven at 95°C. The detection of ketose spots on the paper following heating can be amplified by again viewing the paper under ultraviolet light. The other choice available is to wash or chromatograph the sulfosalicylic acid off the marked paper with ether or dry acetone soon after marking the spots under ultraviolet light and prior to any heating of the paper. This leaves the sugar spots free for elution or for determination by other specific ketose or aldose procedures.

Two ultraviolet light sources were used for these studies. A long-wave lamp with ultraviolet radiation in the 3600-A wavelength region and filtered to exclude visible light was useful as was a low-pressure mercury lamp also filtered to exclude visible light and producing predominantly ultraviolet radiation of 2537 A.

A variety of papers including Whatman No. 1 and Schleicher & Schuell No. 507[2] are suitable for use with this procedure. Prior washing of the paper with water has been found to be beneficial. Papers used for sugar analyses should be dipped in the sulfosalicylic acid solution without delay after development and drying of the chromatograms.

RESULTS AND DISCUSSION

If the chromatogram which was dipped in the sulfosalicylic acid solution and air-dried is heated at 95°C for 5 min, tan spots appear for ketoses. Ribulose, fructose, sorbose, tagatose, sedoheptulose, sucrose, turanose, raffinose, melezitose, and stachyose were detected on the papers in amounts of from 2 to 5 μg. Spots which were nonselectively marked earlier and which contain ketoses are thus detected in daylight by their tan coloration. When viewed again under ultraviolet radiation of 2537-A wavelength, the tan ketose spots which appeared following heating of the paper are amplified as dark spots on the chromatograms. If a 3600-A source is used, an intense golden fluorescent spot appears instead of the tan spot as observed for ketoses under visible light. With either of the ultraviolet sources, all of the ketoses tested could be detected as gray–black or as golden spots in amounts of 5 μg or less. There is no change

[2] Mention of specific products does not imply recommendation by the Department of Agriculture over others of a similar nature not mentioned.

in appearance under either visible or ultraviolet light for compounds other than ketoses, following heating.

The presence of inorganic compounds or of nonvolatile organic compounds in the developing solvents has been found to be deleterious and results in losses of sensitivity due to a masking of the paper (12). All of the materials in the solvent system used for moving the sugars on the papers should volatilize from the papers prior to the sulfosalicylic acid dip.

SUMMARY

A sensitive procedure is described for the selective detection of ketoses in which the developed and dried chromatograms are dipped in a solution of 1% sulfosalicylic acid in acetone and dried at room temperature. When viewed under ultraviolet light, many organic compounds including ketoses, aldoses, sugar alcohols, *O*-methyl sugars, etc. can be detected against the fluorescent background of the papers and the spots can be marked. When the marked papers are then heated, only ketoses develop a tan color visible in daylight which can be further amplified by again viewing the papers under ultraviolet light. As little as 2 μg ketose per spot is detected by this procedure.

REFERENCES

1. MALYOTH, E., *Naturwissenschaften* **38,** 478 (1951).
2. UJEJSKI, L., AND WAYGOOD, E. R., *Can. J. Chem.* **33,** 687 (1955).
3. GRAY, R. A., *Science* **115,** 129 (1952).
4. FORSYTH, W. G. C., *Nature* **161,** 239 (1948).
5. HOUGH, L., JONES, J. K. N., AND WADMAN, W. H., *J. Chem. Soc.* **1950,** 1702.
6. RACHINSKII, V. V., AND KNYAZYATOVA, E. I., *Doklady Akad. Nauk S.S.S.R.* **85,** 1119 (1952); *C. A.* **47,** 448c (1953).
7. PARTRIDGE, S. M., *Biochem. J.* **42,** 238 (1948).
8. KLEVSTRAND, R., AND NORDAL, A., *Acta Chem. Scand.* **4,** 1320 (1950).
9. BEVENUE, A., AND WILLIAMS, K. T., *Arch. Biochem. Biophys.* **34,** 225 (1951).
10. GODIN, P., *Nature* **174,** 134 (1954).
11. BACON, J. S. D., *Biochem. J.* **73,** 507 (1959).
12. ROREM, E. S., *J. Chromatography* **4,** 162 (1960).

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Analysis for Guanidine and Methylguanidine in Uremic Plasma[1]

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Early reports (1–4) on the increased concentration of guanidine and methylguanidine in uremic plasma are equivocal because of the lack of specificity of the analytical methods employed. The Sakaguchi reaction (5), which is specific for monosubstituted guanidinium compounds, cannot be used directly for the assay of these compounds because of interference with the reaction by numerous naturally occurring substances (6) notably ammonia, histidine, tyrosine, tryptophan, and urea. The last is particularly troublesome since blood concentrations of urea nitrogen in excess of 200 mg/100 ml are not uncommon. This level would result in approximately 30 mg urea per color tube. Van Pilsum and co-workers (7) report that as little as 3 mg urea per color tube will interfere with the Sakaguchi reaction.

The present paper describes a method for determining guanidine and methylguanidine in uremic plasma and presents evidence that these compounds are elevated in uremia.

METHOD

Principle

A protein-free, arginine-free, filtrate is prepared from plasma with $Ba(OH)_2$ and $ZnSO_4$ solutions. The methylguanidine and guanidine are adsorbed on a small quantity of a strong cation-exchange resin from which they are eluted with KOH. Guanidine is then converted to methylguanidine with dimethyl sulfate. Reaction of methylguanidine with the

[1] Supported in part by Research Grant H-2363 from the National Heart Institute, U. S. Public Health Service. A preliminary account of this work was presented at the 137th National Meeting of the American Chemical Society at Cleveland, Ohio, April 1960.

Sakaguchi reagents produces a pink color which is measured with a spectrophotometer.

Materials and Reagents

Protein precipitation and Sakaguchi color reagents are prepared as described by Van Pilsum and co-workers (7).

Standards. Guanidine stock solution, 1.0 mg/ml, was prepared by solution of guanidine acetate[2] in distilled water.

Methylguanidine stock solution, 1.0 mg/ml, was prepared by solution of methylguanidine sulfate[2] in distilled water.

Ion-Exchange Resin. Dowex 50 W-X8 (100-200 mesh) in the sodium form.

Potassium Hydroxide. 4 N.

Dimethyl Sulfate.[2]

Preparation of Protein-Free Filtrate

Using 20 ml plasma, a protein-free filtrate is prepared according to the procedure of Van Pilsum and associates (7) except that, after centrifuging, the supernatant is filtered through a small Büchner funnel and the filtrate is divided into equal parts. If the concentrations of guanidine and methylguanidine are known to be high, a smaller quantity of plasma with correspondingly smaller volumes of precipitating reagents may be used.

Chromatography of Filtrates

Each aliquot of filtrate is passed through a small column, approximately 6 mm in diameter, containing 1 cm resin. The resin is washed once with 1 ml of 0.1 *M* NaCl and eluted with 4 *N* KOH. Two milliliters of eluant for methylguanidine and 2.5 ml for guanidine are used. The pH of the methylguanidine eluant is adjusted to 8–9 with 10 *N* HCl (approximately 0.6 ml is required). The volumes are then made up to 3 ml with water.

Methylation of Guanidine

To each of the 2.5-ml portions of KOH eluate in 25-ml Erlenmeyer flasks is added 0.5 ml dimethyl sulfate, and the flasks are shaken under a hood for 40 min at 37°C.

Color Development

Two milliliters of eluate is pipetted into 15 × 85 mm tubes in an ice bath. Reagents are added according to the procedure of Van Pilsum and associates (7).

[2] Eastman Organic Chemicals, Distillation Products Industries, Rochester, N. Y.

Preparation of Standard Curves

Since both guanidine and methylguanidine are adsorbed on the $BaSO_4$-$Zn(OH)_2$ precipitates, standard curves must be prepared by including the protein-precipitating reagents. Dilutions of the stock guanidine and methylguanidine solutions are prepared to contain from 0.05 to 0.20 mg/100 ml. Ten-milliliter aliquots of these dilutions are treated exactly like the plasma samples. Two series of methylguanidine standards are prepared. One is for direct analysis and the second is treated with dimethyl sulfate according to the procedure for guanidine. Since plasma may contain methylguanidine as well as guanidine, the color resulting from the application of the Sakaguchi reaction to the methylated eluate is due to the sum of these two compounds. From the total optical density observed, that portion due to methylguanidine alone, as determined from the curve of the "methylated" methylguanidine, must be subtracted. This difference represents the amount of guanidine which is derived from the graph of optical density–guanidine concentration.

RESULTS AND DISCUSSIONS

Interference with the Sakaguchi reaction by urea was confirmed (7), and the extent of this inhibition was quantified. Figure 1 shows the

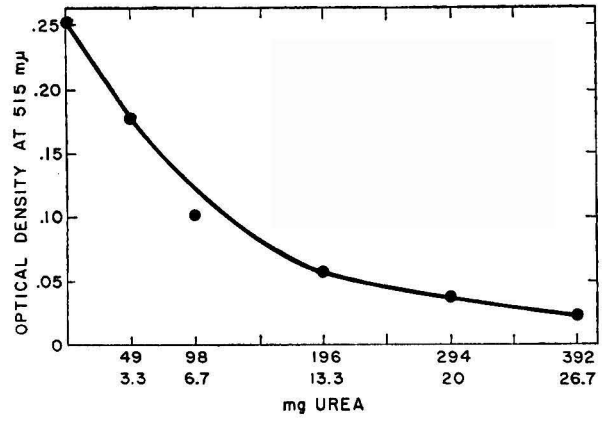

FIG. 1. The influence of urea on the Sakaguchi color reaction. On the abscissa, the upper values represent concentrations of urea per 100 ml blood corresponding to amounts of urea per color tube listed below them.

effect of increasing amounts of urea on the optical density of 0.10 mg methylguanidine/100 ml. Since the concentrations of urea in uremic plasma may approximate 140 mg urea nitrogen per 100 ml blood or about 50 mmoles urea/l, it is apparent that the use of a correction factor would not be feasible. Attempts to remove the urea by treatment with

urease were not successful because of interference by ammonium compounds and possibly by contaminants introduced with the urease. Xanthydrol, which will precipitate urea quantitatively under certain conditions, was also unsatisfactory because of the introduction of large quantities of salts, especially sodium acetate.

Determinations by the method described were found to be reproducible with standard deviations of 0.004 mg/100 ml for methylguanidine and 0.005 mg/100 ml for guanidine. Recoveries of added methylguanidine and guanidine in the physiologic range are shown in Table 1.

TABLE 1
RECOVERIES[a]

Serum	Methylguanidine				Guanidine			
	Added (mg)	Theo. (mg)	Found (mg)	Recov. (%)	Added (mg)	Theo. (mg)	Found (mg)	Recov. (%)
Undiluted	0	—	0.21	—	0	—	0.02	—
Diluted 1:2	0	0.11	0.10	91	0	0.01	0.01	100
	0.05	0.16	0.15	94	0.05	0.06	0.05	83
	0.10	0.21	0.20	95	0.10	0.11	0.11	100
	0.20	0.31	0.32	103	0.20	0.21	0.22	105

[a] Methylguanidine and guanidine were added to pooled serum which had been diluted 1:2 in such concentration that the expected levels would lie in the physiologic range. Values represent mg/100 ml.

Van Pilsum (7) and associates state that the low optical densities found in the determination of guanidine are due either to incomplete methylation or to the formation of some other compound. In order to explore the first of these alternatives, attempts were made to increase the yield of methylguanidine from guanidine by altering the amount of dimethyl sulfate, the concentration of KOH, the temperature, and the length of time and speed of shaking. No essential difference was observed.

Exploration of the second alternative by paper chromatography yielded more information. Using ammoniacal ethanol as a solvent (8), a descending chromatogram showed single spots of methylguanidine and guanidine for the pure compounds. After methylation, two spots were developed from each standard. One had the same R_f as methylguanidine and the second showed greater mobility. When a solution containing guanidoacetic acid, guanidine, methylguanidine, and 1,1-dimethylguanidine was analyzed, the second spot was found to correspond to 1,1-dimethylguanidine.[2] Ferricyanide–nitroprusside reagent (9) was used to develop the spots. Because dimethylguanidine does not react with Sakaguchi reagents, the conversion of a portion of both methylguanidine

and guanidine to this compound must have been responsible at least in part for the low optical densities found.

The extent of this loss was demonstrated by the fact that when methylguanidine was treated with dimethyl sulfate as in the methylation of guanidine, the optical density resulting from application of the Sakaguchi reagents was only 28–33% of that obtained in the original

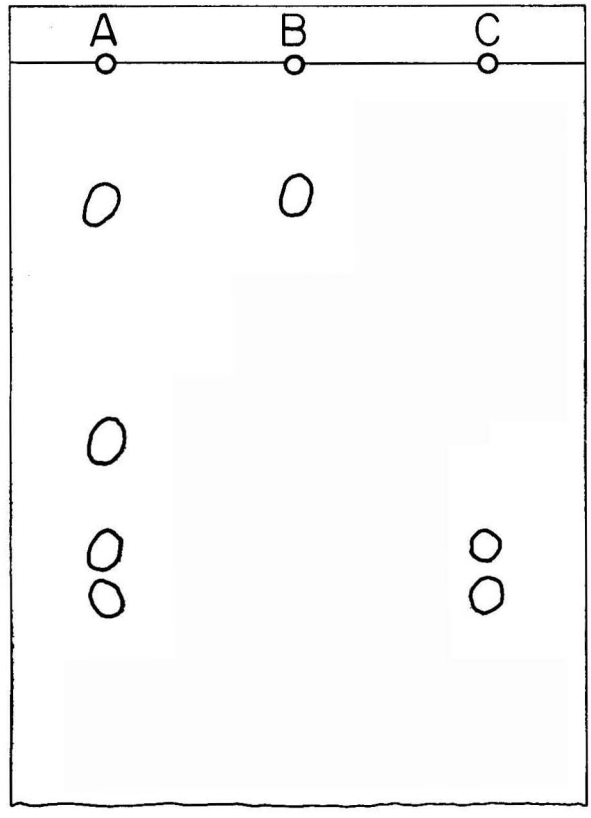

FIG. 2. Reproduction of a chromatogram. *A*, solution containing guanidoacetic acid, guanidine, methylguanidine, and dimethylguanidine; *B*, the effluent from a filtrate of this solution passed through the cation-exchange column; *C*, the eluate of this solution after methylation.

solution. Since the ratio appears to be constant, this fact does not appear to invalidate the method. Figure 2 shows a tracing of a chromatogram of (*a*) a solution consisting of guanidoacetic acid, guanidine, methylguanidine, and dimethylguanidine, (*b*) the effluent from a filtrate of this solution passed through the cation-exchange column, and (*c*) the eluate of this solution after methylation. The mixed standard solution showed

good separation of its components with R_f values which compared favorably with values given in a reference table (8). The presence of guanidoacetic acid only in the effluent from the column showed the efficacy of the resin in the separation of this compound from guanidine and methylguanidine. The absence of guanidine and the presence of methylguanidine and dimethylguanidine in the methylated eluate indicated a high efficiency of methylation. Chromatograms made from uremic plasma identified guanidine and methylguanidine before methylation and methylguanidine and dimethylguanidine after methylation.

The average plasma methylguanidine value of 13 normal mongrel dogs was 0.022 ± 0.004 mg/100 ml (standard deviation), while the average plasma concentrations of nine nephrectomized dogs sacrificed on the fourth day was 0.212 ± 0.031 mg/100 ml. The average plasma guanidine value for these normal dogs was 0.002 ± 0.004 mg/100 ml while this concentration rose to 0.038 ± 0.024 mg/100 ml in the nine animals 4 days after removal of the kidneys. The increases of both of these substances were found to be significant ($P = 0.001$).

While guanidoacetic acid and arginine are the only monosubstituted compounds occurring in significant amounts in normal biological fluids (11, 12), significant increases of guanidine and methylguanidine were found in the plasma of uremic dogs.

The conclusion of Child (4), Andes and associates (10), and Major and co-workers (3) that guanidine and methylguanidine are elevated in uremia has been confirmed and a quantitative appraisal of these elevations has been presented for nephrectomized dogs. The possible toxicologic significance of these increases is being evaluated experimentally and will form the subject of a separate presentation.

SUMMARY

A method for the determination of guanidine and methylguanidine in the presence of urea has been described. These compounds were adsorbed quantitatively on a strong cation-exchange resin from a protein-free, arginine-free filtrate, eluted with 4 *N* KOH, and the quantities were determined by application of the Sakaguchi reaction.

Plasma guanidine and methylguanidine concentrations were found to be elevated significantly in uremic dogs.

ACKNOWLEDGMENTS

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REFERENCES

1. BRADLEY, S. E., *New Engl. J. Med.* **235,** 791 (1946).
2. MASON, M. F., RESNIK, H., JR., MINOT, A. S., RAINEY, J., PILCHER, C., AND HARRISON, T. R., *A.M.A. Arch. Internal Med.* **60,** 312 (1937).
3. MAJOR, R. H., WEBER, C. J., AND RUMOLD, M. J., *A.M.A. Arch Internal Med.* **64,** 488 (1939).
4. CHILD, C. G., *J. Clin. Invest.* **17,** 301 (1938).
5. SAKAGUCHI, S., *J. Biochem.* (Tokyo) **5,** 133 (1925).
6. WEBER, C. J., *J. Biol. Chem.* **86,** 217 (1930).
7. VAN PILSUM, J. F., MARTIN, R. P., KITO, E., AND HESS, J., *J. Biol. Chem.* **222,** 225 (1956).
8. BLOCK, R. J., DURRUM, E. L., AND ZWEIG, G., "A Manual of Paper Chromatography and Paper Electrophoresis," 2nd Ed., p. 129. Academic Press, New York, 1958.
9. BLOCK, R. J., DURRUM, E. L., AND ZWEIG, G., "A Manual of Paper Chromatography and Paper Electrophoresis, 2nd Ed., p. 130. Academic Press, New York, 1958.
10. ANDES, J. E., LINEGAR, C. R., AND MYERS, V. C., *J. Lab. Clin. Med.* **22,** 1209 (1937).
11. CHUNG, W., *Arch. Biochem. Biophys.* **85,** 461 (1959).
12. SIMS, E. A. H., *J. Biol. Chem.* **158,** 239 (1945).

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A Specific Method for the Analysis of Hydroxyproline in Tissues and Urine

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Since nearly all animal hydroxyproline is found in collagen, the measurement of hydroxyproline has been used extensively in studies on collagen formation and metabolism. A number of methods have been reported for the specific assay of the imino acid (1–5), but in general they can be used only with relatively pure solutions. The present paper presents a simple quantitative method which can be applied directly to crude tissue or protein hydrolyzates containing less than 0.01% hydroxyproline, and to urine containing less than 2 μg/ml.

Most of the published methods for hydroxyproline involve the oxidation of the imino acid to pyrrole-2-carboxylic acid or pyrrole, and then formation of a chromophore with *p*-dimethylaminobenzaldehyde (Fig. 1). Although the available methods based on this reaction are sensitive

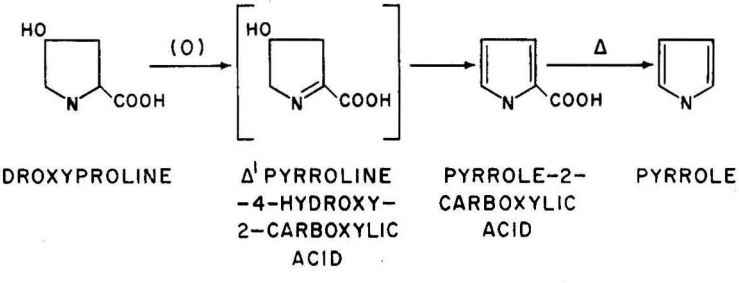

FIG. 1. Oxidation and decarboxylation of hydroxyproline to pyrrole.

to 0.01 μmole hydroxypyroline, results in urine and unpurified solutions are erratic largely because the oxidation step is difficult to control. Other substances which undergo oxidation destroy the oxidant and drastically reduce the yields of pyrrole (4); with an excess of oxidant the desired product polymerizes (6). A further problem in many of the methods is

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that pigments and other products from biological materials obscure the pyrrole chromophore (2).

In the procedure described here, hydroxyproline is oxidized in the presence of a measured excess of alanine, and as a result the varying amounts of amino acids or similar substances in urine or tissue hydrolyzates do not influence the yield of pyrrole. Specificity is achieved by extracting the pyrrole into toluene. Since the first oxidation products [Δ^1-pyrroline-4-hydroxy-2-carboxylic acid and pyrrole-2-carboxylic acid (7)] are not soluble in toluene, materials which might interfere with the color reaction are removed with toluene before the pyrrole is formed. These precautions make it possible to analyze 5 μg hydroxyproline in solutions containing over 50 mg of other amino acids.

MATERIALS

Buffer Solutions. (*a*) Sodium pyrophosphate, pH 8.0: 0.2 *M* solution in distilled water. (*b*) Potassium borate, pH 8.7: 61.84 gm boric acid and 225 gm potassium chloride are mixed into about 800 ml of distilled water. The pH is adjusted to 8.7 with a potassium hydroxide and the final volume made up to 1 l.

Alanine Solution. Ten grams of alanine is dissolved in about 90 ml of distilled water, the pH is adjusted to 8.7 with potassium hydroxide, and the final volume is made up to 100 ml.

Chloramine T Solutions (Eastman Organic Chemicals). (*a*) 0.02 *M* solution in distilled water is prepared daily. (*b*) 0.2 *M* solution in Methyl Cellosolve is prepared daily.

Sodium Thiosulfate. Solution is 3.6 *M* in distilled water and is stored under toluene at room temperature for several weeks.

p-Dimethylaminobenzaldehyde or Ehrlich's Reagent (analytical grade; Matheson, Coleman & Bell). Concentrated sulfuric acid, 27.4 ml, is slowly added to 200 ml of absolute alcohol in a beaker and the mixture cooled. *p*-Dimethylaminobenzaldehyde,[1] 120 gm, is added to 200 ml of absolute alcohol in another beaker, and then the acid–ethanol mixture is slowly stirred into the first beaker. The solution can be stored in the refrigerator for several weeks; the crystals which precipitate on cooling are redissolved by warming the solution briefly.

Culture Tubes, Screw-Capped with Teflon Liners, 30-ml and 70-ml Capacity (Kimax).

Humin Precipitant. Twenty grams of analytical grade cation-exchange resin (AG 1-X8 and 200–400 mesh, chloride form; California Corp. for Biochemical Research) is mixed with 10 gm of Norit A (Fisher Scien-

[1] Occasionally, it is necessary to recrystallize the reagent from an ethanol solution to reduce absorbancy of the blank.

tific Co.). The mixture is washed several times with 6 *N* hydrochloric acid in a coarse sintered-glass funnel, and then dried to a fine powder with ethanol and ether.

*Hydroxy-*L*-proline (CfP; California Corp. for Biochemical Research).*

L*-Tyrosine (Nutritional Biochemicals Corp.).*

Casamino Acids (Difco Laboratories).

DL*-α-Alanine (Nutritional Biochemicals Corp.).*

METHODS

On the basis of the findings reported below (see *Results*), two procedures for the analysis of hydroxyproline were developed: (I) for relatively pure solutions of hydroxyproline, and (II) for crude samples of tissue, proteins, and urine. Procedure I generally can be applied to amino acid mixtures containing 1% or more of hydroxyproline, but with most samples it is necessary to determine the recovery of added internal standards.

Procedure I. Analysis of Hydroxyproline in Purified Solutions

A sample containing 0.01–0.10 μmole hydroxyproline is made up to 5 ml and the pH adjusted to 8 ± 0.5. Two milliliters of the pyrophosphate buffer is added, and the mixture is oxidized at room temperature with 1.0 ml of the 0.02 *M* chloramine T solution. After 20 min the oxidation is stopped by adding 1 ml of the sodium thiosulfate solution; an excess of sodium chloride is also added. If the presence of interfering materials is suspected (*vide infra*), the sample should be shaken vigorously 100 times with about 5 ml toluene, centrifuged at low speed, and the toluene discarded. The tube is then capped and heated in a boiling water bath for 25 min to form pyrrole. After cooling the tube, 5.0 ml toluene is added, the cap replaced, and the tube shaken vigorously 100 times. The tube is centrifuged briefly at low speed, and a 4.5-ml portion of the toluene phase is placed in a separate tube. Two milliliters of the Ehrlich's reagent is quickly stirred into the toluene, and the resulting color is read after 15 min at 560 mμ on a Beckman model B spectrophotometer.

Procedure II. Analysis of Hydroxyproline in Urine and Other Complex Solutions

A. Hydrolysis. From 0.1 to 1.0 ml urine or protein solution (0.2–4.0 mg/ml) is placed in a tube which can be sealed for autoclaving. The volume is made up to 2 ml with water or internal standard, and 2 ml of concentrated hydrochloric acid is added. A maximum yield is obtained from urine or other peptide solutions by autoclaving at 124° for 3 hr;

tissue homogenates or proteins need to be autoclaved for 15–24 hr. To assay more than 1 ml urine or more than 5 mg protein, the sample is autoclaved in a larger volume of acid and then concentrated in a rotatory vacuum evaporator after hydrolysis.

B. Precipitation of Pigments and Neutralization. The hydrolyzed sample or suitable portion containing 0.10–1.0 μmole of hydroxyproline is placed in a graduated centrifuge tube, and the volume made up to 8 ml. The final hydrochloric acid concentration should be 2–6 *N*. The resin–charcoal preparation is added with a spatula as necessary (up to a volume of 1 ml), and the mixture is stirred and then centrifuged at about 5000 × *g* for 10 min. From 2.0 to 4.0 ml of the clear supernatant layer is placed in a large culture tube, and one drop of a 1% phenolphthalein solution is added. The pH is adjusted to a faint pink color by adding first 10 *N* and then 0.1 *N* potassium hydroxide. The tube is cooled to room temperature.

C. Hydroxyproline Determination. The final volume of the sample is made up to about 8 ml, and the solution is saturated with an excess of potassium chloride. The pH is readjusted as necessary, and 2 ml of borate buffer and 1.0 ml of alanine solution are added. The sample is oxidized at room temperature with 2.0 ml of the 0.2 *M* chloramine T solution, and after 20 min the oxidation is stopped with 6 ml sodium thiosulfate solution. The solution is again saturated with potassium chloride, and about 10 ml toluene is added. The tube is capped and shaken vigorously 100 times. The two phases usually can be separated by centrifuging briefly at low speed, but occasionally it is necessary to break emulsions by agitating gently and recentrifuging. The toluene is discarded, and if the presence of large amounts of interfering materials is suspected, the extraction is repeated. The tube is capped and placed in a boiling water bath for 30 min. After cooling under a running tap, 10.0 ml toluene is added and the tube is shaken vigorously 100 times and centrifuged. Of the clear toluene phase, 5.0 ml is placed in a separate test tube and 2.0 ml of the Ehrlich's reagent stirred in rapidly for the final color reaction.

In measuring the free hydroxyproline in urine and other complex solutions, up to 4 ml of the unhydrolyzed sample is placed in a large culture tube. The pH is adjusted, and the sample is oxidized in the same manner as the hydrolyzate.

RESULTS

Analysis of Hydroxyproline in Pure Solutions (Procedure I)

Although hydrogen peroxide (1), sodium hypochlorite (2), and several other oxidants have been used for the analysis of hydroxyproline, chlor-

amine T was employed because it is available in a stable form and because solutions of reproducible strength can be prepared readily (3).

A. pH Optima for the Analysis. Preliminary experiments with procedure I indicated that optimal yields obtained when the oxidation, incubation in boiling water, and final extraction of pyrrole were performed at pH 8.0–8.5. When solutions were oxidized at pH 7.0 or 9.5, the yields were 10–15% less, but standard curves were linear and reproducible. The incubation and extraction steps could be performed safely

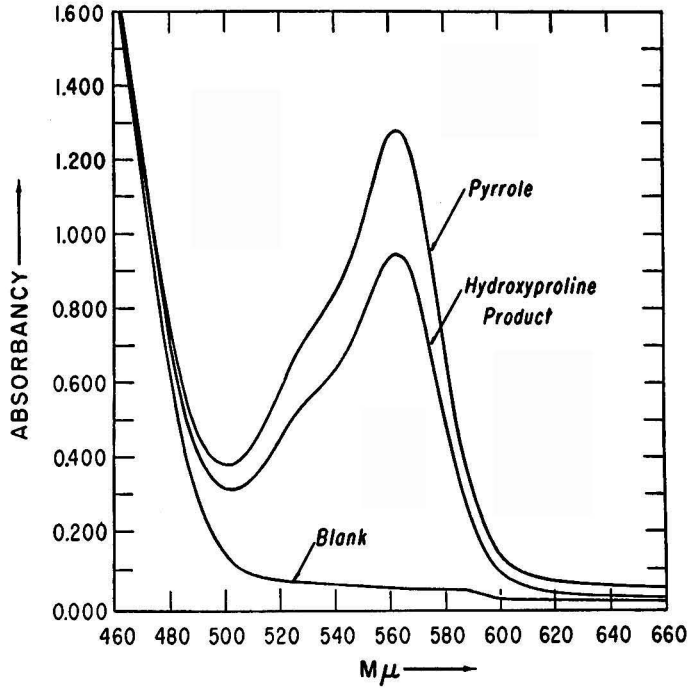

FIG. 2. Spectral absorbancy of chromophore obtained from authentic pyrrole and from the hydroxyproline product in procedure I. All absorbancies were compared to distilled water.

at pH 10, but at pH 12 about 15% of the pyrrole was lost during incubation. When the preliminary extraction was performed at pH 7.0 the final yield of pyrrole was lowered by 5–10%, probably because some of the acid intermediates (pyrrole-2-carboxylic acid and Δ^1-pyrroline-carboxylic acid) were removed by the toluene.

B. Yields of Pyrrole from Pure Solutions of Hydroxyproline. As shown in Fig. 2, the product obtained from hydroxyproline reacted with *p*-dimethylaminobenzaldehyde to give the same chromophore as was obtained with authentic pyrrole. Under the conditions employed, the

color reached maximal intensity 15 min after the addition of *p*-dimethylaminobenzaldehyde, remained constant for 1 hr, and thereafter faded about 1% per hour at room temperature. A concentrated solution of *p*-dimethylaminobenzaldehyde was employed because this improved the sensitivity and proportionality of the color reaction. With analytical grade *p*-dimethylaminobenzaldehyde, reagent blanks yielded an absorbancy of less than 0.050.

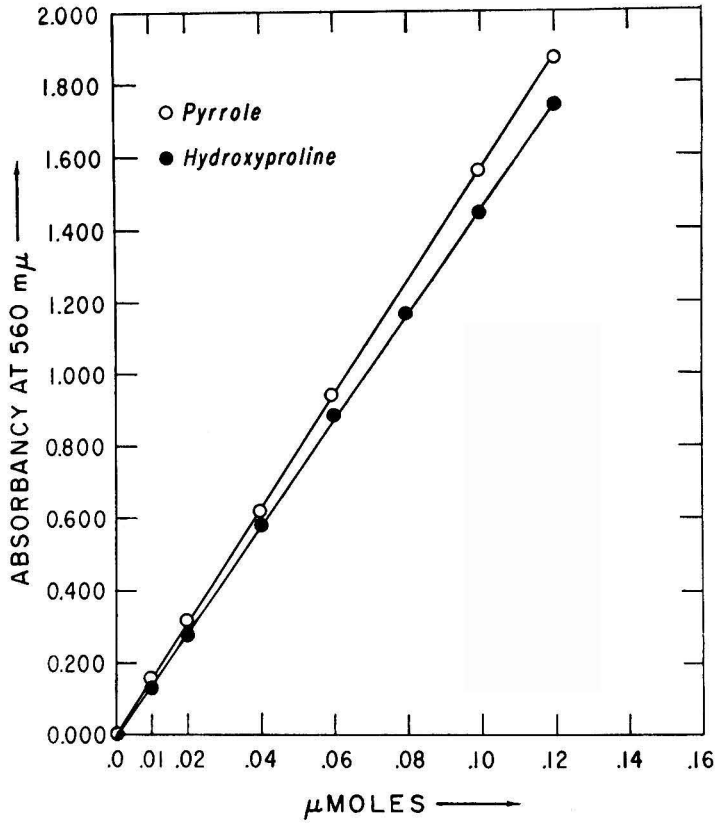

FIG. 3. Molar absorbancy of chromophore of authentic pyrrole and of the hydroxyproline product obtained in procedure I.

With procedure I the method was sensitive to 0.01 μmole (1 μg) hydroxyproline and standard curves were linear over the whole range of the Beckman model B spectrophotometer. Values were reproducible within 1% on successive days and within 4% with the aging of reagents over a period of several weeks. After correction for the 0.5 ml toluene discarded during the final extraction, the over-all yield of pyrrole was 94–97% (Fig. 3).

Analysis of Hydroxyproline in Urine and Amino Acid Mixtures (Procedure II)

As shown in Fig. 4 the concentration of the oxidant is critical to the reaction; excessive amounts reduce the yield of pyrrole, probably because of polymer formation (6), and the presence of other solutes which consume chloramine T drastically reduces the yield of pyrrole. With 2 mg alanine the maximum yield was 65%, and 5–10 times more chloramine T was required than with a pure hydroxyproline solution. The yield with 2 ml of human urine was frequently less, and the optimal amount of oxidant varied widely from one sample to another.

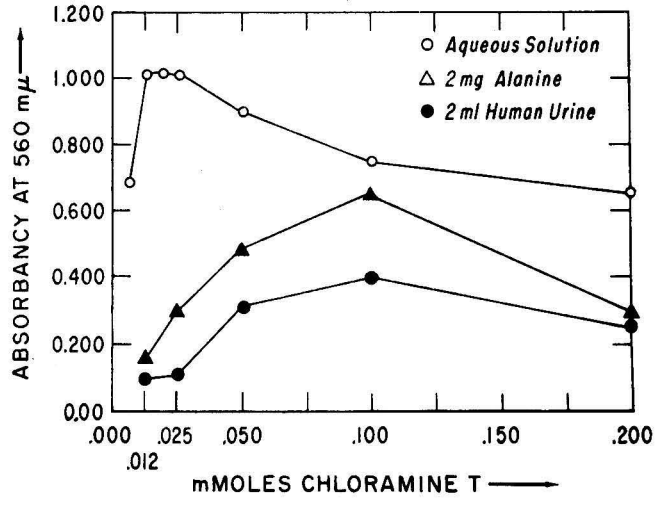

FIG. 4. Effect of varying amounts of chloramine T on the recovery of hydroxyproline from different solutions. Absorbancy of the chromophore from 10 μg hydroxyproline in pure solution (○); net absorbancy from 10 μg hydroxyproline added to 2 mg DL-alanine (△); net absorbancy from 10 μg hydroxyproline added to 2 ml of human urine (●).

A. Stabilizing the Conditions for Oxidation. In procedure II the variable influence of other solutes was overcome by adding a measured excess of alanine and employing stronger oxidizing conditions (Fig. 5). When 100 mg alanine was used to stabilize the oxidation, the conversion to pyrrole was reduced to 57–61%, but the recovery was not appreciably affected by the addition of 50–100 mg more of the amino acid. Standard curves were linear within 1–2%, and values were reproducible within 3% on successive days. Oxidized blanks demonstrated a strong absorbancy at wavelengths greater than 600 mμ, but this did not interfere with

detection of the desired chromophore, and the absorbancy at 560 mμ was less than 0.010 when compared to the color reagent in toluene.

Although hydroxyproline is somewhat more sensitive to oxidation by chloramine T at pH 8.0 than at pH 8.7, the latter pH was employed because a strong unoxidizable pH 8.0 buffer was not available and because the solubility of chloramine T is greater in more alkaline solutions. Methyl Cellosolve was used to enhance the solubility of the reagents, and solutions were neutralized with potassium hydroxide instead of sodium hydroxide for the same reason.

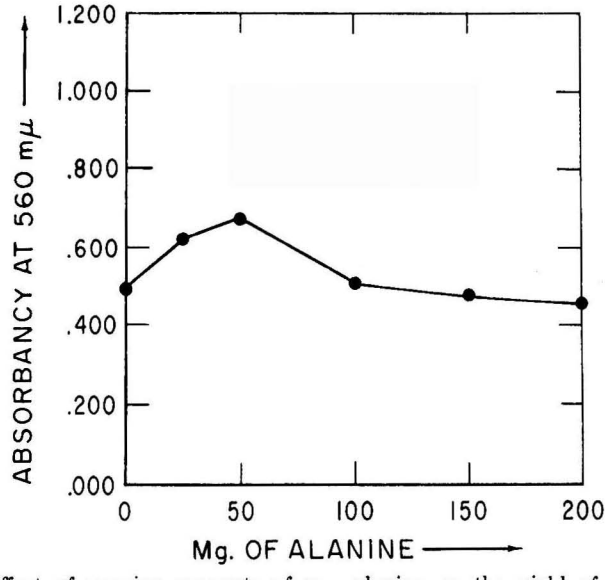

FIG. 5. Effect of varying amounts of DL-α-alanine on the yield of chromophore from 10 μg hydroxyproline in procedure II.

B. Removal of Humin and Other Pigments. Although humin can be removed from acid hydrolyzates with charcoal, it is necessary to neutralize the opaque solutions before adding the charcoal. It occurred to us that the handling of hydrolyzates would be simplified if the pigments were removed directly from the acid media. Empirically it was found that a mixture of charcoal (Norit A) and Dowex 1 exchange resin removed humin as well as other pigments from strong acid solutions. Recoveries of 10 μg hydroxyproline were 98–100% when 2 ml of the mixture was used in 8 ml of 2 *N* to 6 *N* HCl. The mixture was more effective than an equal amount of charcoal in neutralized hydrolyzates, and it separated more readily on centrifuging. After removal of the humin, the hydrolyzates were easily neutralized because an indicator solution could be employed in the clear supernatants.

C. Analysis of Hydroxyproline in the Presence of Other Amino Acids. Previously published methods for hydroxyproline are suitable for analyzing hydroxyproline in gelatin hydrolyzates or in amino acid mixtures containing 1% or more of the imino acid, but the conditions employed are not suitable when larger amounts of other materials are present (Fig. 4). With procedure II recoveries were 90% or better when 25 μg hydroxyproline was analyzed in the presence of 50 mg of an amino acid mixture (Table 1). The sensitivity of the method would allow detection

TABLE 1

RECOVERIES OF HYDROXYPROLINE WITH PROCEDURE II IN PRESENCE OF AMINO ACIDS AND PYRROLE-2-CARBOXYLIC ACID[a]

	Absorbancy$_{560}$	Recovery of 25 μg hydroxyproline (%)
Casein amino acids, 1 mg	0.000	101
Casein amino acids, 2 mg	0.000	99
Casein amino acids, 5 mg	0.010	98
Casein amino acids, 10 mg	0.025	96
Casein amino acids, 20 mg	0.045	93
Casein amino acids, 50 mg	0.045	90
L-Tyrosine, 0.5 mg	0.000	97
L-Tyrosine, 2.5 mg	0.020	96
Pyrrole-2-carboxylic acid, 0.5 mg	0.030	98
Pyrrole-2-carboxylic acid, 2.5 mg	0.112	94
Hydroxyproline, 25 μg	0.750	—

[a] The small absorbancies obtained with casein amino acids (Casamino Acids, Difco), and L-tyrosine did not show the 560-mμ peak characteristic of the pyrrole chromophore.

of less than 5 μg. The small absorbancy obtained with 50 mg of casein hydrolyzate did not show the 560 mμ peak characteristic of pyrrole, and the same was true of the small absorbancy obtained wtih 2.5 mg tyrosine. Pyrrole-2-carboxylic acid (kindly furnished by Dr. Bernhard Witkop, National Institute of Arthritis and Metabolic Diseases) yielded the same chromophore as hydroxyproline, but did not significantly interfere with the assay (Table 1). The aromatic acid is probably overoxidized under the conditions employed (6).

D. Analysis of Hydroxyproline in Human and Rat Urine. As indicated above, recoveries of hydroxyproline from urine are poor largely because other solutes which also undergo oxidation are present (Fig. 4). With the use of procedure II the recovery of hydroxyproline was 87–100% from hydrolyzed human urine (Table 2). Slightly lower recoveries were obtained with hydrolyzed rat urine (Table 2).

E. Analysis of Hydroxyproline in Tissue Homogenates. When 25 μg hydroxyproline was added to a rat liver homogenate, the recoveries

TABLE 2
DETERMINATIONS OF FREE AND TOTAL HYDROXYPROLINE IN HUMAN AND RAT URINE BY PROCEDURE[a]

	Free hydroxyproline		Total hydroxyproline	
	mg/24 hr	Recovery (%)	mg/24 hr	Recovery (%)
Human urine (20 adults)	0.5–1.2	70–92	15–62	87–100
Rat urine (10 young adults)	0.051–0.097	65–96	0.72–1.33	77–92

[a] Recoveries of hydroxyproline were measured by an addition of hydroxy-L-proline approximately equal to the amount initially present. Free hydroxyproline excretions of less than 0.5 mg/24 hr could not be measured in human urine, since only 4 ml was taken for analysis. The human subjects were normal adult volunteers and were on low gelatin diets (less than 3 gm/day); the rats were on gelatin-free diets and were 6-week-old (140–150 gm) Sprague-Dawley males.

ranged from 82 to 95% (Table 3), and the chromophore again showed the typical 560-mμ absorption peak. The value obtained from hydroxyproline indicated a collagen content of 0.2 gm/100 gm tissue (wet weight); this compares well with previously published values (8). Since

TABLE 3
DETERMINATION OF HYDROXYPROLINE IN HYDROLYZED RAT LIVER HOMOGENATE BY PROCEDURE II[a]

Rat liver homogenate (mg)	A_{560}	Recovery of added hydroxyproline 25 μg (%)	Hydroxyproline/100 mg liver (μg)
9.0	0.065	95	25.0
18.0	0.116	92	24.4
45.0	0.285	93	23.7
90.0	0.545	89	23.6
180.0	1.051	82	24.8

[a] Livers of 6-week-old (150 gm) Sprague-Dawley male rats were employed. The weight of homogenate (mg) refers to the amount of initial wet weight of liver present in the final oxidation mixture.

liver contains less collagen than other mammalian organs (8), the method could easily be applied to any other tissue. Analysis of single samples of guinea pig heart and tendon gave recoveries of over 90% for added hydroxyproline.

DISCUSSION

Lang (9) first proposed an analytical method for hydroxyproline involving an oxidation to pyrrole, but the number of modifications published over the past three decades (1–5) attest to the difficulties

encountered with this approach. Since oxidation reactions are notoriously difficult to regulate quantitatively, it is surprising that the conditions for oxidizing hydroxyproline have not received more attention. The use of a second amino acid to standardize the oxidation is a relatively simple solution to the problem, and appears to be adequate for most biological materials. The small blank obtained with alanine does not appreciably reduce the sensitivity of the method, and it should be possible to find substances other than alanine which will act in a comparable way. It is interesting to note that a similar problem in standardizing oxidation conditions has been encountered with another common analytical method, the Sakaguchi reaction for arginine (10). Although we have not investigated the problem, it is likely that the use of a measured excess of a second oxidizable substance would greatly extend the usefulness of that method as well.

The specificity of the present method depends on the ease with which pyrrole-2-carboxylic acid is decarboxylated, and on the solubility of pyrrole in nonpolar solvents. A few other carboxylic acids might decarboxylate under the mild conditions employed, but the results above suggest that little, if any, such materials are present in urine or liver. There is a slight interference with the color reaction when using 50 mg of casein hydrolyzate, but this effect is easily detected by identifying the typical 560-mμ peak of the final chromophore.

The sensitivity (5 μg) of the procedure for hydroxyproline in impure solutions is adequate for most studies, but it can easily be increased several fold by reducing all the volumes employed and reading the final color in smaller cuvettes. Also, a more sensitive method could be developed by extracting the pyrrole from the toluene into a small volume of dilute acid and developing the chromophore in an aqueous medium.

The analysis of protein hydrolyzates was facilitated by the discovery that a mixture of charcoal and ion-exchange resin will remove humin and other pigments from acid solutions. The mixture is more efficient in producing clear supernatants than charcoal alone, and the technique may be useful generally in handling acid hydrolyzates, since many other amino acids are probably not absorbed.

Since proteins containing less than 0.01% hydroxyproline can be analyzed directly, the method could be used for examining the hydroxyproline content of pure proteins. Even with molecular weights of over 300,000 the method should detect one residue of hydroxyproline per molecule of protein. The method should also be applicable to analyzing directly the total hydroxyproline content of tissue cultures, bacteria, and plasma fractions. Because it does not involve any cumbersome procedures, the method can be employed for a large number of samples,

and we have found it particularly useful for measurement of urinary hydroxyproline on a routine basis.

REFERENCES

1. NEUMAN, R. E., AND LOGAN, M. A., *J. Biol. Chem.* **184,** 299 (1950).
2. MITOMA, C., SMITH, T. E., DAVIDSON, J. D., UDENFRIEND, S., DACOSTA, F. M., AND SJOERDSMA, A., *J. Lab. Clin. Med.* **53,** 970 (1959).
3. STEGEMANN, H., *Z. physiol. Chem.* **311,** 41 (1958).
4. GRUMBAUM, B. W., AND GLICK, D., *Arch. Biochem. Biophys.* **65,** 260 (1956).
5. HUTTERER, F., AND SINGER, E. J., *Anal. Chem.* **32,** 556 (1960).
6. CORWIN, A. H., *in* "Heterocyclic Compounds" (R. C. Elderfield, ed.), vol. I, p. 277. Wiley and Sons, New York, 1950.
7. RADHAKRISHNAN, A. N., AND MEISTER, A., *J. Biol. Chem.* **226,** 559 (1957).
8. KAO, K. T., AND MCGAVACK, T. H., *Proc. Soc. Exptl. Biol. Med.* **101,** 65 (1959).
9. LANG, K., *Z. physiol. Chem.* **219,** 148 (1933).
10. BHATTACHARYA, K. R., DATTA, J., AND ROY, D. K., *Arch. Biochem. Biophys.* **84,** 377 (1959).

ANALYTICAL BIOCHEMISTRY, 1, 240–248 (1960)

The Determination of Glyoxylic Acid in Biological Systems[1,2]

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INTRODUCTION

Glyoxylate is a compound of central importance in metabolism. Thus it is rapidly oxidized to formate and carbon dioxide in rat liver slices (1). In at least some higher plants the conversion of glycolate to glycine via glyoxylate seems likely (2). Glyoxylate is an intermediate in the degradation of allantoin by fishes (3) and uric acid by a strain of *Pseudomonas* (4). It may undergo transamination with certain amino acids in plants, animals, and microorganisms and is converted to oxalate under certain conditions (5). Perhaps, however, the glyoxylate cycle has attracted the most attention to the metabolism of glyoxylate (6). As a result of an interest in this metabolic pathway, the analytical method for glyoxylate described herein was developed.

The method described is a quantitative modification of a qualitative test for glyoxylic acid reported by Paget and Desodt (7). Kramer *et al.* (8) have recently reported a similar colorimetric method for glyoxylic acid, but because the procedure described below considerably extends their findings, being especially suitable for biological systems, our results are reported here. The present paper describes the colorimetric determination of glyoxylate as well as a titrimetric method for glyoxylate based on its reaction with sodium sulfite releasing stoichiometric quantities of sodium hydroxide.

[1] Most of the material of this paper was presented to the Biological Chemistry Division of the American Chemical Society, Cleveland, Ohio, April, 1960.

[2] This work was supported by a research grant (No. RG-5904) from the National Institutes of Health, U. S. Public Health Service.

[3] National Defense Education Act Fellow, 1959–60.

MATERIALS AND METHODS

Organic Chemicals

The suppliers of compounds used were Eastman Kodak: D-arabinose, benzaldehyde, *n*-butyraldehyde, *n*-butyric acid (technical; actually used as the sodium salt), fumaric acid, L-malic acid, phenylhydrazine hydrochloride (practical); Mallinkrodt: acetaldehyde, acetone, 37% formaldehyde, glycerol, potassium acetate, trichloroacetic acid; California Corp. for Biochemical Research: *cis*-aconitic acid, glycoaldehyde, *d,l*-isocitric lactone (this was converted to the trisodium salt), α-ketoglutaric acid, oxaloacetic acid, ribose (Cfp); The Matheson Co., Inc: ethyl acetoacetate, glycolic acid, propionic acid; Mann Research Labs.: glucose-6-phosphate (barium), glyceraldehyde, β-hydroxybutyric acid; J. T. Baker: glucose, oxalic acid; Aldrich Chemical Co., Inc.: dihydroxyacetone, 30% pyruvic aldehyde; Nutritional Biochemicals Corp.: sedoheptulosan, sodium pyruvate; U. S. Industrial Chemicals Co.: absolute ethanol; Fisher Scientific Co.: mannose; Merck: succinic acid; Pfizer: potassium citrate; K and K Labs., Inc.: 30% glyoxal.

Glyoxylic acid $\cdot H_2O$ (reagent grade) was obtained from K and K Labs., Inc., and sodium glyoxylate $\cdot H_2O$ was obtained from Sigma Chemical Co.

Titrimetric Procedure

The method used for the titration of glyoxylic acid involved potentiometric titration of the NaOH quantitatively liberated by the reaction of glyoxylate with Na_2SO_3. A similar method for formaldehyde has been discussed by Walker (9). An aliquot portion containing approximately 0.5–1 meq glyoxylic acid (or the salt) was carefully measured into a beaker, and the pH of the solution was adjusted to 9.5 with 0.5 *N* NaOH. Fifty milliliters of 1 *M* Na_2SO_3 which had been adjusted to pH 9.5 was added, and the solution was swirled 1 min and titrated to pH 9.5 with standard 0.1 *N* H_2SO_4. The milliequivalents of acid required are equal to the milliequivalents of glyoxylate in the aliquot portion.

Titrimetric data had an average deviation of about 3 parts/1000. This method proved far more satisfactory than titration to a thymolphthalein fade which occurred at pH 9.2–9.4. The fade was difficult to detect, and an average deviation of 1.3% on six determinations was observed. The titrimetric determination of iodine consumed during the oxidation of bisulfite after decomposition of the bisulfite addition product as described by Clift (10) has proven much less satisfactory in our hands than the method above.

Periodate Oxidative Procedure

The method employed was essentially that of Cantley *et al.* (11) except that a 0.1 *M* acetate buffer of pH 4.8 was used at 28° and the molar ratio of periodate to glyoxylate was about 11. A Warburg respirometer was used to measure CO_2 produced. The average deviation obtained was 0.7%.

Colorimetric Procedure

To 5.0 ml of a solution containing 0.025–0.3 μmole glyoxylate, 1.0 ml of 1 *M* oxalic acid buffer at pH 1 was added followed by 1.0 ml of 1% phenylhydrazine hydrochloride. The solution was heated to boiling and cooled for 5 min. After chilling in an ice bath to approximately room temperature, 4.0 ml concentrated HCl was added followed by 1.0 ml of 5% $K_3Fe(CN)_6$. After thorough mixing, the absorbancy was determined at 7 min (unless otherwise specified) at 520 mμ in a Bausch and Lomb Spectronic 20 colorimeter or in a Beckman DU spectrophotometer using tubes with 1.0-cm light path. All spectral data and molar absorbancy indices (a_M's) were obtained using the latter instrument unless otherwise specified.[4]

All concentrations are defined as final molar concentrations in the 5.0 ml of solution to be tested (see above).

RESULTS

Potentiometric and Titrimetric Methods

Figure 1 presents the results of a typical potentiometric titration of the NaOH released after reaction of glyoxylate with Na_2SO_3. A plot of Δ pH/Δ ml versus ml (see inset, Fig. 1) clearly shows the equivalence point to be between pH 9.5 and 9.6. Glycolaldehyde shows the same equivalence point and this method may therefore prove useful for analytical determinations of glycolaldehyde.

Table 1 shows comparative analytical data for samples of sodium glyoxylate and glyoxylic acid from the same stock in each case. Even though the theoretical and experimental elemental analyses for the acid are in essential agreement and the neutralization equivalent suggests high purity, results of the sulfite and periodate methods, which are in

[4] a_M and absorbancy are equivalent to molar extinction coefficient and optical density, respectively, and are used in accordance with the recommendation of the National Bureau of Standards. See LC 857 "Terminology and Symbols for Use in Ultraviolet, Visible, and Infrared Absorptometry," U. S. Department of Commerce, National Bureau of Standards, Washington, D. C.

good agreement, indicate a purity of only 84%. On the other hand, the experimental carbon and sodium analyses of the salt are in poor agreement with those calculated, while the sulfite and periodate methods suggest high purity.

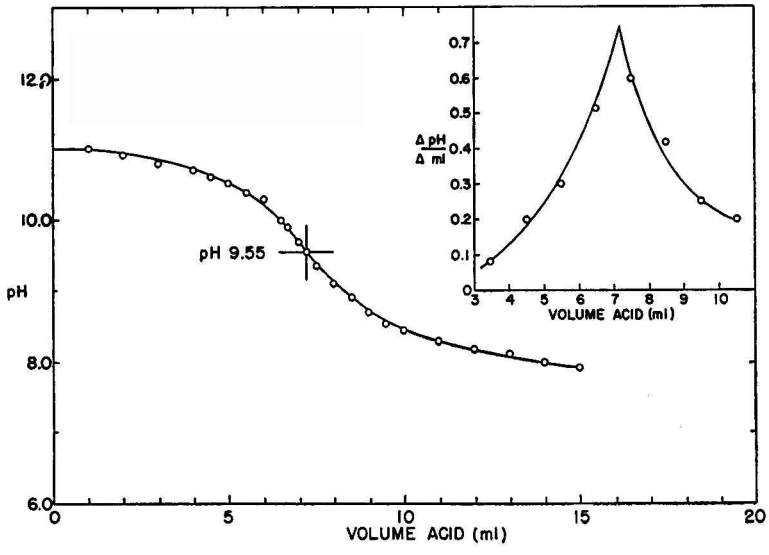

FIG. 1. Potentiometric titration by H_2SO_4 of the NaOH liberated by the reaction of sodium glyoxylate with sodium sulfite.

Because data from the cleavage by periodate substantiated those obtained by the sulfite method, the concentrations defined in reporting the colorimetric data are based upon the sulfite method. For analytical work, routine analysis of glyoxylate or glyoxylic acid reagents by the

TABLE 1
ANALYTICAL DATA

	Elemental analysis[a]		Assay		
	Calculated	Found	Sulfite method (%)	Periodate method (%)	Neutralization equivalent (%)
Glyoxylic acid· H_2O	C: 26.09 H: 4.38	25.83 4.54	84.3	84	102.4
Na glyoxylate· H_2O	C: 21.06 H: 2.65 Na: 20.17	20.53 2.70 19.66	96.6	94	—

[a] Elemental analyses were carried out by Weiler and Strauss Laboratories, Oxford, England.

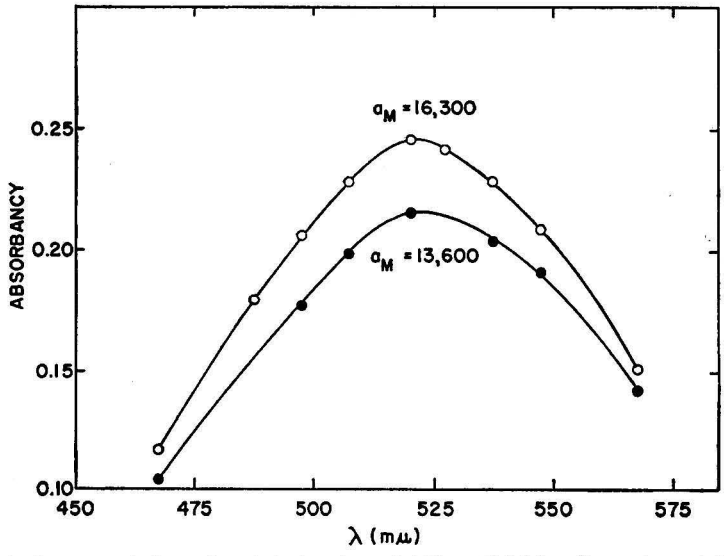

FIG. 2. Spectra of the colored derivative of 1.32×10^{-5} *M* sodium glyoxylate after color development for 7 min ○ and after 1 hr ●.

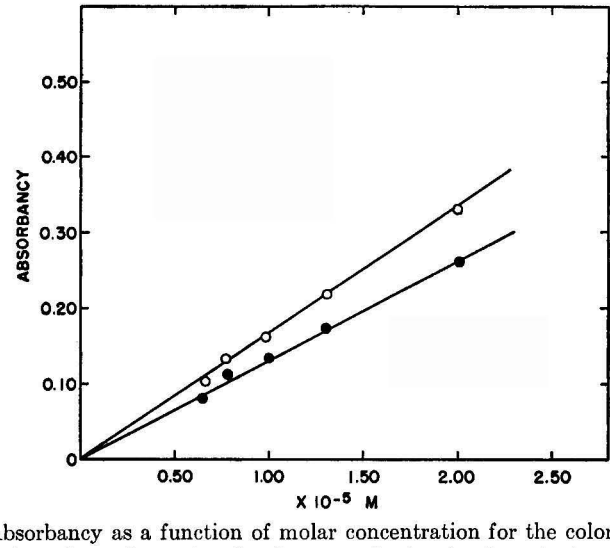

FIG. 3. Absorbancy as a function of molar concentration for the colored derivative of sodium glyoxylate after color development for 7 min ○ and after 1 hr ●.

sulfite method is recommended because of the occasionally incorrect definition of purity of these reagents.

In developing the colorimetric procedure, it was found that more reproducible results could be obtained if oxalic acid buffer was used during the formation of the phenylhydrazone as contrasted to an un-

buffered system. Further, the absorbancy of the buffered system was in general 10% greater than for the unbuffered system. The absorbancy was independent of the concentration of oxalic acid between the limits of 0.01 and 1 M. HCl at equivalent molarities could not be substituted for the buffer. The absorbancy for the reagent blank containing oxalic acid was identical to that without the buffer.

The absorption spectrum of the colored glyoxylate derivative after color development of 7 min and 1 hr reveals an absorption maximum at 520 mμ in both cases (Fig. 2). The absorbancy obeys the Lambert-Beer relationship after both time periods with a_M being 16,300 after 7 min and 13,600 after 1 hr (Fig. 3). Plots of absorbancy versus concentration as determined by the sulfite method for both sodium glyoxylate and glyoxylic acid are superimposable indicating that the colorimetric and sulfite methods principally determine the same compound. This is especially striking when one considers that the impurity (or impurities) in the acid is approximately 15% by the sulfite method.

Noninterfering and Interfering Compounds

A number of compounds do not interfere with the determination of glyoxylate when each is mixed at a concentration of 10^{-3} M with glyoxylate (Table 2). Mixing of glyoxylate with glyceraldehyde or with pyridoxal each at 10^{-3} M or with potassium acetate at 10^{-2} M results in absorbancies 7–9% greater than for glyoxylate alone. Consequently, it

TABLE 2
NONINTERFERING COMPOUNDS[a]

Acetone	Glucose-6-phosphate
cis-Aconitic acid	β-Hydroxybutyric acid
Arabinose	Malic acid
Benzaldehyde	Mannose
n-Butyraldehyde	Potassium acetate
Ethanol	Propionic acid
Ethyl acetoacetate	Ribose
Fumaric acid	Sedoheptulose
Glycerol	Sodium butyrate
Glycine	Succinic acid
Glycolic acid	Trichloroacetic acid[b]
Glucose	Tripotassium citrate
	Trisodium isocitrate

[a] Each of the compounds listed was mixed at 10^{-3} M (unless otherwise specified) with glyoxylate, and the absorbancy after color development for 7 min was compared with that for a glyoxylate control.

[b] At a concentration of 3×10^{-2} M (or 0.5% w/v).

is clear that these compounds would not interfere with the glyoxylate determination unless present in at least 100-fold molar excess. The interference by acetate is presumably due to the oxidation of acetate to glyoxylate by phenylhydrazine as suggested by Paget and Desodt (7), as these workers observed the color reaction for acetic acid (10^{-2} M) which had been highly purified.

Preliminary studies revealed several compounds which might interfere with the quantitative determination of glyoxylate. They are shown in Table 3 with a_M values after color development for 7 min. No attempt

TABLE 3
INTERFERING COMPOUNDS

Compound	M	a_M
Acetaldehyde	10^{-3}	20
Dihydroxyacetone	6×10^{-3}	21
Formaldehyde	10^{-3}	1000
Glycolaldehyde	6×10^{-3}	120
Glyoxal	6×10^{-4}	2300
α-Ketoglutaric acid	6×10^{-3}	14
Oxaloacetic acid	10^{-3}	400
Pyruvic aldehyde	10^{-3}	400[a]
Sodium pyruvate	10^{-3}	630

[a] Determined with a Bausch and Lomb Spectronic 20 colorimeter.

was made to establish whether Lambert-Beer relations obtained for any of these compounds. Assuming that these relations hold, the absorbancies of each compound should be at most 6% of that for an equivalent molarity of glyoxylate with the exception of glyoxal. The a_M of oxaloacetic acid is tentative at best because the absorbancy maximizes and decreases rapidly prior to 7 min after which it is considerably more constant. It is likely that oxalacetic acid undergoes extensive decarboxylation under the conditions of color development. Furthermore, when the reaction mixture is not thoroughly cooled prior to the addition of ferricyanide and hydrochloric acid, the value of $a_M{}^{7\ \mathrm{min}}/a_M{}^{60\ \mathrm{min}}$ for 10^{-3} M oxalacetic acid and 10^{-3} M pyruvate is respectively 1.5 and 2.5 times as great as for glyoxylate alone which has approximately the same $a_M{}^{7\ \mathrm{min}}/a_M{}^{60\ \mathrm{min}}$ as for solutions which have been chilled. This may reflect decarboxylation of pyruvic acid and oxalacetic acid and seems worthy of further exploration as a means of diminishing interference by these compounds when in considerable excess of glyoxylate.

The a_M values for glyoxal and formaldehyde are not in agreement with those reported by Kramer *et al.* for similar concentrations of these compounds (10).

DISCUSSION

Adequate criteria for purity of glyoxylic acid and sodium glyoxylate are difficult to define. For example, sodium glyoxylate monohydrate contaminated with small amounts of monosodium oxalate and sodium glycolate monohydrate would give the expected elemental analysis and equivalent weight. The same is true of the acid monohydrate and the analogous contaminants. It has been established that a commercial reagent-grade glyoxylic acid monohydrate, though seemingly pure by the criteria of elemental analysis and neutralization equivalent, in fact contains 16% contaminating material by two other criteria, i.e., the titrimetric and periodate oxidative methods described. Thus for analytical work, the purity of glyoxylate preparations should be established by at least two independent methods. Elemental analysis is at best a qualitative indication of the degree of purity.

The titrimetric procedure described seems as reliable as any yet reported for the estimation of the purity of glyoxylate samples. The procedure is short and provides a rapid estimation of purity of the stock reagent. Once this has been made, the colorimetric procedure described affords a rapid, highly sensitive, and highly specific determination of glyoxylate solutions. The colorimetric methods of Friedemann and Haugen (12) and Metzler and Snell (13) require extraction procedures and are far less specific than the method described here unless lengthy extraction procedures are employed to separate the phenylhydrazones of keto acids from that of glyoxylate. The method of Olson (14) is limited to measurements in the ultraviolet region and affords an a_M for glyoxylate of about 75% that of the method reported here. Additionally it is rather nonspecific in that of four compounds studied in addition to glyoxylate, the semicarbazones of two, pyruvate and α-ketoglutarate, have a_M's and absorption maxima which are very similar to those for the semicarbazone of glyoxylate.

The colorimetric procedure described is particularly suitable for biological systems. For example, it can be used when trichloroacetic acid has been used as a deproteinizing agent. It has been used in this laboratory repeatedly for assays for isocitritase and malate synthetase. Added sodium glyoxylate has been quantitatively recovered from boiled cell-free preparations of *Pseudomonas indigofera* during malate synthetase assays. When substances are found to interfere extensively with the colorimetric method, the separation of glyoxylate on a Dowex 1 acetate column as described by Zelitch (15) should prove effective. However, of all of the carbonyl compounds examined as possible interfering substances including representative sugars and other metabolites, only

glyoxal forms a colored derivative which absorbs substantially at the same wavelength as that for glyoxylate. Glyoxal is a compound of dubious physiological significance, at least at present. The procedure should be applicable to the study of transamination in either model systems or enzymic systems for at least some, if not all, glycine-α-keto acid combinations.

SUMMARY

A method is described for the determination of glyoxylate (or the acid) which involves potentiometric titration of the NaOH produced as a result of the formation of the sulfite addition compound. Results are in good agreement with those from a less precise assay involving periodate oxidation. Using the sulfite method to determine the concentration of glyoxylate in stock solutions, a highly specific colorimetric procedure is described for the assay of more dilute solutions. Glyoxylate has an a_M of 16,300. A diversity of compounds of biological interest, including carbonyl substances, does not interfere with the determination. Of the compounds tested, only glyoxal should interfere substantially at molar concentrations equivalent to glyoxylate.

REFERENCES

1. NAKADA, H. I., FRIEDMANN, B., AND WEINHOUSE, S., *J. Biol. Chem.* **216,** 583 (1955).
2. TOLBERT, N. E., AND COHAN, M. S., *J. Biol. Chem.* **204,** 649 (1953).
3. LASKOWSKI, M., *in* "The Enzymes" (J. B. Sumner and K. Myrback, eds.), p. 946. Academic Press, New York, 1951.
4. CAMPBELL, L. L., *Biochim. et Biophys. Acta* **18,** 160 (1955).
5. MEISTER, A., *in* "Biochemistry of the Amino Acids," pp. 181, 267. Academic Press, New York, 1957.
6. KORNBERG, H. L., AND KREBS, H. A., *Nature* **179,** 988 (1957).
7. PAGET, M., AND DESODT, J., *Ann. pharm. franc.* **7,** 422 (1949).
8. KRAMER, D. N., KLEIN, N., AND BASELICE, R. A., *Anal. Chem.* **31,** 250 (1959).
9. WALKER, J. F., *in* "Formaldehyde," 2nd Ed., p. 382. Reinhold Publ. Corp., New York, 1953.
10. CLIFT, F. P., AND COOK, R. P., *Biochem. J.* **26,** 1788 (1932).
11. CANTLEY, M., HOUGH, L., AND PITTET, A. O., *Chem. & Ind.* (London) **1959,** 1126.
12. FRIEDMANN, T. E., AND HAUGEN, G. E., *J. Biol. Chem.* **147,** 415 (1943).
13. METZLER, D. E., AND SNELL, E. E., *J. Am. Chem. Soc.* **74,** 979 (1952).
14. OLSON, J. A., *Arch. Biochem. Biophys.* **85,** 225 (1959).
15. ZELITCH, I., *J. Biol. Chem.* **233,** 1299 (1958).

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Procedures for the Analysis of Purines and Pyrimidines by Elution from Columns of Sulfonated Polystyrene with Buffers of pH 4[1]

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INTRODUCTION

The separation of purines and pyrimidines on columns of Dowex 50 using 2 *N* HCl as eluant, one of the numerous chromatographic procedures contributed by Cohn, has proved extremely useful for many purposes [see Fig. 2 in Ref. (1)]. In this eluant, portions of the uracil and thymine probably bear positive charges, because of protonization of the ring, and for the same reason portions of the guanine and adenine may be doubly charged. Accordingly, a large volume of eluant is required to elute all of the constituents from the sulfonated polystyrene, and the emerging peaks are broad and unsuitable for the direct quantitative estimation of microgram quantities of the bases. Columns of smaller diameter would improve the situation from the viewpoint of sensitivity, but they present the general disadvantages of increased tendency to non-laminar flow, of inconvenience in administering the sample and the eluant, of fragility, and of loss in versatility imposed by diminishing the capacity of any given column. The value of pH 4 was chosen for exploratory experiments using Dowex 50 because the p*K* values of the amino groups of the bases most frequently encountered in nucleic acids lie between pH 3.2 and pH 4.7. Initially, the bases were eluted by Na citrate buffers (2), but elution with ammonium formate buffers of pH 4 also proved satisfactory.

Many of the principles utilized in designing the present chroma-

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tographic procedures are based on the experiences of Moore and Stein in their experiments on the analyses of amino acids on columns of sulfonated polystyrene (3, 4). Screened resin of uniform particle size has been used in order to provide sharp peaks at reasonable rates of flow. By applying an eluant of gradually increasing ionic strength, the bases are separated in a single run, thereby avoiding the inconvenience and the uncertainties with respect to base line caused by abrupt changes in the eluant. The use of Dowex 50 beads permits regeneration of the resin directly in the columns in spite of the eluant of increasing concentration applied. The use of strong buffers under conditions where the bases are not highly retarded provides great sensitivity, since the bases are eluted as sharp peaks, confined to small volumes. Resolution depends upon the use of long columns, upon manipulation of the rate of increase of the ionic strength of the eluant, and upon precise control of the temperature and pH.

PROCEDURES

Pretreatment of the Resin

The ammonium formate system will be emphasized for purposes of illustration in this communication because it offers the additional advantage of facilitating the recovery of the bases in a form suitable for measurement of specific radioactivity. About 500 gm of Dowex 50-X4, —400 mesh (obtained from the Dow Chemical Company, Midland, Michigan), was converted to the sodium form, and the fines were removed by repeated settling from 5 l of distilled water for periods of 20–30 min. After 5–10 decantations, when the final supernatant fluid was essentially free from fines, the settled resin was suspended in an equal volume of water and passed through a 200-mesh sieve with the aid of a sharp stream of tap water and collected as prescribed by Moore and Stein (4). For use with Na citrate buffers, the Dowex 50-X4 was at this point screened a second time using a 325-mesh sieve in order to remove finer particles. The "through 200" resin (about 50% in the case of the -X4 resin, and about 10% in the case of the -X2 material, prepared from 200–400-mesh resin) was suspended in 4 l of distilled water, and sufficient NaOH pellets were cautiously added with vigorous mechanical stirring to bring the mixture to 4 N in Na^+. The stirring was continued for 1 hr, after which the resin was allowed to settle for 30 min. The supernatant fluid was decanted and the resin was washed twice by resuspension and decantation using 4 l of distilled water. The resin was then washed successively on a large Büchner funnel under gravity with 2-l

portions of water, 4 *N* HCl, water, 4 *N* NaOH, water,[5] 4 *N* HCl, water, acetone, and water. Finally, the resin was converted to the ammonium form by passing through the resin bed 6 vol of 4 *N* NH_4OH, water, 1 *M* ammonium formate of pH 8,[6] and "starting buffer" (0.2 *N* ammonium formate of pH 4).[7] After the resin was freed of excess buffer by suction, it was suspended in enough of the starting buffer to provide a 1:1 slurry. The slurry was used to pour 0.9 × 60 cm columns (four 15-cm segments) essentially as described in Ref. (4). The yield of "through 200" resin obtained from 500 gm of the original -X4 resin sufficed for the preparation of about six columns.

Operation of the Columns

In general, the columns are operated with the aid of accessory devices (condenser jackets, reservoirs, mixing chambers, stoppers, delivery tubes, air-pressure regulators, etc.) such as have been described by Moore and Stein (3, 4). A roll of silver gauze terminating in a pointed tip (5) is inserted in the bottom of the column in order to promote a regular flow of the effluent. The column is then washed out[8] at room temperature with at least 120 ml of 1 *N* ammonium formate of pH 8, 90 ml of 1 *N* ammonium formate of pH 4,[9] and finally with 90 ml of starting buffer at the temperature and the rate of flow at which the column will subsequently be operated. The latter detail is important since the base line is markedly affected by the rate of flow and the temperature. The

[5] For use in the Na form, the resin was at this point equilibrated with, and suspended in, 0.2 *N* Na citrate, pH 5, and used directly to pour columns.

[6] The 1 *N* ammonium formate of pH 8 is prepared by cautiously titrating one mole of aqueous ammonia (68.3 ml of a 29% solution of ammonia) with 90% formic acid (about 40 ml) and diluting the mixture to 1 l with distilled water. The pH of this solution is not critical.

[7] The 0.2 *N* ammonium formate of pH 4 is prepared by diluting the 1 *N* ammonium formate of pH 4 prepared as described in footnote 9. No attempt is made to compensate for the very slight change in the pH of the ammonium formate solution that may occur on dilution.

[8] When operated with Na buffers, the columns of Dowex 50 are washed out before use with about 75 ml of 0.2 *N* NaOH and 100 ml of 0.2 *N* Na citrate, pH 4. (The 0.2 *N* Na citrate is prepared from a 1 *N* solution, which, in turn, is prepared by diluting to 1 l a mixture of about 800 ml water and 105 gm citric acid, in which 40 gm NaOH and 22.8 ml of 12 *N* HCl has been dissolved.)

[9] The 1 *N* ammonium formate of pH 4 is prepared by cautiously adding 55 ml of 90% formic acid to a mixture of one mole of ammonia (68.3 ml of a 29% solution of ammonia) and 800 ml of distilled water. After the mixture has returned to room temperature, the pH is carefully adjusted to pH 4.0 by the further addition of 8–12 ml of the formic acid, and the volume is brought to 1 l.

buffers should be boiled and cooled before use in order to prevent the development of air bubbles in the resin. After being prepared as described below, the sample (1–4 ml)[10] is washed quantitatively into the column using three successive 0.2-ml portions of starting buffer. The sample is added at a rate of about 4 ml/hr. Starting buffer is then added to the top of the column. In general, a rate of flow of 5–9 ml/hr is maintained throughout the run as well as before the addition of the sample. It is not advisable deliberately to change the rate during an analysis, since this may produce an abrupt change in the base line.

Collection of Fractions

Fractions of 2–4 ml are collected in 150 × 18 mm test tubes. Because the quantitative estimation by means of ultraviolet absorption depends upon the volume of the fractions, recalibrated hematocrit tubes (Kimax 46752) are used to collect every fifth fraction. The volumes of these fractions are recorded at the time the absorbancy is determined. The collection tubes should be stoppered[11] and refrigerated if there occurs an appreciable delay before the measurement of ultraviolet absorption.

Measurement of Ultraviolet Absorption

Aliquots of the fractions are successively transferred from the collection tubes to quartz absorption cells having a 1-cm light path. The use of absorption cells of 0.7 ml capacity[12] permits smaller aliquots of the fractions to be read. The absorbancy of each fraction is determined at the desired wavelength(s) versus distilled water using an ultraviolet spectrophotometer. Direct determination of the amounts of bases present in the individual effluent fractions is regarded as preferable to pooling the fractions or to adjusting the effluent to an extreme value of pH with NaOH or HCl, because such manipulations would detract from the over-all precision and sensitivity of the procedures. When the samples contain about 60 μg of each principal base (as, for example, when

[10] Smaller sample volumes will provide sharper peaks. Samples of somewhat larger volumes could probably be tolerated if the ionic strength and the pH of the sample were adjusted to values sufficiently low to cause strong retention of guanine, cytosine, adenine, and 5-methylcytosine. However, uracil and thymine are so poorly retarded (except perhaps at values of pH where the resin exists in the H^+ form) that their peaks would probably broaden if the sample size were increased unduly.

[11] The polyethylene caps supplied with No. T 17100 disposable tubes, 17 mm × 100 mm (Falcon Plastic Products, Culver City, California) have proved very satisfactory for this purpose. These caps contribute negligible ultraviolet absorption to the contents of the tubes which they are used to cover.

[12] Pyrocell No. UVS504.310, Pyrocell Manufacturing Co., 207–11 East 84th Street, New York 28, N. Y.

TABLE 1

ULTRAVIOLET ABSORPTION DATA FOR SOME PURINES AND PYRIMIDINES

Base	Wavelength (mμ)	$E_{1\text{ cm}}^{0.001\%}$ In 0.1 N HCl		$E_{1\text{ cm}}^{0.001\%}$ In Na citrate,[b] pH 4	Total $A_{m\mu}$ units recovered/10 μg base[c] Na citrate, Dowex 50		Ammonium formate, Dowex 50	
		Found[a]	Literature (10)		−X2	−X4	−X4	−X4
Uracil	260	0.723	0.705	0.696	0.712 (7)		0.697	
	265						0.635	
	275				0.318 (4)			
	280				0.140 (3)		0.132	0.132
Thymine	260			0.602	0.590 (7)		0.593	
	265	0.623	0.63				0.626	
	275			0.487	0.483 (4)			
	280			0.334	0.324 (3)			
	285						0.185	0.185
Guanine	250	0.742	0.73	0.698	0.675 (2)	0.668 (2)	0.659	
	260			0.497	0.467 (5)	0.464 (3)	0.465	
	285				0.377 (1)	0.368 (2)		
	290						0.250	0.252
Cytosine	260			0.561	0.541 (5)	0.553 (2)	0.537	
	275	0.940	0.95	0.876	0.850 (2)	0.845 (2)	0.847	
	290				0.405 (1)	0.413 (2)	0.406	0.408
Adenine	260	0.966	0.96	0.975	0.960 (4)	0.935 (2)	0.925	
	280						0.296	0.296
5-Methylcytosine	260			0.290	0.278 (1)	0.285 (1)	0.257	
	280				0.687 (1)		0.676	
	283	0.731	0.785					
	290				0.614 (1)	0.627 (2)	0.603	
	295							0.467

[a] These data refer to averages of measurements of dilutions of aliquots of one set of the same solutions that was used to obtain the values for total $A_{m\mu}$ units.

[b] For these measurements, the normalities of the Na citrate solutions were adjusted approximately to those estimated to obtain in the effluent containing the corresponding bases after chromatographic analysis by use of the Dowex 50 −X2 system.

[c] With the exception of the last column, which is based on a single experiment in which 300 μg of each base was chromatographed, the values refer to averages found in replicate experiments using samples of 60–120 μg of the bases. The number of replicate experiments (agreement among which was always ±5%) is indicated in parentheses, except in the next to last column, where the number of replicates was 4 throughout.

hydrolyzates of 1 mg nucleate are analyzed), all of the fractions are read at 260 mμ. To achieve greater sensitivity, the fractions are regularly read also at 265 mμ in the region of uracil and thymine, at 250 mμ in the region of guanine, and at both 275 and 280 (or 290) in the region

of cytosine, adenine, and 5-methylcytosine. It is essential that an adequate number of fractions be read both before and after the peaks at the appropriate wavelengths in order reliably to establish the base lines. If the samples contain considerably more than 60 μg of each base, it is necessary to measure the absorbancy of the fractions at wavelengths farther from the maxima.[13] For example, determinations at the wavelengths indicated in the last column of Table 1 were found satisfactory for the analyses of samples containing about 300 μg of each base, since the absorbancy at these wavelengths did not greatly exceed 1.0. The actual choice of wavelength will be dictated by the sharpness of the peaks obtained, which in turn, will depend upon a variety of factors. Therefore, the data in Table 1, particularly with respect to the wavelengths used in the last column, should be regarded merely as a guide to be followed for standardizing the procedures under a particular set of laboratory conditions.

Factors for Converting Absorbancy Units to Micrograms of Bases

The effluent curves at appropriate wavelengths are plotted as shown in Figs. 1–3. If the peaks are not symmetrical, the fractions should be reread promptly in order to determine whether an error has been made in reading or in recording the absorbancy.

Base lines are drawn under each peak by extrapolations from the base lines on either side of the peaks. The absorbancy of the base line at the midpoint of the peak is regarded as the average blank. The average blank is multiplied by the number of fractions comprising the peak, and the product so obtained is subtracted from the sum of the absorbancies throughout the peak at the wavelength in question. The resulting figure, Total $A_{m\mu}$ less total base line, is then multiplied by the average volume of the fractions throughout the peak, thereby giving Total $A_{m\mu}$ units. The average $A_{m\mu}$ units found per 10 μg base for 14 standardization experiments are summarized in Table 1, which is presented in its entirety in view of the fact that spectral data for purines and pyrimidines in buffers of pH 4 are not commonly available. The Total $A_{m\mu}$ units corresponding to an unknown quantity of base is calculated in the same way, and is then converted directly to micrograms of base by means of the factor previously obtained using the standard quantity of base.

Regeneration of the Columns

At the conclusion of a run, the resin in the column is carefully inspected for air bubbles. If no dry spots are visible, the column may be

[13] This procedure is regarded as more accurate and convenient than the alternative possibilities of preparing dilutions of the fractions or transferring the fractions to absorption cells having a shorter light path.

reused directly after being washed out with 1 *N* ammonium formate of pH 8, and of pH 4, and, finally, with starting buffer (or with 0.2 *N* NaOH and 0.2 *N* Na citrate, pH 4) as described above. Otherwise, the resin must be extruded from the column, washed on a Büchner funnel, and used to pour a new column. The characteristics of the columns show no significant change after repeated use of the resin, except, perhaps, a gradually decreasing base line.

Preparation of Samples for Chromatography

Hydrolysis by excess formic acid, as recommended by Wyatt and Cohen (6) has proved extremely satisfactory for routine base analyses of highly purified specimens of sodium deoxyribonucleate. Using 1 ml of 90% formic acid/mg nucleate, the hydrolysis is conducted during 30 min in an evacuated, sealed, heavy-walled Pyrex tube, heated in a bath of silicone oil, which in turn is supported in a bath of boiling *p*-dichlorobenzene [see footnote 2 in Ref. (7)]. After cooling and rinsing with acetone, the tube is centrifuged in order to sediment condensate from the top, and the pressure within the tube is cautiously released by applying a hot oxygen flame to the upper tip of the tube. The formic acid may be removed conveniently by placing the opened tube in an evacuated desiccator over pellets of NaOH. To prepare perchloric acid hydrolyzates, the procedure of Wyatt (8) is followed, except that the hydrolysis is conducted in sealed, evacuated tubes.[14] For the analysis of purines alone, hydrolysis by sulfuric acid is performed in evacuated sealed tubes by the procedure of Vischer and Chargaff (11). The hydrolysis residues are stirred thoroughly and extracted with warm (about 50°C) 1 *N* H_2SO_4 (0.4 ml/mg nucleate) and, if necessary, clarified by centrifugation. Aliquots of the supernatant fluid are pipetted for total P estimation, performed by the method of King (9), on triplicate 0.1-ml aliquots which contain about 20 μg P. For the analysis of the bases, 0.3–0.4-ml aliquots of the solution, corresponding to about 50–90 μg of the principal bases, are diluted to 2–4 ml with water or starting buffer.

[14] Although they are regularly examined in order to verify the absence of uracil from specimens of deoxyribonucleate, perchloric acid hydrolyzates have not been used for routine analyses because of their failure to provide reasonable P analyses. As a rule, 110 to 130 moles of total bases are found per 100 moles of P in extracts of perchloric acid hydrolyzates prepared and analyzed as described in the text, while 100 to 110 moles of total bases per 100 moles of P are usually found for formic acid hydrolyzates. The discrepancy probably results from adsorption of P by the charcoal arising during hydrolysis (10), and this effect may be more pronounced when the perchloric acid hydrolysis is conducted in sealed, evacuated tubes. The difficulty could probably be remedied by pipetting aliquots of a suspension of the hydrolyzate. Although the charcoal would not interfere with the P estimation, or with paper chromatographic analysis, it is undesirable to introduce extraneous, insoluble material into the resin.

In order to standardize the procedures (to obtain factors for Total $A_{m\mu}$ units per 10 μg base such as are shown in Table 1), specimens of the bases are dried (3 hr, 100°C, P_2O_5, 0.1 mm Hg) and weighed by difference into volumetric flasks which are brought to volume with 0.1 N HCl (or, 1.0 N HCl in the case of guanine). Specimens of bases obtained from commercial sources[15] have been used without recrystallization since they gave in 0.1 N HCl absorption spectra and $E^{0.001\%}_{1\text{ cm}}$ values that agreed with values in the literature, with the possible exception of 5-methylcytosine (see Table 1). In general, 25 ml of solutions containing about 15 mg (roughly 0.1 mmole) of base are prepared. From these stock solutions, aliquots of suitable size (0.05–0.5 ml) are withdrawn and combined as desired. The mixtures of bases are diluted with starting buffer, or with water, to volumes of 1–4 ml before addition to the columns.

RESULTS AND DISCUSSION

In Fig. 1 is shown a placement chromatogram obtained with Dowex 50-X2 using as eluant a solution of Na citrate of gradually increasing normality but of essentially constant pH. In a previous experiment using an eluant of constant composition (0.2 N Na citrate, pH 4.25) with a 0.9 $\times$ 50 cm column of "through 120" resin, adenine and cytosine failed to emerge even after 400 effluent milliliters. The latter two bases were, however, eluted abruptly in a single sharp peak when, at this point, the eluant was changed to 0.2 N Na citrate, pH 5.4. In another experiment, when the initial eluant of 0.2 N, pH 4.25 was followed after 76 ml by 0.2 N Na citrate of gradually increasing pH but constant normality (produced by adding 0.2 N, pH 5.25 to a 125-ml mixing chamber initially filled with 0.2 N, pH 4.25), guanine moved to 150 ml, while adenine and cytosine emerged at 320 ml, again unresolved. Subsequently, the use of an eluant of increasing concentration was found to produce the separation shown. Since uracil and thymine bear no charge at pH 4, the first two bases may be resolved because of interaction with the resin through secondary valence forces involving the methyl group of thymine. Operation of the columns at a higher temperature causes both of these bases to be eluted slightly earlier, but affects thymine more than uracil, thereby impairing their separation. To test the sensitivity to pH of the system shown in Fig. 1, experiments were performed using Na citrate buffers of pH 3.8, 4.0, and 4.2. At the lower pH all components were somewhat more retarded than at the higher pH, but the separations remained essentially as shown in Fig. 1. Consequently, pH 4

[15] The bases were obtained from California Corporation for Biochemical Research, Los Angeles 63, Calif., or from Francis Earle Biochemicals, Peekskill, N. Y.

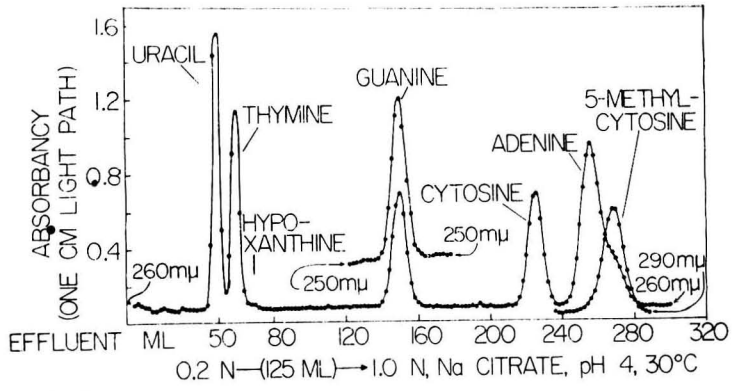

FIG. 1. Elution of purines and pyrimidines from a 0.9 × 60 cm column of Dowex 50-X2 (Na form "through 200" mesh). The eluant consisted of a solution of gradually increasing molarity, produced by adding 1 *N* Na citrate to a 125-ml mixing chamber (4) initially filled with 0.2 *N* Na citrate. Fractions of 2 ml were collected. Each principal peak contained 100–125 μg of base.

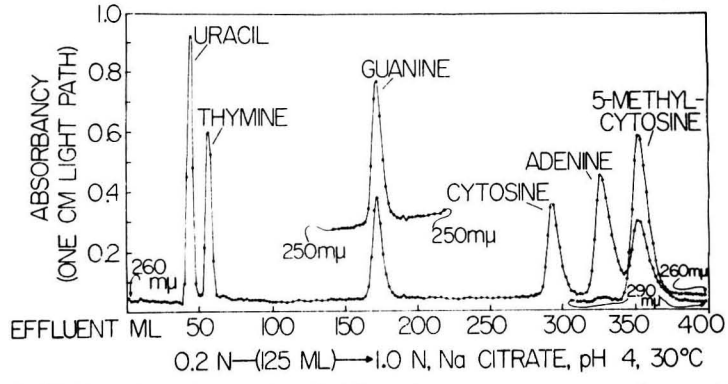

FIG. 2. Elution of purines and pyrimidines from a 0.9 × 62 cm column of Dowex 50-X4 (Na form "through 200" mesh, not "through 325" mesh). The Na citrate eluant was produced as for Fig. 1. Fractions of 2 ml were collected.

was adopted to obtain the standardization data in Table 1 and for the analysis of unknown mixtures. Increasing the size of the mixing chamber from 125 to 250 ml does not greatly improve the poor separation between adenine and 5-methylcytosine. For purposes of estimation, the different absorption spectra of these two bases compensate for the overlap. The trace of hypoxanthine shown in Fig. 1 is derived as a contaminant from one of the bases. When added, hypoxanthine is completely separated from thymine, but xanthine emerges together with thymine. The deoxyribonucleosides of thymine, guanine, cytosine, and adenine emerge in the same order as their parent bases, but deoxyadenosine and deoxycyti-

dine overlap slightly. Tyrosine and phenylalanine are eluted together at 86 ml, well after thymine, while tryptophan emerges at about 275 ml.

Although Dowex 50-X4 was tested because the commercial product provides good yields of "through 200" mesh particles, Fig. 2 shows that the higher cross-linkage greatly improves the separation of 5-methylcytosine and adenine. All constituents, with the exception of uracil and thymine, are somewhat more retarded than when chromatographed on the -X2 resin under the same conditions. This may result partly from the presence, in a column of identical dimensions, of less water and more resin when the degree of cross-linkage is increased and the particle size decreased, in spite of the fact that the exchange capacities of the two resins are about equal on a dry-weight basis. However, the structure of the resin is probably a more important factor. Strictly from the standpoint of the pK's of their amino groups, 4.1 and 4.5, respectively, adenine should be eluted before cytosine. That the reverse occurs reflects the generally greater affinity of purines than of pyrimidines for polystyrene resins, an effect which was originally described by Cohn [see Ref. (1)]. The improved separations in Fig. 2 compared with Fig. 1 probably depend upon enhanced secondary valence interaction. A similar effect of cross-linkage on the behavior of nucleotides on polystyrene resins (Dowex 1-X2 and -X8) has been reported by Sinsheimer (12). The temperature sensitivity of the separations supports this conclusion, since a chromatogram run at 50°C, but otherwise identical to that illustrated in Fig. 2, caused all of the bases to be eluted earlier. However, the effect on adenine was particularly great, causing it to emerge together with cytosine in a single peak.[16] When an experiment was performed with the ammonium form of Dowex 50-X4, and Na citrate was replaced by

[16] The behavior of purines and pyrimidines on columns of Amberlite IR-120, XE-69, has further emphasized the striking influence of resin structure. This sulfonated polystyrene resin is nominally 8% cross-linked, and, on a dry-weight basis, it has about the same exchange capacity as Dowex 50. However, particles of the Amberlite product (available from Rohm & Haas Company, Resinous Products Division, Philadelphia 5, Pa.) are manufactured by crushing and consequently they may be expected to possess surface characteristics different from the beads of Dowex 50. When the elution system used in Figs. 1 and 2 was used at 30°C with a 0.9 × 58 cm column of the XE-69 resin, "through 325" mesh, guanine did not emerge until 300 effluent milliliters. Replacing 0.2 N Na citrate by 0.4 N Na citrate in the elution system did not appreciably affect the position of guanine, and cytosine and adenine emerged as broad peaks at 550 and 620 ml, respectively. When, in addition, the 1 N Na citrate was replaced by 2 N Na citrate, pH 3.7, thymine emerged slightly before xanthine (a separation not achieved by use of Dowex 50), guanine was placed at 260 ml, cytosine at 370 ml, and adenine plus 5-methylcytosine at 445 ml. Use at 50°C of this same elution system moved guanine up to 160 ml, and cytosine and adenine to 270 and 305 ml, respectively.

ammonium acetate under conditions otherwise identical to those shown in Fig. 2, similar separations were obtained and all constituents were found to emerge slightly later. However, use of ammonium formate of pH 4, under the same conditions provided guanine at 210 ml and cytosine at 321 ml, but adenine and 5-methylcytosine emerged in a single symmetrical peak at 370 ml. Thus, by the use of ammonium formate, elution

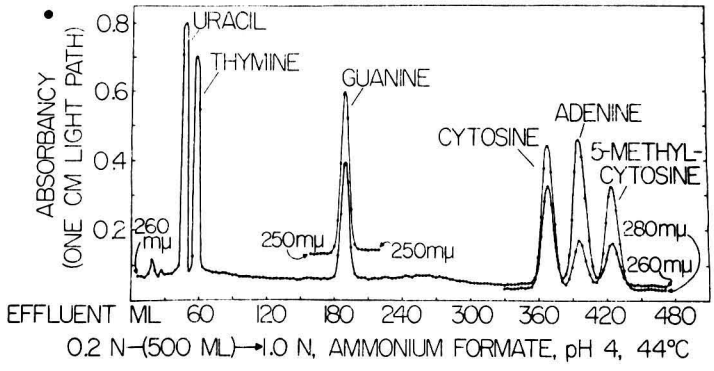

FIG. 3. Elution of purines and pyrimidines from a 0.9 × 60 cm column of Dowex 50-X4 (ammonium form, "through 200" mesh). The ammonium formate eluant was produced by use of a 500-ml mixing chamber. Fractions of 3 ml were collected. Each principal peak contained about 60 μg of base.

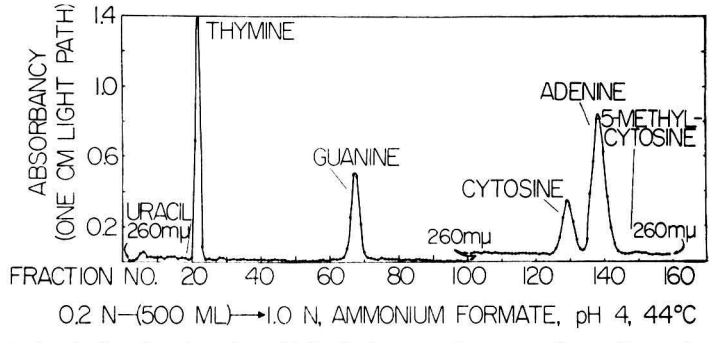

FIG. 4. Analysis of a formic acid hydrolyzate of 1.2 mg deoxyribonucleate from mouse ascites tumor cells. The experimental conditions were the same as for Fig. 3, except that fractions of 2.3 ml were collected until fraction 12, after which fractions of 2.9–3.0 ml were collected. For this reason, fraction numbers are plotted rather than effluent milliliters as in Figs. 1–3.

of the purines is delayed to a relatively greater extent than is the elution of the pyrimidines. As shown in Fig. 3, the difficulty was overcome by increasing the temperature of the operation, which, as has been indicated, affects purines more markedly than pyrimidines. Remarkably enough, the use of a mixing chamber of 500 ml does not greatly delay the elution

of the constituents, but is definitely required for reproducible separation of the last three bases. Although thymine and uracil are brought slightly closer together, they are still completely separated, but temperatures appreciably higher than 44°C will cause them to overlap.

In Fig. 4 is shown a chromatogram of a typical formic acid hydrolyzate of a twice-deproteinized specimen of deoxyribonucleate from mouse ascites tumor cells. As can be seen, there is little material in the position of uracil. The tremendous sensitivity of the procedure, which places directly into 6 to 9 effluent milliliters all of the uracil present in a hydrolyzate of 1.2 mg nucleic acid, suggests that the original specimen of nucleate contains little, if any, uracil (< 0.2 mole %). Chromatography of a larger sample would be required to establish whether the trace of material is indeed uracil. Perchloric acid hydrolyzates of the same samples of deoxyribonucleate give less material in the position of uracil than do formic acid hydrolyzates, although the former agent liberates uracil more efficiently from ribonucleic acid than does the latter (11, 8). This problem will be discussed in a future communication[17] together with results that have thus far been obtained by applying the chromatographic procedures described here to 13 specimens of nucleic acid, prepared from the nucleoprotein fractions insoluble in 6 *M* urea, 1% NaCl, 0.1% Merthiolate of a number of mammalian tissues. In all cases, the amounts of uracil found have been extremely small. With few exceptions, the moles of total bases found in formic acid hydrolyzates of the specimens were close to the gram atoms of P present, and the molar quantities of adenine and thymine, and of guanine and cytosine + 5-methylcytosine were nearly equivalent. When the present procedures are used to analyze hydrolyzates of 1-mg samples of deoxyribonucleate from calf thymus, the values obtained for 5-methylcytosine are only approximate, but more reliable values are obtained when analyses are performed of hydrolyzates of 1-mg specimens which contain this base more abundantly, such as specimens from wheat germ or certain fractions prepared by chromatography on columns of Mg IRC-50 (2). More accurate data for 5-methylcytosine can be obtained readily by increasing the sample load to 300 μg of each principal base, that is, by chromatographing roughly 5 mg of material, since the peaks are not perceptibly broadened by this higher load. The upper limit of the capacity of the resin has not been ascertained. Judging from the spectral data in Table 1, and considering the excellent recoveries obtained with respect to P in hydrolyzates of deoxyribonucleates, the recoveries of bases from the columns appear to be $100 \pm 5\%$ over the range of sample weights used thus far. Moreover, the reasonable values obtained for 5-methylcytosine in hydrolyzates

[17] Crampton, C. F., Greenfield, B., Adair, J., and Frankel, F. R., in manuscript.

indicate that there are no appreciable losses when 3-μg quantities of this base are chromatographed.

The present procedures are, then, recommended for the analysis and estimation of microgram quantities of purines and pyrimidines. For some purposes, the procedures should prove more versatile than analysis by paper chromatography, particularly in structural studies on fragments of the nucleic acids, where the unavoidable presence of extraneous materials, and loads of different size, frequently alter R_f values in an unpredictable way. Such factors scarcely affect the behavior of bases on strongly buffered columns of sulfonic-exchange resins.[18] In the column procedures, the necessity of performing numerous replicates, as must be done to obtain trustworthy analyses by paper chromatography, is minimized, since each point throughout a peak may be regarded as a separate determination. Asymmetry of a peak readily indicates aberrations in the quantitative estimations occurring after the constituents have been separated from one another. Because the fractions are collected directly in tubes using fraction collectors having enclosed turntables, the procedures have proved particularly valuable in a Department of Pathology, where vapors of xylene and thymol in the atmosphere render paper chromatographic analyses of the bases unreliable unless prohibitive precautions are taken. For qualitative analyses, the procedures recommend themselves for use with automatic ultraviolet recording devices, and they could easily be modified for quantitative purposes when more refined

[18] While the use of the carboxylate resin Amberlite IRC-50, XE-64 was not studied exhaustively, application of the elution system used in Fig. 3 to a 0.9 × 61 cm column of "through 200" mesh material gave an encouraging, complete separation of uracil, thymine, 5-hydroxymethylcytosine, cytosine, 5-methylcytosine, and guanine, which emerged in that order when 60-μg quantities of the bases were chromatographed at 30°C. Spectral data indicated that these bases were recovered quantitatively. Although guanine was placed at 220 ml, adenine failed to appear after 450 ml. This surprising result, which was confirmed, suggests that adenine may form an unusually strong bond with the resin, perhaps involving chelation. At pH 4, moreover, hydrogen bonds involving un-ionized COOH groups of the resin may contribute to producing the unexpected elution sequence and the great retardation of adenine. Use of this IRC-50 system for analysis of a formic acid hydrolyzate of deoxycytidine diphosphate (obtained from a diphenylamine hydrolyzate (13) by elution with HCl from Amberlite CG-400) proved disappointing, however, in that the peak obtained for cytosine exhibited pronounced tailing not observed with standardization mixtures. Since pH 4 is close to the pK's both of the carboxyl groups of the resin, and the amino groups of the bases, it is concluded that the interaction between IRC-50 and the bases is more sensitive to the composition of the sample than when resins of sulfonated polystyrene are used. For this reason, and because large volumes of buffer and long periods of time are required to equilibrate the carboxylate resin, attempts to analyze bases using IRC-50 at pH 4 were abandoned.

devices become available. It should be possible to increase greatly the flow rate by use of finer resin and higher pressure as was done by Moore *et al.* in designing systems for automatic amino acid analyses (14).

SUMMARY

The analysis and estimation of microgram quantities of the common purines and pyrimidines by use of 0.9×60 cm columns of sulfonated polystyrene (Dowex 50) operated at pH 4 are described. The advantages of the procedures, a number of observations relating to the development of the procedures, and some principles underlying the analyses are illustrated and discussed.

ACKNOWLEDGMENTS

The authors are indebted to Dr. J. L. Edwards for making available the facilities and the opportunities that made these studies possible, and to Drs. S. Moore and W. H. Stein, whose chromatographic studies on protein derivatives established many precedents which were used to advantage in the present investigations. The assistance of Mrs. J. L. Rodeheaver in the early phases of the work is gratefully acknowledged.

REFERENCES

1. Cohn, W. E., *in* "The Nucleic Acids" (Chargaff, E., and Davidson, J. N., eds.), Vol. 1, p. 211. Academic Press, New York, 1955.
2. Crampton, C. F., Benson, A. M., Rodeheaver, J. L., and Wade, A. E., *Federation Proc.* **17,** 206 (1958).
3. Moore, S., and Stein, W. H., *J. Biol. Chem.* **192,** 663 (1951).
4. Moore, S., and Stein, W. H., *J. Biol. Chem.* **211,** 893 (1954).
5. Hirs, C. H. W., Moore, S., and Stein, W. H., *J. Biol. Chem.* **200,** 493 (1953).
6. Wyatt, G. R., and Cohen, S. S., *Biochem. J.* **55,** 774 (1953).
7. Crampton, C. F., and Petermann, M. L., *J. Biol. Chem.* **234,** 2642 (1959).
8. Wyatt, G. R., *Biochem. J.* **48,** 584 (1951).
9. King, E. J., *Biochem. J.* **26,** 292 (1932).
10. Wyatt, G. R., *in* "The Nucleic Acids" (Chargaff, E., and Davidson, J. N., eds.), Vol. 1, p. 243. Academic Press, New York, 1955.
11. Vischer, E., and Chargaff, E., *J. Biol. Chem.* **176,** 715 (1948).
12. Sinsheimer, R. L., *J. Biol. Chem.* **208,** 445 (1954).
13. Burton, K., and Peterson, G. B., *Biochim. et Biophys. Acta* **26,** 667 (1957).
14. Moore, S., Spackman, D. H., and Stein, W. H., *Anal. Chem.* **30,** 1185 (1958).

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Differential Density Separation of Cellular Suspensions[1,2]

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INTRODUCTION

Biochemical investigations frequently require that cells be separated from their suspending medium prior to analysis. In many cases, simple centrifugation followed by the removal of the components of the experimental medium by washing is adequate. In many other circumstances, however, such washing is not permissible as, for example, in the study of ion exchange and transport, and in the determination of permeability with isotopically labeled substances. One approach has been to measure the amount of substance which is removed from the suspending medium, but this has the attendant disadvantage that the measurement of the amount taken up by the cells is the difference between two large numbers. In the course of our investigations on the accumulation of the alkaline-earth ions by *Tetrahymena gelii,* we have developed a method which accomplishes the separation of the cells from a suspension in a single rapid step leaving them essentially free of the suspending medium. Further, by the application of I^{131}-labeled serum albumin (RISA, Abbott Laboratories) coupled with a γ-ray Scintillation Crystal Spectrometer, it is possible to measure accurately whatever small amount of contamination of the cell suspension by the medium does occur. In principle[3] the method consists of allowing the cells during centrifugation to pass through a nonmiscible liquid whose density is

[1] This work was supported in part by Contract AT(30-1)-1822 between the U. S. Atomic Energy Commission and the Johns Hopkins University. The isotopes used for this work were obtained from the Oak Ridge National Laboratories of the U. S. Atomic Energy Commission and the Abbott Laboratories, Inc., at Oak Ridge.

[2] This is contribution No. 303 from the McCollum-Pratt Institute.

[4] One of the authors, R. B., was introduced to the basic principles of differential density centrifugation by Dr. Arthur K. Parpart, Princeton University. We are indebted to him for turning the idea over to our laboratory for development and use.

chosen to lie between that of the cells and that of the suspending medium. For convenience, we have called this method the Differential Flotation Method (DFM).

MATERIALS AND METHODS

Density Separators

In addition to having a suitable density, the liquids used as density separators between the cells and the medium should be characterized by (*a*) negligible solubility in aqueous solution, (*b*) physiological inertness, and (*c*) low viscosity and low volatility. We have found the diesters of *o*-phthalic acid to be very satisfactory for this purpose. Table 1

TABLE 1
DENSITIES AT 25°C FOR VARIOUS COMPOUNDS AND MEDIA

Substance	Density
Diethyl phthalate	1.1133
Di-*n*-butyl phthalate	1.0416
Diisobutyl phthalate	1.0326
Octoil-S	0.9098
Weight % Octoil-S in di-*n*-butyl phthalate	
3.462	1.0362
7.739	1.0296
14.73	1.0204
28.53	1.0003
Weight % diethyl phthalate in di-*n*-butyl phthalate	
3.592	1.0442
17.01	1.0536
28.57	1.0614
43.01	1.0717
49.81	1.0767
74.95	1.0950
2% Bacto Proteose–Peptone medium	1.0043
0.90% Sodium chloride	1.0037

presents the densities of several of the commercially available esters of phthalic acid.[4] These were determined picnometrically at 25°C. Solutions of intermediate density can readily be compounded by making mixtures of di-*n*-butyl phthalate with (*a*) Octoil-S or (*b*) diethyl-

[4] The following diesters of orthophthalic acid are to be obtained from the Distillation Products Division, Eastman Kodak Co., Rochester. No. 124 diethyl phthalate, b.p. 172–173°/14 mm; No. 1403 diisobutyl phthalate, b.p. 142–144°/1 mm. These compounds were used as obtained from the supplier. Octoil-S is available from the Rochester Division, Consolidated Electrodynamics Corporation, Rochester, N. Y.

phthalate. The densities are additive to the third decimal place on the basis of weight percentage.

Procedure

Conical glass centrifuge tubes of 12 ml capacity are first rendered hydrophobic either by coating with paraffin, or better, with Dri-Film.[5] One-half milliliter of the desired separator (we have employed diisobutyl phthalate in the separation of *Tetrahymena*) is most conveniently run into the tube from a buret. Ten milliliters of the cell suspension is then added, and the mixture is centrifuged at $2000 \times g$ in a swinging bucket centrifuge, such as the Size 2 International (speed: 3200 rpm). In the case of *Tetrahymena* (an ellipsoidal cell whose dimensions are 17 $\mu \times$ 50 μ and whose density is equal to 1.041–1.036), sedimentation under the above conditions is essentially complete in 1 min. Since the density separator effectively prevents any resuspension of the cells, the centrifuge may be rapidly braked to a stop and the supernatant liquid aspirated off. Any small droplets of aqueous solution adhering to the inside of the centrifuge tube are removed by carefully swabbing with absorbent paper. Special care should be taken to remove any small droplets suspended in the boundary layer of the density separator. After this, the bulk of the organic liquid can be removed by gently tipping the tube and absorbing the liquid in absorbent paper.

The extreme packing of the cells and their consequent exposure to anoxic conditions should be considered if the cells are to be employed in physiological experiments, such as respirometry and enzymology. In this case it is essential to remove the remaining density-separating liquid. This can be done by resuspending the cells in a proper aqueous buffering medium, adding 1 ml of Octoil-S, gentle mixing, then recentrifuging. The organic phase may now be aspirated from the top of the aqueous phase. For those types of experiments in which the cells are to be subjected to destructive analysis, the organic phase may be most conveniently removed by repeated dilution with α,α-dimethyl butane.[6] Finally, the solvent may be simply removed in a vacuum desiccator in less than an hour.

Quantitative Estimation of Residual Intercellular Fluid

As is demonstrated in Table 2, the residual intercellular fluid carried along by the cells and collected in the pellet under the density separator

[5] Dri-Film, General Electric Dri-Film No. SC-87, is used in a 10% solution in CCl_4. It is imperative for safety that this solution be used in a hood. This probably corresponds to the original Beckman Desicote. The *new* Desicote formulation is unsatisfactory in this procedure.

[6] Personal communication from Dr. Malcolm Steinberg.

TABLE 2

TEST OF THE DIFFERENTIAL FLOTATION METHOD (DFM) ON *Tetrahymena* CULTURES

Test	Number of cells/10 ml	Volume of cells/10 ml (ml)	Volume contamination (ml)	Per cent pellet as cells
Expt. 1[a]				
Conventional centrif.	3.54×10^6	0.0578[b]	0.129	31
DMF-RISA	3.54×10^6	0.0578	0.0034	94.5
Expt. 2[a]				
DFM-RISA	2.28×10^6	0.0422[b]	0.0027	94.0
Expt. 3[c]				
DFM-RISA	2.83×10^6	0.0668[d]	0.00453 (±.00019)[e]	93.7
DFM Iodide-I^{131}	2.83×10^6	0.0668	0.00438 (±.00010)	93.8
DFM Diodrast-I^{131}	2.83×10^6	0.0668	0.00476 (±.00012)	93.3
DFM-RISA plus Co^{60} background[f]	2.83×10^6	0.0668	0.00449 (±.0016)	93.7
Mean ± variance of *mean* for above 16 samples			0.00454 (±.00005)	93.6±.07
Uncoated tube: DFM-RISA			0.00725 (±.00078)	90.3
Conventional centrifuge			0.0437 (±.0059)	60.4
Expt. 4[g]				
DFM-RISA, Set 1	1.81×10^6	0.0427[d]	0.00275 (±.00022)	93.8
Set 2	1.81×10^6	0.0427	0.00319 (±.00060)	93.0
Set 3	1.81×10^6	0.0427	0.00300 (±.00028)	93.4
Set 4	1.81×10^6	0.0427	0.00239 (±.00009)	94.7
Set 5	1.81×10^6	0.0427	0.00301 (±.00050)	93.0
Sum all 5 sets			0.00287 (±.00040)	93.6

[a] Centrifuge tubes uncoated in these experiments.

[b] Volume estimated by centrifuging (DFM) cells from 50 ml of the suspension in a graduated tube.

[c] Entries are the mean value of four samples.

[d] The cell volume in 10 ml calculated from (cell number) × (cell volume). The *cell number* was determined by direct counting in a Fuchs-Rosenthal Cytometer. The *cell volume* was determined from phase-contrast photographs of 100 cells in the living state, and treating the cells as elipsoids of revolution.

[e] The values in parentheses (±.xxx) are the standard deviations of the *individual observations.*

[f] Co^{60} as cobaltiethylenediaminetetracetic acid was added at an activity equivalent to the I^{131} present. The radioassay was by gamma-scintillation spectrometry.

[g] Entries are the mean value of eight samples in each set.

constitutes a very small percentage (about 5%) of the pellet volume. Nevertheless, for certain analytical purposes an accurate estimation of this volume is essential. This can be directly estimated by adding I^{131} iodinated serum albumin to the cell suspension prior to centrifugation. For an accurate estimate, $2–3 \times 10^6$ counts/min should be incorporated in 10 ml of cell suspension. Upon completion of the centrifugation and

decantation, simple counting of an aliquot of the supernatant and the cell pellet in a well-type gamma scintillation counter permits an accurate estimate of the contamination of the cell pellet by the residual suspending medium. In the case of *Tetrahymena,* a cell which is almost completely anion-impermeable in the resting state, this estimation can also be carried out using P^{32} as phosphate, S^{35} as sulfate, I^{131} as iodide, and Co^{60} as the cobaltiethylenediamine tetraacetate. Undoubtedly many other large molecules containing suitable radioactive tracer atoms, such as inulin-C^{14}, could be used. We have found I^{131}-labeled Diodrast (3,5 diiodo-4-pyridone-*N*-acetic acid) a useful compound.

RESULTS

The results of the application of this method are presented in Table 2 where we have limited ourselves to presenting the data on *Tetrahymena,* since our studies have been mainly concerned with this organism. Very preliminary studies have indicated that it is also applicable to dissociated embryonic tissues,[6] bacteria (*Escherichia coli*) and red blood cells.

A comparison is made in Table 2 between conventional centrifugation and DFM. For *Tetrahymena,* the degree of contamination is lowered nearly 5–30-fold. An additional improvement of 2–10-fold has been found with reference to coated (paraffin or Dri-Film) tubes versus uncoated. This improvement in freedom from contamination by the suspending medium in a DFM separation as compared to classical centrifugation is probably due to a variety of factors. Perhaps the most important one is the fact that the supernatant may be removed to the last microliter. Secondly, when the cells pass through the immiscible separating layer, this liquid acts something like a squeegee, stripping off the adhering liquid layer surrounding the cell. Finally, the resuspending effects of convection during the centrifugation are prevented by the immiscible barrier. Simple observation with a stroboscope of the liquid motions during centrifugation provides a convincing demonstration that this convective effect may be one of the more seriously limiting factors in rapid quantitative centrifugation, and its negation by the organic phase is probably the major contribution to the vast increase in speed of separation of *Tetrahymena* using the DFM method; i.e., their quantitative collection can be made in less than 1 min as compared to 20 min conventionally. One should remark that in addition to their resuspension by convection, alone, *Tetrahymena* are highly motile organisms and under conventional conditions in the time required to stop the centrifuge they resuspend themselves by swimming, unless they have been packed for prolonged periods of centrifugation at 5000–10,000 $\times$ *g*.

It was pointed out above that this method of separating cells (DFM) places them in a closely packed and anaerobic environment, and hence the question arises whether sufficient damage to the cells occurs while they are confined in the pellet to cause release of cell contents which then pass back through the immiscible isolating layer, thus leading to anomalous results. To test this, we grew *Tetrahymena* in the presence of phosphate P^{32}, separated the cells from the culture medium by DFM, and then carefully layered distilled water over the isolating liquid. After centrifuging for 30 min, aliquots of this supernatant were assayed for beta activity, the results of which indicated that no loss of cell contents had occurred by this route.

It may again be emphasized that the DFM technique completely isolates the sedimented material from its original environment in such a way that no further exchange between the cells and the suspending medium is possible. This has permitted us to carry out studies, to be reported elsewhere, on the calcium and strontium exchange in *Tetrahymena,* and by the use of the RISA technique, coupled with Karl Fischer water analysis and glycerol-C^{14} distribution, to make the measurements of total and solvent intracellular water space.

SUMMARY

A method is described for the rapid separation of cells from their suspending medium with only $\frac{1}{5}$th to $\frac{1}{30}$th the amount of contamination from the medium as with conventional centrifuging. The principle is to interlayer between the cells and the medium upon centrifuging an immiscible liquid whose density lies between that of the cells and medium. Quantitative measurement of the amount of contamination of the cells by the medium which does occur is achieved by adding a nonpermeable radioactively labeled substance to the suspension prior to centrifugation.

ANALYTICAL BIOCHEMISTRY, **1**, 269–277 (1960)

SHORT COMMUNICATIONS

A Specific, Sensitive Assay of Glycerol and L-α-Glycerophosphate[1]

The difficulties of the quantitative determination of glycerol in biological materials are well known and have recently been discussed by Hanahan and Olley (1). Available chemical methods lack specificity and require tedious separations. A two-stage enzymic assay procedure based on the reactions

$$\text{glycerol} + \text{ATP} \xrightarrow{\text{glycerokinase}} \text{L-}\alpha\text{-glycerophosphate} + \text{ADP} \quad (1)$$

$$\text{L-}\alpha\text{-glycerophosphate} + \text{DPN}^+ \xrightarrow[\text{dehydrogenase}]{\text{glycerophosphate}} \text{dihydroxyacetone phosphate} + \text{DPNH} + \text{H}^+ \quad (2)$$

and involving the spectrophotometrical determination of DPNH was suggested by Bublitz and Kennedy (2). On the basis of this principle, an improved one-stage assay was developed by combining reactions (1) and (2) and by replacing the rat liver glycerokinase with a purified extract from glycerol-grown *Mycobacterium tuberculosis* strain 607.

While this work was in progress, a similar method, involving the use of crystalline glycerokinase from pigeon liver, was published (3, 4). However, our extensive studies on the kinetics of the coupled system and on the influence of the various parameters[2] showed that reproducible results and theoretical yields were obtained only by observing a number of critical conditions which had not been defined in the previous work. Thus, it is essential to maintain reaction (2) at maximal velocity lest it

[1] This investigation was supported by research grant No. E-1500 from the Division of Research Grants of the National Institutes of Health, U. S. Public Health Service.

[2] To be published elsewhere.

become limiting for the over-all reaction rate. This was found to require (*a*) a large excess of L-α-glycerophosphate dehydrogenase (GDH), (*b*) addition of albumin to prevent the extremely rapid inactivation of crystalline GDH by the hydrazine used as trapping agent (Table 1),

TABLE 1
EFFECT OF ALBUMIN CONCENTRATION ON FIRST-ORDER INACTIVATION RATE OF GDH AT 37° AND pH 9.0[a]

Albumin concentration (mg/ml)	Time (min) required for 50% inactivation
2.44	6.5
1.25	3.5
0.58	1.0
0	$\ll 0.5$

[a] The reaction mixture (1.0 ml final volume) contained the indicated concentrations of albumin, 0.25 μg GDH, and in micromoles: DPN, 10; L-α-glycerophosphate, 2; N_2H_4 buffer, 400. Blank without L-α-glycerophosphate.

(*c*) DPN concentration in excess of 5 μmoles/ml because of the high Michaelis constant of GDH with respect to DPN ($K_m = 7 \times 10^{-4} M$) and because of the strong competitive inhibition by the reaction product DPNH ($K_i = 7 \times 10^{-5} M$). Finally, it was important to stabilize the DPNH by the addition of ethanol at the end of the reaction. Under the conditions described, theoretical yields were obtained consistently over a concentration range of 0.05–1.0 μmole glycerol/0.1 ml, and the rates of DPNH formation were linear during most of the reaction period.

METHODS AND RESULTS

Preparation of Glycerokinase

Batches of 2 gm of moist cells of *Mycobacterium tuberculosis*, strain 607, grown on the surface of a synthetic glycerol medium (5) were suspended with 2 gm of acid- and water-washed Pyrex powder in 45 ml of 0.005 *M* phosphate buffer pH 6.7, sonicated for 10 min in a Raytheon 10-kc oscillator, and centrifuged for 15 min at 12,000 rpm in a Servall centrifuge. A 1.8-gm portion of lyophilized supernatant dissolved in 50 ml of 0.05 *M* Tris buffer of pH 8.1 containing 0.05% EDTA, was dialyzed for 30 min against 10 l of the same Tris-EDTA buffer.[3] In a subsequent fractionation, the first precipitate with 11 gm of solid $(NH_4)_2SO_4$ was discarded; the second precipitate with an additional 8.5 gm of $(NH_4)_2SO_4$ was dissolved in 5 ml water and lyophilized. The

[3] This step was essential for the elimination of DPN reduction in blanks without glycerol and for improvement of subsequent fractionation with $(NH_4)_2SO_4$. Tris-tris(hydroxymethyl)aminomethane. EDTA-ethylenediaminetetraacetic acid.

yield of dry residue was 762 mg; it contained 16% protein (6) and had a specific activity of 600 units/mg protein.[4]

Glycerol Assay

The following quantities of reagents in 0.1 ml are pipetted into small test tubes in the order given: bovine serum albumin (Armour), 1.25 mg; aqueous hydrazine (95+ %), adjusted with HCl to pH 9.1, 400 μmoles; Mg^{++}, 1 μmole; KF, 25 μmoles; adenosine triphosphate (ATP) (Sigma 98+ %), neutralized to pH 7, 10 μmoles; diphosphopyridine nucleotide (DPN) (Sigma 98+ %), stored in 0.001 *M* HCl and neutralized to pH 7 just prior to use, 10 μmoles; glycerokinase in 0.005 *M* phosphate buffer pH 6.5, 100 units; GDH from muscle, crystalline suspension in $(NH_4)_2SO_4$ (Boehringer & Sons, Mannheim, Germany), 50 μg, or at least 6000 units;[5] glycerol, 2× distilled, 0.05–1.0 μmole; water, to 1.0 ml. The blank contained water instead of glycerol. After incubation in a water bath at 37° for 60 min, 0.1-ml aliquots are removed and added to tubes containing 0.9 ml of 50% aqueous ethanol. In this way, the DPNH concentration remains constant for several hours; it is determined spectrophotometrically with an extinction coefficient of 6.22×10^6 sq cm/mole at 340 mμ (7).

The results in Table 2 show linearity up to 1 μmole glycerol and close to 100% yields. Assays carried out at different times and with different kinase preparations were reproducible within less than 5%. Rate studies showed that the reaction with 1 μmole glycerol was complete after about 45 min. Quantitative yields with 1.0 μmole glycerol were obtained even in the presence of 20 μmoles glucose. Neither did galactose, arabinose, and inositol cause reduction of DPN. Correct values for glycerol were also obtained in the presence of L-α-glycerophosphate by subtraction of the DPNH formed in the absence of glycerokinase. Similarly, the system may be used for the quantitative assay of L-α-glycerophosphate or DPN.

The specificity of the assay is mainly determined by the specificity of the crystalline L-α-glycerophosphate dehydrogenase from muscle which is essentially free of other glycolytic enzymes and is considered to be specific for L-α-glycerophosphate (9). With an unknown assay mixture, however, reduction of DPN by substrates other than glycerol cannot be

[4] One unit of glycerokinase is defined as that amount of enzyme which causes an increase in absorbance at 340 mμ of 0.001 unit/min at 30° under the specified conditions.

[5] One unit of L-α-glycerophosphate dehydrogenase is defined as that amount of enzyme which causes an increase in absorbance at 340 mμ of 0.001 unit/min at 30° under the specified conditions. A fresh crystalline preparation (Boehringer) usually had a specific activity of about 120 units/μg.

TABLE 2
QUANTITATIVE ASSAY OF GLYCEROL

Number of determinations	Glycerol added (μM)	Per cent of input found	
		Enzymic assay	Periodate oxidation[a]
	A[b]		
4	0.200	97	93
3	0.400	96	98
3	0.600	99	97
3	0.800	95	97
10	1.00	96	97
mean $\pm \sigma$ (standard deviation)		96.5 $\pm$ 2	[d]
	B[c]		
3	0.400	98	
3	0.600	100	
3	0.800	98	
3	1.00	100	
mean		99	

[a] Microtitration procedure based on Dyer method (8).

[b] Reagent-grade glycerol was used. The values in col. A were obtained after correction on basis of the manufacturer's specification of 96% purity. The analytical results, however, indicate only about 93% purity.

[c] The values in col. B were obtained with reagent-grade glycerol which was further purified by two distillations.

[d] The percentage figures represent averages from two series of periodate oxidations.

excluded, since the partially purified kinase might contain other DPN-linked enzymes. Inclusion of a control without GDH permits elimination of such an interference. Corrections for the possible presence of other interfering substances or inhibitors may be made by the addition to the unknown samples of a known amount of glycerol as an internal standard.

REFERENCES

1. HANAHAN, D. J., AND OLLEY, J. N., *J. Biol. Chem.* **231,** 813 (1958).
2. BUBLITZ, C., AND KENNEDY, E. P., *J. Biol. Chem.* **211,** 951 (1954).
3. WIELAND, O., *Biochem. Z.* **329,** 313 (1957).
4. WIELAND, O., AND SUYTER, M., *Biochem. Z.* **329,** 320 (1957).
5. SUTER, E., ERLENMEYER, H., SORKIN, E., AND BLOCH, H., *Schweiz. Z. Pathol. u. Bakteriol.* **11,** 193 (1948).
6. LOWRY, O. H., ROSENBROUGH, N. J., FARR, A. L., AND RANDALL, R. J., *J. Biol. Chem.* **193,** 265 (1951).
7. HORECKER, B. L., AND KORNBERG, A., *J. Biol. Chem.* **175,** 385 (1948).
8. DYER, J. R., *Methods of Biochem. Anal.* (D. Glick, ed.) 124 (1956).
9. BEISENHERZ, G., BÜCHER, T., AND GARBADE, K. H., *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. I, p. 391. Academic Press, New York, 1955.

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A Modification in the Synthesis of N′-Methylnicotinamide Chloride (3-Carbamyl-1-methyl-pyridinium Chloride)

Analytical methods for the determination of *N′*-methylnicotinamide (NMN) chloride, the excretion product of nicotinic acid and nicotinamide metabolism, required this substance in pure form for use as a standard. Attempts to synthesize the compound in our laboratory gave low yields. A modification of the synthesis resulted in a significant increase in the yield of the desired product.

The synthesis of *N′*-methylnicotinamide chloride has been reported by Huff and Perlzweig (1) in which the iodide of this compound is converted to the chloride by the method of Karrer *et al.* (2). Attempts to reproduce the synthesis gave good yields of the iodide with low yields of the chloride. The application of a method, described in two patents (3), gave higher yields of the chloride when the iodide was the starting material.

The *N′*-methylnicotinamide iodide was prepared according to the procedure of Huff and Perlzweig (1). The iodide, 1.6 gm, was dissolved in 50 ml water, and 35 ml carbon tetrachloride was added. The intimate mixing of the two phases was maintained by use of a magnetic stirrer while the temperature was kept below 10°C. Chlorine gas was passed through the mixture until the carbon tetrachloride layer became clear and violet in color. Extending the reaction beyond this point resulted in the formation of a yellow precipitate which had the same melting point as the original starting material. On separation of the two layers, the aqueous portion was washed several times with a few milliliters of carbon tetrachloride. The water was then evaporated under vacuum,

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the temperature not going above 35°C. The residue was recrystallized from a small amount of methanol. The yield was 43%. Increased yields may be obtained by the addition of ether and by placing the solution in a freezer. The absorption spectrum has a $\lambda_{max} = 264.5$ mμ and a $\lambda_{min} =$ 241–242 mμ with a molecular extinction coefficient of 4438. This is in agreement with the spectrum and molecular extinction coefficient reported by Cantoni (4).

The m.p. is 243–244°C. (uncorr.)

Anal. Calcd. for $C_7H_9ON_2Cl$: C, 48.70; H, 5.26; N, 16.23; Cl, 20.54; found: C, 48.75; H, 5.17; N, 17.12; Cl, 22.30.

REFERENCES

1. HUFF, J. W., AND PERLZWEIG, W. A., *J. Biol. Chem.* **150,** 395 (1943).
2. KARRER, P., SCHWARZENBACH, G., BENZ, F., AND SOLMSSEN, U., *Helv. Chim. Acta* **19,** 826 (1936).
3. CARNAHAN, R. E., U. S. Patent 2,762,803; 2,678,312.
4. CANTONI, G. L., *J. Biol. Chem.* **189,** 203 (1951).

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Laboratory Film Evaporator for Rapid Concentration of Dilute Solutions[1]

The recovery of solute from dilute solutions in organic solvents is a procedure commonly carried out in the laboratory. A particular case may present one or more of several problems, such as the necessity of handling relatively large volumes or the possibility of decomposing the desired solute by the necessary heating. These problems are often met only unsatisfactorily by the usual apparatus. There are available, however, at least two noteworthy designs of apparatus for this purpose: that of Barthel and Haller (1), and that of Craig, Gregory, and Hausmann

[1] This work was supported in part by a Research Grant, RG-3615, given by the Division of Research Grants of the National Institutes of Health.

(3). The former is especially adapted to concentrating large volumes, the latter small volumes. Modifications of the latter are now available commercially[2] for continuous operation, and thus are adaptable to large volumes.

Both these designs of apparatus are relatively complicated and expensive. It is the purpose of this report to describe a simple, cheap apparatus which has certain advantages over those previously described, but which is also complementary to the batch apparatus of Craig, Gregory, and Hausmann (3). It bears a slight resemblance to the evaporator described by Smith (6). Very recently Kasman and Rubin (4) have suggested a procedure and setup vaguely resembling those to be precisely described here.

The present apparatus can be made in a variety of forms, but that indicated in Fig. 1 is perhaps the most satisfactory of several we have made. The funnel *F* represents a reservoir of dilute solution which is allowed to flow through the stopcock (a Teflon plug[3] at *P* is helpful here) into the heated tube *C*. Here evaporation takes place and the vapor and concentrated solution continue to flow *cocurrentwise* until they separate in the connecting tube *T*; the concentrated solution accumulates in flask *S* and the vapors proceed to the condenser *B*. For operation under reduced pressure this condenser is, of course, suitably connected to a receiver.

All parts except the piece *CJT* are arranged from commonly available apparatus. A ground joint connection in the vicinity of *J* would permit the assembly of this piece from commercially available parts; however, the combined piece *CJT* is cheaper, more compact, and not less versatile (it can also be used as part of a reflux-stirring apparatus, or as a distilling column). The heating tube shown is 14 mm inside diameter and 50 cm long, but these dimensions are not critical. A tube of 8 mm bore by 30 cm length has been very useful in this laboratory for many years. The helical wire spiral on the inside of the heating tube is wound carefully on a suitable mandrel so that it fits the tube snugly, and serves the purpose of spreading the flowing liquid in a fairly uniform film on the wall, as in Podbielniak's earlier distilling columns (5). In our experience the most satisfactory compromise between high chemical resistance of the wire and its cost is met by tantalum. A spiral of 1.0 mm glass rod is quite satisfactory.[3]

The heating of tube *C* can be done by the excellent apparatus of Barthel and Haller (1), who used electrically generated steam; the author has built and used a similar apparatus and circulated vapor from

[2] Instrumentation Associates, 138 New Haven Avenue, New York 32, N. Y.
[3] Scientific Glass Apparatus Company, Bloomfield, N. J.

boiling alcohol or carbon tetrachloride or toluene. The elegant Bronwill Constant Temperature Circulator[4] is superior to both these methods. But by circulating steam or hot water from a tap, whichever is appropriate, we have more simply met most of our needs. These temperatures, say 100° and 60°, respectively, in conjunction with reduced pressures, should encompass most of the common heating problems. An electrically conducting heating tube (Corning LGl-9340) would probably be satisfactory.

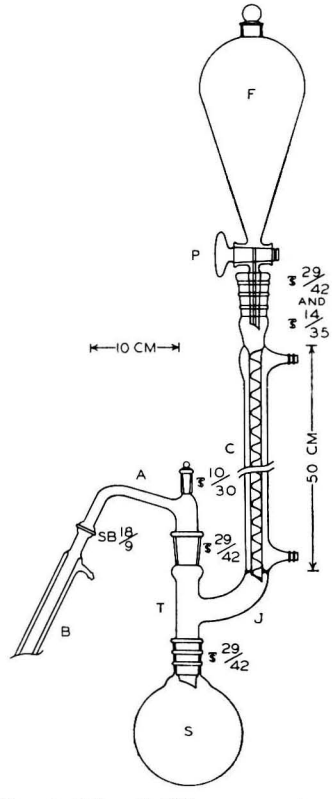

FIG. 1. Film-distilling apparatus.

As an example of a typical problem, we wished to recover solute from 30 l of a dilute extract in petroleum ether. By using steam in the evaporator and circulating ice water in a 100-cm West-type condenser (*B*) (12 mm bore), this solution was concentrated to 3 l in about 5 hr. A further tenfold concentration was accomplished in another hour by reprocessing, and the solute was finally isolated in a Craig-Gregory-Hausmann evaporator.

[4] Will Corporation, Atlanta, Georgia.

The rate of evaporation attainable depends upon the solvent; lower rates are thus attained with ethyl acetate or isopropyl acetate than with ethyl ether. The former two already boil high enough so that reduced pressure is helpful. In operating the still it is important, if solute is to be exposed minimally to heat, to coordinate heating temperature, rate of inflow, and degree of evacuation so that the heating tube remains just wet at the bottom with flowing concentrated solution.

It is not possible to remove the last of the solvent from a solid solute in this apparatus, although a stable liquid solute can be stripped of solvent (6). Precipitation or crystallization of solute on the wall of the evaporator may limit the degree of concentration which can be achieved; in an occasional instance it may be useful to bring about this deposition by completely evaporating the solvent (4). Final evaporation of solvent from concentrated or saturated solutions of solid solutes can usually best be done by the elegant apparatus of Craig *et al.* (3) or by an "Impingement evaporator" (2).

The removal of solvent from aqueous solutions is not satisfactory with the apparatus of Fig. 1, because such solutions do not wet the evaporator wall.

If a solvent contains a desired solute which itself is appreciably volatile, a suitable fractionating column can be inserted between *T* and *A*.

REFERENCES

1. BARTHEL, W. F., AND HALLER, H. L., *Ind. Eng. Chem. Anal. Ed.* **17,** 529 (1945).
2. BUSH, M. T., *Microchem. J.* **1,** 109 (1957).
3. CRAIG, L. C., GREGORY, J. D., AND HAUSMANN, W., *Anal. Chem.* **22,** 1462 (1950).
4. KASMAN, S., AND RUBIN, L. E., *J. Chem. Ed.* **37,** 292 (1960).
5. PODBIELNIAK, W. J., *Ind. Eng. Chem., Anal. Ed.* **3,** 180 (1931).
6. SMITH, F. H., *Ind. Eng. Chem., Anal. Ed.* **14,** 255 (1942).

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Toxic Phosphorus Esters

Chemistry, Metabolism, and Biological Effects

By RICHARD D. O'BRIEN

Pesticide Research Institute, Canada Department of Agriculture, London, Ontario

September 1960, 434 pp., illus., $14.50

This book gives a full account of the mode of action of phosphorus anticholinesterases in mammals and insects, and of their metabolism in mammals, insects, and plants. A chapter is also devoted to their chemical properties, with emphasis both on properties which contribute to an understanding of the biochemical aspects and on those of which a knowledge is necessary for the utilization of these compounds.

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ANALYTICAL BIOCHEMISTRY, 1, 279–285 (1960)

A Simple Efficient Liquid Scintillator for Counting Aqueous Solutions in a Liquid Scintillation Counter

GEORGE A. BRAY[1]

From the Laboratory of Kidney and Electrolyte Metabolism, National Heart Institute, Bethesda, Maryland

Received August 2, 1960

The use of liquid scintillation counters has increased the efficiency and ease with which samples containing radioactive carbon-14 or tritium can be assayed. Most of the solvents which can be used effectively in liquid scintillation counters are nonpolar organic liquids, like toluene (1). Such solvents have imposed a limitation on the ability to count radioactive materials in biological fluids since most of these compounds are polar, and thus insoluble in toluene. A number of approaches have been tried to solve the problem of counting radioactive compounds in aqueous solutions using a liquid scintillation counter (2–6). Among the most recent of these are ones using a solid-phase scintillator mixed with a liquid radioactive sample and a wetting agent (7); a method of preparing tissue samples by dissolving them in alcoholic potassium hydroxide or hyamine hydroxine and counting the dissolved material in toluene–2,5-diphenyloxazole (PPO) (8) or diotol (8, 9) liquid scintillator; and a method of producing a gel or suspension of an aqueous phase in an organic liquid scintillator (10). The system reported here is a modification of the naphthalene–dioxane system (2, 11, 12) that has a relatively high efficiency for both carbon-14 and tritium and can be used for counting a wide variety of aqueous and organic solutions without freezing or separation of phase when maintained at —8°C.

METHODS AND MATERIALS

Reagents

The reagents used in preparing the liquid scintillator described in this paper were obtained from the following sources: *p*-dioxane (purified)

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from Fisher Scientific Co. or from Distillation Product Industries; naphthalene (recrystallized) from Distillation Product Industries or Fisher Scientific Co.; 2,5-diphenyloxazole (PPO) and 1,4-di-2-(5-phenyloxazolyl)benzene (POPOP) from Pilot Chemicals Inc.; methanol and ethylene glycol from Fisher Scientific Co. These reagents were used as supplied.

Counting Procedures

The Packard Tri-Carb Liquid Scintillation Spectrometer equipped with an automatic changer was used for all counting. The photomultiplier tube was maintained at —8°C. Radioactive samples were prepared by adding a known volume of solution to be counted to 10 ml of liquid scintillator in a 20-ml low-potassium glass vial obtained from the Wheaton Glass Co.

Radioactive Materials

Absolute efficiencies were determined by using benzoic acid-C^{14} or tritiated water obtained from New England Nuclear Corp. Urea-C^{14} and methanol-C^{14} from Volk Radiochemical Co. and alanine-C^{14} from New England Nuclear Corp. have also been used in testing this system.

Liquid Scintillator

The liquid scintillator described below was arrived at by systematic attempts to increase the miscibility of naphthalene–dioxane–PPO mixtures with various aqueous solutions and to prevent their freezing or separating into two phases at —8°C. The composition of the liquid scintillator which met these criteria with the highest counting efficiency is as follows:

Naphthalene	60	gm
PPO	4	gm
POPOP	0.2	gm
Methanol (absolute)	100	ml
Ethylene glycol	20	ml
p-Dioxane	to make 1	l

No stabilizers were added to this liquid scintillator, and it has been found to be stable over periods of up to 1 month.

USES AND LIMITATIONS

The absolute efficiency of this system for carbon-14 was determined using benzoic acid-C^{14}. In Table 1 the efficiency for counting benzoic acid-C^{14} added in dioxane or in 1.0 ml water is shown for each voltage setting. The maximum efficiency for benzoic acid-C^{14} in the absence of

water is 65.6% obtained with an operating voltage of 1020 and a discriminator setting of 10–100 v. In the presence of 1.0 ml water the pulse height distribution shifts, giving a maximum efficiency of 60.6% at an operating voltage of 1100 and a discriminator setting of 10–100 v. This shift in pulse height distribution and drop in maximum efficiency in the presence of water was seen with every radioactive compound tested in this liquid scintillator.

The efficiency with which this liquid scintillator counts tritium was determined by adding 1.0 ml of tritiated water to 10 ml of liquid scintil-

TABLE 1

ABSOLUTE COUNTING EFFICIENCY FOR BENZOIC-C^{14} ACID IN THE PRESENCE AND ABSENCE OF WATER AS A FUNCTION OF PHOTOMULTIPLIER TUBE VOLTAGE[a]

Photomultiplier tube voltage	Absolute efficiency in %			
	Discriminator setting 10–50 v		Discriminator setting 10–100 v	
	No water	1.0 ml water	No water	1.0 ml water
870	39.4	21.8	39.4	22.2
950	54.6	40.6	59.3	41.2
1020	45.9	47.1	65.6	56.0
1100	33.8	40.3	60.5	60.6
1180	20.2	28.8	45.6	54.1

[a] 2153 dpm added to each vial in 0.52 mg benzoic acid-C^{14}.

lator. An efficiency of 10.0% was obtained at an operating voltage of 1330 and a discriminator setting of 10–100 v. At a discriminator setting of 10 to infinity, the maximum efficiency of 11.7% was obtained using 1400 v.

The effects of radioactive isotope concentration, of nonradioactive carrier concentration, and of increasing volumes of water on the count rate were investigated using both alanine-C^{14} and tritiated water. With a 1-ml volume of radioactive solution, a linear relationship was found between the radioactive isotope concentration and the observed count rate for alanine-C^{14}. When the radioactive isotope concentration was held constant and the concentration of nonradioactive alanine varied from 0.001 to 0.1 M, there was no change in the count rate. The effect of water on the counting of tritium and carbon-14 in this liquid scintillator was determined by adding increasing volumes of radioactive alanine or radioactive tritiated water to the liquid scintillator. The results are plotted in Fig. 1. It will be seen that the increase in count rate with increasing amounts of alanine-C^{14} solutions is nearly linear up to 1.5 ml. For tritiated water, however, the increment in count rate decreases with increasing volumes of water.

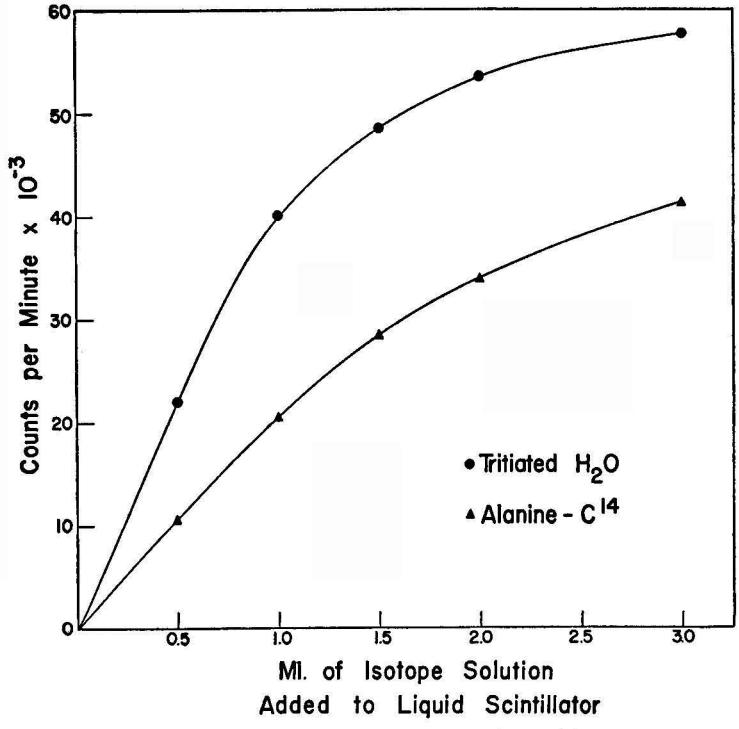


FIG. 1. Effect of volume on count rate of alanine-C^{14} or tritiated water. Increasing volumes of radioactive sample containing the same tritium or C^{14} isotope concentration were added to 10.0 ml of liquid scintillator in triplicate.

A variety of aqueous solutions have been used with this liquid scintillator. In the left-hand column of Table 2 the maximum volume of each aqueous solution which will remain in solution with 10 ml of the mixture at —8°C is indicated. This volume was 3.0 ml for all solutions tested

TABLE 2

SOLUBILITY AND QUENCHING FOR SEVERAL SOLUTIONS

Solutions	Maximum volume which remains in one phase with 10 ml of liquid scintillator at —8°C	Per cent quenching when 1.0 ml of solution is added to liquid scintillator
1 *M* sucrose	3.0	11.7
1 *N* HCl	3.0	69.5
1 *N* H_2SO_4	3.0	58.7
2% NaCl	3.0	21.3
0.5 *N* NaOH	2.0	24.3
0.2 *M* Tris pH 8.2	—	2.8
0.15 *M* acetate pH 5.0	—	2.7

except 0.5 *N* NaOH. Quenching was determined for each of these solutions by adding 0.1 ml of radioactive sample and 1.0 ml of each aqueous solution to 10 ml of liquid scintillator and comparing the count rate observed to that found when 0.1 ml of radioactive sample was added to 1.0 ml water and 10 ml of the liquid scintillator. These results are seen in the right-hand column of Table 2. With the salt, sucrose, and alkaline solutions the quenching was less than 25%, while with acidic solutions it was greater than 50%, suggesting the desirability of neutralizing acidic solutions, where possible, before attempting to count them with this system.

This liquid scintillator has also been used to count some radioactive compounds in biological fluids. Tritium and C^{14} have both been counted by adding 1.0 ml of undiluted radioactive urine to the liquid scintillator. Quenching which can be as high as 80% under these circumstances has been shown to depend on the amount of color of the urine (9). This color can be almost completely removed by adsorption onto charcoal. However, this method can only be used if the charcoal does not adsorb the radioactive material too. Quenching in urine can also be reduced or eliminated by diluting the urine 100 times. When undiluted urine is added to the liquid scintillator, a precipitate forms and settles to the bottom of the vial. This sediment does not appear to interfere with counting efficiency, as long as the radioactive material is not part of the precipitate. Undiluted plasma has also been counted by this method. However, when a sample of plasma was added to the liquid scintillator, quenching was about 25% due in part to the protein that precipitated.

The fact that protein precipitated when plasma was added to the liquid scintillator led to studies on the effect of protein precipitants on the counting characteristics of this system. A 20% homogenate of guinea pig liver was prepared in isotonic potassium chloride, and urea-C^{14} was added. To an aliquot of the radioactive homogenate an equal volume of water or protein precipitant was added. The mixture was centrifuged and 1.0 ml of the supernatant pipetted into the counting vials. Quenching was determined by adding 0.1 ml of alanine-C^{14} with approximately 10,000 counts/min to each vial and comparing the observed increase in count rate for each solution with that to be expected when 0.1 ml of alanine-C^{14} was added to a water blank. The results of these experiments are summarized on the left-hand side of Table 3. Similar studies were done adding the protein precipitants to water and determining their quenching effect in the absence of protein. The results are shown on the right-hand side of Table 3. In the absence of protein the only precipitants that produce any significant quenching are the perchloric acid and the 20% trichloroacetic acid (TCA). When tissue is present, however,

the 10% acidic $ZnSO_4$–0.75 *N* NaOH or heat appear to be most effective in removing the quenching material. The addition of 2.5% trichloroacetic acid decreased quenching from 42 to 26.5%, while 5% TCA only decreased quenching to 32%. The explanation for the fact that 2.5% TCA produces less quenching than 5% TCA may be that under the conditions of these experiments no more quenching material was removed by 5% TCA than by 2.5% TCA, but that the 5% TCA increased the quenching due to the extra quenching material added in the protein precipitant.

This liquid scintillator has been used to count numerous radioactive

TABLE 3

EFFECT OF PROTEIN PRECIPITATING REAGENTS ON COUNTING EFFICIENCY

Protein precipitant	Per cent decrease in count rate after adding 1 ml of treated liver homogenate to 10 ml of liquid scintillator	Per cent decrease in count rate in the absence of protein when protein-precipitating reagent is added to liquid scintillator
2.5% TCA	26.5	3.6
5.0% TCA	32.0	5.8
20% TCA	—	43.3
10% acidic $ZnSO_4$ 0.75 *N* NaOH	13.7	2.0
5% perchloric acid	56.8	26.3
Boiled homogenate	10.0	—
Untreated homogenate	42.0	—

samples. It has, for example, been used to count glycolytic intermediates on paper chromatograms. It has also been used to determine radioactive glucose incorporation into glycogen by counting the acid hydrolyzate of the isolated glycogen. It will keep 1.0 ml water and 1.0 ml benzene in one phase with 10 ml of liquid scintillator at —8°C and can be used to count isotopic compounds which are soluble in either benzene or dioxane. The preceding examples of radioactive samples counted in this liquid scintillator are by no means exhaustive and are included to present its characteristics and versatility. Other adaptations of this method can be made for counting radioactive samples in aqueous or biological fluids. For each application, however, it is advisable to determine the presence and amount of quenching.

SUMMARY

A modification of the naphthalene–dioxane–PPO liquid scintillator has been described which will allow up to 3.0 ml of an aqueous solution to be counted. The efficiency of this method in the presence of 1.0 ml water is 65.6% for C^{14} and 11.7% for tritiated water. This liquid scintillator has been used to count carbon-14 and tritium in urine, plasma, and

liver homogenate. The effect of isotope concentration, solute concentration, and the presence of acids and bases on the count rate has been investigated.

ACKNOWLEDGMENT

The author wishes to thank Dr. Steinberg for his many helpful suggestions during the course of this investigation.

REFERENCES

1. DAVIDSON, J. D., AND FEIGELSON, P., *Intern. J. Appl. Radiation and Isotopes* **2**, 1 (1957).
2. HAYES, F. N., AND GOULD, R. G., *Science* **117**, 480 (1953).
3. CHEN, P. S., *Proc. Soc. Exptl. Biol. and Med.* **98**, 546 (1958).
4. FUNT, B. L., AND HETHERINGTON, A., *Science* **125**, 986 (1957).
5. PASSMAN, J. M., RADIN, N. S., AND COOPER, J. A. D., *Anal. Chem.* **28**, 484 (1956).
6. VAUGHN, M., STEINBERG, D., AND LOGAN, J., *Science* **126**, 446 (1957).
7. STEINBERG, D., *Anal. Biochem.* **1**, 23 (1960).
8. HERBERG, R. J., *Anal. Chem.* **32**, 42 (1960).
9. KINARD, F. E., *Rev. Sci. Instr.* **28**, 293 (1957).
10. SHAPIRA, J., AND PERKINS, W. H., *Science* **131**, 414 (1960).
11. FARMER, E. C., AND BERSTEIN, I. A., *Science* **115**, 480 (1953).
12. LANGHAM, W. H., EVERSOLE, W. J., HAYES, T. N., AND TRUJILLO, T. T., *J. Lab. Clin. Med.* **47**, 819 (1956).
13. OKITA, G. T., SPRATT, J., AND LEROY, G. V., *Nucleonics* **14** (4), 76 (1956).

ANALYTICAL BIOCHEMISTRY 1, 286–290 (1960)

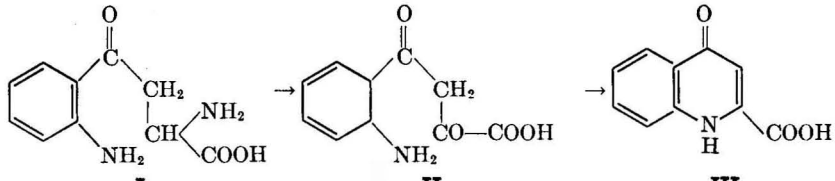
Rapid Spectrophotometric Assays for Snake Venom L-Amino Acid Oxidase Based on the Oxidation of L-Kynurenine or 3,4-Dehydro-L-proline

H. WEISSBACH, A. V. ROBERTSON, B. WITKOP, AND S. UDENFRIEND

From the Laboratory of Clinical Biochemistry, National Heart Institute and Laboratory of Chemistry, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, United States Department of Health, Education and Welfare, Bethesda, Maryland

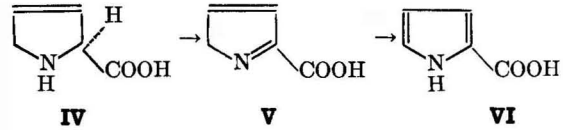
Received August 4, 1960

A recent report from this laboratory described a simple and rapid spectrophotometric assay for monoamine oxidase based on the rate of disappearance of kynuramine (1). It was suggested that a similar procedure could be used with L-amino acid oxidase, using L-kynurenine (**I**) as a substrate. Such a reaction would result in a decrease of absorption in the 360-mμ absorption peak of kynurenine during its metabolism, and a concomitant increase in absorption in the 331-mμ region due to the formation of kynurenic acid (**III**) presumably via the intermediate **II**.



I **II** **III**

In the course of our studies on the metabolism of amino acids and their derivatives, 3,4-dehydro-L-proline (**IV**) was tested as a substrate for snake venom L-amino acid oxidase and was shown to be converted to pyrrole 2-carboxylic acid (**VI**) presumably via the intermediate **V**.



IV **V** **VI**

These observations form the basis of two rapid spectrophotometric assays for L-amino acid oxidase based either on the formation of kynu-

renic acid (**III**) from L-kynurenine (**I**) or on the formation of pyrrole 2-carboxylic acid (**VI**) from dehydroproline (**IV**).

MATERIALS AND METHODS

Dried rattle snake venom (*Crotaleus adamanteus*) obtained from the Ross Allen Reptile Institute was used as the source of L-amino acid oxidase. A 20-fold purified preparation was prepared by the procedure of Ratner (2). L-Kynurenine sulfate was obtained from Mann Research Laboratories Inc. 3,4-Dehydro-L-proline (**IV**) was prepared by enzymic resolution of 3,4-dehydro-DL-proline amide with L-amino acid amidase from hog kidney (3). The racemic dehydroproline amide was obtained by a modification (4) of the reduction of pyrrole-2-carboxamide according to the procedure of Fischer and Gerlach (5).

Measurements of absorption were made in a Beckman DU spectrophotometer at room temperature. The reactions were performed in 1.5-ml capacity silica cuvettes having a light path of 1 cm. Spectra were obtained in a Cary model 14 recording spectrophotometer.

RESULTS

1. Assay of L-*Amino Acid Oxidase Based on the Rate of Formation of Kynurenic Acid*

Incubation of snake venom with L-kynurenine resulted in the formation of a compound with absorption characteristics similar to those of kynu-

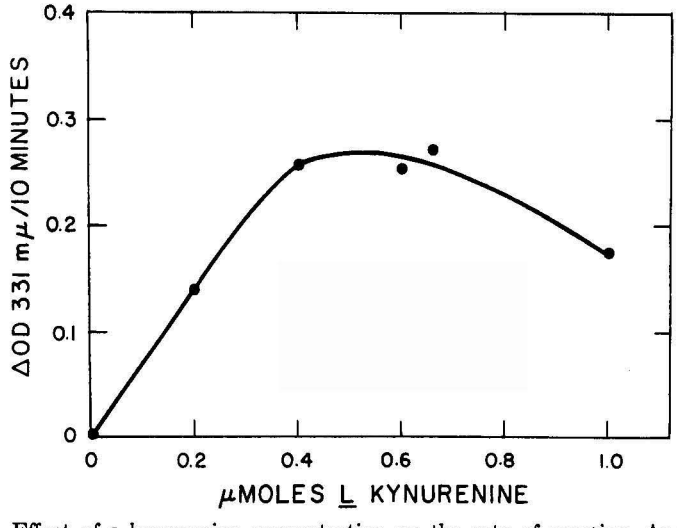


FIG. 1. Effect of L-kynurenine concentration on the rate of reaction. Assay conditions are described in text.

renic acid. The formation of the metabolite could conveniently be followed by the increase in absorption at 331 mμ.

Figure 1 shows the effect of kynurenine concentration on the initial rate of reaction. Maximum rate occurred at a concentration of 4×10^{-4} *M*. The K_m for this substrate was estimated to be about 2×10^{-4} *M*. Routine assays therefore employed 0.5 μmole kynurenine, 100 μmoles tris(hydroxymethyl)aminomethane (Tris) buffer at pH 8.0, enzyme, and water to a total volume of 1 ml. Under these conditions the rate of reaction was dependent on enzyme concentration (Fig. 2). One difficulty

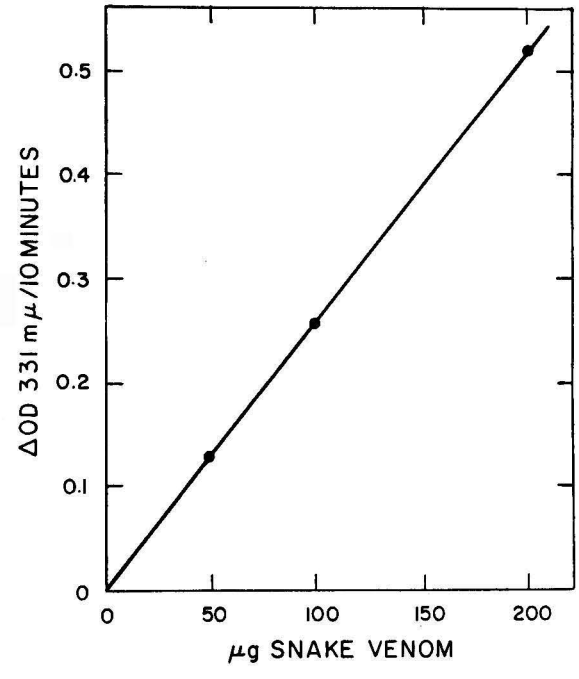


FIG. 2. Effect of enzyme concentration on the rate of reaction using L-kynurenine as substrate.

was that the high concentration of substrate employed resulted in appreciable absorption at 331 mμ, so it was most convenient to run an appropriate blank containing kynurenine with no enzyme. This was found to be adequate since the corresponding enzyme blank (enzyme without any substrate) showed no change in absorption at 331 mμ. The routine blank contained, therefore, 0.5 μmole kynurenine, 100 μmoles Tris buffer, pH 8.0, and water in a total volume of 1 ml.

2. Assay of L-*Amino Acid Oxidase Based on the Conversion of Dehydroproline (IV) to Pyrrole-2-carboxylic Acid (VI)*

This reaction was conveniently followed by the increase in absorption at 261 mμ. The substrate, at a concentration of 3 mg/ml, has little absorption at this wavelength. The effect of substrate concentration indicated that a maximum rate of reaction was obtained at a concentration of 2×10^{-2} M, and the K_m value for this substrate was about $9 \times 10^{-3} M$. The standard incubation mixture contained enzyme, 30 μmoles substrate, 50 μmoles Tris buffer at pH 8.0, and water in a total volume of 1 ml. The corresponding blank contained water in place of substrate. Under these conditions, as shown in Fig. 3, the reaction rate was de-

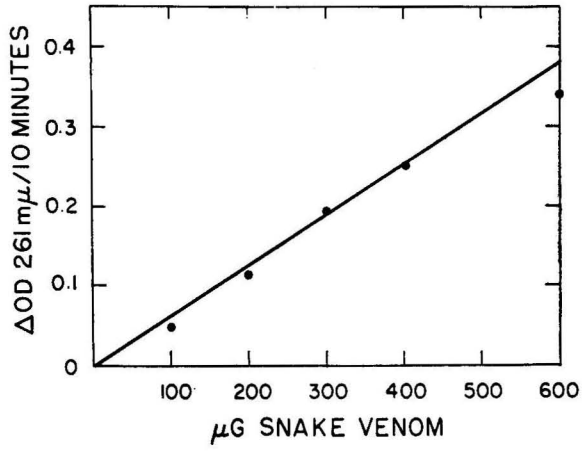


FIG. 3. Effect of enzyme concentration on the rate of reaction using 3,4-dehydro-L-proline as substrate.

pendent on enzyme concentration. The reaction product was identified as pyrrole-2-carboxylic acid both by its absorption spectrum and by its behavior on paper chromatograms.

Since proline derivatives have not been observed to be substrates for snake venom L-amino acid oxidase (6, 7), the possibility had to be examined that another enzyme was responsible for the oxidation dehydroproline. The following findings indicate that the oxidation of dehydroproline was due to L-amino acid oxidase. L-Amino acid oxidase was purified according to the procedure of Ratner (2) to yield a preparation having a specific activity towards kynurenine 17 times that of the crude snake venom. A similar increase in activity was observed with dehydro-

proline as substrate. It was also shown that L-leucine, an excellent substrate for this enzyme, caused an 80% inhibition (at 5×10^{-3} M) of the oxidation of dehydroproline.

DISCUSSION

The assays described here for snake venom L-amino acid oxidase appear to have many advantages over the more commonly employed manometric technique and compare favorably with the spectrophotometric assay based on the formation of a borate-enolphenylpyruvate complex (8, 9). They are relatively simple, sensitive, and can be completed in a matter of minutes. Although we have not attempted to assay D-amino acid oxidase with the corresponding D isomers which were unavailable, or to assay L-amino acid oxidase from other sources, the same principles could be employed.

Each of these techniques has equal merit, but the assay with L-kynurenine would be the method of choice if the enzyme preparation had appreciable absorption in the 260-mμ region. The major difficulty with the assay using kynurenine is the high absorption of kynurenine at 331 mμ, although this can be easily corrected for by a suitable blank tube.

SUMMARY

Spectrophotometric assays for snake venom L-amino acid oxidase have been developed based on the conversion of L-kynurenine (**I**) to kynurenic acid (**III**) and of 3,4-dehydro-L-proline (**IV**) to pyrrole-2-carboxylic acid (**VI**).

REFERENCES

1. WEISSBACH, H., SMITH, T. E., DALY, J., WITKOP, B., AND UDENFRIEND, S., *J. Biol. Chem.* **235,** 1160 (1960).
2. RATNER, S., *in* "Methods in Enzymology," (S. P. Colowick and N. O. Kaplan, eds.), Vol. 2, p. 204.
3. BIRNBAUM, S. M., *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. 2, p. 397. Academic Press, New York, 1955.
4. ROBERTSON, A. V., AND WITKOP, B., *J. Am. Chem. Soc.* **82,** 5008 (1960).
5. FISCHER, E., AND GERLACH, F., *Ber.* **45,** 2453 (1912).
6. ZELLER, E. A., AND MARITZ, A., *Helv. Chim. Acta* **27,** 1888 (1944).
7. ZELLER, E. A., AND MARITZ, A., *Helv. Chim. Acta* **28,** 365 (1945).
8. PITT, B. M., *J. Am. Chem. Soc.* **80,** 3799 (1958).
9. WELLNER, D., AND MEISTER, A., *J. Biol. Chem.* **235,** 2013 (1960).

ANALYTICAL BIOCHEMISTRY 1, 291–306 (1960)

Free Diffusion Measured by Biological Assay in Multilayered Cells:

I. Tables of the Average Concentration in Successive Layers and the Effect of Assay Errors[1]

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INTRODUCTION

The diffusion coefficient, D, is usually computed from the distribution of concentration obtained when Brownian motion is the only phenomenon changing the distribution from some known initial condition. Optical methods for recording the distribution (1) require initial solutions of known purity unlike analytical methods specific for a particular solute. Both can yield exceedingly precise data. For many years D of KCl determined by Cohen and Bruins (2) [see Gordon (3)] in a four-layer cell by a chemical method was accepted as a standard. Analytical methods have also been applied to biological agents. Bourdillon (4) determined D of catalase to verify his fractionation technique and then studied several virus systems (5). Polson diffused viruses using a two-layered cell (6). The most recent use of a multilayered cell, again for a virus system, was by Moore *et al.* (7).

To reduce convection several workers have introduced porous media as in recent work by Berne and Weill (8) and by Lauffer (9). An extremely precise method, which combines the assay into the diffusion apparatus, is that of Polson (10), a variation of which was employed by Allison and Humphrey (11). Here the assay was antigen-antibody precipitation, and the result gave the ratio of diffusion coefficients. For agents without immunochemical properties or for an absolute determination of D their method is not applicable.

[1] Presented at the 4th Annual Biophysical Society Meeting, Philadelphia, Feb. 1960.

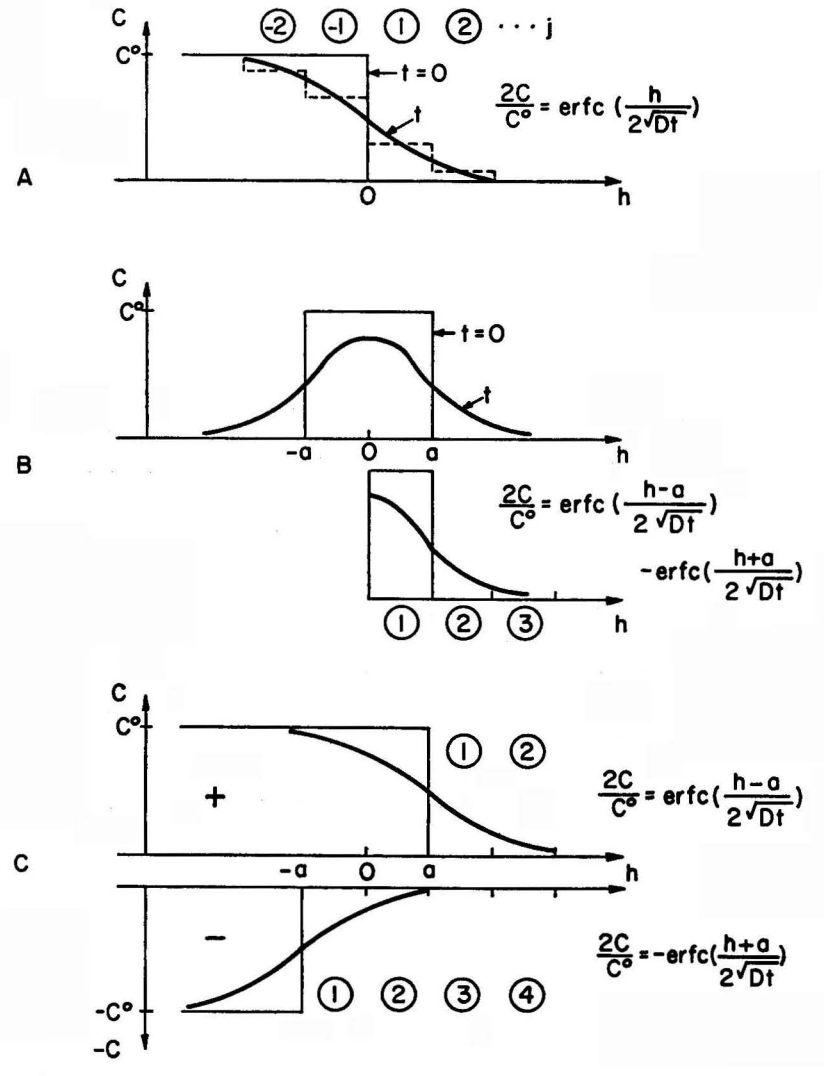

FIG. 1. Formulations for boundary and zone diffusion. *A*. Initial, infinitely sharp boundary is marked at $t = 0$, smooth curve is for time t, dotted steps indicate average concentration in successive layers, numbered above. *B*. Two types of zone diffusion with the same mathematical formulation. Upper curve is for diffusion from initial zone marked $t = 0$ both to the right and left, lower curve is for diffusion only to the right from half the quantity of solution at the same initial concentration. *C*. Separation of zone diffusion into its component double-infinite boundary diffusion terms.

This paper reconsiders the classical situation of free-solution diffusion in a multilayered cell. Emphasis is placed on the use of biological agents for which the assay error is the order of ±100% and so must not be ignored in the analysis of the data.

THEORY AND COMPUTATIONS

Double Infinite

The mathematical theory of simple diffusion for many physical arrangements has been worked out in great detail and is reviewed by Jost (12) and by Crank (13). The theory is only sketched here as it applies to one-dimensional diffusion in which a precision of 10–20% in D is sufficient. Hence, having no concern over coupled diffusion or concentration-dependent coefficients, we assume free solution in which D is constant. In Fig. 1*A* the initial infinitely sharp boundary at $h = 0$ is indicated by $t = 0$; the initial solution of concentration c° extending to $-\infty$ and the pure solvent extending above to $+\infty$. This is defined herein as a double-infinite arrangement which, experimentally, would have the h axis vertical. The concentration c at any level h, either positive or negative, at any time t is given by (12, 13)

$$c/c^\circ = (1/2)\ \mathrm{erfc}\ y \tag{1}$$

where

$$y \equiv h/(2\sqrt{Dt}) \tag{2}$$

and the error function and its complement are

$$\mathrm{erf}\ y \equiv \frac{2}{\sqrt{\pi}} \int_0^y e^{-\eta^2}\, d\eta \tag{3}$$

$$\mathrm{erfc}\ y \equiv 1 - \mathrm{erf}\ y \tag{4}$$

In order to determine D, the concentration is measured at some level h after a diffusion period t. Using tables of Eq. (1) and the measured c/c°, y is determined and then D is computed using Eq. (2).

One variant of this arrangement employed by Polson (10) and by Lauffer (9) uses a porous medium on the solvent side and agitation of the solution. Thus the concentration at the initial boundary position is c° for all t. In the free diffusion case the concentration there is always $c^\circ/2$, as can be seen from Eq. (1) since at $h = 0$ erfc $0 = 1$. Hence, as they point out, Eq. (1) needs be multiplied only by 2 to describe their situation for $h \geqslant 0$.

Assay Error

Since the relative concentration, c/c°, cannot be determined without error, it is necessary to determine what influence its error will have on the computed D. With biological assays based on the titration technique, unlike optical registrations, errors are generally constant on a percentage rather than absolute basis. Thus we choose to determine, theoretically, the percentage error in D caused by a given percentage error in c/c°. It is convenient to do this in terms of e, defined as the ratio of these errors in differential form as follows.

$$e \equiv \frac{(dD)/D}{(dc/c^\circ)/(c/c^\circ)} = \frac{d \ln D}{d \ln (c/c^\circ)} \tag{5}$$

Equations (1) and (2) substituted in Eq. (5) yield

$$e = \frac{-2d \ln y}{d \ln (c/c^\circ)} = \frac{2 \operatorname{erfc} y}{y \, \mathrm{i}^{-1}\operatorname{erf} y} \tag{6}$$

where

$$\mathrm{i}^{-1}\operatorname{erf} y \equiv d (\operatorname{erf} y)/dy = (2/\sqrt{\pi})e^{-y^2} \tag{7}$$

The functional dependence of e on y or c/c° is shown in Fig. 2. Entering with a measured c/c°, marked along the curve, the value of y can be obtained from the abscissa and the value of e from the ordinate. If c/c° is about 10^{-1}, a 100% error in c/c° will yield a 100% error in D. For $y > 0.8$ the error in D is less than the error in c/c°. In fact, if $y \approx 3$ where c/c° is only 10^{-5}, then a 100% error in the assay causes only a 10% error in D. This is a quantitative account for related observations made by Bourdillon (4) that the best experimental conditions will be realized when the material can be determined in very low relative concentration and far from the initial boundary.

Finite Sampling Layer

With nonoptical assays it is necessary to take a fraction of finite volume. Thus the average concentration in a layer, rather than the actual concentration at a given level, is determined after the diffusion period. Following Stefan (14) we shall determine such an average concentration in successive layers at any time in free diffusion. But, whereas he employed numerical integration, the relationship given by Hartree (15) for the integral of the error function complement will be used:

$$2 \operatorname{ierfc} y \equiv \mathrm{i}^{-1}\operatorname{erf} y - 2y \operatorname{erfc} y \tag{8}$$

where

$$\operatorname{ierfc} y \equiv \int_y^\infty \operatorname{erfc} \eta \, d\eta \tag{9}$$

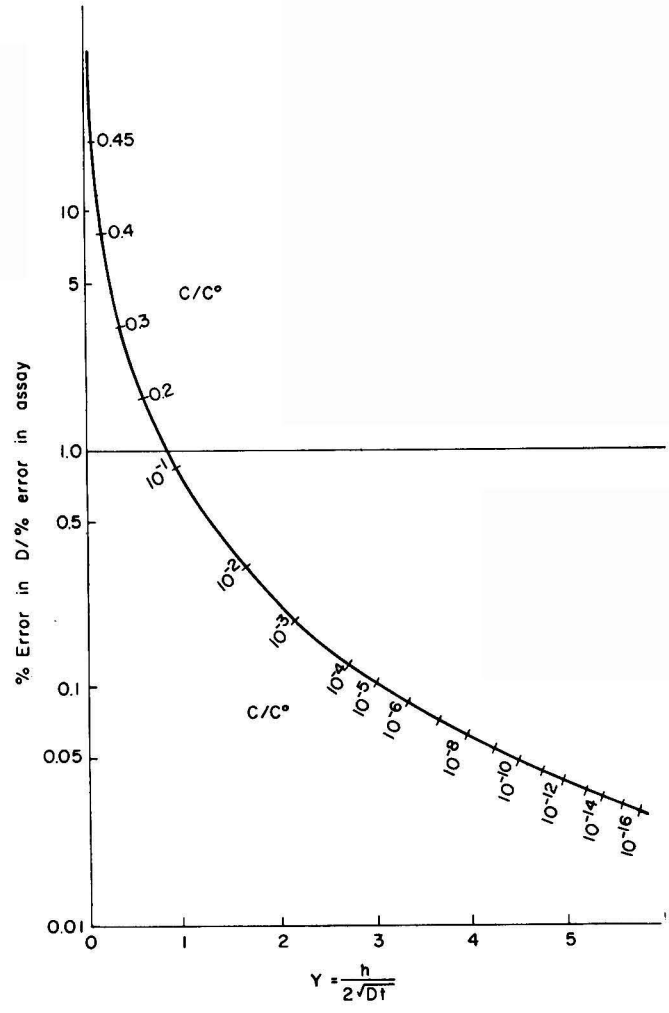

FIG. 2. Assay error characteristic of boundary diffusion. See text for meaning of symbols.

Since Hartree's table did not cover a sufficient range for our purpose, 2 ierfc y was computed over a 10^{-16}-fold range using the WPA table (16) and Eq. (8). This is presented as Table 1.

The average concentration $\bar{c}/c^\circ$ in a layer of thickness $\Delta h = h_2 - h_1$ for the double-infinite case, depicted as the dashed step lines in Fig. 1*A*, is

$$\frac{\bar{c}}{c^\circ} \equiv \int_{h_1}^{h_2} \left(\frac{c}{c^\circ}\right) dh/\Delta h \qquad (10)$$

TABLE 1
INTEGRAL OF ERROR FUNCTION COMPLEMENT

x	2 ierfc x	x	2 ierfc x	x	2 ierfc x	x	2 ierfc x
0.00	1.1283792	1.50	1.724573 (−2)	3.00	6.71007 (−6)	4.50	4.17615 (−11)
0.05	1.0311989	1.55	1.413913	3.05	4.81668	4.55	2.60175
0.10	9.396442 (−1)[a]	1.60	1.154389	3.10	3.44159	4.60	1.61313
0.15	8.536729	1.65	9.385534 (−3)	3.15	2.44770	4.65	9.95380 (−12)
0.20	7.732158	1.70	7.598600	3.20	1.73276	4.70	6.11252
0.25	6.981773	1.75	6.125845	3.25	1.22096	4.75	3.73561
0.30	6.284370	1.80	4.917529	3.30	8.56326 (−7)	4.80	2.27203
0.35	5.638511	1.85	3.930677	3.35	5.97792	4.85	1.37522
0.40	5.042552	1.90	3.128371	3.40	4.15366	4.90	8.28398 (−13)
0.45	4.494656	1.95	2.479086	3.45	2.87262	4.95	4.96604
0.50	3.992825	2.00	1.956045	3.50	1.97738	5.00	2.96268
0.55	3.534924	2.05	1.536641	3.55	1.35476	5.05	1.75899
0.60	3.118707	2.10	1.201890	3.60	9.23833 (−8)	5.10	1.03930
0.65	2.741849	2.15	9.359406 (−4)	3.65	6.27017	5.15	6.11114 (−14)
0.70	2.401965	2.20	7.256314	3.70	4.23563	5.20	3.57603
0.75	2.096645	2.25	5.600944	3.75	2.84778	5.25	2.08246
0.80	1.823473	2.30	4.304049	3.80	1.90564	5.30	1.20684
0.85	1.580054	2.35	3.292741	3.85	1.26918	5.35	6.96013 (−15)
0.90	1.364034	2.40	2.507820	3.90	8.41291 (−9)	5.40	3.99466
0.95	1.173118	2.45	1.901461	3.95	5.55022		
1.00	1.005091	2.50	1.435241	4.00	3.64428		
1.05	8.578278 (−2)	2.55	1.078459	4.05	2.38150		
1.10	7.293075	2.60	8.06711 (−5)	4.10	1.54889		
1.15	6.176211	2.65	6.00707	4.15	1.00259		
1.20	5.209789	2.70	4.45280	4.20	6.45890 (−10)		
1.25	4.377144	2.75	3.28567	4.25	4.14114		
1.30	3.662864	2.80	2.41341	4.30	2.64245		
1.35	3.052792	2.85	1.76461	4.35	1.67810		
1.40	2.534004	2.90	1.28432	4.40	1.06060		
1.45	2.094785	2.95	9.30467 (−6)	4.45	6.67120 (−11)		

[a] Entries alongside and below in the same column are to be multiplied by 10 to the power indicated in parentheses.

Substituting Eqs. (1) and (2) in Eq. (10), using Eq. (9), yields for $j > 0$

$$(\bar{c}/c^\circ)_j = [1/(4x)]\{2 \text{ ierfc } [(j-1)x] - 2 \text{ ierfc } (jx)\} \tag{11}$$

and for $j < 0$

$$(\bar{c}/c^\circ)_j = 1 - (\bar{c}/c^\circ)_{|j|} \tag{12}$$

where

$$x \equiv \Delta h/(2\sqrt{Dt}) \tag{13}$$

and j is the number of the layer above the initial boundary, as indicated in Fig. 1*A*.

Tables 2*A* and 2*B* were constructed from Table 1 using Eq. (11). Entering with the observed value of $\bar{c}/c^\circ$ for a particular layer, x is determined from which D is computed using Eq. (13). Note that x should be essentially constant from fraction to fraction. This is in contrast to the use of Eq. (2) when optical measures of point-by-point concentration are made.

The effect of an assay error in $\bar{c}/c^\circ$ can be determined in terms of e, the ratio of percentage errors, using Eq. (5) with c/c° replaced by $\bar{c}/c^\circ$. Differentiating Eq. (11) and substitution in Eq. (5) gives

$$e = \frac{8x(\bar{c}/c^\circ)_j}{\text{i}^{-1}\text{erf } [(j-1)x] - \text{i}^{-1}\text{erf } (jx)} \tag{14}$$

We prefer to tabulate $1/e^2$ instead of e to give a convenient weighting factor, as will be explained in another section. The value of e for layers 6 and above (Table 2*B*) are very close to e for layer 5 (Table 2*A*) at the same value of $\bar{c}/c^\circ$ (not at the same x). These are also very close to the e of Fig. 2 at $y = (j - \frac{1}{2})x$.

It should be noted that Tables 2*A* and 2*B* can be used for the porous medium case in which the lower solution is stirred (9, 10). Merely divide all the experimental $\bar{c}/c^\circ$ values by 2 before entering the tables to determine x and $1/e^2$.

Finite Initial Solution

Figure 1*B* depicts two arrangements for zone diffusion: In the upper one diffusion can proceed both to the left and right, whereas in the lower, diffusion is only to the right. The same formula applies to both arrangements, since there is no concentration gradient at $h = 0$ and thus no net material crosses that plane. This is the situation treated by Stefan (14, 12), and one defined herein as "semi-infinite." In Fig. 1*C* we give the expression, as in *A*, for double-infinite diffusion for boundaries located, respectively, at $h = a$ and $h = -a$. Comparison of these expressions

TABLE 2A

AVERAGE RELATIVE CONCENTRATION AND WEIGHTING FACTOR IN FIRST FIVE SUCCESSIVE LAYERS ABOVE INITIAL BOUNDARY IN DOUBLE-INFINITE DIFFUSION, FIG. 1A

	Layer No. 1		2		3		4		5	
x^a	$\bar{c}/c^\circ$	$1/e^2$	$\bar{c}/c^\circ$	$1/e^2$	$\bar{c}/c^\circ$	$1/e^2$	$\bar{c}/c^\circ$	$1/e^2$	$\bar{c}/c^\circ$	$1/e^2$
.00	5.000(−1)[b]	—	5.000(−1)	—	5.000(−1)	—	5.000(−1)	—	5.000(−1)	—
.05	4.859	2.10(−4)	4.578	2.11(−3)	4.299	6.51(−3)	4.023	1.41(−2)	3.752	2.58(−2)
.10	4.718	8.85	4.161	9.84	3.619	3.33(−2)	3.105	7.88	2.624	1.55(−1)
.15	4.578	2.09(−3)	3.754	2.56(−2)	2.983	9.40	2.293	2.38(−1)	1.703	4.98
.20	4.440	3.88	3.362	5.19	2.405	2.05(−1)	1.619	5.51	1.023	1.21(+0)
.25	4.302	6.31	2.989	9.19	1.896	3.87	1.092	1.09(+0)	5.674(−2)	2.48
.30	4.166	9.43	2.638	1.49(−1)	1.462	6.61	7.025(−2)	1.94	2.904	4.53
.35	4.032	1.33(−2)	2.312	2.25	1.103	1.05(+0)	4.317	3.18	1.372	7.63
.40	3.901	1.79	2.012	3.24	8.141(−2)	1.58	2.535	4.93	5.992(−3)	1.20(+1)
.45	3.772	2.32	1.739	4.49	5.882	2.28	1.423	7.28	2.421	1.81
.50	3.645	2.93	1.494	6.02	4.163	3.16	7.645(−3)	1.03(+1)	9.063(−4)	2.60
.55	3.522	3.61	1.275	7.86	2.888	4.26	3.936	1.42	3.149	3.62
.60	3.402	4.36	1.082	1.00(+0)	1.966	5.59	1.944	1.90	1.017	4.90
.65	3.285	5.18	9.137(−2)	1.25	1.313	7.18	9.225(−4)	2.48	3.056(−5)	6.48
.70	3.172	6.05	7.673	1.51	8.621(−3)	9.05	4.206	3.17	8.549(−6)	8.40
.75	3.062	6.98	6.414	1.85	5.562	1.12(+1)	1.845	4.00	2.227	1.07(+2)
.80	2.956	7.95	5.338	2.21	3.529	1.37	7.783(−5)	4.98	5.404(−7)	1.35
.85	2.854	8.95	4.424	2.60	2.203	1.71	3.160	6.12	1.220	1.68
.90	2.755	9.97	3.652	3.03	1.354	1.99	1.234	7.45	2.565(−8)	2.06
.95	2.661	1.10(−1)	3.005	3.50	8.186(−4)	2.35	4.639(−6)	8.99	5.014(−9)	2.51
1.00	2.570	1.20	2.464	4.00	4.873	2.77	1.677	1.08(+2)	9.110(−10)	3.04
1.05	2.482	1.31	2.014	4.55	2.856	3.24	5.826(−7)	1.28	1.538	3.64
1.10	2.399	1.41	1.641	5.14	1.647	3.77	1.946	1.51	2.411(−11)	4.32
1.15	2.319	1.51	1.333	5.78	9.350(−5)	4.36	6.243(−8)	1.77	3.507(−12)	5.11
1.20	2.242	1.60	1.080	6.47	5.223	5.02	1.925	2.06	4.733(−13)	5.99
1.25	2.169	1.69	8.726(−3)	7.21	2.870	5.76	5.696(−9)	2.39	5.925(−14)	6.99
1.30	2.100	1.78	7.028	8.01	1.551	6.57	1.618	2.77	6.877(−15)	8.12
1.35	2.033	1.86	5.645	8.87	8.245(−6)	7.47	4.411(−10)	3.18	7.398(−16)	9.37
1.40	1.970	1.93	4.521	9.80	4.310	8.47	1.153	3.64	—	—
1.45	1.909	2.00	3.609	1.08(+1)	2.214	9.56	2.893(−11)	4.14	—	—

[a] $x = \Delta h/(2\sqrt{Dt})$.

[b] Entries alongside and below in the same column are to be multiplied by 10 to the power indicated in parentheses.

TABLE 2B

AVERAGE RELATIVE CONCENTRATION IN LAYERS 6–28 ABOVE INITIAL BOUNDARY IN DOUBLE-INFINITE DIFFUSION, FIG. 1A[a]

x[b]	Layer No., Entries[a] are $\bar{c}/c^\circ$ 6	7	8	9	10	11	12	13	14	15
.00	5.000(−1)[c]	5.000(−1)	5.000(−1)	5.000(−1)	5.000(−1)	5.000(−1)	5.000(−1)	5.000(−1)	5.000(−1)	5.000(−1)
.05	3.487	3.229	2.980	2.739	2.510	2.289	2.081	1.885	1.699	1.527
.10	2.185	1.792	1.446	1.149	8.972(−2)	6.895(−2)	5.208(−2)	3.868(−2)	2.822(−2)	2.024(−2)
.15	1.221	8.437(−2)	5.614(−2)	3.595(−2)	2.214	1.310	7.447(−3)	4.064(−3)	2.129(−3)	1.070(−3)
.20	6.052(−2)	3.345	1.725	8.283(−3)	3.702(−3)	1.538(−3)	5.936(−4)	2.126(−4)	7.067(−5)	2.178(−5)
.25	2.653	1.112	4.170(−3)	1.396	4.166(−4)	1.107(−4)	2.615(−5)	5.489(−6)	1.023(−6)	1.693(−7)
.30	1.027	3.096(−3)	7.926(−4)	1.719(−4)	3.152(−5)	4.878(−6)	6.366(−7)	6.998(−8)	6.472(−9)	5.034(−10)
.35	3.517(−3)	7.227(−4)	1.186	1.549(−5)	1.607(−6)	1.322(−7)	8.604(−9)	4.428(−10)	1.799(−11)	5.768(−13)
.40	1.066	1.417	1.400(−5)	1.025(−6)	5.546(−8)	2.211(−9)	6.487(−11)			
.45	2.864(−4)	2.338(−5)	1.308(−6)	5.000(−8)	1.300(−9)	2.293(−11)				
.50	6.841(−5)	3.256(−6)	9.705(−8)	1.801(−9)	2.073(−11)					
.55	1.455	3.835(−7)	5.721(−9)	4.798(−11)						
.60	2.757(−6)	3.822(−8)	2.682(−10)	9.450(−13)						
.65	4.664(−7)	3.226(−9)	9.993(−12)							
.70	7.039(−8)	2.304(−10)								

x	Layer No. 16	17	18
.05	1.366(−1)	1.217(−1)	1.080(−1)
.10	1.426(−2)	9.863(−3)	6.703(−3)
.15	5.155(−4)	2.382(−4)	1.055(−4)
.20	6.222(−6)	1.646(−6)	4.037(−7)
.25	2.483(−8)	3.230(−9)	3.723(−10)

x	Layer No. 19	20	21	22	23	24	25	26	27	28
.05	9.546(−2)	8.402(−2)	7.363(−2)	6.426(−2)	5.584(−2)	4.832(−3)	4.163(−2)	3.571(−2)	3.050(−2)	2.594(−2)
.10	4.473(−3)	2.931(−3)	1.885(−3)	1.191(−3)	7.381(−4)	4.491(−4)	2.682(−4)	1.571(−4)	9.036(−5)	5.099(−5)
.15	4.480(−5)	1.823(−5)	7.105(−6)	2.652(−6)	9.484(−7)	3.248(−7)	1.065(−7)	3.344(−8)	1.005(−8)	2.893(−9)
.20	9.166(−8)	1.927(−8)	3.748(−9)	6.748(−10)	1.124(−10)	1.732(−11)	2.470(−12)	3.256(−13)	3.971(−14)	—

[a] Use $1/e^2$ of fraction 5, Table 2A at the same $\bar{c}/c^\circ$ (not x) as the entry in this table for the weighting factor.

[b] $x = \Delta h/(2\sqrt{Dt})$

[c] Entries alongside and below in the same column are to be multiplied by 10 to the power indicated in parentheses.

reveals that the semi-infinite zone diffusion case can be considered as made up of two double-infinite boundary diffusions, one being negative. A table for the semi-infinite case can thus be constructed from the double-infinite one (Tables 2*A* and 2*B*). For example, the average concentration in the 3rd layer of Fig. 1*B*, counting the initial zone as the first layer, would be the average concentration in the double-infinite case of layer 2 minus 4. Such a table is not given here because it is so easily constructed for the x values of Tables 2*A* and 2*B*. It should be noted that Stefan's table covers values of x spaced at 0.01 intervals, however does not go beyond $\bar{c}/c^\circ$ of 10^{-3} with more than two significant figures. Also, the reverse computation of the double-infinite case from Stefan's semi-infinite table cannot be made.

Finite Cell (Five Layer)

Stefan (14) also considered the effect of an upper limit on the cell. The procedure for generating the solution for such a case is known as the method of reflection and is illustrated in Fig. 3*A*. Without the upper boundary, represented by the right-hand vertical line, the concentration would be given by the dashed line extending to the right. With the boundary, the same curve is merely reflected back into the cell. Figure 3*A* also shows the average concentration as the solid histogram. Following Stefan, Jost (12) gave a table for a 16-layer cell and Kawalki (17, 12, 18) for four layers. We have arbitrarily chosen a five-layer cell to be filled initially with solution in the bottom layer and solvent in the upper four layers, and have constructed a $\bar{c}/c^\circ$ table from the double-infinite case as illustrated in Fig. 3*B*. Here the method of reflection of Fig. 3*A* is used for both component boundaries of Fig. 1*C* represented, respectively, by the dotted strips in Fig. 3*B*. For example, the average concentration in the fourth layer is the sum of double-infinite fractions 3, 6, 13, 16, 23, 26, 33 . . . minus the sum of fractions 5, 8, 15, 18, 25, 28, 35. . . . The results are given as Table 3.

Since we have chosen to give a complete double-infinite table (2*A* and 2*B*) for boundary spreading rather than a complete semi-infinite table (for zone spreading), the method of reflection involves both the positive and negative terms indicated in Fig. 3*B*. If the semi-infinite table were used, then one would merely add the values of $\bar{c}/c^\circ$ from layers reflected back into the cell. This was done from Stefan's table and agreed with Table 3 in the regions where the tables overlap.

By comparing the figures in the columns for fractions 2 and 5, Table 3, it can be seen that a given percentage error in the concentration measurement will have a much smaller effect on x in fraction 5. In fact, fraction 2 contains almost no information about x, and hence D, since $\bar{c}/c^\circ$ is nearly

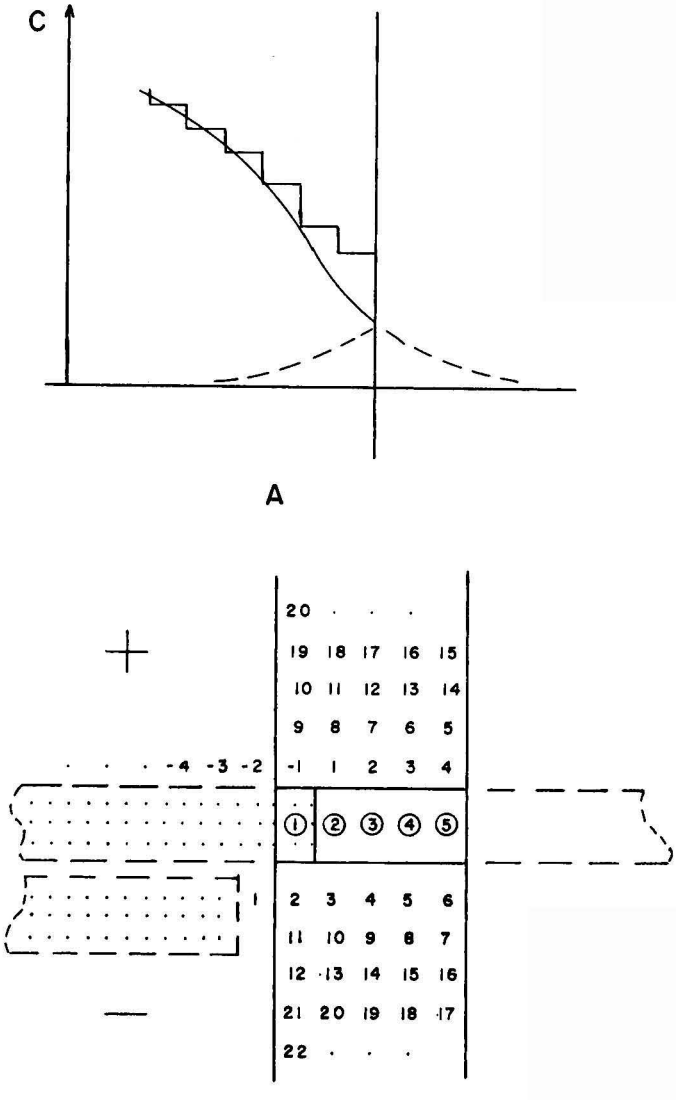

FIG. 3. Method of reflection. *A*. Effect of an upper boundary at right. Solid curve is for free diffusion, dotted curve shows reflected part, solid steps indicate average concentration after reflection. *B*. Five-layer diffusion cell. Solute is initially in layer one, solvent in layers two–five. The upper dotted region and numbers indicate the positive component boundary or upper curve of Fig. 1*C*. Numbers below refer to the negative boundary diffusion as in lower curve of Fig. 1*C*.

TABLE 3

AVERAGE RELATIVE CONCENTRATION AND WEIGHTING FACTOR IN FIVE LAYER RESTRICTED DIFFUSION CELL

	Layer No.[a] 1		2		3		4		5	
x[b]	$\bar{c}/c^{o}$	$1/e^{2}$	$\bar{c}/c^{o}$	$1/e^{2}$	$\bar{c}/c^{o}$	$1/e^{2}$	$\bar{c}/c^{o}$	$1/e^{2}$	$\bar{c}/c^{o}$	$1/e^{2}$
.00	2.000(−1)[d]	—	2.000(−1)	—	2.000(−1)	—	2.000(−1)	—	2.000(−1)	—
.10	2.002	—	2.001	—	2.000	—	2.000	—	2.000	—
.20	2.294	8.3(−2)	2.184	3.5(−2)	2.000	9.0(−6)	1.817	5.8(−2)	1.703	1.8(−1)
.25	2.726	1.73(−1)	2.445	7.33(−2)	1.994	4.87(−4)	1.552	2.04(−1)	1 283	7.31
.30	3.198	1.94	2.712	7.37	1.965	6.85(−3)	1.266	4.35	8.593(−2)	1.79(+0)
.35	3.656	1.84	2.930	5.14	1.887	3.72(−2)	9.998(−2)	7.78	5.265	3.36
.40	4.087	1.69	3.087	2.76	1.760	1.11(−1)	7.647	1.29(+0)	3.014	5.48
.45	4.489	1.52	3.184	1.04	1.597	2.36	5.668	2.00	1.634	8.14
.50	4.860	1.36	3.230	1.46(−3)	1.418	4.10	4.079	2.93	8.480(−3)	1.14(+1)
.55	5.203	1.21	3.233	4.55(−4)	1.236	6.28	2.858	4.09	4.236	1.53
.60	5.516	1.07	3.205	5.51(−3)	1.063	8.78	1.956	5.48	2.044	2.00
.65	5.801	9.38(−2)	3.154	1.51(−2)	9.045(−2)	1.16(+0)	1.310	7.11	9.526(−4)	2.57
.70	6.061	8.25	3.086	2.83	7.632	1.47	8.613(−3)	9.00	4.291	3.25
.75	6.297	7.18	3.006	4.25	6.396	1.81	5.560	1.12(+1)	1.867	4.07
.80	6.510	6 28	2.921	5.69	5.330	2.18	3.528	1.37	7.837(−5)	5.03
.85	6.704	5.49	2.832	7.22	4.421	2.59	2.203	1.66	3.172	6.16
.90	6.879	4.82	2.742	8.63	3.651	3.02	1.354	1.99	1.237	7.47
.95	7.039	4.25	2.653	1.01(−1)	3.005	3.49	8.186(−4)	2.36	4.648(−6)	9.00
1.00	7.184	3.75	2.565	1.15	2.464	4.00	4.873	2.77	1.678	1.08(+2)
1.05	7.317	3.30	2.479	1.27	2.014	4.55	2.856	3.24	5.828(−7)	1.28
1.10	7.437	2.92	2.397	1.37	1.641	5.15	1.647	3.77	1.946	1.51
1.15	7.548	2.64	2.318	1.50	1.333	5.79	9.350(−5)	4.36	6.244(−8)	1.77
1.20	7.650	2.37	2.241	1.59	1.080	6.46	5.223	5.02	1.925	2.06
1.25	7.744	2.11	2.169	1.65	8.726(−3)	7.21	2.870	5.76	5.696(−9)	2.40
1.30	7.830	1.92	2.100	1.77	7.028	8.02	1.551	6.57	1.618	2.72
1.35	7.911	1.75	2.033	1.86	5.645	8.87	8.245(−6)	7.47	4.410(−10)	3.12
1.40	7.985	1.60	1.970	1.94	4.521	9.81	4.310	8.47	1.153	3.64
1.45	8.055	—	1.909	—	3.609	—	2.214	—	2.893(−11)	—

[a] Initially ($x = \infty$), solution c^{o} in layer 1, solvent in layers 2–5.

[b] $x = \Delta h/(2\sqrt{Dt})$.

[c] Last digit may be in error by ±1, for all $\bar{c}/c^{o}$.

[d] Entries alongside and below in the same column are to be multiplied by 10 to the power indicated in parentheses.

[e] Last digit may be in error by ±2, for all $1/e^{2}$.

constant for all x. To quantitate this property, we again use e as the ratio of percentage error in D to the percentage error in the assay, but must compute it from Table 3 itself since an analytical expression is not available. To do this e is expressed as

$$e = \frac{(\Delta D)/D}{(\Delta \bar{c}/c^\circ)/(\bar{c}/c^\circ)} = -\frac{2(\Delta x)/x}{2.303\Delta \log\ (\bar{c}/c^\circ)} \tag{15}$$

We prefer to tabulate $1/e^2$, computed from Eq. (15), for each entry using the values on either side ($\Delta x = 0.10$).

Weighting Factors

From Fig. 2 and Tables 2 and 3, it can be seen that if the concentration is measured with the same relative precision for all levels in a given experiment, the D value will be much more precise the further the fraction is away from the initial boundary, where the concentration is also the lowest. It then would be clearly inconsistent to merely average the computed D values without regard for their weight. In general, if

$$p = f(q) \tag{16}$$

the relationship between the variance v of p and q is

$$v_p = (\partial f/\partial q)^2 v_q \tag{17}$$

and the weight w to attach to each of a series of p's in computing a mean is inversely proportional to their variance (19). Thus

$$w_p = 1/v_p \tag{18}$$

For our particular case D is a function of $\bar{c}/c^\circ$ so, symbolically from Eqs. (17) and (18).

$$w_D = \left[\frac{d(\bar{c}/c^\circ)}{dD}\right]^2 \Big/ v_{\bar{c}/c^\circ} \tag{19}$$

This can be rearranged to

$$w_D = \left(\frac{1}{D^2}\right)\left(\frac{1}{e^2}\right) \Big/ \left[\frac{v_{\bar{c}/c^\circ}}{(\bar{c}/c^\circ)^2}\right] \tag{20}$$

where the term in the square brackets is the square of the assay error expressed as a fraction. Thus, the weight to assign any value of D computed from the argument of the table is proportional to $1/e^2$, and accounts for the decision to tabulate this quantity as a weighting factor. The weighted mean $\bar{D}$, for a series, j, of values, which may or may not be from the same experiment, is

$$\bar{D} = (\Sigma w_j D_j)/\Sigma w_j \tag{21}$$

where w_j is computed from Eq. (20). The variance of the weighted mean is (19)

$$v_{\bar{D}} = 1/\Sigma w_j \tag{22}$$

DISCUSSION

This paper presents some of the theoretical properties of the error function applied to certain diffusion cell arrangements and suggests exploitation of a favorable range of c/c° of about 10^{-4} to 10^{-5} if the assay error is as great as $\pm 100\%$. Convective disturbances further increasing the error of c/c°, can amount to only 1 part in 10^4–10^5 if a $\pm 10\%$ error in D is acceptable. This is not unlike a similar stability in the diffusion apparatus required in refined optical measurements in which D is reported to 1 part in 10^3 or 10^4 using data obtained from concentration ratios greater than 10^{-1} where $e > 1$.

The exploitation of the range of c/c° of 10^{-4}–10^{-5} requires that initial solutions have concentrations of 10^4–10^5 times that of the most dilute fraction measureable. This is particularly possible with infectivity measurements of a virus. In effect, high titer is exchanged for precision in the assay.

The use of nonoptical methods for specific solutes allows small molecules to be used to provide a density gradient for convective stability. Alternatively, a porous medium could be used. In this case it might be difficult to cut the precisely equal segments implied in Tables 2 and 3. The procedure of Lauffer (9) using the complete error function table as though the segment were infinitesimally thin, and then correcting the position of the center of the segment has the advantage of not requiring equal width slices.

In practice it is not possible to have a truly double-infinite cell. If fractions will be taken in a prearranged manner, an appropriate table of $\bar{c}/c^\circ$ vs. x can be constructed in advance from Table 2 using the method of reflection for any cell arrangement to be used. It is easier to make the table if all the layers have the same width and if the depth of the initial solution is an integral multiple of this width. The actual width Δh in cm is used only in converting an x value to D. The effect of the top and bottom is illustrated in Tables 1*A* and 1*B* of the succeeding paper (20) where the actual cell could be considered a 14-layer cell in which the initial solution occupied the lower nine layers. The main effect of the top is to alter the values of $\bar{c}/c^\circ$ from those for the double-infinite cell given here only at small values of x (long t), especially in the layer next to the top [Table 1*A* Ref. (20)]. The finite depth of solution prevents any layer from maintaining the initial concentration. The pool of the lower "layers"

which would be considered to exist in the large base unit has, of course, an average concentration very close to $c°$, and the slight correction necessary in using this as a measure of $c°$ can be obtained from Table 1*B* of Ref. (20).

Recently, Berne and Weill (8) have used the gel technique in another variation. They put a very thin layer of solution on top of the gel in a cut-off syringe. After the diffusion period, the plunger is used to push out the gel for slicing. The formulas for diffusion from an infinitely thin initial zone are somewhat simpler than for the finite layer; however, the same considerations apply as to the weighting. Since the initial and final quantity of diffusing solute in any layer are zero, the quantity in each layer must reach a maximum for some value of the argument. Operation of the cell near this value will yield little information as to D. However, in the region where the quantity recovered is $< 10^{-3}$ of the initial quantity used, the curves are similar to the $\bar{c}/c°$ curves of the double-infinite case. This, in effect, shows that it is the particles nearest the initial boundary that are the ones which get out ahead. Once an appropriate table is constructed, e.g., Table 2 or 3, it is not necessary to experimentally use an infinitely thin layer, with its resulting requirement of high concentration.

SUMMARY

The expressions for free diffusion from an initial infinitely sharp boundary were considered in terms of the type of concentration measurement made and its error. The percentage error in the computed diffusion coefficient D will be less than the percentage concentration error if $c/c° < 0.1$ and $h/(2\sqrt{Dt}) > 0.8$. Here $c°$ is the initial concentration below the boundary, t the time and h the height of the measurement plane above the boundary. If the concentration can be measured where $c/c° \approx 10^{-5}$, then a 100% error in $c/c°$ causes only a 10% error in D.

A table is given of the integral of the error function complement (ierfc x) to seven figures for 0.05 increments in the argument to 2.55 and six figures to 5.40. The average relative concentration $\bar{c}/c°$ of successive, finite volume fractions was computed from ierfc x for a multilayered, double-infinite cell intended for nonoptical diffusion measurements. Using this table and the method of reflection, an appropriate table can be constructed for any finite cell. A special case of such restricted diffusion is presented for a five-layer cell in which the initial solution occupies the lowest layer. For both the double-infinite and the five-layer cells the weighting factor for each entry in the table of $\bar{c}/c°$ is given for use in obtaining a weighted mean diffusion coefficient from all the data from several fractions in one experiment or replicate experiments.

REFERENCES

1. GOSTING, L. J., *Advances in Protein Chem.* **11,** 430 (1956).
2. COHEN, E., AND BRUINS, H. R., *Z. physik. Chem.* **113,** 157 (1924).
3. GORDON, A. R., *Ann. N. Y. Acad. Sci.* **46,** 285 (1945).
4. BOURDILLON, J., *J. Gen. Physiol.* **24,** 459 (1941).
5. BOURDILLON, J., *J. Gen. Physiol.* **25,** 263 (1941).
6. POLSON, A., *Proc. Soc. Exptl. Med. Biol.* **67,** 294 (1948).
7. MOORE, D. H., LASFARGUES, E. Y., MURRAY, M. R., HAAGENSEN, C. D., AND POLLARD, E. C., *J. Biophys. Biochem. Cytol.* **5,** 85 (1959).
8. BERNE, E. AND WEILL, M., *J. chim. phys.* **56,** 103 (1959).
9. LAUFFER, M., *in* Abstracts, p. 45. 4th Annual Meeting Biophysical Society, Philadelphia, 1960.
10. POLSON, A., *Biochim. et Biophys. Acta* **29,** 426 (1958).
11. ALLISON, A. C., AND HUMPHREY, J. H., *Nature* **183,** 1590 (1959).
12. JOST, W., "Diffusion in Solids, Liquids, Gases." Academic Press, New York, 1952.
13. CRANK, J., "The Mathematics of Diffusion." Oxford University Press, Oxford, 1956.
14. STEFAN, J., *Sitzber. Wiener Akad. Wissensch. II* **79,** 161 (1879).
15. HARTREE, D. R., *Mem. Manchester Phil. Soc.* **80,** 85 (1936).
16. "Tables of the Error Function and Its Derivative," National Bureau of Standards Applied Mathematics Series 41, Washington, D. C., 1954 (prepared by the Works Projects Administration, 1941).
17. KAWALKI, W., *Ann. Physik* **52,** 166 (1894).
18. LONGSWORTH, L. G., *Ann. N. Y. Acad. Sci.* **46,** 211 (1945).
19. FINNEY, D. J., "Statistical Methods in Biological Assay," p. 365. Hafner Pub. Co., New York, 1952.
20. BREESE, S. S., JR., AND TRAUTMAN, R., *Anal. Biochem.* **1,** 307 (1960).

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Free Diffusion Measured by Biological Assay in Multilayered Cells:

II. Diffusion Coefficient of Foot-and-Mouth Disease Virus Determined by Infectivity[1]

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INTRODUCTION

A multilayer ground-glass diffusion cell designed by Dan H. Moore to estimate the size of a mammary tumor agent in mice (1) was adapted for use in the determination of the diffusion coefficient of foot-and-mouth disease virus (FMDV) type A, strain 119. The diffusion coefficient D was desired since the sedimentation coefficient of FMDV has been previously determined by means of infectivity assay (2). Combining the two coefficients with other data allows an estimation of the molecular weight of the infective particle; or D alone determines the diameter of the equivalent sphere, which should be comparable to that determined by electron microscopy (3, 4).

The particular cell arrangements used were suitable for work with small quantities of relatively impure virus suspensions. Quantities of virus pure enough to do standard optical diffusion studies have not yet become available. Since infectivity was to be the measure of concentration, other macromolecules in the fluids would not interfere with the assay. The range of infectivity that may be assayed, approximately a 10^5 difference between starting material and most dilute final fraction, was well suited to the development of the method since here a 100% error in the assay effects only a 10% error in D.

METHODS

Infectivity

FMDV, type A, strain 119 was used as collected from infected cultures of bovine kidney epithelial cells. In some experiments virus in the infec-

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tious fluid was partially purified by alcohol precipitation followed by collection and dialysis of the precipitate as described by Bachrach (5). The virus fluids were of greater density than the solvent (0.2 *M* NH_4OAc, pH 7.6) into which the virus diffused so that no artificial increase in density was needed.

The plaque assay method (6) was used to measure infectivity. Samples at appropriate dilutions were inoculated into 4-oz prescription bottles containing confluent cultures of bovine kidney cells. After incubation for 90 min at 37°C an agar overlay was applied. Plaques were counted after approximately 72 hr incubation at 37°C.

Cell Arrangements[2]

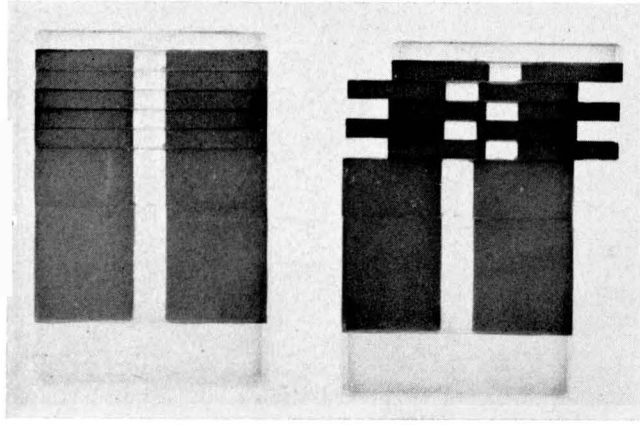
In early experiments, the initial boundary was formed between a virus solution in a single base unit 25 mm high and an upper compartment consisting of assorted movable layers 2 and 3 mm in height. The channel of the diffusion cell was 5 mm square so that the volumes of the movable layers were 0.05 and 0.075 ml, respectively. Subsequently a standard cell was devised which consisted of a single base unit 27 mm high with five 3-mm movable layers above it. This cell (Fig. 1) is equivalent to a finite 14-layer cell with the lower nine layers initially filled with virus fluid.

The base unit of the assembled cell was filled from the top with virus fluid (Fig. 1). After initial temperature equilibration the base unit was displaced to one side. The upper layers were then thoroughly rinsed and filled with solvent. After further temperature equilibration the solvent-solution sections were aligned so diffusion could proceed. A Spinco model H electrophoresis-diffusion apparatus water bath was used at 4.00 ± 0.05°C. Fractionation at the end of the experiment was done by alternate displacement of the layers (Fig. 1).

Another cell arrangement was designed to use a smaller volume of starting virus fluid. It was a five-layer cell made up of 2-mm layers; the lower section contained 0.05 ml of virus fluid, and the upper four layers contained a total of 0.20 ml solvent (0.2 *M* NH_4OAc, pH 7.6). In this case the cell was filled from the top and bottom by reversing the cell in order to avoid rinsing the upper layers. The arrangements are shown in Fig. 2. Fractionation was effected by displacement of the layers as before.

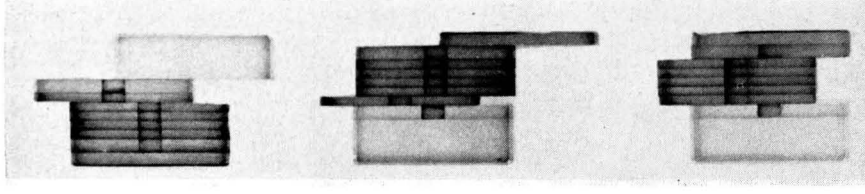
In all cases fractions were removed with finely drawn Pasteur pipets, the compartment layer being rinsed several times with the fluid in the first tube of the dilution set. The concentration measured was assumed to be the average concentration $\bar{c}$ of any particular layer prior to fractionation.

[2] All glass component parts for the various cell arrangements were manufactured by Pyrocell Manufacturing Co., 207–11 East 84th St., New York 28, N. Y.



A B

FIG. 1. Double-infinite cell arrangement. *A*. Layers aligned for diffusion run. *B*. Layers displaced for fraction collection.



A B C

FIG. 2. Five-layer cell arrangement. *A*. Cell inverted for loading of virus sample. *B*. Cell with virus layer isolated and upper layers open for solvent loading. *C*. Cell layers aligned for diffusion run. After run, layers are displaced as in Fig. 1*B*.

Calculation of Diffusion Coefficient D

The methods and tables for the double-infinite case and for the five-layer cell appear in the preceding paper (7). For the standard 14-layer cell described above, the theoretical values which differ from the double-infinite case are given in Table 1*A*. The values for the average concentration for a pool of the lower six or nine layers of the standard cell appear in Table 1*B*. Such a sample can be used as a measure of c° to check against a control tube held in the water bath during the experiment. When required, tables were computed for the other essentially equivalent double-infinite cell arrangements in which experiments were done.

For a series of replicate runs the mean of $\bar{c}/c^\circ$ and its standard deviation (assay error) were determined for each layer, and the corresponding

TABLE 1*A*

AVERAGE RELATIVE CONCENTRATION AND WEIGHTING FACTOR FOR THE FIVE LAYERS ABOVE INITIAL BOUNDARY IN DIFFUSION FROM LOWER NINE LAYERS IN FOURTEEN-LAYER CELL

	Layer No. 10		11		12		13	
x^a	$\bar{c}/c^\circ$	$1/e^x$	$\bar{c}/c^\circ$	$1/e^x$	$\bar{c}/c^\circ$	$1/e^x$	$\bar{c}/c^\circ$	$1/e^x$
.10	5.57(−1)[b]	—	5.28(−1)	—	5.05(−1)	—	4.88(−1)	—
.15	4.80	2.69(−2)	4.11	1.03(−1)	3.54	1.92(−1)	3.14	4.72(−1)
.20	4.48	1.18	3.44	9.91(−2)	2.58	3.65	1.95	9.19
.25	4.31	8.05(−3)	3.00	1.11(−1)	1.94	5.15	1.20	1.50(+0)
.30	4.17	9.71	2.64	1.54	1.45	7.12	7.34(−2)	2.29
.35	[c]		[d]		1.10	1.02(+0)	4.39	3.42
.40							2.55	5.06
.45							1.43	7.34
.50							7.65(−3)	1.03(+1)
							[f]	

Layer 14								
x	$\bar{c}/c^\circ$	$1/e^x$	x	$\bar{c}/c^\circ$	$1/e^x$	x	$\bar{c}/c^\circ$	$1/e^x$
.10	4.80(−1)	—	.40	7.06(−3)	1.37(+1)	.70	8.62(−6)	8.47(+1)
.15	2.92	6.57(−1)	.45	2.71	1.98	.75	2.24	1.08(+2)
.20	1.63	1.58(+0)	.50	9.75(−4)	2.77	.80	5.41(−7)	1.35
.25	8.33(−2)	3.16	.55	3.29	3.77	.85	1.22	1.68
.30	3.93	5.58	.60	1.04	5.02	.90		
.35	1.72	9.03	.65	3.10(−5)	6.57			

[a] $\Delta h/(2\sqrt{Dt})$.

[b] Entries alongside and below in the same column are to be multiplied by 10 to the power indicated in parentheses.

[c–g] For $x \geq$ this layer, $\bar{c}/c^\circ$ is given in the double-infinite table for layers 1–5, respectively.

TABLE 1*B*

AVERAGE RELATIVE CONCENTRATION BELOW INITIAL BOUNDARY IN DIFFUSION FROM LOWER NINE LAYERS IN FOURTEEN-LAYER CELL

Entries are $\bar{c}/c^\circ$

x^a	Pool of layers 1–6	Pool of layers 1–9	x	Pool 1–9
.10	.761	.716	.60	.948
.15	.876	.794	.70	.955
.20	.935	.844	.80	.961
.25	.965	.875	.90	.965
.30	.981	.896	1.00	.969
.35	.990	.910	1.10	.972
.40	.996	.922	1.20	.974
.45	.997	.937	1.30	.976
.50	.999	.943	1.50	.979

[a] $x = \Delta h/(2\sqrt{Dt})$.

values for x and $1/e^2$ were read from the tables. The value of D was calculated from

$$D = \frac{(\Delta h)^2}{4tx^2} \tag{1}$$

where Δh is the layer thickness in cm and t is the time in sec. (1) This D, the assay error, and the weighting factor, $1/e^2$, were combined to give the weight w, for each layer according to

$$w = \left(\frac{1}{D^2}\right)\left(\frac{1}{e^2}\right)\Big/\left[\frac{v_{\bar{c}/c^\circ}}{(\bar{c}/c^\circ)^2}\right] \tag{2}$$

where v is the variance. The weighted mean diffusion coefficient D and its variance $v_{\bar{D}}$ were calculated from

$$\bar{D} = (\Sigma w_j D_j)/\Sigma w_j \tag{3}$$

$$v_{\bar{D}} = 1/\Sigma w_j \tag{4}$$

where j represents any layer in the series.

In order to calculate particle diameter, the standard diffusion equation

$$D = RT/6\pi N\eta r \tag{5}$$

was used where R is the gas constant, T the absolute temperature, N Avogadro's number, r the particle radius, and η the viscosity in poises, taken as 1.75×10^{-2} poise for 0.2 M NH_4OAc at 4°C.

RESULTS

Double-Infinite Cell

Two double-infinite cell systems were used which differed only slightly from the standard 14-layer cell. The first arrangement had a 25-mm and a 2-mm layer below the initial boundary and 2, 3, 3, 3, and 4 mm layer

TABLE 2
DIFFUSION COEFFICIENT OF FMDV-A119 BY INFECTIVITY ASSAY IN A DOUBLE-INFINITE CELL

Expt. No.	Layer No., entries are $\bar{c}/c°$				
	(9–10)′	11′	12′	13′	14′
7	3.43(−1)[a]	4.50(−2)	3.63(−3)	5.09(−4)	3.27(−4)
8	4.98	5.04	1.47	1.93	1.45
9	3.98	4.15	2.11	5.18	1.60
10	4.56	3.87	3.32	1.34	0.68
11	3.50	2.87	3.41	7.57	2.82
12	5.08	7.73	2.31	0.91	0.26
13	7.39	4.37	2.41	0.94	0.085
20	6.06	3.32	0.58	0.69	0.098
21	3.47	1.67	2.89	6.73	3.86
Mean $\bar{c}/c°$	4.72(−1)	4.17(−2)	2.46(−3)	3.38(−4)	1.57(−4)
$\sigma\,\bar{c}/c°$	1.34	1.67	0.99	2.75	1.44

	Calculation outline					
Layer No.	Mean $\bar{c}/c°$	x[b]	D[c]	$1/e^{2}$ [b]	σ/Mean	w[d]
(9–10)′	4.72(−1)	—	—	0.00	0.284	0.00
11′	4.17(−2)	0.386	0.496(−7)	2.33	0.400	14.52
12′	2.46(−3)	0.328	0.687	15.15	0.402	93.32
13′	3.38(−4)	0.264	1.060	33.39	0.814	50.42
14′	1.57(−4)	0.204	1.775	41.21	0.917	49.04

$\bar{D} = 1.02 \times 10^{-7}$ sq cm/sec; $\sigma_{\bar{D}}/D = 7.0\%$; $2r = 22.7$ mμ

[a] Entries alongside and below in the same column are to be multiplied by 10 to the power indicated in parentheses.

[b] x and $1/e^{2}$ are read from table similar to Table 1 at the value of mean $\bar{c}/c°$ for the particular cell layer.

[c] Calculated by Eq. (1).

[d] Calculated by Eq. (2).

above. The 27 mm below the initial boundary can be considered nine layers of 3 mm each, and the 15 mm above as five layers. Since they are not of equal width, they will be denoted by primes to distinguish them

from the corresponding fractions in the standard 14 layer cell of Fig. 1. A series of nine replicate 94-hr diffusion runs were made, and the data are given in Table 2. The $\bar{c}/c^\circ$ value calculated from the infectivity measurements was determined for each layer in each run. Since these were replicate experiments, the assay error $\sigma_{\bar{c}/c^\circ}/\bar{c}/c^\circ$, given in the next to the last column in the lower half of Table 2, was determined from the mean and standard deviation of the nine runs given in the rows just above the calculation outline. The values of x and $1/e^2$ at the mean value of $\bar{c}/c^\circ$ were taken from the appropriate theoretical table and are given in the 3rd and 5th columns of the lower half of Table 2. The calculation of D and w for each layer was done by substitution in Eqs. (1) and (2), respectively. The value of $\bar{D}$ was calculated from Eq. (3), and gave for this series 1.02×10^{-7} sq cm/sec with $\sigma_{\bar{D}}/D = 7.0\%$. The corresponding equivalent sphere diameter, $2r$, would be 22.7 ± 1.6 mμ from Eq. (5).

The second cell arrangement, which had a 25-mm and a 2-mm layer below the initial boundary and 2, 4, 3, 3, and 3 mm above, more closely approached the standard cell. Using this system, seven runs were made using a single virus source, but the diffusion time varied from 91 to 95 hr. This time variation of approximately 5% was neglected in determining the assay error which, due to other difficulties, was as high as $\pm$ 190%. The values of D were calculated for each experiment individually. The over-all weighted mean value of $\bar{D}$ was 1.21×10^{-7} and $\sigma_{\bar{D}}/\bar{D}$ was 10% giving an equivalent particle diameter of 19.1 ± 1.9 mμ.

With both of the cell arrangements used, the two 2-mm layers on either side of the initial boundary were pooled for assay purposes. This sample should have a value of $0.5c^\circ$ for all diffusion times and serves as an internal check on the run. The data in Table 2 show that this layer $(9\text{–}10)'$ had a mean $\bar{c}/c^\circ$ of 0.472 which was close to the expected. For the second series the value was 0.38 which was an indication of greater errors in the cell manipulation.

Five-Layer Cell

The five-layer cell was more advantageous in determining the diffusion coefficient since it required only 0.05 ml of starting virus suspension. Preliminary sets of experiments run for 48, 72, and 94 hr indicated that difficulties in cell manipulation and relatively high percentage errors in recovering such small fractions would necessitate a series of replicate runs. The preliminary experiments did, however, indicate a particle diameter ranging between 18 and 31 mμ.

Ten replicate 66-hr diffusion runs were made on aliquots of a single suspension using two five-layer cells simultaneously. Comparison between the results showed no bias for either cell. In each case the starting

virus was assayed along with the fractions collected after the run. The data for this series are shown in Table 3. The values of x and $1/e^2$ were taken from the tables prepared for this type of cell (7). The calculations were the same as for the double-infinite cell. The over-all weighted mean diffusion coefficient was 1.01×10^{-7}, the $\sigma_{\bar{D}}/\bar{D}$ was 21%, and the equivalent particle diameter was 23.0 ± 4.9 mμ. This high error was evidently due to the fact that the assay error ranged up to $\pm 100\%$ for all but the top fraction where it was $\pm 290\%$. These high assay errors are probably due to difficulties in cell manipulation and incomplete recovery of the fractions since the assay error for the ten replications of the starting virus was only $\pm 72\%$.

DISCUSSION

Other methods for measuring diffusion have recently been discussed (8, 9) which involve the movement of the virus or antigen through a semisolid medium such as agar. Lauffer (10) has also pointed out that corrections have to be made to reconcile the diffusion in a gel with diffusion in free solution.

The method used here permits measurement of free diffusion with a minimum of virus suspension and with simple equipment. Two cell arrangements gave comparable results when a series of replicate runs were made. Manipulation of the two cell types is similar, so that the five-layer cell is more advantageous when only small quantities of virus suspension are available.

The combined data from Tables 2 and 3 gives a value for the diameter of FMDV which is consistent with the values previously determined by electron microscopy, 23 ± 2 mμ (3, 4). Whether or not this would occur with other agents has not been investigated. The effects of hydration of the virus have also not been considered.

The weighting of the data was undertaken in order to adjust for the differences which were known to occur in the plaque assay and in the change in the computed value of D with assay error for samples removed at greater and greater distances above the initial boundary. Each layer in each experiment gives a single estimate of D, and the over-all arithmetic mean of these individual estimates agreed very closely with the weighted mean. If, therefore, the number of runs is large, the effect of the weighting is not as significant as it would be if D were to be determined by a single experiment. An examination of each experiment separately showed that the error could be due to variations in infectivity assay, mechanical disturbances in the manipulation of the cell, and incomplete removal of the contents of small layers.

TABLE 3
DIFFUSION COEFFICIENT OF FMDV-A119 BY INFECTIVITY ASSAY IN A FIVE-LAYER CELL

Expt. No.[f]	Layer No., entries are $\bar{c}/c^\circ$				
	1	2	3	4	5
47	—	0.78(−1)	0.63(−2)	1.40(−3)	0.053(−4)
48	1.36(−1)[a]	—	0.75	0.83	0.195
49	2.33	1.63	3.22	3.98	2.33
50	2.38	0.99	1.37	2.59	3.33
51	1.38	0.46	9.77	13.20	3.18
52	2.96	0.42	11.00	12.60	3.72
53	8.20	4.29	5.35	4.69	1.51
54	3.15	2.51	0.91	1.21	0.29
55	0.81	1.02	3.37	3.02	0.76
56	2.74	1.15	2.28	2.43	0.41
Mean $\bar{c}/c^\circ$	2.81(−1)	1.47(−1)	3.87(−2)	4.60(−3)	1.58(−4)
$\sigma\,\bar{c}/c^\circ$	1.05	1.24	3.75	4.54	4.56

Calculation outline

Layer No.	Mean $\bar{c}/c^\circ$	x[b]	D[c]	$1/e^2$[b]	σ/Mean	w[d]
1	2.81(−1)	0.259	6.27(−7)	1.77(−1)	0.375	1.26
2	1.47(−1)	—[e]	—	(2.17(−2)[e]	0.840	(0.031)[e]
3	3.87(−2)	0.885	0.537	2.90	0.968	3.10
4	4.60(−3)	0.772	0.706	1.23(+1)	0.987	12.67
5	1.58(−4)	0.757	0.734	4.08(+1)	2.887	4.90

$\bar{D} = 1.01 \times 10^{-7}$ sq cm/sec; $\sigma_{\bar{D}}/\bar{D} = 21\%$; $2r = 23.0$ mμ.

[a] Entries alongside and below in the same column are to be multiplied by 10 to the power indicated in parentheses.

[b] x and $1/e^2$ are read from Table 3, Ref. (7), at the value of mean $\bar{c}/c^\circ$ for the particular cell layer.

[c] Calculated by Eq. (1).

[d] Calculated by Eq. (2).

[e] Fraction 2 values for x are indeterminate and, furthermore, would contribute little to the weighted mean $\bar{D}$ since $1/e^2$ and w values are small.

[f] Runs with odd numbers in one cell. Even-numbered runs in the other.

SUMMARY

A multilayered glass diffusion cell was used to measure the diffusion coefficient of foot-and-mouth disease virus (FMDV) type A, strain 119, propagated in bovine kidney tissue culture. Diffusion was followed by

infectivity changes rather than by optical methods. The cell was arranged either for free diffusion in an essentially double-infinite system or for restricted diffusion in a closed system separable into five layers. Infectivity of each fraction was measured by plaque assay to determine the concentration of virus in each layer after diffusion. The influence of the assay error was reduced by using fractions at a distance from the initial boundary where the concentration was several orders of magnitude less than the starting material. All data were weighted to adjust for errors of assay and the location of the layer in the cell. The weighted mean diffusion coefficient $\bar{D}$, expressed in 10^{-7} sq cm/sec at 4°C, was determined for the various replicate experiments.

In nine replicate runs in the double-infinite cell, with a diffusion time of 94 hr and an assay error as high as ±90%, $\bar{D}$ was 1.02. In a similar series of 10 runs in the five-layer cell, with a diffusion time of 66 hr and an assay error of about ±100%, $\bar{D}$ was 1.01. The weighted mean value of $\bar{D}$ for this total of 19 runs, representing 76 individual values, was 1.020 ± .067. This corresponds to a particle diameter for FMDV of 22.7 ± 1.5 mμ which compares favorably with the electron microscope value of 23 ± 2 mμ.

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REFERENCES

1. Moore, D. H., Lasfargues, E. Y., Murray, M. R., Haagensen, C. D., and Pollard, E. C., *J. Biophys. Biochem. Cytol.* **5**, 85 (1959).
2. Breese, S. S., Jr., Trautman, R., and Bachrach, H. L., *Arch. Biochem. Biophys.* **87**, 1 (1960).
3. Bachrach, H. L., Breese, S. S., Jr., *Proc. Soc. Exptl. Biol. Med.* **97**, 659 (1958).
4. Breese, S. S., Jr., and Bachrach, H. L., *Proc. Intern. Congr. Electron Microscopy, 4th Congr.*, Vol. II, pp. 619–21. Springer Verlag, Berlin, 1960.
5. Bachrach, H. L., *Biophys. Biochem. Research Communs.* **1**, 356 (1959).
6. Bachrach, H. L., Callis, J. J., Hess, W. R., and Patty, R. E., *Virology* **4**, 224 (1959).
7. Trautman, R., and Breese, S. S., Jr., *Anal. Biochem.* **1**, 291 (1960).
8. Neff, J. C., and Becker, E. L., *J. Immunol.* **78**, 5 (1957).
9. Polson, A., *Biochim. et Biophys. Acta* **29**, 426 (1958).
10. Lauffer, M. A., *in* Abstracts, p. 45. 4th Annual Meeting Biophysical Society, Philadelphia, 1960.

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The Determination of Lactic Dehydrogenase with a Tetrazolium Salt[1]

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Methods for the measurement of lactic dehydrogenase use manometric (1–3), spectrophotometric (4–10), and colorimetric techniques. One of the earliest reports was that of Green and Brosteaux (1) in which oxygen uptake was measured using methylene blue as the intermediate electron carrier between reduced flavoprotein and molecular oxygen. Two spectrophotometric types of assay were devised, one which measured the appearance of DPNH[2] upon the oxidation of lactate (4–6), and another which measured the disappearance of DPNH upon the enzymic reduction of pyruvate (7–10). The colorimetric methods utilized two quite different principles; (*a*) the color change upon reduction of oxidation–reduction dyes (11–13), and (*b*) the decrease in pyruvate concentration upon enzymic reduction of the substrate, as measured with 2,4-dinitrophenylhydrazine (14).

The advantages of tetrazolium salts as colorimetric indicators of dehydrogenase activity have been described by a number of investigators (15–21). Although a wide variety of these compounds have been utilized in histochemical studies (22–26), only one, 2,3,5-triphenyltetrazolium chloride has been used for the assay of lactic dehydrogenase in tissue homogenates (17). Since the publication of this work, newer tetrazolium salts have become available, which are better acceptors of electrons than 2,3,5-triphenyltetrazolium chloride, due to the incorporation of electron-donor groups into the N-2 and N-3 phenyl groups of this tetrazole (16,

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[2] The abbreviations used are: DPN and DPNH, the oxidized and reduced forms of diphosphopyridine nucleotide; INT, 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride; PMS, phenazine methosulfate; LDH, lactic dehydrogenase; Tris, tris(hydroxymethyl)aminomethane.

27). One of the best agents for this purpose is 3-*p*-nitrophenyl-2-*p*-iodophenyltetrazolium chloride (INT). The use of INT as hydrogen acceptor with phenazine methosulfate as intermediate electron carrier (28) permitted the development of a sensitive and simple colorimetric method for measuring lactic dehydrogenase activity. Description of the method forms the basis of this paper.

MATERIALS AND METHODS

Enzyme Preparations

Three sources of lactic dehydrogenase were used in this study (serum, purified enzyme, and tissue homogenates). Serum was obtained from normal individuals and assayed on the same day. A commercial preparation of crystalline LDH[3] from rabbit muscle (50,000 units/mg) was dissolved in 0.1 *M* cold phosphate buffer (pH 7.4) as a stock solution. Homogenates were prepared from the tissues of freshly killed animals (rats, mice, guinea pigs, or dogs) using a ground-glass motor-driven homogenizer for 1–3 min, while the tube was immersed in an ice bath during the homogenization. The large particles were removed by centrifugation for about 20 sec at 2000 rpm. The opalescent supernatant as well as the clear solution of the commercial preparation and serum could be stored at —20°C for as long as 1 week, without significant loss of activity.

Preparation of Reagents

The buffered substrate solution was prepared by mixing 4.8 ml of 60% *sodium lactate* solution (Fisher) and 95.2 ml of 0.1 *M* Tris buffer (pH 9.0). *DPN*[3] was dissolved in water, neutralized with 0.1 *N* sodium hydroxide to raise the pH to about 7.0, and diluted with water to give a stock solution of 10 mg/ml. A 5% solution of *gelatin* was made and stored in the gel state in 1-ml aliquots. When needed, the gelatin was dissolved in warm water to a concentration of 0.1%. A stock solution of INT[4] was made up at 4 mg/ml. PMS[3] was dissolved in distilled water at a concentration of 0.2 mg/ml. When the solution was stored in a dark bottle at 4–6°, it was stable for several weeks. Decomposition was easily recognized by a change in color from a clear yellow to a muddy green. Hydrochloric acid (0.35 *M*) is also required.

Measurement of Lactic Dehydrogenase Activity

The enzymic assay was performed in a reaction mixture containing the buffered substrate solution (1.5 ml), DPN (0.5 ml), INT (1 ml), and

[3] Obtained from Sigma Chemical Company, St. Louis, Mo.

[4] Obtained from Dajac Laboratories, 5000 Langdon Street, Philadelphia 24, Pa.

gelatin (0.5 ml).[5] The 3.5-ml mixture was warmed to 37°, 0.5 ml of the stock PMS solution was added, and this was followed immediately by the addition of the enzyme to be assayed in a volume of 0.5 ml. The mixture was incubated at 37° for 15 min and the reaction was terminated by the addition of 0.5 ml of 0.35 *M* hydrochloric acid, with a drop in pH to 4.5. A control tube was also used in which the enzyme preparation and the hydrochloric acid were added to the reagent mixture after the 15-min incubation period was over. The colorimeter (Klett-Summerson) was adjusted to zero reading with the control tube, using a 540-mμ filter, and the color densities of the assay tubes were determined within the hour. The color is stable for at least 12 hr.

Calibration Curve

Graded increments of INT (from 0.04 to 0.24 mg) were placed in a series of colorimeter tubes, containing freshly prepared DPNH (2.5 mg), gelatin (1 ml), and Tris buffer (pH 9.0) (1 ml), and the volume in each tube was brought to 4.0 ml with water. PMS (0.5 ml) was added to each tube, and, after 2–3 min when all of the INT had been reduced, hydrochloric acid (0.5 ml) was added bringing the volume of each tube to 5 ml. The color densities were determined and found to be strictly proportional to the formazan concentrations. If DPNH were not available, an equally satisfactory alternate method was to prepare a series of assay tubes containing all of the reactants except PMS, and to use crystalline lactic dehydrogenase (0.01 mg) as the enzyme source. After 15 min of incubation, an excess of DPNH was present and upon addition of PMS, the tetrazolium salt in each tube was reduced. The reason why other reducing agents cannot be used to produce the formazan has been described in a previous paper (21). One *unit* of lactic dehydrogenase activity is defined as that amount of enzyme which will yield 1 μg formazan under the conditions of the assay method.

EXPERIMENTAL

Comparison of Substitutes for DPNH Diaphorase

Although a preparation of Straubs' "soluble diaphorase"[6] is commercially available, the use of such a reagent as an intermediate electron carrier between DPNH and the tetrazolium salt has several disadvantages. The enzyme is obtained from pig heart in an impure state, so

[5] For convenience it is possible to mix all the reagents except the PMS in the proportions given, and transfer 3.5-ml aliquots to colorimeter tubes. These may be kept at 4° and used any time within a week. When the reagents are stored separately at 4°, they are stable for at least a month.

[6] Worthington Biochemical Company, Freehold, N. J.

that some variation in activity might be expected with different samples. This material is quite costly for routine use and cannot be stored for any length of time. Finally, "soluble diaphorase" does not dissolve readily in water at the concentrations needed in the enzymic assay. For these reasons, a suitable substitute was sought which could transfer electrons

TABLE 1

COMPARISON OF DIAPHORASE WITH CERTAIN STRUCTURALLY SIMILAR HETEROCYCLIC COMPOUNDS AS SUBSTITUTE ELECTRON CARRIERS[a]

Electron carrier	Chemical class	Formazan produced	
		DPNH (μg)	LDH and DPN (μg)
Diaphorase	Flavoprotein	186	167
Phenazine methosulfate	Phenazine	214	195
Neutral Red O	Phenazine	35	28
Safranin O	Phenazine	24	28
Methylene blue	Thiazine	243	51
Thionin	Thiazine	215	158
Gallocyanin	Oxazine	2	59
Nile blue A	Oxazine	157	87
Janus Green B	Azo-safranin	131	75
Riboflavine	Flavine	112	33
Flavine mononucleotide	Flavine	68	32
Acriflavine	Flavine	22	34
2,6-Dichloro–benzeneoneindophenol	Indophenol	7	0

[a] The reaction mixture contained INT, gelatin, Tris buffer (pH 9.0), and the various agents listed above at a concentration of 6.5×10^{-4} *M*. Diaphorase was used at a final concentration of 0.2 mg/ml. For the chemical transfer study, DPNH (7.5×10^{-4} *M*) was added, and the optical densities were determined on the photoelectric colorimeter at 30 min. For the enzymic transfer, 2 μg of crystalline LDH was introduced into a series of tubes which contained the standard assay medium except that PMS was replaced with one of the electron carriers listed above. Incubation was carried out for 15 min at 37°C. Because of interference of colored agents, the amount of formazan produced was measured after ethyl acetate extraction by subtraction of the color density in the control tube and by referring to a standard calibration curve.

from the DPNH to the tetrazolium salt. PMS has been reported to act as an electron carrier in both the succinic and glucose-6-phosphate dehydrogenase systems (21, 28). Furthermore, certain oxidation–reduction dyes have been noted to accept hydrogens from dehydrogenase systems in both colorimetric and histochemical studies (28–30), and in some instances the reduced form of the dyes transferred their hydrogens to tetrazolium salts resulting in the facilitation of increased formazan production (29). Therefore, PMS, certain oxidation–reduction dyes, and other reagents with structures resembling PMS were tested as inter-

mediate electron carriers in both enzymic and non-enzymic systems. The findings (Table 1) indicate that many of the compounds studied can transfer electrons from DPNH to INT in a 30-min period and that methylene blue and thionin are as effective as PMS. However, when these agents were studied in an enzyme system all were less effective, and the largest amount of formazan was produced with PMS. Thionin had less of an inhibitory effect on the enzyme than methylene blue, while PMS was not inhibitory. To confirm the role of PMS in the enzymic assay, the following observations were made:

(*a*) The reaction mixture plus enzyme gave no color in the absence of PMS. When PMS was added, formazan appeared and increased in amount as the incubation period lengthened.

(*b*) DPNH plus tetrazolium salt gave no color change. When PMS was added, formazan was noted immediately.

(*c*) The combination of enzyme, DPN, tetrazolium salt, and PMS yielded no formazan until sodium lactate was introduced.

Thus, the findings support the view that PMS acts as an efficient intermediate transfer agent between DPNH and tetrazolium salt, thus making the formazan produced in the recommended reaction mixture a measure of the dehydrogenase activity.

Influence of Proteins on the Enzymic Assay

Since the formazans produced by reduction of most tetrazolium salts are markedly insoluble in water, colorimetry requires that the formazan be extracted into an organic solvent or suspended in colloidal solution in the aqueous medium. Since the latter procedure yields more convenient methodology, it was accomplished by the use of emulsifying agents such as Tween 20 or by taking advantage of the previously described substantive properties of certain formazans for protein (25). Although Tween 20 did maintain the formazan of INT in colloidal suspension, so that the concentrations of the formazan were proportional to the optical densities, this agent produced a change in color of the formazan, and it was not as satisfactory as the use of gelatin for the reasons which follow.

When gelatin was added to the reaction mixture containing lactate, DPN, tetrazolium salt, PMS, and tissue homogenate, a 2- to 3-fold increase in enzymic activity was noted over that with medium in which the gelatin was lacking (Table 2). This activating effect was also observed with crystalline bovine albumin, human albumin, and egg albumin. However, when serum served as the enzyme source no such activa-

tion was noted with either gelatin or bovine albumin, presumably since the albumin of the serum exerted the optimal effect. When soluble diaphorase served as the intermediate electron carrier, more formazan was produced than with PMS alone, but less formazan than with PMS plus gelatin (Table 2). These findings were observed with both rat liver homogenate and crystalline lactic dehydrogenase. They suggest that PMS alone is not as efficient in transferring electrons to tetrazolium salts as is diaphorase. However, PMS in the presence of non-specific

TABLE 2
INFLUENCE OF PROTEINS ON ELECTRON TRANSFER BY PHENAZINE METHOSULFATE AND DPN DIAPHORASE[a]

Additions to reaction mixture	Formazan produced	
	By liver homogenate (μg)	By cryst. LDH (μg)
1. None	11	0
2. PMS	117	39
3. PMS and gelatin	192	194
4. PMS and albumin	167	154
5. Diaphorase	136	98
6. Diaphorase and gelatin	160	134
7. Diaphorase and albumin	154	127

[a] The reaction mixture contained sodium lactate, DPN, INT, and either rat liver homogenate or crystalline LDH. The incubation was carried out at 37° for 15 min. The results given are those of a typical experiment noted with each enzyme preparation.

protein apparently furnishes a complex more nearly resembling the natural flavoprotein so that electrons are transferred equally well or better to the tetrazolium salt. The increased yield of formazan with diaphorase plus gelatin is probably due to better colloidal suspension of the dye. Therefore, in the assay of lactic dehydrogenase in tissue homogenates or crystalline preparations, gelatin was added to the reaction mixture as called for in the procedure. Although gelatin is not needed in the assay of serum enzyme, it is convenient to use the same reaction mixture since it further stabilizes the colloidal suspension of formazan.

Enzyme Kinetics

The rate of the reaction is linear with time (up to the 30 min tested), and the reaction rate is proportional to concentration of homogenate of heart muscle (0.5–3.0 mg). The influence of pH was studied over the pH range of 5–11. The reported pH optimum of 10 (5) was not confirmed with this assay system. An optimal range from pH 8.9 to 9.3 was

noted using a glycine–sodium hydroxide buffer system. Over their effective buffering ranges, no significant differences were found between 0.1 M phosphate, Tris, Veronal, or glycine buffers; whereas, borate buffer caused complete inhibition. An extended range of temperatures was not studied, but it was noted that the activity of lactic dehydrogenase at 37°C was more than twice that at room temperature.

Activators and Inhibitors

Among the large variety of agents which have been studied for their effect on LDH, sodium cyanide, adrenalin, and amytal have been reported to be activators (26, 31), whereas pyruvate, oxalate, nicotinamide, and *p*-chloromercuribenzoate have been observed to produce inhibition (1, 5, 11, 12, 32, 33). These potential activators were tested with this colorimetric method to see whether the sensitivity could be increased by their use. The only effects noted with the substances tested were inhibitory. The inhibitors were examined as agents for stopping the reaction quickly at the end of the incubation period. Thus, sodium fluoride (7.2×10^{-3} M) and N-ethyl maleimide (2×10^{-3} M) showed slight inhibition; mercurous acetate (6×10^{-3} M) and sodium iodate (6×10^{-3} M) showed moderate depression; while oxalic acid (1.2×10^{-2} M) and borate buffer gave complete inhibition. The most satisfactory method for stopping enzymic activity was by dropping the pH to about 4.5 with 0.35 M hydrochloric acid.

Sensitivity of the Method and Normal Values

When two samples of crystalline LDH were tested, 1 μg of each commercial preparation resulted in the production of 140 and 310 μg formazan. Similar amounts of formazan were produced with 0.1 mg of rat liver homogenate and 0.1 ml of pooled human serum.

Forty normal sera were assayed. The range with the new method varied from 50 to 100 units per 0.05 ml serum.

Influence of Hemolysis

Red blood cells have been known to contain abundant amounts of LDH (9, 36, 37). Caution against the use of hemolyzed serum is advised with each method. The extent of the error introduced was investigated by adding a 1% solution of hemolyzed red blood cells to pooled serum in increasing amounts, after the cell membranes had been removed by centrifugation. When gross hemolysis could be recognized in the sera, the activity was at least 20% higher than the control sera. However, it was interesting to note that 10–15% error could be introduced by the

presence of hemolysis unrecognizable grossly. The rate of unrecognizable hemolysis was tested in ten normal patients. Fresh blood was placed in several tubes, and the serum removed for testing immediately, after 1, 2 and 4 hr. No significant error was introduced by allowing the serum to remain in contact with clot at room temperature for a 4-hr period. However, serum should be discarded if it is grossly hemolyzed, and interpretation should be guarded if the serum is left in contact with clot for longer periods than tested here. Serum may be stored at 4°C. for 48 hr without significant loss in activity.

DISCUSSION

The diagnostic usefulness of methods for serum LDH has stimulated modification and simplification of the techniques and development in instrumentation for the enzyme. The methods in common use today for LDH measure the rate of appearance of DPNH or of the disappearance of either DPNH or pyruvate. Although expensive ultraviolet spectrophotometers are becoming available in more and more hospital laboratories, the necessity for taking multiple timed readings allows the determination of activity in only one serum at a time at 5-min intervals (9). The colorimetric method which involves the measurement of pyruvate requires a 30-min incubation, after which two timed reactions have to be performed (an additional 25 min) before the test is completed. The assay method described in this paper has the advantages that the activities in many sera can be determined simultaneously, the reagents are inexpensive and readily obtainable, the procedure is simple and of short duration, and the instrument required (photoelectric colorimeter) is presently available in considerable numbers in all clinical and research laboratories. The method is also adaptable to automation equipment.

SUMMARY

A colorimetric method for the measurement of lactic dehydrogenase activity has been described, utilizing phenazine methosulfate as an intermediate agent for electron transfer from DPNH to a tetrazolium salt (INT). The use of gelatin to keep the formazan in colloidal suspension provided the added advantage of improving electron transfer via a phenazine methosulfate gelatin complex over that obtained with soluble diaphorase or phenazine methosulfate alone. Several compounds were studied for their ability to act as intermediate electron-transfer reagents, but none were superior to phenazine methosulfate. The sensitivity of the new method is comparable to others measuring either DPNH or pyruvate; its advantages are those of brevity and simplicity. Normal

human sera were found to contain from 50 to 100 units of lactic dehydrogenase. The importance of avoiding hemolysis of the blood has been re-emphasized, and the possibility has been demonstrated that a 10–15% overestimation may occur due to hemolysis even when gross hemolysis cannot be recognized.

REFERENCES

1. Green, D. E., and Brosteaux, J., *Biochem. J.* **30,** 1489 (1936).
2. Quastel, J. H., and Wheatley, A. H. M., *Biochem. J.* **32,** 936 (1938).
3. Straub, F. B., *Biochem. J.* **34,** 483 (1940).
4. Euler, H. V., Adler, E., and Hellström, H., *Z. physiol. Chem.* **241,** 239 (1936).
5. Neilands, J. B., *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan eds.), Vol. 1, p. 449. Academic Press, New York, 1955.
6. Wacker, W. E. C., Ulmer, D. D., and Vallee, B. L., *New Engl. J. Med.* **255,** 449 (1956).
7. Kubowitz, F., and Ott, P., *Biochem. Z.* **314,** 94 (1943).
8. Kornberg, A., *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. 1, p. 441. Academic Press, New York, 1955.
9. Wroblewski, F., and LaDue, J. S., *Proc. Soc. Exptl. Biol. Med.,* **90,** 210 (1955).
10. White, L. P., *New Engl. J. Med.* **255,** 948 (1956).
11. Quastel, J. H., and Woolridge, W. R., *Biochem. J.* **22,** 689 (1928).
12. Hopkins, F. G., Morgan, E. J., and Lutwak-Mann, C., *Biochem. J.* **32,** 1829 (1938).
13. Katagiri, H., and Kitahara, K., *Biochem. J.* **32,** 1654 (1938).
14. Berger, L., and Broida, D., Tech. Bull. #500, Sigma Chemical Co., April, 1958.
15. Kun, E., and Abood, L. G., *Science* **109,** 144 (1949).
16. Atkinson, E., Melvin, S., and Fox, S. W., *Science* **111,** 385 (1950).
17. Jensen, C. O., Sacks, W., and Baldauski, F. A., *Science* **113,** 65 (1951).
18. Glock, E., and Jensen, C. O., *J. Biol. Chem.* **201,** 271 (1953).
19. Glick, D., and Nayyar, L. N., *J. Histochem. and Cytochem.* **4,** 389 (1956).
20. Shelton, E., and Rice, M. E., *J. Natl. Cancer Inst.* **18,** 117 (1957).
21. Nachlas, M. M., Margulies, S. I., and Seligman, A. M., *J. Biol. Chem.* **235,** 499 (1960).
22. Seligman, A. M., and Rutenberg, A. M., *Science* **113,** 317 (1951).
23. Shelton, E., and Schneider, W. C., *Anat. Record* **112,** 61 (1952).
24. Farber, E., Sternberg, W. H., and Dunlap, C. E., *J. Histochem. and Cytochem.* **4,** 254 (1956).
25. Nachlas, M. M., Tsou, K. C., DeSouza, E., Cheng, C. S., and Seligman, A. M., *J. Histochem. and Cytochem.* **5,** 420 (1957).
26. Scarpelli, D. G., Hess, R., and Pearse, A. G. E., *J. Biophys. Biochem. Cytol.* **4,** 747 (1958).
27. Nachlas, M. M., Karmarkar, S. S., and Seligman, A. M., *Proc. Soc. Exptl. Biol. Med.* **104,** 407 (1960).
28. Dickens, F., and McIlwain, H., *Biochem. J.* **32,** 1615 (1938).
29. Farber, E., and Louviere, C. D., *J. Histochem. and Cytochem.* **4,** 347 (1956).
30. Singer, T. P., and Kearney, E. B., *Methods of Biochem. Anal.* **4,** 307 (1957).
31. Green, D. E., and Richter, D., *Biochem. J.* **31,** 596 (1937).
32. Feigelson, P., Williams, J. N., Jr., and Elvehjem, C. J., *J. Biol. Chem.* **189,** 361 (1951).

33. ALIVISATOS, S. G. A., AND DENSTEDT, O. F., *J. Biol. Chem.* **199,** 493 (1952).
34. CORRAN, H. S., GREEN, D. E., AND STRAUB, F. B., *Biochem. J.* **33,** 793 (1939).
35. MCKEE, R. W., ORMSBEE, R. A., ANFINSEN, C. B., GEIMAN, Q. M., AND BALL, E. G., *J. Exptl. Med.* **84,** 569 (1946).
36. HSIEH, K. M., AND BLUMENTHAL, H. T., *Proc. Soc. Exptl. Biol. Med.* **91,** 626 (1956).

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A Simplified and Specific Method for the Estimation of Choline[1]

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INTRODUCTION

The reineckate procedure as outlined by Engel (1) and by Glick (2) for the estimation of choline in biological tissues is extensively used. That the specificity of the reineckate procedure may be enhanced if choline reineckate is precipitated from strongly alkaline solutions has been pointed out by others (3–5). Street, Kenyon, and Watson (6) reported, however, that choline reineckate tended to decompose during filtration from strongly alkaline solutions. Huerga and Popper (7) preferred to precipitate choline reineckate at the thymolphthalein end point and then wash the precipitate with 0.1 *N* NaOH that had been saturated with choline reineckate.

Various reagents have been suggested for the hydrolysis of tissue samples or extracts prior to analysis. An investigation of the hydrolysis and precipitation steps in the analysis of choline has led to a simplified and specific procedure for the estimation of choline in plant or animal tissues. The details of the procedure are presented in this paper.

METHODS

Materials and Equipment

Samples for analysis were prepared from animal and plant tissues as follows: the liver, kidneys, spleen, heart, and leg muscles from an adult rat were dried and ground to a fine powder. To this was added an equal

[1] Approved by the Director of the Agricultural Experiment Station of Auburn University. The data are from a dissertation submitted by C. J. Ackerman to Auburn University in partial fulfillment of the requirements for the Ph.D. degree. The investigation was supported in part by research grants from the National Cancer Institute, U. S. Public Health Service and from the American Cancer Society.

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weight of a 1:1 mixture of soybean oil meal and peanut meal. One gram of dried egg yolk was then added, and the entire mixture was thoroughly mixed by grinding. This mixture will be referred to as the "test sample."

Choline chloride was recrystallized four times from hot ethanol, and a concentrated stock solution was prepared in 0.01 *N* HCl. The concentration of this solution was determined by the micro-Kjeldahl procedure for nitrogen, and suitably diluted aliquots were used for analysis.

Choline reineckate for use in chromatographic studies was precipitated from aqueous solution and recrystallized three times from acetone–water. The precipitate was stored over Drierite and shielded from light. From reagent-grade nitric acid (sp. gr. 1.42) "25% nitric acid" was prepared by diluting 25 ml to 100 ml with water. Ammonium reineckate (Krishell Lab., Inc.) was used without further purification.

Paper chromatography was carried out in a chromatocab, the window of which was covered with heavy wrapping paper. One-inch strips of Whatman #1 filter paper (specially prepared for chromatography) were used. Acetone solutions of reineckate samples were repeatedly applied in a small spot until a definite pink color was visible. After development, the pink reineckate spot could be detected visually. Choline was detected by exposure of the developed strips to iodine vapors (9) or treated with Dragendorf's $KBiI_4$ reagent as described by Bregoff *et al.* (10).

Analysis

From 0.1 to 0.3 gm of the test sample was added to 15 ml of 25% nitric acid in a 50-ml beaker. The beaker was covered with a watch glass, and the temperature was kept just below the boiling point for 5.5 hr. After hydrolysis, the watch glass was removed and the sample allowed to stand at room temperature for 12–24 hr. The yellow acid solution was then filtered with suction into a 50-ml suction flask, and the residue was washed with water until the volume of the filtrate was approximately 25 ml. The solution was cooled and then slowly made alkaline with NaOH pellets or 10 *N* NaOH to the pink of phenolphthalein. Then 0.4 ml of 10 *N* NaOH was added to bring the pH above 12. This solution was again chilled before adding 3 ml of a 5% solution of ammonium reineckate in methanol. The flask was stored at 5°C for 3 hr in a dark refrigerator. The red precipitate was filtered out on a pad of acetone-washed Supercel contained in a sintered-glass funnel of medium porosity. The reineckate crystals, suction flask, and walls of the funnel were washed four times with 2 ml *n*-propanol. During this filtration, the precipitate was shielded from light by inverting a small paper box over the funnel. Excess propanol was removed by continued aspiration. The filtrate and propanol washings were discarded. Acetone was then slowly drawn through the

funnel and collected in a 5- or 10-ml volumetric flask which was set below the funnel inside of a large suction flask. The flask was filled to the mark, a sample was removed, and the absorbancy was measured in a Beckman model DU spectrophotometer at 526 mμ. The concentration of choline chloride was calculated from the molar absorbancy index of choline reineckate. This was reported by Glick (2) to be 111 and confirmed in this laboratory.

Samples of the test material were also hydrolyzed with 15 ml of 9 *N* HCl for a period of 12 hr. Subsequent steps were exactly as described for HNO_3 hydrolysis.

For alkaline hydrolysis, 1–1.5 gm of solid $Ba(OH)_2$ was added to the sample with 15 ml water. This mixture was heated for 4–8 hr. After cooling and filtering, methanolic ammonium reineckate was added directly to the flask and treated as for HNO_3 hydrolysis.

Effect of NaOH Concentration. The effect of the NaOH concentration on the precipitation of choline reineckate was determined. Suitably diluted samples of the stock solution were made up to 15 ml with water or 0.4 *N* NaOH or 0.8 *N* NaOH. Methanolic ammonium reineckate was added, and the samples were filtered and measured as described above.

Chromatography

The purity of the reineckates obtained after HNO_3 and HCl hydrolysis was determined by paper chromatography by use of *n*-butanol saturated with water as the developing solvent. Paper strips spotted with recrystallized choline reineckate were run at the same time for comparison. Duplicate strips were also treated with $AgNO_3$ before development according to the procedure of Bregoff *et al.* (10). Insoluble silver reineckate is formed on the paper, and choline nitrate moves from the origin during development. After development, the strips were dried at room temperature and then exposed to iodine vapors (9) or treated with Dragendorf's $KBiI_4$ reagent (10).

The reineckate obtained after HNO_3 hydrolysis was further investigated by use of an acid and a basic solvent system for paper chromatography. These consisted of *n*-butanol–acetic acid–water (100:30:satd. with water) and 95% ethanol-NH_4OH (95:5).

Specificity

Two mixtures of certain basic compounds were prepared. These consisted of (*a*) 0.5 mg/ml each of cinchonine, brucine, benzidine, caffeine, urea, and guanidine; and (*b*) 1 mg/ml each of guanidine, urea, adenine, guanine, with 1.5 mg/ml of 2-dimethylaminoethanol. From 1 to 3 ml of these solutions were diluted with and without choline, to 25 ml with 0.2

N NaOH. They were then treated with methanolic ammonium reineckate and analyzed as for choline.

Effect of Light

Paper chromatography of reineckates in all-glass chambers set in a sunlit room frequently resulted in the complete disappearance of the pink reineckate spot. Other observations suggested that light may cause decomposition of choline reineckate. To test this, an acetone solution of choline reineckate was divided equally among six 25-ml volumetric flasks. Two of these flasks contained 1.3 ml of 2 *N* NaOH; two contained 1.3 of 2 *N* HCl, and two contained 1.3 ml water. After filling to the mark with acetone, one of each pair of flasks was stored in a dark cabinet at room temperature (26–32°C), and the remaining flasks were placed 70 cm from a 60-w, tungsten light bulb that burned continuously. Samples were removed from each flask at the beginning of the experiment, and the absorbancy was measured at 526 mμ. At 24-hr intervals thereafter samples from each flask were removed, and the absorbancy was measured and recorded as per cent of the value at the beginning of the experiment.

RESULTS AND DISCUSSION

Preliminary experiments had indicated that the precipitation of choline reineckate at pH 9 often resulted in erroneously high values. 2-Dimethylaminoethanol was observed to precipitate as the reineckate from

TABLE 1

PRECIPITATION OF CHOLINE REINECKATE IN THE PRESENCE OF CERTAIN BASES AND ALKALOIDS

Sample (ml)[a]	Choline added (mg)	Absorbance	Choline recovered (mg)
1 (A)	0	0.001	
2 (A)	0	0.000	
3 (A)	0	0.001	
1 (A)	1.06	0.177	1.11
2 (A)	1.59	0.250	1.57
3 (A)	2.12	0.346	2.17
1 (B)	0	0.001	
1 (B)	0	0.002	
1 (B)	1.27	0.203	1.27
1 (B)	1.27	0.206	1.29

[a] Sample A: 0.5 mg/ml each of cinchonine, brucine, benzidine, caffeine, urea, and guanidine. Sample B: 1 mg/ml each of guanidine, adenine, urea, guanine, and 1.5 mg/ml of 2-dimethylaminoethanol. Volume was made to 25 ml with 0.2 *N* NaOH before adding 3 ml of 5% methanolic ammonium reineckate. Precipitate dissolved in 5 ml acetone and absorbance measured at 526 mμ.

solutions if the pH was 12 or less, and methylaminoethanol to precipitate from solutions at approximately pH 10. Table 1 shows that choline was quantitatively precipitated from 0.2 *N* NaOH solutions and that 2-dimethylaminoethanol and certain other bases did not interfere with this procedure. As indicated in Table 2, higher concentrations of NaOH (0.4 and 0.8 *N*) had no effect on the recovery of choline reineckate. This permitted a considerable range in the amount of NaOH that may be added to samples before the addition of ammonium reineckate. This enhances the value of this procedure for routine analysis since it is not necessary

TABLE 2

PRECIPITATION OF CHOLINE REINECKATE FROM ALKALINE SOLUTIONS[a]

Choline Cl added (mg)	NaOH (*N*)	Recovery	
		Mg	%
2.392	0	2.389	99.8
1.794	0	1.848	103.0
1.196	0	1.163	97.2
2.392	0.4	2.364	98.8
1.794	0.4	1.707	95.1
1.196	0.4	1.203	100.5
2.392	0.8	2.427	101.4
1.794	0.8	1.823	101.6
1.196	0.8	1.213	101.6

[a] Volume of samples were 25 ml; 3 ml of 5% methanolic ammonium reineckate added. Precipitate was dissolved in acetone and measured colorimetrically at 526 mμ. Recovery was calculated as choline Cl as described in text.

to measure accurately the volume of the filtrate after hydrolysis or to measure the pH by other means. However, it has been observed that ammonium reineckate may decompose suddenly in 1 *N* NaOH solutions. A curdy, bright orange-red precipitate forms which is not easily dissolved in *n*-propanol. In practice, the volume of the filtrate is estimated and a calculated amount of NaOH is added to the sample so that the concentration is between 0.1 and 0.2 *N* NaOH.

Preliminary experiments had indicated that HNO_3 hydrolysis had little effect on the recovery of pure choline chloride. Table 3 summarizes the results when samples of plant and animal tissues were analyzed for choline after hydrolysis with 25% HNO_3 and then made alkaline with NaOH. The average of 10 analyses was 6.418 mg/gm choline chloride with a standard deviation of 0.139 mg/gm. The average value was used to calculate the recovery of choline chloride added to the test sample. The average recovery of six analyses was 100.9% with a standard deviation

of 3.4.% These results indicate that the test sample used here contained no material which interfered with the analysis.

Hydrolysis with HNO_3 has certain disadvantages. It was observed that hydrolysis with 30% HNO_3, as recommended by Ducet and Kahane (8), resulted in low recovery values. Hydrolysis with 25% HNO_3 was found to be more satisfactory, but hydrolysis for 8 or 10 hr resulted in low

TABLE 3

ANALYSES OF THE TEST SAMPLE AND RECOVERY OF ADDED CHOLINE[a]

Weight of test sample (gm)	Choline Cl added (mg)	Choline Cl found	
		Mg	Mg/gm
0.1449	0	0.905	6.245
0.1530	0	0.977	6.392
0.1740	0	1.150	6.609
0.1902	0	1.220	6.415
0.2110	0	1.314	6.227
0.2221	0	1.426	6.422
0.2324	0	1.471	6.329
0.2539	0	1.629	6.415
0.2623	0	1.748	6.664
0.2975	0	1.924	6.467
			Av. 6.418 ± 0.139 mg/gm[b]
			Recovery (%)[c]
0.1031	1.932	2.590	99.8
0.1244	1.932	2.854	106.4
0.1378	1.288	2.175	100.6
0.1627	1.288	2.384	104.0
0.1772	0.644	1.761	96.8
0.1958	0.644	1.886	97.8
			Av. 100.9 ± 3.4%[b]

[a] Test sample consisted of dried rat tissues, peanut meal, soybean oil meal, and egg yolk. Hydrolyzed with 25% nitric acid, and choline reineckate was precipitated from 0.1 *N* NaOH solutions.

[b] Standard deviation.

[c] Calculated on the basis of 6.418 mg choline Cl/gm of the test sample.

recovery of choline. Optimum hydrolysis time may vary for different tissues. Maximum yield of choline from rat liver tissues alone has been observed within a 3-hr hydrolysis period.

Results from hydrolysis of the test sample with $Ba(OH)_2$, 25% HNO_3, and 9 *N* HCl are compared in Table 4. Low values were obtained with $Ba(OH)_2$ and this procedure was discarded. Hydrolysis with 9 *N* HCl for 12 hr resulted in higher values than when the samples were hydrolyzed with HNO_3. Therefore, the reineckates obtained from HNO_3

TABLE 4

HYDROLYSIS OF A TEST SAMPLE WITH $Ba(OH)_2$, HCl, AND HNO_3[a]

Test sample (gm)	Reagent	Hydrolysis (hr)	Choline found	
			Mg/sample	Mg/gm
0.1478	$Ba(OH)_2$	4	0.704	4.763
0.1901	$Ba(OH)_2$	4	0.855	4.497
0.1513	$Ba(OH)_2$	8	0.918	6.067
0.1962	$Ba(OH)_2$	8	1.050	5.351
				Av. 5.133
0.1281	25% HNO_3	5	0.962	7.509
0.1424	25% HNO_3	5	1.075	7.549
0.2064	25% HNO_3	5	1.546	7.490
				Av. 7.516
0.2135	9 *N* HCl	12	1.924	9.011
0.2031	9 *N* HCl	12	1.810	8.911
0.2337	9 *N* HCl	12	2.112	9.037
				Av. 8.986

[a] The test sample consisted of a dried mixture of rat tissues, peanut meal, soybean oil meal, and egg yolk. Three milliliters of 5% methanolic ammonium reineckate was added to the hydrolyzates made alkaline to 0.1 *N* NaOH or added directly to the $Ba(OH)_2$ hydrolyzate.

TABLE 5

PAPER CHROMATOGRAPHY OF REINECKATES OBTAINED FROM HCl AND HNO_3 HYDROLYZATES[a]

Reineckate	R_f
A. HNO_3 hydrolysis	
Choline[b]	0.00
Test sample[c]	0.00
Choline[b] + $AgNO_3$	0.00; 0.14
Test sample[c] + $AgNO_3$	0.00; 0.14
B. HCl hydrolysis	
Choline[b]	0.00
Test sample[c]	0.00; 0.20
Choline[b] + $AgNO_3$	0.00; 0.13
Test sample[c] + $AgNO_3$	0.00; 0.14; 0.22

[a] Solvent system: 1-butanol satd. with water. Spots were detected by treatment with $KBiI_4$ or I_2 vapors.

[b] Recrystallized choline reineckate.

[c] Reineckate obtained from HNO_3 or HCl hydrolyzate of dried rat and plant tissues and precipitated from 0.1 *N* NaOH.

and HCl hydrolyzates were compared with recrystallized choline reineckate on paper chromatograms (Table 5).

After development with the butanol-water solvent, only one pink spot could be observed at the origin on chromatograms of the reineckate obtained from an HNO_3 hydrolyzate of the test sample. No additional spot could be detected after exposure of the paper strip to iodine vapors or treatment with $KBiI_4$. Pretreatment of the reineckate spot with $AgNO_3$ resulted in the appearance of choline nitrate at R_f 0.14. These results compared favorably with the behavior of recrystallized choline reineckate. Similar treatment of the reineckate obtained from a HCl hydrolyzate revealed the presence of an unknown substance having an R_f of 0.20–0.22.

The reineckate obtained after HNO_3 hydrolysis was further examined by paper chromatography with an acid and a basic solvent (Table 6). In

TABLE 6
PAPER CHROMATOGRAPHY OF REINECKATE OBTAINED FROM HNO_3 HYDROLYSIS OF TEST SAMPLE

Reineckate[a]	Solvent[b]	R_f
Choline	1	0.00; 0.47; 0.83
Test sample	1	0.00; 0.40; 0.81
Choline + $AgNO_3$	1	0.00; 0.44
Test sample + $AgNO_3$	1	0.00; 0.45
Choline	2	0.00; 0.69
Test sample	2	0.00; 0.65
Choline + $AgNO_3$	2	0.00; 0.44; 0.64
Test sample + $AgNO_3$	2	0.00; 0.44; 0.62

[a] Recrystallized choline reineckate or reineckate obtained from 25% HNO_3 hydrolyzate of rat tissues, peanut meal, soybean meal, and egg yolk.

[b] Solvent 1: 1-butanol–acetic acid–water (100:30:satd. with H_2O). Solvent 2: 95% ethyl alcohol–NH_4OH (95:5). The spots were detected by exposure to iodine vapors.

these systems some decomposition of the reineckate occurred which was not evident when the neutral solvent was used (Table 5). After development with the butanol-acetic acid-water system, a faint blue spot could be detected at R_f 0.8 after exposure to iodine vapors. This decomposition could not be detected with $KBiI_4$. Choline appeared as a brown spot at R_f 0.4. When the spots were pretreated with $AgNO_3$, only the silver reineckate at the origin and the choline spot (R_f 0.44-0.45) could be detected.

When the ethanol-NH_4OH solvent was used (Table 6), decomposition was evident by the appearance of a faint blue spot (R_f 0.65–0.69). Choline was not detectable, but the behavior of the sample reineckate

compared favorably with that of recrystallized choline reineckate. No evidence was obtained to indicate that the reineckate obtained after HNO_3 hydrolysis of the test sample was not choline reineckate.

Additional attempts to detect the presence of impurities in the reineckate precipitates by scanning in the ultraviolet range with the Beckman spectrophotometer were unsuccessful. Choline reineckate in methanol exhibits two intense absorption maxima at 235 and 309 mμ. In water, the

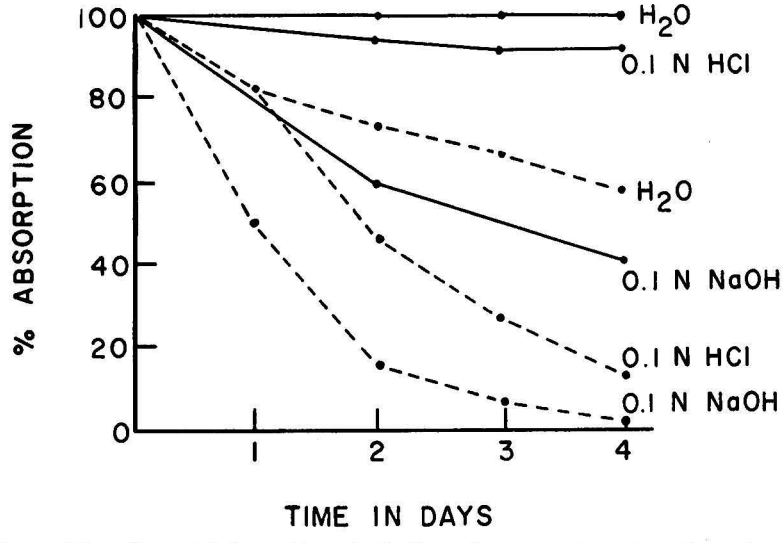

FIG. 1. The effect of light, acid, and alkali on the absorption of choline reineckate in acetone.

Acetone solutions of choline reineckate were made 0.1 *N* with respect to HCl or NaOH and then exposed to light--or stored in a dark cabinet—. Absorbancy at 526 mμ was measured and compared with the initial reading.

maxima are 232 and 302 mμ. The reineckates obtained after HNO_3 and HCl hydrolysis of the test sample had identical ultraviolet absorption curves to that of recrystallized choline reineckate in methanol and water.

The effect of light, HCl, and NaOH on acetone solutions of choline reineckate is shown in Fig. 1. Neutral acetone solutions exhibited no decrease in absorption at 526 mμ when stored in a dark cabinet. However, the presence of light, HCl, or NaOH resulted in a marked decrease in absorption, particularly when NaOH was present. This effect of acid or bases probably accounts for the decomposition observed on paper chromatograms when an acid or basic solvent system was used and may account in part for the variability in the analyses of the test sample.

SUMMARY

The estimation of choline by the reineckate method was investigated. A simplified and specific procedure for the estimation of choline in biological tissues was devised. This procedure required no preliminary extraction of the samples to be analyzed. Direct hydrolysis of the sample with 25% HNO_3, followed by precipitation of choline reineckate from 0.1 to 0.2 *N* NaOH solutions resulted in a quantitative and specific precipitation of choline. Hydrolysis of samples with $Ba(OH)_2$ resulted in inconsistent and low values while hydrolysis with 9 *N* HCl resulted in high values. Paper chromatography of the reineckate obtained after HCl hydrolysis revealed the presence of an unknown substance. No impurity could be detected in the reineckate obtained from HNO_3 hydrolzates. It was found that light and acid and alkaline conditions may cause a decomposition of choline reineckate in acetone solution.

REFERENCES

1. ENGEL, R. W., *J. Biol. Chem.* **144,** 701 (1942).
2. GLICK, D., *J. Biol. Chem* **156,** 643 (1944).
3. STRACK, E., AND SCHWANEBERG, H., *Z. physiol. Chem.* **245,** 11 (1936).
4. NEUBERGER, A., AND SANGER, F., *Biochem. J.* **36,** 662 (1942).
5. BANDELIN, F. J., AND TUSCHKOFF, J. W., *J. Am. Pharm. Assoc.* **40,** 245 (1951).
6. STREET, H. E., KENYON, A. E., AND WATSON, G. M., *Biochem. J.* **40,** 869 (1946).
7. HUERGA, J. DE LA, AND POPPER, H., *J. Clin. Med.* **30,** 463 (1951).
8. DUCET, G., AND KAHANE, E., *Bull. soc. chim. biol.* **28,** 794 (1946).
9. BRANTE, G., *Nature* **163,** 651 (1949).
10. BREGOFF, H. M., ROBERTS, E., AND DALWICHE, C. D., *J. Biol. Chem.* **205,** 565 (1953).

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A Sensitive Analytical Method for Choline

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A sensitive and generally applicable procedure for the estimation of choline in biological tissues is still lacking. The reineckate procedure is specific if choline reineckate is precipitated from alkaline solution (1) but it is insensitive. The periodide method is a sensitive procedure and has been used successfully for the estimation of choline in blood plasma (2, 3) and isolated phospholipid fractions (4–6). In such samples, interference from other substances is negligible but the method is not applicable to urine or other tissues.

Attempts to use the Smits (3) modified periodide procedure for the estimation of choline in liver homogenates were unsuccessful in this laboratory. Since it has been observed that choline reineckate exhibited an intense absorption maxima in ultraviolet light (1), the reineckate method was investigated as a possible micro method for the estimation of choline. This paper describes a reliable and sensitive procedure for the estimation of choline in tissue homogenates.

METHODS

Reagents

Choline chloride was recrystallized three times from absolute ethanol. A concentrated stock solution was prepared and analyzed for nitrogen by the micro-Kjeldahl procedure. This was diluted with 0.01 *N* HCl so as to contain 27 μg choline chloride/ml. Choline reineckate was precipitated from aqueous methanol and then recrystallized three times from acetone by the addition of water. Five per cent ammonium reineckate in methanol was freshly prepared before use. Fifty per cent ammonium hydroxide was prepared by diluting concentrated NH_4OH (sp. gr. 0.898) to 100 ml with water. Nitric acid (sp. gr. 1.4) was reagent-grade and should be colorless. Nitric acid colored yellow by oxides of nitrogen should not be used. Choline methyl-C^{14} bromide (1 mc/mmole) (Volk Radiochemical Co.)

[1] Present address: Eli Lilly and Co., Indianapolis, Indiana.

was not purified for these experiments. The radiocholine content was determined by precipitating the reineckate and counting the precipitate. 2-Dimethylaminoethanol-1,2-C^{14} hydrobromide (0.47 mc/mmole) (Tracerlab, Inc.) was distilled into dilute HCl.

Standard Curve

Choline reineckate in 50% NH_4OH exhibits an intense absorption maxima at 303 mμ. The specific absorbancy index (a_s) was determined as follows: 16.0 mg choline reineckate were dissolved in 50 ml of conc. NH_4OH and then made to 100.0 ml with water. This was used for preparing a series of solutions ranging in concentration from 1.0 to 16 μg/ml in 50% NH_4OH. The absorbancy (A) of each of these solutions was measured at 303 mμ with a Beckman model DU spectrophotometer.

Analysis of Tissue Homogenates

Weighed samples of rat tissues were homogenized for 1 min in 0.15 *M* tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.4, with a Virtis homogenizer. The homogenate was centrifuged at 3000 rpm for 5 min, and aliquots of the supernatant were transferred to 20-ml beakers. Water was added to bring the volume to 3 ml, followed by 0.53 ml of conc. HNO_3. The beakers were covered by a watch glass and allowed to simmer on a hot plate for 2.5 or 4.5 hr in subdued light.

After hydrolysis, the beakers were cooled to room temperature and then neutralized slowly to the pink of phenolphthalein (one drop in each beaker), first with NaOH pellets and then with 10 *N* NaOH. The volume of the solutions in the beakers was estimated, and a calculated amount of 2 *N* NaOH was added to bring the concentration to 0.1–0.2 *N* NaOH. After cooling to room temperature, the solutions were filtered with suction through a small sintered-glass funnel of medium porosity. A graduated centrifuge tube was placed below the funnel inside of the suction flask to collect the filtrate. The beaker and the wall of the funnel were washed with water until the volume of the filtrate plus washings was 5 ml. The centrifuge tube was then chilled before adding 2 ml of 5% ammonium reineckate solution. The centrifuge tube was again chilled for 4 hr.

The reineckate precipitate was centrifuged out at 3000 rpm for 10 min and the supernatant discarded. The precipitate was washed three times by suspending it in 1 ml of cold 1-propanol and centrifuging. The washings were decanted and discarded. The precipitate in the centrifuge tube was dried over H_2SO_4 in a dark cabinet.

The dry precipitate was dissolved in 1.0 ml acetone and then centrifuged to separate any insoluble material. A 0.1-ml aliquot of the acetone solution was transferred to a 10-ml volumetric flask. The acetone was

evaporated, and 5.0 ml of conc. NH_4OH was added to dissolve the dried residue. The flask was filled to the mark with water, shaken, and the absorbancy measured at 303 mμ (A_{303}). The total amount of choline chloride in the original sample was calculated from the equation:

$$\mu g \text{ choline Cl in the sample} = \frac{A_{303}}{0.041} \times \frac{139.6}{422.7} \times \text{vol.} \times 10$$
$$= 8.09 \times A_{303} \times \text{vol.} \times 10$$

where A_{303} is the observed absorbancy at 303 mμ in a light path of 1.0 cm; 0.041 is the specific absorbancy index for choline reineckate when the concentration is expressed in μg/ml; and 139.6 and 422.7 are the molecular weights of choline chloride and choline reineckate, respectively. "Vol." is the volume in milliliters of ammonium hydroxide used to dilute the 0.1-ml aliquot, and "10" indicates that one-tenth of the sample was used for absorbance measurements.

When radioactivity measurements were made, 0.8 ml of the remaining acetone solution was plated on a planchet and counted in a Nuclear-Chicago thin-window gas-flow counter. Total radioactivity in the sample was calculated and the specific radioactivity was calculated from the total choline chloride found in the sample.

RESULTS AND DISCUSSION

The usual method for the estimation of choline depends on the solution of choline reineckate in acetone which is then measured colorimetrically. For measurements in ultraviolet light, it was necessary to replace acetone by a solvent system which exhibits little or no absorbance in ultraviolet light, but which readily dissolves choline reineckate. This compound is practically insoluble in water and alcohol but readily dissolves in concentrated NH_4OH. This is an objectionable solvent, and it was observed that choline reineckate dissolves slowly in more dilute solutions of NH_4OH. Therefore, it is desirable to dissolve the reineckate first in concentrated NH_4OH and then dilute with water so that the final concentration is 50% NH_4OH. In addition, the absorbance of choline reineckate varies slightly with changes in NH_4OH concentration, and it is necessary that this be constant when a series of samples are to be assayed.

The relationship of absorbancy at 303 mμ to concentration of choline reineckate in 50% NH_4OH is shown in Table 1. This compound obeys Beer's law in the range measured, 1–16 μg/ml. When the concentration is expressed in μg/ml, the specific absorbancy index is 0.0408. The molar absorbancy index was calculated to be 17.2×10^3.

The feasibility of adapting this reineckate procedure to small samples

TABLE 1
ABSORBANCY OF CHOLINE REINECKATE MEASURED AT 303 mμ

Concentration of Choline Reineckate (μg/ml)[a]	A_{303}	Absorbancy index (a_s)
1	0.040	0.040
2	0.082	0.041
4	0.165	0.041
8	0.328	0.041
16	0.642	0.040

[a] Dissolved in 50% NH_4OH.

of tissue was tested on a 1:1 mixture of rat brain and liver homogenates. The results are shown in Table 2. Hydrolysis for 4.5 hr with 15% HNO_3 did not increase the yield of choline when compared to a hydrolysis time of 2.5 hr, and more consistent results were obtained with the shorter hydrolysis time. In separate experiments, hydrolysis with 20% HNO_3 for 2.5 hr also failed to increase the yield of choline from liver–brain

TABLE 2
EFFECT OF HYDROLYSIS TIME ON CHOLINE ANALYSIS BY THE REINECKATE METHOD[a]

Sample No.	Hydrolysis time (hr)	A_{303}	Choline Cl in sample (μg)
1	2.5	0.275	223
2	2.5	0.279	226
3	2.5	0.281	227
1	4.5	0.282	228
2	4.5	0.275	223
3	4.5	0.260	210

[a] Each sample consisted of 1.6 ml of a 5% rat liver and brain (1:1) homogenate, hydrolyzed with 15% HNO_3.

homogenates. With the exception of *O*-phosphocholine, it is assumed that maximum yield of choline is obtained under these conditions of hydrolysis. Phosphocholine is stable to acid hydrolysis (7), and treatment of this compound with 15% HNO_3 for 2.5 hr failed to liberate any choline. It does not precipitate as the reineckate from 0.1 *N* NaOH solutions. However, the hydrolysis of lecithin by 15% HNO_3 for 2.5 hr was found to be quantitative.

The range of the method is limited in part by the size of the sample which can be accurately measured. In Table 3, aliquots of a liver–brain homogenate, ranging from 0.2 to 2.0 ml were analyzed for choline. The choline content of the smallest aliquot (0.2 ml) was 26.2 μg or 131 μg/ml.

TABLE 3

RANGE OF THE REINECKATE METHOD AND RECOVERY OF CHOLINE CHLORIDE ADDED TO TISSUE HOMOGENATES

Homogenate (ml)[a]	Choline Cl found per sample (μg)	Choline Cl μg/ml of Homogenate	Recovery (%)
0.2	26	131	
0.4	54	135	
0.4	53	131	
0.6	78	130	
0.6	79	132	
0.8	110	137	
0.8	108	135	
1.2	155	129	
1.2	163	136	
1.6	210	131	
1.6	215	134	
2.0	285	142	
2.0	275	137	
		Av. 133 ± 3.6[c]	
1.6[b]	270	133	103[d]
1.6[b]	267	133	98[d]
1.6[b]	275	133	112[d]

[a] The homogenate consisted of a 5% rat liver and brain mixture (1:1). The volume was made to 3 ml with water and hydrolyzed with 15% HNO_3 for 2.5 hr.

[b] Choline chloride, 54 μg, added to these samples.

[c] Standard deviation.

[d] Recovery was based on the average value of 133 μg choline Cl/ml homogenate.

The average of thirteen samples was 133.8 μg choline chloride/ml homogenate with a standard deviation of ±3.6 μg/ml.

The recovery of 54 μg choline chloride added to three samples of 1.6 ml of the homogenate is also shown in Table 3. On the basis that the homo-

TABLE 4

ANALYSIS OF LIVER HOMOGENATES CONTAINING RADIOACTIVE CHOLINE[a]

Sample No.	A_{303}	Choline Cl (μg/sample)	Radioactivity		
			Cpm/sample	Cpm/μg choline Cl	Recovery (%)
1	0.146	118	768	6.5	97.2
2	0.143	116	711	6.1	90.0
3	0.149	121	751	6.2	95.0

[a] Each sample contained 0.6 ml of a 10% rat liver homogenate plus 790 cpm choline methyl-C^{14} bromide. The volume was made to 3 ml and hydrolyzed with 15% HNO_3 for 2.5 hr. Calculations are described in the text.

genate contained 133 μg choline chloride/ml, recovery ranged from 98.0 to 112% with an average of 104%.

The recovery of choline methyl-C^{14} bromide added to 0.6 ml of a rat liver homogenate is summarized in Table 4. The recovery of radioactivity varied more than did the analysis for total choline. However, the calculation for specific radioactivity yielded satisfactory results. This method has been used successfully for enzymic studies where small amounts of tissue were used and the addition of carrier choline was not necessary.

In normal animal tissues at least, 2-dimethylaminoethanol is the most likely substance to interfere with choline analysis. Previous experiments (1) had demonstrated that this compound did not interfere with the analysis of choline. However, it was desirable to examine the behavior of this compound under the slightly modified conditions of this procedure. Table 5 demonstrates that 2-dimethylaminoethanol-1,2-C^{14} hydrobromide could not be detected spectrophotometrically or by its radioactivity.

TABLE 5
THE SPECIFICITY OF THE REINECKATE METHOD

Sample	Radioactivity recovered (cpm)	Choline Cl recovered (μg)	Recovery (%)
80 μg DME[a] plus 1 ml DME-C^{14} [b]	0	0	0
40 μg DME plus 1 ml DME-C^{14} plus 135 μg choline Cl	0	134	99.3
80 μg DME plus 1 ml DME-C^{14} plus 135 μg Choline Cl	0	135	100

[a] 2-Dimethylaminoethanol-HCl.

[b] 2-Dimethylaminoethanol-1,2-C^{14} hydrobromide, 6000 cpm/ml. Each sample was made to 5 ml with water and hydrolyzed with 15% HNO_3 for 2.5 hr.

The method described here and previously (1) has the advantage that the requirements for reproducible and quantitative results are not rigid except, that in the presence of possible interfering substances, the pH should be greater than 12 when choline reineckate is precipitated. Good technique is necessary for handling acetone solutions, but if radioactivity measurements are not to be made, the choline reineckate precipitate may be dissolved directly in NH_4OH. The method is sufficiently flexible to assay samples with low choline content by using larger aliquots for absorbancy measurements instead of 0.1 ml.

This method was devised for the estimation of choline in small volumes of tissue homogenates used for enzyme studies, but it should be applicable to the analysis of plant tissues as well.

SUMMARY

A reliable and sensitive method has been devised for the estimation of choline in small amounts of tissue. This method depends on the precipitation of choline reineckate from small volumes of an alkaline solution. The choline reineckate precipitate is dissolved in 50% ammonium hydroxide and measured spectrophotometrically at 303 mμ. The specific radioactivity of the choline reineckate may be determined by counting the radioactivity of an aliquot of the choline reineckate solution. In 50% ammonium hydroxide solution, choline reineckate obeys Beer's law and has a molar absorbancy index of 17.2×10^3 when measured at 303 mμ.

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REFERENCES

1. ACKERMAN, C. J., AND SALMON, W. D., *Anal. Biochem.* **1,** 1 (1960).
2. APPLETON, H. D., LADU, B. N., LEVY, B. B., STEELE, J. M., AND BRODIE, B. B., *J. Biol. Chem.* **205,** 803 (1953).
3. SMITS, G., *Biochim. et Biophys. Acta* **26,** 424 (1957).
4. HAYAISHI, O., AND KORNBERG, A., *J. Biol. Chem.* **206,** 647 (1954).
5. WEBSTER, G. R., *Biochim. et Biophys. Acta* **20,** 432 (1956).
6. KUSHNER, D. J., *Biochim. et Biophys. Acta* **20,** 554 (1956).
7. MEYERHOF, O., AND KIESSLING, W., *Biochem. Z.* **264,** 40 (1933). See also Cardini, E. C., and L. F. Leloir, *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. III, p. 848. Academic Press, New York, 1957.

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Gas Chromatography of the *N*-Trifluoroacetylmethyl Esters of the Amino Acids

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Gas chromatography of some amino acids after conversion to the corresponding aldehydes with ninhydrin (triketohydrindene) has been reported by Hunter, Dimick, and Corse (1), Zlatkis and Oro (2), and Bier and Teitebaum (3). Bayer, Reuther, and Born (3a) succeeded in analyzing a few of the amino acids after conversion into the methyl ester. Youngs (4) chromatographed some amino acids in the gaseous phase by employing the *N*-acetylbutyl esters. The acetylated *n*-amyl esters have been used with success by Johnson and Meister (4a). An amino acid derivative which appears useful for gas chromatography is the *N*-trifluoroacetylmethyl ester described by Weygand and Geiger (5), who employed this derivative for gross distillations of protein hydrolyzates.

This paper reports on the preparation and gas chromatography of the *N*-trifluoroacetylmethyl esters of the amino acids.

EXPERIMENTAL

Preparation of the N-trifluoroacetyl-methyl esters was accomplished by preparing the methyl ester of the amino acids and then acylating the amino group. Our early method of preparing the methyl ester followed the procedure of Weygand and Geiger using dry hydrochloric acid gas and methyl alcohol. This method of preparation was found unsatisfactory for glycine. Glycine derivatives prepared with dry HCl and methanol gave, on gas chromatography, an additional less volatile component which appeared as a shoulder on the emergence curve of glycine on chromatography. When glycine was included in a mixture of amino acids and the mixture converted into the derivative with dry HCl gas as a catalyst in the esterification step, the glycine was found to react with some of the other amino acids in a manner to decrease the yields. The presence of

the second glycine component and interference with the other amino acids were eliminated by the use of the insoluble acid, sulfonated polystyrene resin (Dowex 50) in the hydrogen form as a catalyst (6–8). The sulfonated polystyrene resin not only catalyzed the formation of the methyl ester but also bound the amino acid permitting easy removal of the water of esterification by decantation of the excess methyl alcohol followed by a methyl alcohol rinse.

A typical preparation of the *N*-trifluoroacetylmethyl ester was carried out as follows. The amino acid (0.002 mole) was added to 10 ml of dry methyl alcohol in which were suspended 2.5 gm of 50–100-mesh sulfonated polystyrene resin (0.013 mole H^+). The mixture was usually refluxed for 30–45 min, cooled, and methyl alcohol decanted. (Occasional amino acids required longer reflux times because of limited solubility in methyl alcohol.) The resin was then washed once with dry methyl alcohol. To the washed resin were added 10 ml of dry methyl alcohol and 2.1 gm (0.016 mole) of the methyl ester of trifluoroacetic acid. This solution was made alkaline by adding 3.3 gm (0.033 mole) of triethylamine or by bubbling trimethylamine into the mixture for 10 min. The resulting alkaline solution was then refluxed for 15 min, cooled, and decanted from the resin, and a sample was injected without further treatment into the gas chromatographic apparatus.

The methyl ester of trifluoroacetic acid was prepared by refluxing a solution of 3 moles of methyl alcohol with 1 mole of trifluoroacetic acid. The methyl ester, distilled from the resulting solution at 40°C, was dried over anhydrous magnesium sulfate and redistilled.

Triethylamine, purchased from Eastman Kodak Company, was dried over potassium hydroxide and distilled.

Trimethylamine (anhydrous) was purchased from the Matheson Company, Inc., Rutherford, N. J.

Methyl alcohol was the J. T. Baker Chemical Company, reagent-grade, containing 0.06% water.

Gas chromatographic column packings consisted mainly of polyethylene glycol adipate adsorbed on chromosorb W, acid-washed, 80–100 mesh, purchased from Applied Science Laboratories, Inc., State College, Pa. Other column packings employed were polyvinyl acetate (Union Carbide, polyvinyl acetate AYAC), polyethylene glycol succinate,[1] and polyethylene glycol isophthalate,[1] all adsorbed on chromosorb W, acid-washed.

Detectors used were of the ionization type. The argon ionization detector (9) with a radium foil as radiation source was used for most of the determinations. A hydrogen-flame ionization detector (9) was also used in some measurements.

[1] We wish to thank Dr. E. Horning for furnishing these polymers.

RESULTS

General

The *N*-trifluoroacetylmethyl esters of 14 of the more common amino acids gave single peaks when analyzed in the polyester columns. Table 1 lists the derivatives prepared from each of these amino acids with their retention times on the polyethylene glycol adipate columns expressed as the ratio of the retention times of the derivatives compared to that of methyl stearate under similar conditions. When chromatographed on

TABLE 1

RETENTION TIMES OF *N*-TRIFLUOROACETYLMETHYL ESTERS OF AMINO ACIDS COMPARED TO METHYL STEARATE ON POLYETHYLENE GLYCOL ADIPATE COLUMNS

Amino acid	Approximate temperature (°C)	Derivative time/Methyl sterate time
Alanine	160	0.059
Valine	160	0.059
Isoleucine	160	0.076
Leucine	160	0.10
Glycine	160	0.12
Proline	160	0.22
Aspartic acid	190	0.34
Threonine	190	0.41
Methionine	190	0.71
Serine	190	0.79
Glutamic acid	190	0.82
Phenylalanine	190	0.96
Hydroxyproline	215	1.4
Lysine	215	2.7

polyethylene glycol adipate columns, these amino acid derivatives are easily resolvable with the exception of the pairs, alanine-valine and glutamic acid-serine. Separations on the other polyester columns was found to be similar to that for the adipate, although the retention times compared to methyl stearate were greater on the more polar succinate column.

Figures 1 and 2 illustrate gas chromatographs of the amino acids in the 160° and 190° temperature groups of Table 1. The amino acids in each group were mixed together and then esterified and acylated.

Threonine, serine, and lysine required closer control of the preparative procedure than that required for the other amino acids included in Table 1. Short reflux times in the acetylation step with methyl trifluoroacetate gave multiple peaks (3 for threonine and serine, 2 for lysine) on chromatographic analysis. We interpret the multiple peaks for threonine and

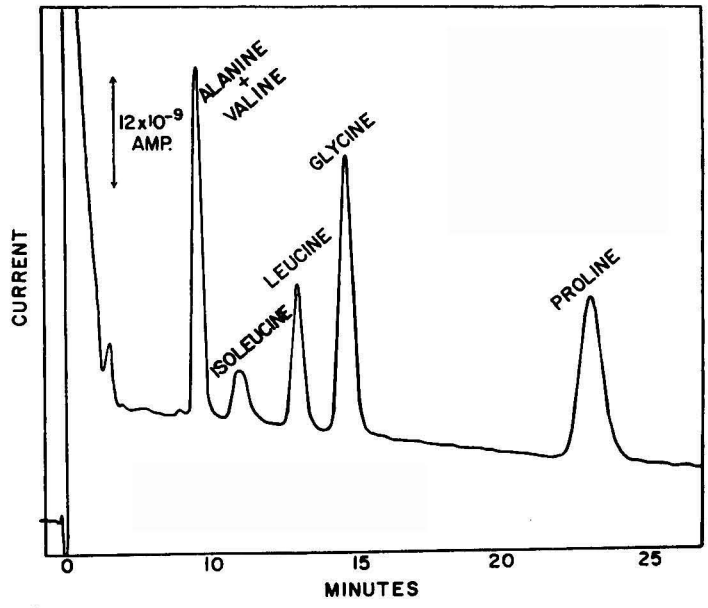

FIG. 1. Gas chromatogram from the amino acids: alanine, valine, isoleucine, glycine, and proline mixed and then converted into the *N*-trifluoroacetylmethyl esters. Column was ¼ in. × 6 ft, packed with polyethylene glycol adipate 22% by weight on chromosorb W; temperature was 162°C; carrier gas was argon at a flow rate of 80 cc/min; detector was argon ionization. The sample of the reaction mixture injected represented 16 × 10^{-6} gm of each amino acid.

serine to be derived from the N-O acyl shift to yield finally the diacetylated derivative on longer reaction times. The single final peak for lysine we also interpret to be the diacetylated derivative.

Tyrosine and arginine derivatives gave no peaks when chromatographed with the polyethylene glycol adipate columns at temperatures of about 205–210°C. The more stable polyethylene glycol isophthalate column was employed at 220°C for the study of these derivatives. The tyrosine derivative gave four peaks with retention times of 13, 17, 28, and 40 min. The arginine derivative gave two peaks—a small one at 12 min and a larger one at 26 min. There is room to believe that these peaks represent decomposition of the derivatives at the preheater block of the column.

Cystine, histidine, and tryptophan derivatives yielded negative results in our present study.

Detectors

Ionization detectors were chosen for this work because of their high sensitivity for organic substances and because of the requirement for

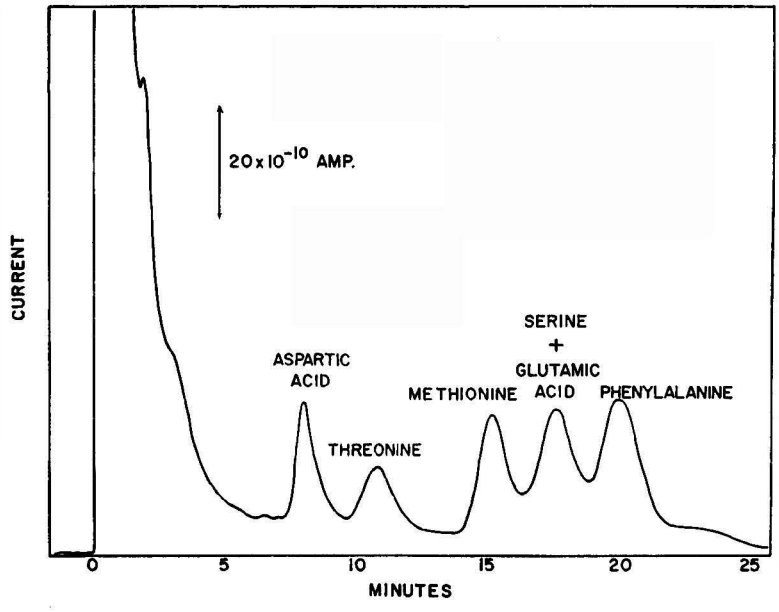

FIG. 2. Gas chromatogram from the amino acids: aspartic acid, threonine, methionine, serine, glutamic acid, and phenylalanine mixed and then converted into the *N*-trifluoroacetylmethyl esters. Column was ¼ in. × 6 ft, packed with polyethylene glycol adipate 22% by weight on chromosorb W; temperature was 181°C; carrier gas was nitrogen at a rate of 50 cc/min; detector was hydrogen-flame ionization. The sample of the reaction mixture injected onto the column represented 78 × 10^{-6} gm of each amino acid.

high sensitivity in many biological applications. The use of these detectors tends to complicate the quantitation of the results. It was difficult to determine whether failure to obtain similar peak areas for equal amounts of different amino acids represented unequal yields in the synthesis of the amino acid derivatives or represented a variation in the detector response to the different amino acid derivatives. The hydrogen-flame ionization detector showed unequal responses to supposedly equal quantities of different derivatives, and the behavior of the argon ionization detector was largely determined by its operating parameters. Analysis of the same mixture of amino acid derivatives shown by the two detectors is illustrated in Fig. 3. The ratio isoleucine:leucine:glycine is 1:2.6:1.8 for the argon detector compared to 1:2.9:0.59 for the hydrogen-flame detector. The argon ionization detector in this instance was operated at 700 v. Varying the voltage across this detector from 300 to 700 v caused the peaks to change from negative or quenching peaks at 300 v to minimal responses at 500 v and to large positive peaks at 700 v. With this same

detector, the methyl esters of fatty acids gave positive peaks (increases in current) at each of these voltage settings. At one voltage setting, different compounds derived from the same amino acid (tyrosine) showed responses that were positive, flat, and negative. These varied responses were probably a function of the fluorine content of the derivatives.

The possibility of utilizing the qualitatively discriminatory response properties of the argon ionization detector for the different derivatives containing fluorine remains to be investigated.

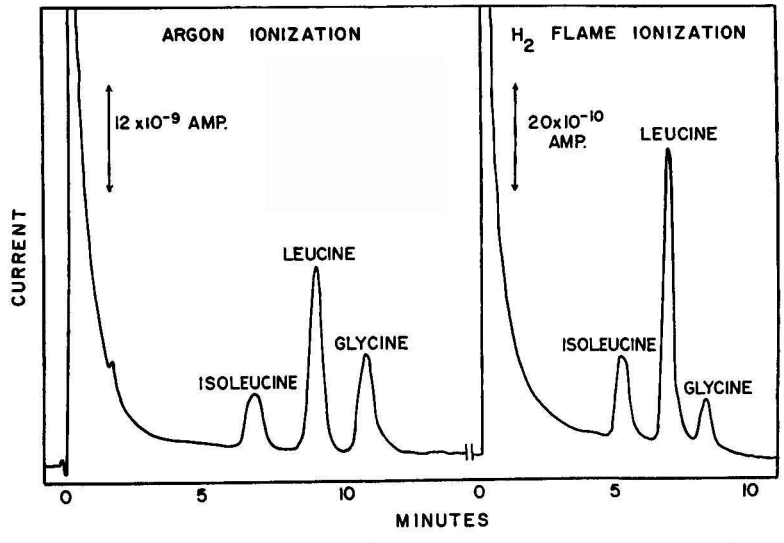

FIG. 3. Comparison of two identical samples of the derivatives of isoleucine, leucine, and glycine (19×10^{-6} gm of each amino acid) both analyzed on polyethylene glycol adipate columns, one with an argon ionization detector, the other with a hydrogen-flame detector.

It is necessary to determine the quantitative response for each individual derivative before quantitative comparisons of different amino acid derivatives can be made. An obvious alternative to the use of the ionization detectors is the use of the less sensitive but also less discriminatory thermal conductivity detectors, gas-density balances, or detectors based on the measurement of the speed of sound, all of which give a response more proportional to the molecular weight rather than to factors dependent upon the chemical nature of the derivatives.

DISCUSSION

The advantages of gas chromatography over the present methods of paper and ion-exchange chromatography lie in the decrease in time for analysis and increase in sensitivity. Gas chromatography offers a time

advantage of about one order of magnitude over the present methods. Free amino acids (in water solution) may be adsorbed on a sulfonated polystyrene resin, converted to the derivative and analyzed in a period of 1–3 hr. Ionization detectors, despite some difficulties with quantitation, offer a sensitivity which lies in the region of 10^{-3} to 10^{-4} μmole amino acid with the present equipment. Thus, 0.10 μmole leucine gave, on the hydrogen-flame detector, a signal of 4×10^{-8} coulomb (a peak with a maximum of 10.5 mv measured across a 10^{7}-ohm resistance). This peak was measured on a base line, caused by volatile material leaking off the column packing, of about 40×10^{-10} amp. A sensitivity of this order of magnitude makes possible an amino acid analysis of a hydrolyzate of a peptide contained in a single spot on an ordinary paper chromatogram.

SUMMARY

The *N*-trifluoroacetylmethyl esters were found to yield single peaks on gas chromatography for the amino acids: alanine, valine, isoleucine, leucine, glycine, proline, aspartic acid, threonine, methionine, serine, glutamic acid, phenylalanine, hydroxyproline, and lysine. Improved yields of the derivatives were prepared by the use of sulfonated polystyrene resin in the hydrogen form as a catalyst for the formation of the methyl ester.

ACKNOWLEDGMENTS

It is a pleasure for the authors to express their indebtedness to Mrs. J. W. Healy, Laura Giuffrida, and Irmgarde McCaffrey for their detailed technical assistance in the course of this work.

REFERENCES

1. HUNTER, I. R., DIMICK, K. P., AND CORSE, J. W., *Chem. & Ind.* (London) **1956**, 294.
2. ZLATKIS, A., AND ORO, J. F., *Anal. Chem.* **30**, 1156 (1958).
3. BIER, M., AND TEITEBAUM, P., *Federation Proc.* **17**, 191 (1958).
3a. BAYER, E., REUTHER, K. H., AND BORN, F., *Angew. Chem.* **69**, 640 (1957).
4. YOUNGS, C. G., *Anal. Chem.* **31**, 1019 (1959).
4a. JOHNSON, D. E., AND MEISTER, A., *Abstr. Papers, Am. Chem. Soc., New York* 1960, p. 59C.
5. WEYGAND, F., AND GEIGER, R., *Chem. Ber.* **92**, 2099 (1959).
6. SUSSMAN, S., *Ind. Eng. Chem.* **38**, 1228 (1946).
7. LEVESQUE, C. L., AND CRAIG, A. M., *Ind. Eng. Chem.* **40**, 96 (1948).
8. TSUDA, K., AND MATSUMOTO, T., *J. Pharm. Soc. Japan* **68**, 235 (1948).
9. LOVELOCK, J. E., *J. Chromatog.* **1**, 35 (1958).
10. MCWILLIAM, I. G., AND DEWAR, R. A., "Gas Chromatography" (D. H. Desty, ed.), p. 13. Butterworth, London, 1957.

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The Ammonia Content of Blood and Plasma

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INTRODUCTION

A fundamental question that still remains unanswered is whether the ammonia level of normal human blood is zero or has a definite value. This question exists despite the work of Conway (1, 2), who used his ingenious technique of microdiffusion, despite the work of Seligson *et al.* (3, 4), who used a substantially different form of microdiffusion, and despite the work of later workers (5–23), who obtained conflicting results.

In the present paper, Conway's own suggestion for reducing the complexity of his method has led to a simple and sensitive procedure that has been used to clarify further some of the artifacts influencing the determination of blood ammonia levels.

EXPERIMENTAL

Materials

Conventional Conway microdiffusion units (Arthur H. Thomas No. 4472-7) with heavy ground-glass covers were used. Titrations were carried out with a Rehberg microburet of 0.225 ml capacity (Macalaster Bicknell No. 3095).

All reagents with exception of mineral oil and paraffin were of analytical grade purity. Saturated potassium carbonate was prepared by the method of Conway (1). Boric acid absorbent (0.1%) was made by dissolving 1.0 gm boric acid in distilled water, adding 6.0 ml of 0.1% ethanolic methyl red, 4.0 ml of 0.1% ethanolic bromocresol green, and distilled water to 1 l. The color was adjusted with dilute alkali to reddish brown with a faint tinge of green (pH 5.7). This reagent was stable for several weeks but needed occasional readjustment of pH to counteract ammonia absorption from the atmosphere. Hydrochloric acid, 0.001 *N* was prepared every two weeks by pipetting 10.0 ml of 0.100 *N* hydrochloric acid into a liter volumetric flask and diluting with neutralized water containing indicator; this contained respectively 6.0 and 4.0 ml of

0.1% ethanolic solutions of methyl red and of bromocresol green in 990 ml water and was neutralized to pH 5.7. The solution was stored in a brown bottle at 3°C. A standard solution containing 300 μg NH_3 N/100 ml was prepared by dissolving 0.471 gm ammonium sulfate in 1 l of distilled water and diluting 3.0 ml to 100 ml. The fixative was made by adding 200 ml of mineral oil to 100 gm of melted paraffin wax, mixing, and cooling. Conway units were cleaned scrupulously as described by Conway (1).

Procedure

The ground-glass covers were smeared uniformly with fixative. Then 1.0 ml of boric acid absorbent was pipetted into the inner chamber and the dish tilted gently until all the inner surface was wetted. Now 1.0 ml of saturated potassium carbonate was pipetted into the far side of the outer chamber and the cover placed in position. For each blood ammonia determination, four dishes were pipetted: duplicates for blood and duplicate blanks. For routine clinical use, venous blood was drawn into a 2-ml all-glass syringe sparingly wetted with heparin solution, 5 mg/ml of isotonic saline. (Concentrated heparin solution was tested first for absence of free ammonia, as described below.) Within 10 min after drawing, the lid of a unit was slid partly open, and 1.0 ml blood was delivered directly from the syringe and its attached needle into the side of the outer chamber directly opposite from the side containing potassium carbonate. In research experiments, blood or standard solutions were delivered into the unit from a 1-ml graduated pipet; duplicate blanks were run at each time point when time was a variable. The lid was closed firmly, the time noted, and the contents of the outer chamber mixed by 15 gentle rotations given in as many seconds by tilting the unit slightly from side to side (1). Subsequent units and blanks were pipetted at 1-min intervals, and the laboratory temperature was noted. Exactly 10 min after mixing, the lid was removed and the contents of the inner chamber titrated at once to the first definite shade of pink, using 0.001 N hydrochloric acid contained in the Rehberg buret.

Calculation

The Rehberg buret is calibrated in lambda units (1 $\lambda = 0.001$ ml = 1 μl). The ammonia nitrogen content per 100 ml of whole blood is given by

$$\mu\text{g } NH_3 \text{ N/100 ml} = F(x - b) - N \qquad (1)$$

where F is the recovery factor (see Tables 1 and 2), x is the mean buret reading for blood (λ), b is the mean buret reading for blanks (λ), and N is the correction for nonspecific ammonia liberation by concentrated alkali (see Table 2). When the present procedure was used in a clinical labora-

tory, the recovery of ammonia from 1.0 ml of working standard containing 300 μg NH_3 N/100 ml was checked, using the recovery figures in Tables 1 and 2 referring to distilled water, whenever new reagents were prepared.

Special Precautions

No ammonia-containing cleaning fluid was used in the laboratory, nor were bottles containing ammonia or concentrated acid opened in the room where analyses were done. Adequate cleaning of units was very important. A slow alkaline color change was usually observed when absorbent was pipetted into the inner chamber of "clean" units. This was caused mainly by ammonia absorption from air, but also by potassium carbonate trapped in an invisible film of fixative covering the units. For experiments in which low blanks were important, the inner chamber of clean units was filled with approximately 3 ml absorbent, and titrated to the end point with 0.001 *N* hydrochloric acid at least twice during 90 min standing. After the last titration, the absorbent was sucked out. The units were now prepared as usual with 1 ml absorbent and 1 ml of saturated potassium carbonate; but just before closing each unit, its absorbent was titrated to the end-point color. When time permitted, each unit was reopened, retitrated, and closed again immediately before a run was begun. A constant temperature of 24.0 $\pm$ 1.0°C was maintained unless stated otherwise. This eliminated need for a temperature correction when comparing different experiments. No such precautions were taken during routine clinical use of the method.

RESULTS

Modification of the Absorbent Medium

The buffering action of the boric acid absorbent has the advantage of ensuring that ammonia is absorbed at an acid pH, but the disadvantage that it makes the end point of the titration of absorbed ammonia against standard acid insensitive. Conway (1) used boric acid (1%) only as absorbent for relatively high concentrations of ammonia; for low concentrations, he used back-titration of ammonia absorbed in a known aliquot of dilute acid. However, Conway suggested (1) that a more convenient procedure might be worked out, using absorption into a low concentration of boric acid and direct titration of absorbed ammonia with standard acid.

Figure 1 shows the effect of boric acid concentration on the change in pH due to ammonia absorbed in it, following diffusion in Conway units. From an equation given by Conway (1), it was calculated that after prolonged diffusion, 98.8% of the ammonia liberated is absorbed by an

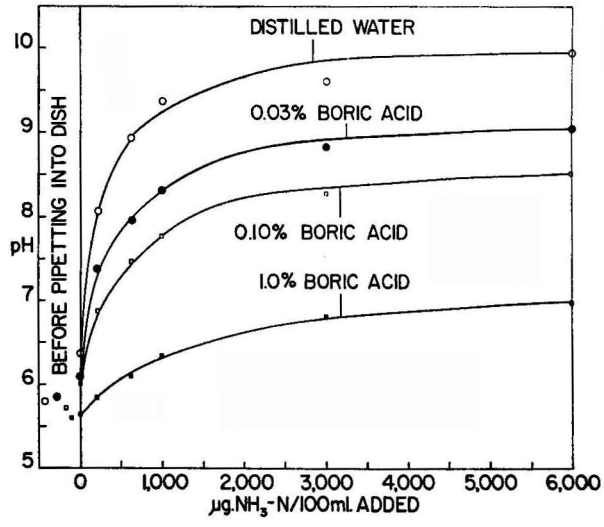

FIG. 1. The final pH of 1 ml boric acid absorbent in the inner chamber of a Conway unit, following diffusion of standard $(NH_4)_2SO_4$ solutions for 10 min at 24°C.

absorbent at pH 7.7. Figure 1 shows that the 0.1% boric acid absorbent remains below pH 7.7 up to 900 µg NH_3 N/100 ml, i.e., for all conceivable clinical situations.

Figure 2 illustrates ammonia recovery in Conway units. As in Fig. 1,

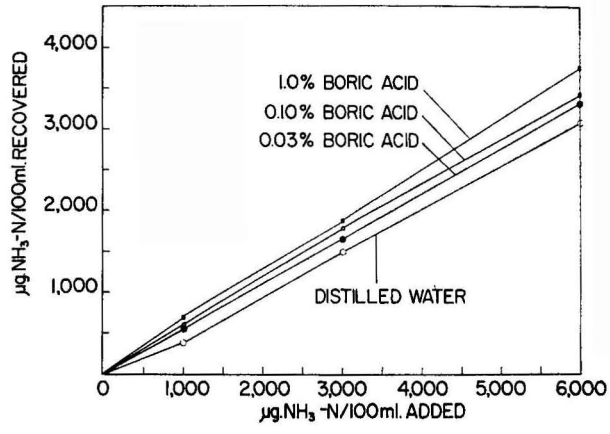

FIG. 2. Recovery of added NH_3 N in 1 ml boric acid absorbent in the inner chamber of a Conway unit, following 10 min diffusion of $(NH_4)_2SO_4$ solutions at 24°C.

higher ammonia concentrations than the maximum of about 900 µg NH_3 N/100 ml found in hepatic coma were used, to show that absorption does

not break down due to a rise in pH of the absorbent fluid. However, the percentage recovery was higher at higher boric acid concentrations.

The indicator composition was chosen after trial of various mixtures of bromocresol green and methyl red.

Effect of Diffusion Time on Ammonia Recovery

Recovery of ammonia from blood (Fig. 3) was determined as the difference between the ammonia recovered from 19 parts fresh blood mixed with 1 part ammonium sulfate dissolved in isotonic saline, and from 19 parts of the same blood mixed with 1 part of isotonic saline. In two ex-

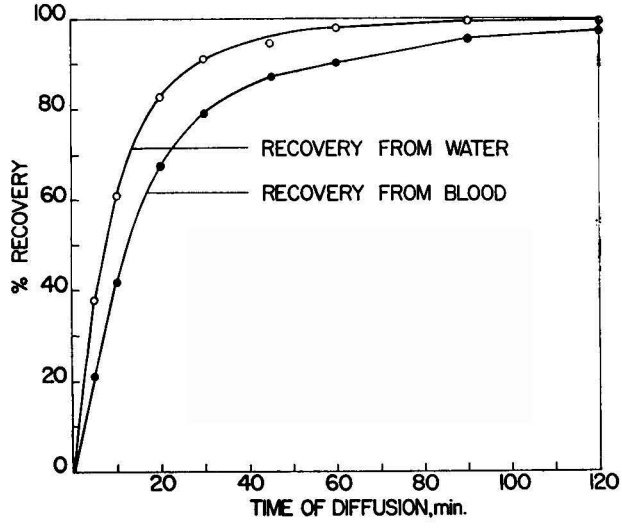

FIG. 3. Recovery of 3 μg of added NH_3N from 1 ml water or from 1 ml of rabbit blood following diffusion in Conway units for various time periods at 24°C.

periments, the maximum difference from the mean recovery figure obtained with triplicate determinations was 2.0%. Table 1 gives recovery factors corresponding to Fig. 3. The lower rate of diffusion of ammonia from blood is ascribed to slower diffusion within the viscous mixture of denatured blood and alkali.

Effect of Temperature on Ammonia Recovery

Determination of ammonia recovery at different temperatures proved unexpectedly difficult, since the temperature within the units lagged as much as 8°C behind air temperature while the laboratory was being heated. Temperature was determined by a thermocouple sealed within a Conway unit and immersed in the absorbent in the inner chamber. Above

TABLE 1

RECOVERY OF AMMONIA NITROGEN FROM WATER AND BLOOD AT 24°C

Time of diffusion (min)	Values for recovery factor F^a	
	Distilled water	Whole blood
5	3.88	6.70
10	2.38	3.41
20	1.70	2.11
30	1.56	1.78
45	1.48	1.62
60	1.43	1.54
90	1.41	1.45
120	1.40	1.42
360	1.40	1.41

[a] F = (100/% recovery) × 1.4.

about 27°C, the paraffin fixative began to lose its adhesiveness and was replaced by Lubriseal.

The results for ammonia recovery from aqueous ammonium sulfate solutions were averaged, a smooth curve was drawn through the experimental points, and the data read from it were recorded in Table 2. The

TABLE 2

TEMPERATURE DEPENDENCE OF FACTORS FOR ESTIMATING RECOVERY OF AMMONIA FROM DISTILLED WATER OR HUMAN BLOOD FOLLOWING DIFFUSION IN CONWAY UNITS FOR A PERIOD OF 10 MINUTES

Laboratory temperature during diffusion (°C)	F^a Factor for recovery of NH_3 N in a diffusion period of 10 min from		N Nonspecific ammonia production
	Distilled water	Blood	μg NH_3 N/100 ml/10 min
30	2.18	3.11	23
29	2.21	3.15	21
28	2.24	3.20	19
27	2.27	3.25	17
26	2.30	3.30	16
25	2.34	3.35	14
24	2.38	3.41	13
23	2.42	3.47	12
22	2.46	3.53	11
21	2.51	3.60	10
20	2.56	3.67	9
19	2.61	3.74	8
18	2.66	3.82	7
17	2.72	3.90	7
16	2.78	3.98	6
15	2.84	4.06	5

[a] F = (100/% recovery) × 1.4.

data were further corrected by 0.5% to coincide with a recovery of 58.8% at 24°C, which was obtained in the experiments recorded in Table 1. The data on ammonia recovery from blood in Table 2 are based on the experimentally determined recovery of 41.1% at 24°C and were calculated for other temperatures using Conway's assumption (1) that the relative change with temperature is the same for ammonia recovery from aqueous solution or from blood. The factor N in Table 2, for nonspecific ammonia production, was based on the value determined at 24°C and was calculated for other temperatures on the basis that its temperature dependence is as given by Conway (1).

Stability of Ammonium Sulfate in Fresh Blood

Within 5 min of drawing, 1 part of ammonium sulfate solution in isotonic saline was added to 24 parts of human blood to give an additional concentration of 300 μg NH_3 N/100 ml blood. At 10, 40, 70, and 145 min after this, recovery of ammonia nitrogen was respectively 100.3, 95.4, 96.3, and 102.0% relative to a control consisting of 1 part saline added to 24 parts blood. Thus, human blood does not destroy ammonium sulfate appreciably under these conditions.

Recovery from Blood at Low Ammonia Concentrations

To test the validity of the method, aliquots of isotonic buffered saline containing various known concentrations of ammonium sulfate were mixed with aliquots of a freshly drawn heparinized human blood sample, in the ratio 1 part saline: 9 parts blood. The ammonia content of the resulting mixture was immediately determined, and results were calculated using the recovery factors in Table 2. Mean results for three such experiments with buffered saline or blood are shown in Fig. 4 and indicate that recovery from blood remains proportional to ammonia addition at low ammonia concentrations.

Effect of Concentrated Alkali on Heparin

Conway (1) thought heparin unsatisfactory as an anticoagulant since alkali releases ammonia from it. In the present study, the potassium carbonate-labile NH_3 N content of 1 ml of isotonic saline containing 5000 U.S.P. units (approximately 50 mg) of heparin (Testagar & Co., Detroit, Michigan) was 1.47 μg. Since at most 0.10 ml of a 1:10 dilution was used to heparinize 2 ml blood, the maximum error due to use of heparin was less than 1 μg NH_3 N/100 ml blood.

Effect of Concentrated Alkali on Blood and Serum

Figures 5 and 6 illustrate the continuous rate of ammonia release from freshly drawn blood or serum during diffusion, confirming the data of

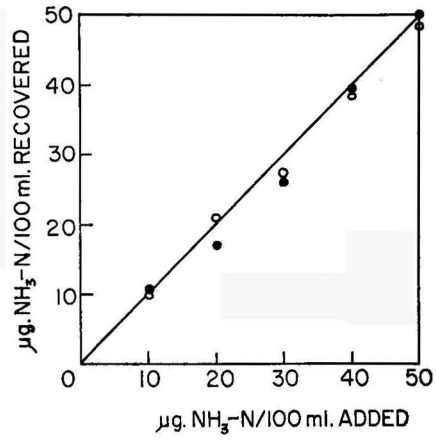

FIG. 4. Recovery of small amounts of NH_3 N added to human blood (solid circles) or to isotonic saline (open circles) in Conway units.

Conway (1) and of Jacquez *et al.* (5). These data illustrate why a correction [factor N in Eq. (1)] must be subtracted from the determined blood ammonia value, to correct for nonspecific liberation of ammonia. This correction gives essentially the same result as extrapolation of the curves

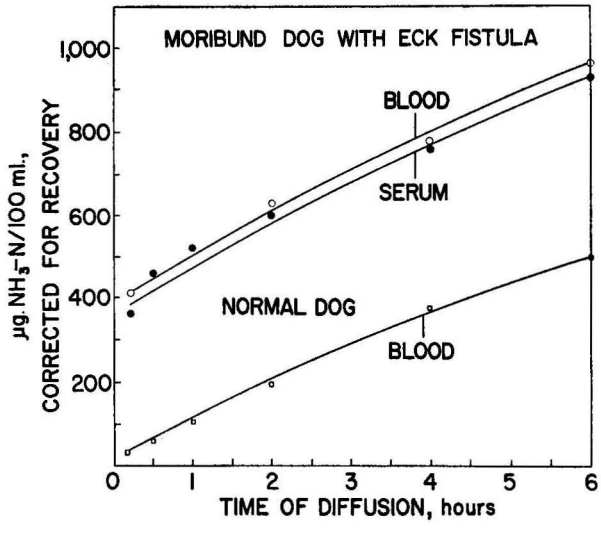

FIG. 5. Ammonia liberated from fresh normal dog blood, or from fresh blood or serum of a moribund dog with an Eck fistula, on prolonged diffusion in Conway units. Corrected for recovery, but not corrected for nonspecific ammonia production.

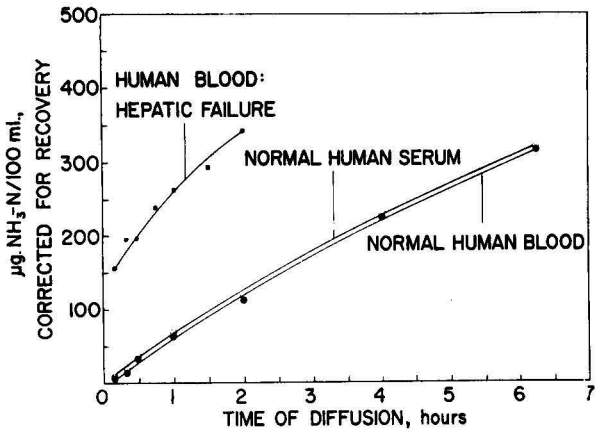

FIG. 6. Same experiment as in Fig. 5, but with blood from human beings.

in Figs. 5 and 6 to zero time of diffusion. Such extrapolation gave corrected blood ammonia levels that could lie below 10 μg/100 ml for normal human or dog blood. Equally important, Figs. 5 and 6 show that the elevated levels of blood ammonia found in liver disease are not an artifact but signify the presence of excess alkali-labile base.

Effect of Concentrated Alkali on Blood Constituents

Table 3 summarizes experiments done to investigate which blood constituents are responsible for nonspecific ammonia production on contact with concentrated alkali. Experiments A1 and A2 indicate that the rate of ammonia evolution is smallest in freshly drawn blood. Experiment A3 shows this rate is similar for dog and for human blood. Experiments A5 and A6 illustrate that both red cells and hemoglobin give a definite rate of nonspecific ammonia release.

The experimental series B in Table 3 indicates that serum has a similar rate of nonspecific ammonia production as does whole blood. In all experiments, dialysis removed most or all the ammonia content; the only exception was acid-denatured hemoglobin (Expt. A7), possibly due to its content of polypeptides of varying size that resist dialysis but are more sensitive to alkali than the intact hemoglobin molecule. Neither dialyzed rabbit serum (Expts. B4 and B5) nor dialyzed albumin (Expts. C3 and C4) replenished their ammonia content on standing for 4 weeks at 3°C.

Series C shows that L-glutamine (ammonia content less than 0.02%; Nutritional Biochemicals Corp.) was the only normal nonprotein serum constituent tested that gave a significant though small evolution of ammonia throughout the diffusion period. The rate of ammonia produc-

TABLE 3

LIBERATION OF "NONSPECIFIC" NH_3 N FROM BLOOD AND SERUM CONSTITUENTS

Solution in Conway unit	Ammonia evolved during diffusion[a]		
	During first 10 min	Av. between 10 min and 2 hr	Av. between 2 and 6 hr
	μg NH_3 N/100 ml/10 min		
A. Blood and hemoglobin			
1. Fresh nonfasting human blood	20	13	11
2. As *1.*, stood 18 hr at 23°C	218	24	16
3. Fresh fasting dog blood	16	15	13
4. Fresh nonfasting rabbit blood	118	15	
5. 3× washed 50% rabbit RBC	51	4	7
6. 2½% solution of hemoglobin	503	9	12
7. As *6.*, denatured, then dialyzed	40	5	3
B. Serum			
1. Fresh human serum	23	14	12
2. As *1.*, dialyzed	10	20	14
3. Fresh fasting dog serum	20	15	13
4. As *3.*, dialyzed	15	9	8
5. As *4.*, then stored 4 weeks at 3°C	5	12	6
6. Reconstituted lyophilized guinea pig serum	278	8	6
7. As *6.*, dialyzed	17	13	4
8. As *7.*, then stored 1 week at 3°C	39		
C. Active constituents of serum			
1. L-Glutamine, 8 mg/100 ml[b]	18	2	1
2. Albumin, 5.0% (Dade)	470	6	7
3. Albumin, 25.1%, dialyzed	11	19	14
4. As *3.*, then stored 4 weeks at 3°C	0	18	12
5. γ-Globulin, 3.9% (Dade)	852	8	4
6. As *5.*, then dialyzed	4		

[a] Corrected for recovery, but not corrected for nonspecific ammonia production.
[b] Normal level in serum (24).

tion from serum albumin and γ-globulin was similar to that from whole serum or whole blood. This suggests that "nonspecific" ammonia liberation" is due to the nonspecific action of concentrated alkali on proteins, be they serum proteins, red cells, or hemoglobin.

Increase in Blood Ammonia After Drawing

Figure 7 shows typical results obtained when freshly drawn dog blood was held in a sparingly heparinized syringe for different time intervals before determination of ammonia by diffusion for 10 min. There was a

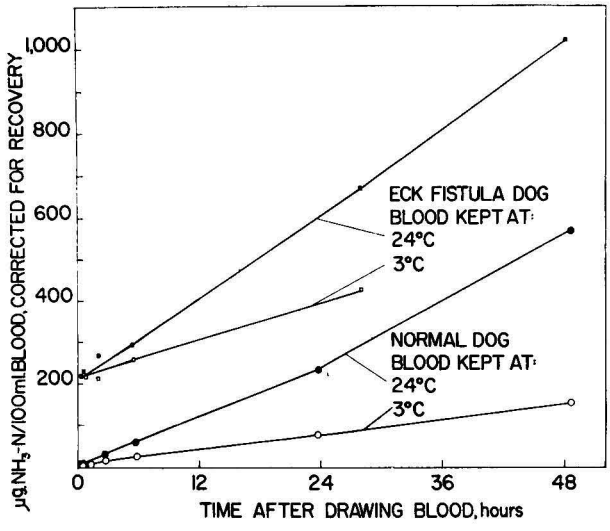

FIG. 7. Increase in the ammonia content of bloods stored both at 24°C and at 3°C for varying times after drawing. Corrected both for recovery and for nonspecific ammonia production.

steady increase of ammonia content with time, which was much higher at room temperature (averaging 1.4 μg NH_3 N/100 ml) than at 3°C. A similar rate of increase (averaging 1.0 μg NH_3 N/100 ml) was obtained with normal human blood standing at 24°C. Thus, storage at 3°C reduced but did not eliminate the increase in ammonia content of freshly drawn blood.

Blood ammonia values immediately after drawing the blood were determined with normal nonfasting young adults. All possible precautions to ensure accuracy were taken; syringes and receiving vessels were rinsed with fresh hemagglutination buffer (Difco) immediately before each experiment. With seven subjects, the rise in blood ammonia between 45 sec and 3 min after drawing was respectively 10, 9, 8, 3, 0, —3, and —3 μg NH_3 N/100 ml. The mean increase of 3 μg NH_3 N/100 ml was statistically insignificant by the standard t test. There were no significant changes in similar experiments performed with normal dogs, nor in the elevated blood ammonia levels of four patients with liver disease and one dog with an Eck fistula.

The same experiments were repeated using dry syringes containing a few crystals of ammonia-free potassium oxalate (1) instead of heparin. With blood drawn from one human being, four dogs, and two rabbits, the maximum rise in blood ammonia between 45 sec and 6 min after drawing

was 10 μg NH_3 N/100 ml. Similar results were obtained in two experiments in which normal human blood was collected in dry syringes and immediately expelled into open Erlenmeyer flasks containing either oxalate crystals or heparin solution; the final ammonia level at the end of 10 min did not exceed 25 μg NH_3 N/100 ml in flasks with oxalate, or 10 μg NH_3 N/100 ml in flasks with heparin.

Normal Blood Ammonia Values

The mean corrected blood ammonia value determined for ten normal nonstarving human adults aged 20–35 years was 7.4 μg NH_3 N/100 ml, with a range of 2–17 and a standard deviation of 5 μg NH_3 N/100 ml. With normal dogs starved 18 hr, results seemed to depend on kennel temperature. When the temperature was 22°C or less, the mean corrected value (seven dogs) was 2 μg NH_3 N/100 ml, rising to 47 (four dogs) at 28°C. The blood ammonia level tended to be higher if the dog became excited during the blood drawing procedure. With four normal-fed rabbits, the mean corrected blood ammonia value was 103 μg NH_3 N/100 ml, with a range of 48–132. Normal plasma ammonia values in human beings were essentially the same as the normal blood ammonia values.

Routine Clinical Blood Ammonia Values

The present method has been in routine clinical use in a separate laboratory for over 2 years. Without employing the special precautions outlined, blood ammonia levels in 12 patients with hepatic coma averaged 187 μg NH_3 N/100 ml, with a range of 55–418; in 27 patients with cirrhosis, the average was 128 with a range of 50–272. Under these conditions of determination, and with patients of varying healths and ages, values above 75 μg NH_3 N/100 ml were taken to be elevated.

COMPARISON WITH OTHER BLOOD AMMONIA METHODS

Seligson-Hirahara Method

The method of Seligson and Hirahara (4) was examined critically because it gives substantially higher blood ammonia levels than Conway's method (1, 2). Their claim of negligible nonspecific ammonia formation with the action of solid $K_2CO_3 \cdot 1\frac{1}{2}H_2O$–$KHCO_3$ on blood (4) was disproved by experiments in which this mixture was rotated for 20 min in a closed bottle exactly as described (4). At this point, aliquots of the blood–solid alkali mixture were pipetted to rest in the empty outer chamber of Conway units. The subsequent release of ammonia occurred at approximately the same rate as from an equivalent quantity of blood treated with saturated potassium carbonate as in Conway's method. This

experiment confirms the finding of Jacquez *et al.* (5) that nonspecific ammonia formation occurs in the Seligson-Hirahara method. Failure of the latter authors to find nonspecific ammonia formation (4) was traced to an error in their recovery equation $X = (C/X)/[(B/X) + A]$, which should read correctly $X = C/[(B/X) + A]$, or more simply $X = (C - B)/A$, where A is μg NH_3 N added, B is μg NH_3 N found in blood, C is μg NH_3 found in blood to which A was added, and X is the fraction of NH_3 N recovered from the total amount estimated to be present. Using the correct recovery equation, nonspecific ammonia formation averaging 13 μg NH_3 N/100 ml/10 min was calculated from their data (4) for subjects Nos. 7, 8, and 9 in their Table 1.

For plasma, their average of 48 μg NH_3 N/100 ml from Table 3 for presumably healthy human beings (4) is in line with present values when corrected for the rate of nonspecific nitrogen formation. As a close estimate of this rate, the value of 11 μg NH_3 N/100 ml/10 min determined for dog plasma by Jacquez *et al.* (5) can be used. Then during a period of of 3 min for initial preparation and mixing and 20 min for diffusion (4), the correction totals 25 μg NH_3 N/100 ml plasma, which subtracted from Seligson and Hirahara's value of 48 gives 23 μg/100 ml plasma as the corrected value.

For whole blood, no reliable data for nonspecific ammonia formation during Seligson-Hirahara diffusion are available. Even after such a correction (5), the results probably remain in excess of those obtained by Conway's method. To test whether this discrepancy is due to failure to obtain adequate disruption of red cells in Conway's procedure, photomicrographs were taken of whole blood treated by various procedures (Fig. 8). Surprisingly, a high percentage of red cells treated by any of the methods assayed appeared to have essentially intact cell membranes. Following gentle swirling with saturated K_2CO_3 during Conway's procedure (1), red cells were highly clumped, with individual cells badly crenated (Fig. 8*a*). Following prolonged agitation with solid alkali during Seligson's procedure (4), there was less clumping and apparently more disruption, but individual cells were similarly crenated (Fig. 8*b*). After vigorous shaking for 30 sec with 20% trichloroacetic acid in Nathan and Rodkey's method (6), there was little clumping and cells were sickle-shaped as well as crenated (Fig. 8*c*).

Experiments with Lysed Blood

Since the above experiment indicated that in Conway's procedure a proportion of red cells fail to mix well with alkali, experiments with lysed blood were done. Undiluted lysed blood was unsuitable, since it was highly viscous and caked on addition of saturated K_2CO_3. A 1:2 mixture

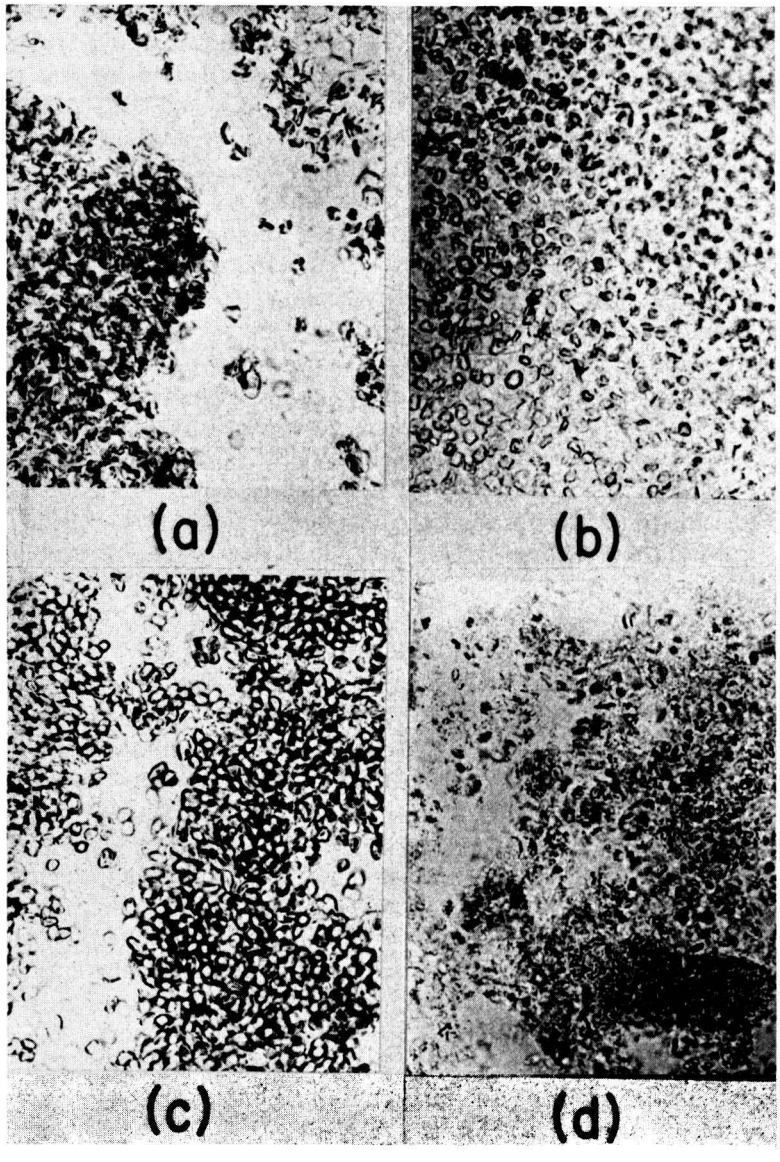

FIG. 8. Photomicrographs (×800) of human blood following treatment in various blood ammonia methods.

(*a*) Blood–saturated K_2CO_3 mixture in Conway's method (1).

(*b*) Blood–solid $K_2CO_3 \cdot 1\frac{1}{2}H_2O$–$KHCO_3$ mixture in Seligson's procedure (4).

(*c*) Blood–20% TCA mixture before addition of alkali in Nathan and Rodkey's procedure (6).

(*d*) Diluted (1:2) lysed blood–saturated K_2CO_3 mixture in Conway's method (1).

of lysed blood and distilled water had approximately the same soluble protein content as plasma. But it mixed less easily with saturated K_2CO_3 in Conway units, and the mixture contained clumps of protein (Fig. 8*d*) larger than those found in plasma–alkali mixtures. To prepare it, freshly shed blood was collected in an iced container, diluted with two parts water at 0°C in an Erlenmeyer flask, rapidly frozen-thawed three times using Dry Ice–alcohol, and warmed to room temperature. Compared to control blood held at 0°C during the intervening 15 min, its ammonia content was higher by 64 μg NH_3 N/100 ml of undiluted blood. However, this difference was due mainly to the very rapid rate of ammonia accumulation in lysed blood standing at room temperature, which varied from 36 to 61 μg NH_3 N/100 ml/10 min in three experiments. When both lysed blood (1:2 parts water) and whole blood (1:2 parts saline)were pipetted into Conway units without prior warming to room temperature, the difference in ammonia contents essentially disappeared. The rate of nonspecific NH_3 N formation was only slightly higher in lysed than in whole blood.

Brown et al. *Adaptation of Seligson-Hirahara Method*

The work of Brown *et al.* (7) is vital in resolving the discrepancies between the Conway and Seligson methods, since they used the Seligson technique but substituted a solution of a relatively mild alkalinizing agent for solid alkali. Using essentially 2 vol of 25% Na_2CO_3 to liberate ammonia from 1 vol plasma, they obtained complete recovery of added ammonium ions during 20 min diffusion, but failed to correct for nonspecific ammonia formation. Employing their mixture of plasma and Na_2CO_3 in Conway units in this study, a rate of nonspecific ammonia formation of 10 μg NH_3 N/100 ml/10 min was obtained. (To obtain this figure, the recovery factor F for ammonium ions added to the plasma–Na_2CO_3 mixture was determined for diffusion periods of 10 min and 2 hr at 24°C as respectively 5.37 and 1.50.) This rate is probably higher under the dynamic mixing conditions of Seligson's method; however, even this minimum correction reduces their results for plasma drawn from healthy young males from 39 to 19 μg NH_3 N/100 ml.

DISCUSSION

Definition of Blood Ammonia

In order to avoid confusion as to what constitutes an artifact, plasma and blood ammonia must be defined. For plasma, three of the possible definitions for ammonia content are (*a*) the free ammonium-ion concentration, (*b*) the total (free plus bound) ammonium-ion concentration, and (*c*) the alkali-labile ammonium-ion concentration. For whole blood, the

same three definitions apply to plasma plus formed elements, as well as (*d*) the alkali-labile ammonium-ion concentration of lysed blood. For precise correctness, "base" should replace "ammonium ion" in these definitions.

Blood ammonia methods that purport to offer a serious estimate of normal blood ammonia values must be judged by their success in avoiding or compensating for artifacts.

Artifacts Giving High Ammonia Values

(*A*) Ammonia formation in the time between drawing blood and the determination of blood or plasma ammonia.

(*B*) Nonspecific ammonia formation due to the continuous action of the agent liberating ammonia on blood or plasma.

(*C*) Nonspecific ammonia formation on the initial contact of the ammonia-liberating agent with blood or plasma.

(*D*) Failure to use healthy resting young adults for normal values.

(*E*) Absorption of ammonia present in air on equipment used in the determination.

(*F*) Specific experimental problems, such as failure to remove all K_2CO_3 from the inner chambers of Conway units.

(*G*) Presence of nonprotein material which alkali can degrade with liberation of ammonia.

Regarding artifact (*A*), Conway (1, 2) and White *et al.* (8) have found a rapid increase of blood ammonia from zero to about 40 μg NH_3 N/100 ml in the first 3 min after shedding, when potassium oxalate was used as anticoagulant and blood was exposed to air. Conway and Cooke (2) attributed this behavior to deamination of adenylic acid by its specific deaminase, which could be inhibited by carbon dioxide, resulting in elimination of this rapid increase (2); a similar inhibitory action has been ascribed to heparin (9). In the present study, as in those of Singh *et al.* (10) and Sherlock (11), low initial blood ammonia values were obtained. However, neither in the present study nor in those of Calkins (12) or DeGroote and Vandenbroucke (13) was there a sharp initial increase (1, 2, 8) in blood ammonia using oxalate as anticoagulant, perhaps because adequate aeration of blood did not occur. The data of Hulme and Cooper (14) are not strictly comparable since they begin only 1 min after shedding; their high variability suggests presence of an artifact. Both the methods of Seligson and Hirahara (4) and of Nathan and Rodkey (6) fail to show an increase in blood ammonia within the first few minutes after shedding.

Present results further confirm (1, 2, 5, 13) the importance of correcting

for artifact (*B*), which has been neglected by several workers (3, 4, 7, 12, 15, 16). A claim to have avoided nonspecific ammonia formation (4) has been disproved by Jacquez *et al.* (5) and in this study. Perhaps most important, the data of Brown *et al.* (7) yield low plasma ammonia levels when corrected for artifact (*B*). Finally, with the vacuum steam-distillation method of Parnas and Heller (17), normal blood ammonia values of below 50 μg NH_3 N/100 ml were obtained at a time when the accepted blood ammonia levels were very much higher.

After correction for other artifacts, presence of artifact (*C*) is revealed by comparison with another method. Thus, Brown *et al.* (7) found that three deproteinization methods, including use of trichloroacetic acid (6), gave plasma ammonium values from 25 to 175% higher than obtained without deproteinization. Since they obtained quantitative recovery of ammonium ions added to plasma, they concluded that deproteinization procedures entail a varying degree of glutamine or other amide hydrolysis. Similar conclusions were drawn by Parnas and Heller (17) following trial of protein-free tungstic acid filtrates. Thus, such deproteinization procedures (6, 18) give artifactually high values for the free ammonium-ion concentration. Similarly, use of solid alkali (4) is suspect, since its heat of solution in whole blood may cause amide hydrolysis, leading to higher blood ammonia values by artifact (*C*). Thus, results for blood ammonia of patients with diseases other than liver disease were higher by 59 μg NH_3 N/100 ml when determined using solid alkali (4) than using saturated sodium carbonate (19), despite use of the same diffusion technique in both instances.

Concerning artifact (*D*), the present incidental observations on the *in vivo* increase of blood ammonia due to a high kennel temperature or excitement of the animal fall in line with those of Schwartz *et al.* (20), that muscular exercise greatly increases the blood ammonia levels in man and dogs. Until this question has been delineated further, it seems relevant to note the physiologic state of the subjects when blood ammonia levels are reported. Regarding artifact (*E*), particular stress on blanks and on observation of special precautions are essential to obtaining results relevant to the present objectives.

Evidence that ammonia is present in the expired air of normal anesthetized dogs does not prove that ammonia was present in their blood; for trachael cannulation, which was done to exclude ammonia formed in the mouth by bacterial action (21), does not exclude ammonia formation deeper in the respiratory tree [artifact (*F*)]. Regarding the role of glutamine [artifact (*G*)], Nathan and Warren (22) have shown that it contributes significantly to ammonia levels in brain as determined by their

method. However, little or no elevation of glutamine is found in patients with high blood ammonia values (10, 23), in line with the present results that the presence of glutamine is not normally a significant artifact in the Conway procedure.

Artifacts Giving Low Ammonia Values

(*a*) Application of recovery data obtained with water to recovery from serum or blood.

(*b*) Failure to obtain complete mixing between plasma and the ammonia-liberating agent.

(*c*) Failure to obtain complete contact between the formed elements of blood and the ammonia-liberating agent.

(*d*) Failure to lyse red cells or otherwise to extract their entire content of ammonium ions.

(*e*) Recovery data obtained with added ammonium ions apply only to free ammonium ions in plasma. Such data may give low values if applied to the recovery of free ammonium ions in red cells, or of bound ammonium ions in plasma or red cells.

Regarding artifacts giving low ammonia values, a more common artifact (23) than artifact (*a*) has been risked by all investigators who used Conway's method both for blood and for plasma, including this investigator. This risk involves use of recovery data obtained with blood to estimate recovery from plasma. Happily, the most likely result is overestimation rather than underestimation of plasma ammonium-ion content.

Granted 100% recovery of ammonium ions added to plasma, either in fact or by application of a recovery factor, than artifacts (*b*) and (*e*) are relevant only if the objective is to determine the hypothetical bound plasma ammonium ion content. Since Conway's procedure gives results for plasma similar to those obtained by the method of Brown *et al.* (7) that involves thorough mixing, artifact (*b*) does not apply. In this connection it is irrelevant that the need for a higher recovery factor for ammonium ions added to plasma rather than to water may be due to the trapping of some ammonium ions in protein precipitates, in addition to slower diffusion within the alkalinized mixture.

Demonstration (Fig. 8*a*) that red cells remain strongly clumped in Conway's procedure makes it questionable that recovery of the free ammonium-ion content within the cells is complete [artifacts (*c*) and (*d*)]. Close correspondence between ammonia content of plasma and whole blood may be a coincidence of higher ammonia content (4) and lower ammonia recovery from the red cells. However, even with severe mixing as in Seligson's procedure, the formed elements were not lysed (Fig. 8*b*). While the solution may lie in using lysed blood, present ex-

periments with it were not decisive. Difficulties encountered include a rapid increase in ammonia content after lysing, failure to obtain good mixing with saturated K_2CO_3, and decrease in sensitivity through working with dilutions of lysed blood. In addition, artifactual increase in ammonia content caused by the lysing procedure is a possibility.

Determination of the hypothetical bound ammonium-ion content of plasma or blood is virtually impossible due to artifact (*e*). Hence by necessity, the free ammonium-ion concentration of plasma is the only definition of fundamental value. Unfortunately, the same definition applied to whole blood can be interpreted in several ways.

The actual property of plasma determined in Conway's procedure is the concentration of alkali-labile base; it measures all the free ammonium-ion concentration, and presumably includes some nonspecific ammonia, some "bound" ammonia, and some other volatile bases. It therefore represents an upper limit for the free ammonium-ion concentration. In this context, the present results represent a temporary solution of the problem of the ammonia content of normal plasma; for whole blood, even a temporary solution awaits further work.

SUMMARY

1. Conway's own suggestion was followed in adapting his method to permit direct titration of small amounts of diffused ammonia.

2. After correction for nonspecific ammonia formation from plasma proteins, the free ammonium-ion concentration of fresh normal resting human plasma lies below 20 μg NH_3 N/100 ml. This figure represents an upper limit of the true concentration. Much of the conflicting literature regarding this value has been resolved.

3. The corresponding value for whole blood lies below 10 μg NH_3 N/100 ml as determined by Conway's procedure. However, severe clumping queries the completeness of ammonia recovery from red cells. Nevertheless, coincidence of ammonia values, whether determined on whole blood or on plasma, make Conway's method a convenient tool.

4. In human patients with liver disease and in dogs with Eck fistulae, elevated blood ammonia levels are due to a real increase in alkali-labile ammonia.

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REFERENCES

1. Conway, E. J., "Microdiffusion Analysis and Volumetric Error," 4th Ed. Crosby Lockwood & Son, London, 1957.
2. Conway, E. J., and Cooke, R., *Biochem. J.* **33,** 457 (1939).
3. Seligson, D., and Seligson, H., *J. Lab. Clin. Med.* **38,** 324 (1951).
4. Seligson, D., and Hirahara, K., *J. Lab. Clin. Med.* **49,** 962 (1957).
5. Jacquez, J. A., Jeltsch, R., and Hood, M., *J. Lab. Clin. Med.* **53,** 942 (1959).
6. Nathan, D. G., and Rodkey, F. L., *J. Lab. Clin. Med.* **49,** 779 (1957).
7. Brown, R. H., Duda, G. D., Korkes, S., and Handler, P., *Arch. Biochem. Biophys.* **66,** 301 (1957).
8. White, L. P., Phear, E. A., Summerskill, W. H. J., Sherlock, S., and Cole, M., *J. Clin. Invest.* **34,** 158 (1955).
9. Diamond, E. G., *J. Lab. Clin. Med.* **46,** 807 (1955).
10. Singh, I. D., Barclay, J. A., and Cooke, W. T., *Lancet* **226,** 1004 (1954).
11. Sherlock, S., *Am. J. Med.* **24,** 805 (1958).
12. Calkins, W. G., *J. Lab. Clin. Med.* **47,** 343 (1956).
13. DeGroote, J., and Vandenbroucke, J., *Am. J. Digest. Diseases* **3,** 502 (1958).
14. Hulme, W. A., and Cooper, A. C., *J. Med. Lab. Technol.* **13,** 543 (1956).
15. McDermott, W. V., Jr., and Adams, R. D., *J. Clin. Invest.* **33,** 1 (1954).
16. McDermott, W. V., Jr., *New Engl. J. Med.* **257,** 1076 (1957).
17. Parnas, J. K., and Heller, J., *Biochem. Z.* **152,** 1 (1924).
18. Schweigert, G., and Rapoport, S., *Voprosy Med. Khim.* **3,** 65 (1956).
19. Bessman, S. P., and Bessman, A. N., *J. Clin. Invest.* **34,** 622 (1955).
20. Schwartz, A. E., Lawrence, W., Jr., and Roberts, K. E., *Proc. Soc. Exptl. Biol. Med.* **98,** 548 (1958).
21. Jacquez, J. A., Poppell, J. W., and Jeltsch, R., *Science* **129,** 269 (1959).
22. Nathan, D. G., and Warren, K. S., *Arch. Biochem. Biophys.* **81,** 377 (1959).
23. Seegmiller, J. E., Schwartz, R., and Davidson, C. S., *J. Clin. Invest.* **33,** 984 (1954).
24. Sunderman, F. W., and Boerner, F., "Normal Values in Clinical Medicine," p. 110. W. B. Saunders Co., Philadelphia, 1950.

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A Rapid, Quantitative Determination of Total and Free Cholesterol with Anthrone Reagent[1]

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There has long been need for a rapid and quantitative colorimetric method for routine determination of free and esterified cholesterol in biological materials. A previous report from this laboratory described the preparation and use of a stable anthrone–sulfuric acid reagent for indirect determination of digitonin-precipitable 3β-hydroxy sterols (1). In addition, various points regarding the specificity and advantages of anthrone reagent were discussed.

Most commonly employed methods involving digitonin precipitation of cholesterol and other 3β-hydroxy sterols are lengthy due to the time required for quantitative precipitation of the sterol as the digitonide complex. In 1937, Obermer and Milton reported the use of aluminum hydroxide as a gathering agent for rapid precipitation of cholesterol digitonide (2). Brown *et al.* (3) have more recently compared the use of aluminum hydroxide and aluminum chloride for cholesterol digitonide precipitation from serum extracts, in combination with ferric chloride reagent for color development.

The method presented here involves rapid precipitation (15 min) of 3β-hydroxy sterols with digitonin and aluminum chloride in the presence of dilute hydrochloric acid. The sterol digitonide is then recrystallized twice from methanol–aluminum chloride (1:1), each precipitation requiring 20 min. Finally the purified sterol digitonide is dissolved in glacial acetic acid and determined colorimetrically with anthrone reagent.

In general, the major digitonin-precipitable 3β-hydroxy sterol of most animal tissues, such as liver, serum, and lymph, is cholesterol. β-Cholestanol comprises only about 1–2% of the total 3β-hydroxy sterols. Thus, the proposed anthrone method can be used for rapid microcolorimetric routine analysis of cholesterol in serum, lymph, and most other tissues,

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and in *in vitro* systems. From 30 to 40 aliquots of tissue extracts, containing between 20 and 200 μg sterol, are routinely determined in a total time of 4 hr.

EXPERIMENTAL

Materials

1. *Anthrone Reagent.* Anthrone, 0.05% in 66% sulfuric acid (v/v), containing 3% thiourea is prepared as previously described (1), except that the reagent need not be heated to dissolve the anthrone and thiourea.

2. *Cholesterol Standard.* (*a*) *Stock Solution.* One hundred milligrams of pure cholesterol is dissolved in 100 ml of acetone–ethanol (1:1).

(*b*) *Working Standard.* Five milliliters of the stock solution is diluted to 100 ml with acetone–ethanol. Two milliliters of this solution, containing 0.1 mg cholesterol, is used as standard.

3. *Digitonin Standard.* One milliliter of glacial acetic acid containing 0.318 mg digitonin is prepared as described by Webster, Nichols, and Chaikoff (4).

4. *Aluminum Chloride, 10% Aqueous Solution.* One hundred grams of aluminum chloride hexahydrate is dissolved in distilled water and made to 1 l.

5. *Hydrochloric Acid, 3.5% Aqueous Solution.* Ten milliliters of concentrated hydrochloric acid (35%) is diluted to 100 ml with distilled water.

6. *Methanol.*

7. *Other Reagents.* These are described by Sperry and Webb (5).

Procedure

Acetone–ethanol (1:1) has been used for the extraction of total sterols from tissues (25 parts solvent: 1 part tissue) as described by Sperry and Webb (5). For determination of total sterols, two drops of 50% potassium hydroxide (w/v) is added to 1 ml of the acetone–ethanol extract in a centrifuge tube, and the solution is mixed with a tube buzzer.[2] The tubes are collectively placed in a 45°C tube heater[2] for 30 min with intermittent buzzing. Following hydrolysis of esterified sterols, excess potassium hydroxide is neutralized with 3.5% hydrochloric acid with phenolphthalein as indicator, and all solutions are made to 2 ml with acetone–ethanol. For determination of free sterols, 2-ml aliquots of the extract are pipetted into centrifuge tubes. At this point, a standard containing 100 μg cholesterol/2 ml acetone–ethanol is added to the series. One drop of 3.5% hydrochloric acid and 1 ml of 0.5% digitonin in 50% aqueous ethanol (5)

[2] Biochemical Associates.

are added to all tubes, and the mixtures are stirred by buzzing. One milliliter of 10% aluminum chloride is added, the contents are mixed as above, and then heated at 45°C for 15 min. After cooling for 1 min, the tubes are centrifuged for 20 min at 2500 rpm, the supernatant is decanted and discarded, and the tubes are drained for 1 min by inversion. Two milliliters of methanol is added to each tube, and the sterol digitonides are dissolved by buzzing and gentle warming.[3] One drop of 3.5% hydrochloride acid and 2 ml of 10% aluminum chloride are added, and, following buzzing, the tubes are heated at 45°C for 20 min. The tubes are then cooled for 1 min, centrifuged at 2500 rpm for 20 min, and decanted and drained as in the previous step. To insure complete removal of digitonin, the methanol–aluminum chloride recrystallization step is repeated. Finally, 2 ml acetone is added, and the precipitates are dispersed thoroughly by buzzing. These are centrifuged for 5 min at 2500 rpm, and the solvent is decanted. One milliliter of glacial acetic acid is added immediately, and the precipitates are suspended by vigorous buzzing and warming at 45°C if necessary. A blank, containing 1 ml of glacial acetic acid is prepared, and a standard containing 0.318 mg digitonin in 1 ml of glacial acetic acid is added to the series if a standard of 100 μg cholesterol was not carried through the procedure. Ten milliliters of anthrone reagent is forcefully blown directly into the glacial acetic acid suspension, and the tubes are capped with air condensers and collectively heated in a boiling water bath for 12 min. These are then immediately cooled in cold tap water for 10–15 min, transferred to colorimeter tubes, and read at a wavelength of 600 mμ.

DISCUSSION OF RESULTS

Anthrone Reagent

As discussed previously (1), the addition of 30 gm thiourea, an antioxidant, to a solution of 500 mg anthrone in 1 l of 66% sulfuric acid gives a mixture which is chromogenically stable for 4 weeks at 25°C. Originally, this mixture was heated to 80°C to effect solution of anthrone and thiourea. However, in the present method, it has been found more satisfactory to dissolve these substances in sulfuric acid by continuous stirring for 1 hr. Anthrone reagent prepared in this manner remains stable for longer periods and gives exactly the same results as the previous reagent. Since anthrone is sensitive to fluorescent light, it is necessary to preserve the reagent in a closed container in the dark.

[3] With certain tissue sterols, including liver-free cholesterol, it is difficult to dissolve the sterol digitonide in cold methanol. Vigorous buzzing and heating of the methanol solution aids in this dissolution.

Color Development

Glacial acetic acid is used as a solvent for 3β-hydroxy sterol digitonides prior to addition of anthrone reagent. Normally, sterol digitonides are readily soluble in this acid. However, in the present procedure, minute amounts of aluminum chloride are present in the digitonide precipitates, and although the digitonide dissolves in glacial acetic acid, insoluble aluminum acetate is formed and causes the final mixture to be cloudy. This suspension is rapidly dissolved upon addition of anthrone reagent, and the presence of aluminum salts has no effect on wavelength of maximum absorption (600 mμ) or on the stability of the color produced by anthrone reagent and digitonides. To check the effect of aluminum ion on the rate of maximum color development, 24 standards containing 100 μg cholesterol in 2 ml acetone–ethanol were precipitated with digitonin and recrystallized as described under *Procedure*. After addition of glacial acetic acid and anthrone reagent, the tubes were collectively placed in a boiling water bath. Duplicate tubes, together with a blank, were removed at 2.5-min intervals, immediately cooled, and read at 600 mμ. Optical density readings attain a peak after 7.5 min of heating and remain constant for approximately 10 min of heating, thereafter. In addition, the optical density readings after complete color development occurs are identical to those of 0.318 mg digitonin standards, showing that aluminum chloride does not affect the intensity of color developed with anthrone reagent and digitonides. The results are identical to those obtained previously (1), and a standard heating time of 12 min is again employed in the present procedure.

Rapid Precipitation of Sterol Digitonides with Aluminum Chloride

Brown *et al.* (3) have reported that the addition of two drops of 30% aluminum chloride to serum extracts containing digitonin gave complete precipitation of cholesterol digitonide after 5 min of heating at 50°C. In our laboratory, this procedure was not found satisfactory for the quantitative precipitation of cholesterol over the wide ranges of sterol found in various tissues. The addition of a volume of 10% aluminum chloride equivalent to the volume of 0.5% digitonin added, and heating at 45°C for 15 min, gives consistent and quantitative precipitation of cholesterol digitonide. This precipitation must be carried out in the presence of acid to prevent the formation of insoluble aluminum hydroxide, which interferes with subsequent purification of the sterol digitonide. Hydrochloric acid is most effective for this purpose by maintaining aluminum as the chloride salt. Acetic acid, which is commonly used to provide an acidic medium for sterol digitonide formation, gives inconsistent and erratic results.

Rapid Recrystallization of Sterol Digitonides

In our previously reported anthrone method (1), boiling acetone–water (1 : 1) was used to selectively remove excess digitonin from the sterol digitonide complex. Although quantitative recoveries of sterol digitonides are obtained, the procedure is tedious and, upon centrifugation, results in a loose precipitate which requires extremely careful decantation of the solvent. Webster, Nichols, and Chaikoff reported that sterol digitonides could be separated from excess digitonin by recrystallization overnight from methanol–water (1 : 1). This separation and recrystallization procedure is quantitatively complete in 24 hr. Since aluminum chloride has been used to precipitate cholesterol as the digitonide, it seemed that this reagent might also be valuable for recrystallizing the digitonide from a methanol solution more rapidly. This was checked using 100 μg cholesterol standards precipitated with digitonin and aluminum chloride and centrifuged as described in the procedure. The sterol digitonides were dissolved in 2 ml methanol and recrystalled by addition of 2 ml of 10% aluminum chloride and heating at 45°C. Quantitative recrystallization of cholesterol digitonide was obtained after 15 min at 45°C. However, the cholesterol values obtained by this procedure were between 5 and 10% higher than those obtained by our previous method, the method of Webster *et al.* (4), or the Sperry-Webb procedure (5), indicating incomplete removal of digitonin. Complete purification of cholesterol digitonide was obtained with two methanol–aluminum chloride recrystallizations, each step taking 20 min at 45°C. Further recrystallization does not result in additional purification of sterol digitonide or removal of digitonin. To check the precipitation and recrystallizations of cholesterol digitonide with aluminum chloride, 5.45 mg digitonin in 1 ml aqueous ethanol (1 : 1) was added to 100 μg cholesterol in 2 ml of acetone–ethanol, and the mixture was carried through the outlined procedure. After the initial precipitation and each subsequent recrystallization, aliquots of the supernatants were evaporated to dryness. A blank for each supernatant, containing the same amounts of solvent and aluminum chloride, was also prepared and an aliquot was dried. One milliliter of glacial acetic acid was added to each sample and to the final digitonide precipitate, and the amount of digitonin in each sample was determined with anthrone reagent. Table 1 shows the results of the recovery. Approximately 93% (5.05 mg) of the added digitonin was recovered in the first supernatant after precipitation of cholesterol digitonide. The supernatant from the first methanol–aluminum chloride recrystallization contained 210 μg digitonin (equivalent to 66 μg cholesterol), while the second recrystallization removed approximately 20 μg digitonin (equivalent to 6.4 μg cholesterol). Thus, this latter step is necessary if a high degree of accuracy is required. The recovery of 0.32

TABLE 1
QUANTITATIVE RECOVERY OF DIGITONIN AND CHOLESTEROL DIGITONIDE[a]

	mg	%
Digitonin added for precipitation of cholesterol	5.45	
Digitonin recovered from supernatant after initial precipitation from acetone–ethanol	5.05	92.6
Digitonin recovered from first methanol recrystallization	0.21	3.8
Digitonin recovered from second methanol recrystallization	0.02	0.3
Digitonin recovered as cholesterol digitonide	0.32	5.9
Total recovery of digitonin	5.60	102.6
Theoretical amount of digitonin complexed to 0.1 mg of free cholesterol	0.318	

[a] To 0.1 mg cholesterol in 2 ml of acetone–ethanol (1:1) was added 5.45 mg digitonin. The sterol digitonide was precipitated and handled as described in the text. All washings were appropriately diluted, and aliquots were evaporated to dryness, dissolved in glacial acetic acid, and determined with anthrone reagent.

mg digitonin complexed to 100 μg cholesterol (theoretical = 0.318 mg/100 μg cholesterol) shows that there was no loss of cholesterol digitonide in the precipitation from acetone–ethanol and recrystallizations from methanol with aluminum chloride.

Free cholesterol standards in 2 ml acetone–ethanol, ranging between 10 and 200 μg, were carried through the entire anthrone procedure and compared to a standard of 0.318 mg digitonin in glacial acetic acid. The percentage recovery of cholesterol in these samples is shown in Table 2. The low recovery of the 10 μg standard is due primarily to the difficulty

TABLE 2
RECOVERY OF PURE CHOLESTEROL STANDARDS

Cholesterol present (μg)	Optical density	Cholesterol found (μg)	Recovery (%)
10	0.031	9.4	94
25	0.084	25.3	101
50	0.168	50.6	101
75	0.250	75.3	100
100	0.334	100.6	101
125	0.425	128.0	102
150	0.516	152.4	102
200	0.681	205.1	102
0.318 mg digitonin standard (equivalent to 100 μg cholesterol)			

in handling extremely small quantities of cholesterol digitonide. Similar samples containing 15 and 20 μg have given quantitative recoveries.

Precision and Accuracy

To check the reproducibility and accuracy of the proposed method, 10 ml of a pooled human serum sample was extracted with 250 ml of boiling acetone–ethanol and filtered. Twenty 1-ml aliquots were saponified with potassium hydroxide, neutralized with hydrochloric acid, and made to

TABLE 3

COMPARISON OF HUMAN SERUM CHOLESTEROL LEVELS DETERMINED BY THE PROPOSED ANTHRONE METHOD AND THE SPERRY-WEBB METHOD[a]

	Anthrone method		Sperry-Webb method		Difference	
Sample No.	μg analyzed[b]	mg %	μg analyzed[b]	mg %	μg	mg %
Total sterol						
1	79	198	75	188	4	10
2	116	290	117	292	1	2
3	76	190	75	188	1	2
4	168	420	171	426	3	6
5	121	302	116	290	5	12
6	100	251	103	257	3	6
7	75	188	75	188	—	—
8	97	242	96	241	1	1
9	79	198	82	205	3	7
10	79	198	76	190	3	8
Avg.	99	248	98	247	2.5	5
Free sterol						
1	28	35	32	40	4	5
2	43	54	43	54	—	—
3	28	35	27	34	1	1
4	76	95	81	101	5	6
5	43	54	45	56	2	2
6	53	66	54	68	1	2
7	26	33	28	35	2	2
8	42	52	41	51	1	1
9	40	49	39	48	1	1
10	30	37	28	35	2	2
Avg.	41	51	42	52	2	2

[a] Serum samples were extracted by boiling with 24 vol of acetone–ethanol (1:1). Comparable aliquots were analyzed for total and free cholesterol by the two methods.

[b] The optical density readings for cholesterol analyzed by the anthrone procedure ranged between 0.091 and 0.553 using 1 ml of the 1:25 extract for total cholesterol and 2 ml for the free fraction. Using 1 and 3 ml, respectively, for total and free cholesterol by the method of Sperry and Webb, the reading ranged between 0.047 and 0.191 optical density units.

2 ml with acetone–ethanol. Ten samples were analyzed for total cholesterol by the method of Sperry and Webb (5), giving an average value of 252 mg % with a standard error of the mean of ±2.6 mg %. Ten comparable samples, analyzed for total 3β-hydroxy sterols by the anthrone procedure, gave an average value of 251 mg % with a standard error of ± 1 mg %. The data show that the proposed anthrone method has an accuracy comparable to that of the Sperry-Webb procedure, and a greater degree of precision.

Ten samples of human sera were analyzed for total and free sterols by the anthrone and Sperry-Webb methods. Table 3 shows the agreement in results between single routine analysis by the two methods when expressed as micrograms determined, or as mg/100 ml serum. A similar comparison with duplicate determinations of total and free sterols on sera from six atherosclerotic patients is summarized in Table 4. Agreement of duplicate

TABLE 4
COMPARISON OF SERUM CHOLESTEROL FROM ATHEROSCLEROTIC PATIENTS

	Anthrone method (mg %)[a]		Sperry-Webb method (mg %)[a]	
Serum No.	Sample 1	Sample 2	Sample 1	Sample 2
		Total sterols		
1	235	235	246	248
2	304	311	310	310
3	180	180	181	176
4	198	197	198	196
5	265	273	277	281
6	164	163	163	161
		Free sterol		
1	64	64	64	65
2	82	79	71	70
3	45	46	48	47
4	53	52	45	45
5	72	72	66	—
6	46	45	39	41

[a] The amount of sterol determined in the two methods ranged from 31 to 124 μg.

analysis within each method and between the two methods is excellent. It is obvious from the data on sera that cholesterol is the major digitonin-precipitable 3β-hydroxy sterol present in measurable amounts; thus the proposed anthrone procedure can be used as a routine clinical method for determination of total and free serum cholesterol levels.

Comparative data on the recovery of cholesterol from 1-ml aliquots of 1 : 100 extract of rat liver are shown in Table 5. As in the caes of serum, the total cholesterol value, measured by the Sperry-Webb method, and the

TABLE 5
RECOVERY OF CHOLESTEROL ADDED TO LIVER LIPID EXTRACTS[a]

	Cholesterol		3β-Hydroxy sterol	
	Amount determined (μg)	Added cholesterol recovered (μg)	Amount determined (μg)	Added cholesterol recovered (μg)
Total sterols	48 (.4)		49 (.4)	
Total sterol + 100 μg cholesterol	148 (.6)	100	149 (.4)	100
Free sterols	43 (.7)		42 (.4)	
Free sterol + 100 μg cholesterol	146 (1.3)	103	140 (.6)	98

[a] One-milliliter aliquots of a 1:100 acetone–ethanol liver extract were analyzed for total and free cholesterol and total and free 3β-hydroxy sterols by the Sperry-Webb method (5) and the anthrone method, respectively. One hundred micrograms of cholesterol was added to comparable aliquots of the liver extract and determined by the respective methods. The results represent the means of 3–4 analyses. The figures in parentheses are ± standard error of the mean. Standard error of the mean = $\pm \sqrt{\frac{\Sigma(X - X)^2}{(n - 1)(n)}}$.

total 3β-hydroxy sterol value, determined with anthrone, are comparable. The recovery of 100 μg cholesterol from the liver extract was quantitative in both methods, whether analyzed for total or free cholesterol. A comparable study on the recovery of cholesterol and its saturated analog, β-cholestanol, from serum extracts is summarized in Table 6. The average serum cholesterol value before additions of sterol, and the precision of replication, was identical for both methods. Since the proposed anthrone method measures all digitonin-precipitable 3β-hydroxy sterols, there was quantitative recovery of added cholesterol (100 μg) and of 101 μg of added total 3β-hydroxy sterols, which consisted of a 1 : 1 mixture of cholesterol and β-cholestanol. The recovery of 100 μg of added cholesterol by the Sperry-Webb method was less satisfactory (96%) than by the proposed anthrone method (100%). Since the Liebermann-Burchard reagent does not give color with β-cholestanol digitonide, only cholesterol in a mixture of the two sterols is measurable by the Sperry-Webb method. The difference between the recovered sterol values obtained by the two methods will, therefore, represent the saturated 3β-hydroxy sterol, β-cholestanol, provided no other interfering substances are present in measurable amounts.

Value of the Proposed Method

The present method has several advantages over previous methods for determination of total and free cholesterol in biological materials. The desirable but lengthy procedure for preliminary separation of sterols by

TABLE 6

RECOVERY OF CHOLESTEROL AND β-CHOLESTANOL FROM HUMAN SERUM EXTRACTS[a]

Additions	Total sterol determined (μg)	Recovery of added sterol	
		μg	%
Anthrone method			
None	112 (1)		
100 μg cholesterol	212 (0.4)	100	100
101 μg total sterol (50 μg cholesterol + 51 μg β-cholestanol)	215 (0.3)	103	102
Sperry-Webb method			
None	113 (0.9)		
100 μg cholesterol	209 (2.9)	96	96
101 μg total sterol (50 μg cholesterol + 51 μg β-cholestanol)	164[b] (0)	51[b]	102

[a] The values represent the averages of 4–5 analyses. The figures in parentheses are ± standard error of the mean (see Table 5).

[b] Since the Sperry-Webb method will not determine saturated 3β-hydroxy sterols, the sterol determined and the recovery of added sterol refer only to the cholesterol present in the mixture of sterols added.

precipitation with digitonin has been shortened by the addition of aluminum chloride, the precipitation being quantitatively complete in 15 min at 45°C. Likewise, the purification of the sterol digitonide by recrystallizations from methanol replaces steps which previously required 24 hr. In the presence of aluminum chloride, total or free sterol digitonides pack extremely well upon centrifugation and do not need to be dried prior to addition of the reagent, nor must conditions be kept anhydrous during color development. The anthrone reagent introduced previously (1) and employed in the present method is stable for 4–6 weeks at room temperature when kept in a closed container in the dark. In addition, the color developed with digitonin is stable for 24 hr, provided the tubes are stoppered and are not left in the presence of fluorescent light. Thus, it is possible to analyze large numbers of samples for 3β-hydroxy sterols without special precautions for color changes due to slight variations of temperature, time, or humidity.

In general, the values for total and free cholesterol obtained by the anthrone method either closely duplicate or are slightly higher than those obtained with acetic anhydride–sulfuric acid reagent. This is to be expected since blood and tissue sterols usually contain 1–2% saturated sterols. Parallel determinations of tissue sterols by the anthrone and Sperry-Webb methods indicate that the anthrone procedure has the same

accuracy as, and a slightly greater sensitivity and precision than the latter method.

The present conditions have also been applied to the microgravimetric determination of cholesterol-4-C^{14} and for plating prior to radioactive assay. The minute amount of aluminum chloride in the isolated cholesterol digitonide does not interfere with either of these determinations.

SUMMARY

A simple, rapid microcolorimetric determination for total and free 3β-hydroxy sterols in serum and tissues has been described. Following extraction of total sterols and saponification of the esterified fraction by established procedures, the sterol is precipitated in 15 min at 45°C as the digitonide using 10% aluminum chloride. The isolated sterol digitonide is purified by twice recrystallizing from methanol–10% aluminum chloride (1 : 1), each step requiring 20 min at 45°C. The precipitate is washed with acetone and suspended in glacial acetic acid, and the digitonin moiety is determined with a stable anthrone reagent. Multiple analyses of sterol concentrations as low as 15–20 μg are routinely determined with an accuracy comparable to the method of Sperry and Webb, and with a greater sensitivity and precision than obtained with the latter method.

REFERENCES

1. Vahouny, G. V., Mayer, R. M., Roe, J. H., and Treadwell, C. R., *Arch. Biochem. Biophys.* **86**, 210 (1960).
2. Obermer, E., and Milton, R., *J. Lab. Clin. Med.* **22**, 943 (1937).
3. Brown, H. H., Zlatkis, A., Zak, B., and Boyle, A. J., *Anal. Chem.* **26**, 397 (1954).
4. Webster, W. W., Jr., Nichols, C. W., Jr., and Chaikoff, I. L., *Arch. Biochem. Biophys.* **82**, 195 (1959).
5. Sperry, W. M., and Webb, M., *J. Biol. Chem.* **187**, 97 (1950).

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A Method for the Study of the Incorporation of Inorganic Radiophosphorus into Leukocyte Deoxyribonucleic Acid[1]

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Radioactive phosphorus (P^{32}) has been used by a number of investigators to study leukocyte kinetics (1–12). The rates of incorporation of inorganic radiophosphorus into the several constituents of cells and the constancy of the level of tagging vary (2, 4, 13–17). It has been shown that tagging of deoxyribonucleic acid (DNA) occurs in some types of cells only during mitotic interphase when new DNA is being synthesized (3, 6, 17–19). Although there is evidence suggesting some turnover of DNA (16, 20), most work supports the thesis that DNA is quite stable and that when labeled with an isotope retains that label until cell division or death occurs (2, 3, 7, 16, 19, 21). The stability of DNA and the relative ease of working with radiophosphorus make this type of label a useful tool for studying leukocyte dynamics.

The Schmidt-Thannhauser (22) method for isolating nucleic acids and various modifications of it have been used widely (2, 5, 7–9, 11, 15) in studying leukocyte kinetics. This technique has been criticized by Davidson *et al.* and others as yielding DNA contaminated by other phosphorus-containing compounds (8, 14, 23). Numerous techniques for the digestion of organic matter and the quantitative determination of phosphorus have been employed, and there is some disagreement as to the optimum procedure (7, 13, 24–26). It is the purpose of this paper to present an adaptation and modification of existing methods for the determination of the incorporation of inorganic radiophosphorus into leukocyte deoxyribonucleic acid and to present data obtained with this method in studying normal rabbits.

METHODS

Leukocytes are obtained from whole blood by the method of Athens *et al.* (27). The red cells are sedimented from heparinized whole blood in

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dextran and hemolyzed with gramicidin–lysolecithin. Leukocytes are washed in saline, and platelets are removed by differential centrifugation. The remaining leukocyte suspension is fractionated as shown in Fig. 1.

FIG. 1. Fractionation scheme.

A. Homogenize leukocytes in 10% TCA	Supernatant 1
B. Homogenize residue thrice in 5% TCA	Supernatants 2, 3, 4
C. Homogenize residue twice in 70% ethanol	Supernatants 5, 6
D. Extract residue with ethanol–ethyl ether	Supernatant 7
E. Wash residue with ether	Supernatant 8
F. Hydrolyze residue in 1 *N* NaOH and precipitate DNA with 0.5 *N* sulfuric acid	Supernatant 9
G. Wash residue twice with cold 0.5 *N* sulfuric acid	Supernatants 10, 11

These cells are homogenized in 10 vol of cold 10% trichloroacetic acid (TCA) and centrifuged at 4°C for 15 min at 700 $\times$ *g*. The supernatant is decanted and the residue homogenized three times with 10-vol aliquots of cold 5% TCA. These supernatants contain the acid-soluble constituents. The residue is homogenized twice in 70% ethanol, the supernatant containing phospholipids and residual TCA being decanted after each wash. A 3 : 1 mixture of ethanol–ethyl ether is added to the residue. After homogenization this is boiled for 3 min on a water bath, and the volume of solvent lost is replaced. After centrifugation the supernatant is decanted and the residue washed once with ether. The remaining residue is dried by gentle heating as required in a water bath. For each 0.1 ml of initial leukocyte volume, 0.2 ml of 1 *N* sodium hydroxide is added and the mixture is incubated at 37°C for 40 hr. Following incubation there is routinely present a small amount of alkali-insoluble gelatinous material (14) which is separated from the solution by centrifugation. The supernatant is decanted and neutralized with ice-cold 0.5 *N* sulfuric acid and acidified by the addition of 1 ml excess. This is allowed to stand at 4°C for 1 hr, and the precipitated DNA is separated by centrifugation and washed twice with cold 0.5 *N* sulfuric acid. The DNA is hydrolyzed into soluble components by heating for 20 min at 90°C in 0.2 ml of 1 *N* sulfuric acid/0.1 ml of initial cell volume. Any visible residue of denatured protein is removed by centrifugation, and quantitative aliquots of the solution are taken for phosphorus determination and for counting of radioactivity.

Samples for counting are pipetted onto Mylar-covered scintillating plastic planchets (27), and the sulfuric acid is neutralized with an equal volume of 1 *N* sodium hydroxide to facilitate drying. Counting is done with a deep-well photomultiplier attachment and an Atomic Instruments Company decade scaler. Samples are counted to a 3% accuracy level, and all counts are corrected for background and physical decay.

To aliquots (0.1–0.2 ml) for phosphorus determination is added 0.5 ml of 10 *N* sulfuric acid, and the side of the tube is washed with 1.0 ml of phosphorus-free water. This is heated uncovered for 3 hr in a boiling water bath and diluted to 7.5 ml with phosphorus-free water.

Orthophosphate is determined by an adaptation of the method of Berenblum and Chain (28). Optimum sample size is 5–7 μg phosphorus. To the 7.5 ml of solution of the above is added 2.5 ml of 5% ammonium molybdate reagent, and the reaction between orthophosphate and molybdate is allowed to occur at room temperature in 0.5 *N* sulfuric acid. Phosphomolybdate is extracted into 10 ml isobutyl alcohol, and the butanol extract is twice washed with 5-ml portions of 1 *N* sulfuric acid. Reduction of the phosphomolybdate in isobutyl alcohol is accomplished by 8–10 ml of dilute stannous chloride reagent. The butanol phase is transferred quantitatively to a 10-ml volumetric flask and diluted to the mark with absolute ethanol. An appropriate reagent blank is prepared for each series of determinations. Optical density is determined in a Beckman DU spectrophotometer at a wavelength of 730 mμ in matched and calibrated 1 sq cm Corex cuvettes precisely 30 min following reduction.

Experimental animals (rabbits of 3+ kg weight) were given intravenously 2 μc/kg radiophosphorus[3] as sodium salts diluted in sterile isotonic saline. At the time of sacrifice each was subdued by intravenous injection of 3–4 ml of 3.5% sodium pentobarbital and then bled by cardiac puncture. Usually 120 ml blood was obtained. The leukocytes obtained from this blood were divided into two portions. One portion was used to determine the specific activity as related to nitrogen content (27) and the other was processed as described above for the determination of the specific activity of the DNA phosphorus.

RESULTS

Separation of Leukocytes

The reliability of the method as performed in this laboratory was tested by adding either platelets or red cells which had been tagged *in vivo* with diisopropyl fluophosphate-P^{32} [4] (DFP) to dextran supernatant containing untagged leukocytes. The addition of tagged platelets resulted in 2.7 counts/min/mg of leukocyte nitrogen representing less than 2% of the added platelet activity. The addition of tagged red cells resulted in 17 counts/min/mg of leukocyte nitrogen representing 2.5% of the added red cell activity. Since leukocytes from tagged animals are much more

[3] E. R. Squibb and Sons, New York, N. Y.

[4] Manning Research Laboratory, Waltham, Mass.

radioactive (Table 3), this amount of contamination is of little consequence. No effort was made to separate lymphocytes from granulocytes.

Fractionation of Leukocytes

Various aspects of the fractionation scheme were evaluated. Inorganic P^{32} was added to blood *in vitro* in amounts ranging from 0.04 to 0.12 μc/20 ml of untagged blood. These amounts represent a sizable excess to what one might expect to find in the blood of animals receiving 2 μc/kg. In one experiment using 0.04 μc/20 ml blood, only the DNA fraction was examined for activity and none was found. In subsequent experiments various fractions were analyzed for the specific activity of the phosphorus, and it was apparent that the bulk of the inorganic P^{32} had been removed by the procedure used to isolate the leukocytes. In three such examinations less than 0.1% of the added activity was recovered in the DNA fraction. These results suggest that contamination of DNA with inorganic phosphorus is probably not significant under the circumstances described.

In another experiment four rabbits were given intravenously 2 μc/kg of inorganic P^{32} and two were sacrified on days 1 and 4 post injection. The leukocytes were isolated and fractionated, and all the resulting fractions were separately analyzed for radioactivity and in most instances for phosphorus content also. The more pertinent findings were the absence of significant activity in the last acid extracts, phospholipid extracts, and DNA washes (supernatants 3, 4, 7, 8, 10, and 11: Fig. 1), and the absence of phosphorus in the DNA washes (supernatants 10 and 11). Significant phosphorus and radioactivity were found in supernatants 1, 2, 5, 6, and 9 (Fig. 1).

To evaluate the removal of ribonucleic acid (RNA) from DNA, the nucleic acid–protein fraction from pooled leukocytes of four rabbits tagged 4 days previously was dissolved in 8 ml of 1 *N* sodium hydroxide and divided immediately into eight portions. Purified untagged RNA[5] was dissolved in 1 *N* sodium hydroxide and added to six of these samples in 50-, 100-, and 200-μg amounts. Following incubation at 37°C for 40 hr, the DNA in each was obtained and the specific activity of the DNA phosphorus was determined. The mean specific activity of the two control samples was 6.75 counts/min/μg DNA-P. The mean specific activity of all eight determinations was 7.2 counts/min/μg DNA-P. No dilution of the DNA label was demonstrated. Further, the gelatinous material noted after alkaline hydrolysis of RNA was analyzed for phosphorus and found not to contain any.

Initial precipitation and hydrolysis of DNA with TCA according to

[5] Schwartz BioResearch, Inc., Mount Vernon, N. Y.

Schneider (29) was abandoned as it was found that TCA produced high phosphate blanks unless removed by complete evaporation and/or digestion with concentrated sulfuric acid, the latter being attended by considerable "bumping" and requiring subsequent hydrolysis of pyrophosphate. Sulfuric acid of appropriate normality was selected to replace TCA in this step. Following the alkaline hydrolysis of RNA, a sample was divided into two quantitative aliquots. The DNA of one sample was precipitated and washed with 10% TCA and hydrolyzed by heating at 90° in 10% TCA for 20 min. Quantitative portions of this were digested in concentrated sulfuric acid, and the DNA-P content was determined. The second sample was precipitated and washed with 0.5 *N* sulfuric acid and hydrolyzed with 1 *N* sulfuric acid at 90°C for 20 min, and the DNA-P content was determined. In the first sample 63.13 μg DNA-P was recovered, and in the second sample 65.65 μg DNA-P was recovered. Thus the use of sulfuric acid to precipitate, wash, and hydrolyze DNA compares favorably with the use of TCA and is to be preferred because of its simplicity.

Stock solutions of a purified commercially available DNA[6] were prepared in water. Portions of these were subjected to various types of hydrolysis and digestion. Table 1 presents data pertinent to the method

TABLE 1
DNA DIGESTION ON BOILING WATER BATH[a]

Digestion time (hr)	Number of determinations	DNA-P in sample (μg)[b]	Mean DNA-P recovered (μg)	Mean % DNA-P released	Mean % DNA-P released for entire group ± one standard error of mean
1	8	12.26	8.81	71.9	71.9 ± 2.9
2	2	5.02	4.10	81.7	
	9	12.26	10.94	89.2	87.7 ± 1.9
	2	12.56	10.93	87	
3	3	5.02	5.17	103	
	9	12.26	11.83	96.5	97.9 ± 1.2
	3	12.56	12.21	97.2	
4	6	5.02	4.72	94	97.2 ± 1.6
	9	12.26	12.17	99.3	
5	6	5.02	4.67	93	95.4 ± 1.7
	6	12.26	12.01	98	

[a] All samples digested with 5 meq H_2SO_4 in 1.7 ml volume on boiling water bath. Evaporation unrestricted.

[b] Total phosphorus determined by wet ashing with concentrated sulfuric acid.

of digestion adopted finally. Inspection of this table reveals that measurable DNA phosphorus reached the maximum after 3 hr of digestion in an

[6] Worthington Biochemical Corporation, Freehold, N. J.

uncovered tube in an initial 1.7-ml volume containing 5 meq sulfuric acid. With larger volumes of more dilute acid more time is required for DNA hydrolysis. Comparison of this procedure with digestions in concentrated sulfuric acid at 180°C for 1–6 hr and at 280°C for 2–5 hr revealed that neither of these latter two released more measurable DNA-P than the digestion at 100°C as described above. The adopted procedure also eliminated several steps.

Counting

Self absorption by sodium sulfate produced by neutralization of the acid on the planchet was checked for increasing amounts of the salt. The amount routinely present in experimental samples produced 5–6% absorption.

Phosphorus Determination

Reduced phosphomolybdic acid in isobutyl alcohol has a rather broad absorption peak at 730 mμ. Compliance with Beer's law was found to be satisfactory up to a concentration of 2.2 μg phosphorus/ml isobutyl alcohol, the highest concentration tested. The data in Table 2 demonstrate

TABLE 2
PHOSPHORUS DETERMINATION

Conc. P (μg/ml)	Time after reduction (optical density ± standard error of mean)[a]			Per cent change	
	20 min	30 min	60 min	20–30 min	20–60 min
0.212	0.117 ± .0007	0.115 ± .001	0.111 ± .0006	1.7	5.1
0.424	0.240 ± .003	0.236 ± .004	0.228 ± .004	1.7	5.0
0.848	0.486 ± .003	0.471 ± .002	0.457 ± .002	3.1	6.0
1.059	0.614 ± .010	0.597 ± .019	0.569 ± .014	2.8	7.3

[a] Each sample represents 3–5 determinations.

a gradual decay of color intensity and the high degree of reproducibility obtainable with the method. Concentrations producing absorption too great for determination can be diluted with absolute ethanol to a color intensity suitable for reading.

Incorporation of Inorganic P^{32} into Leukocyte DNA

Table 3 presents the findings in normal rabbits given 2 μc/kg of inorganic P^{32} and bled on the indicated day following injection. Schneider's (29) technique for DNA precipitation and hydrolysis followed by digestion in concentrated sulfuric acid was used in obtaining most of

TABLE 3
LEUCOCYTE TAGGING WITH INORGANIC P^{32}

Day post injection	Counts/min/mg leucocyte nitrogen[a]	Counts/min/μg DNAP[a]
1	227 ± 30.5	1.25 ± .143
2	452 ± 44.5	1.43 ± .28
3	535 ± 61.9	4.44 ± 1.14
4	673 ± 57.4	9.43 ± 1.20
5	676 ± 59.9	6.81 ± .60
6	539 ± 83.6	6.25 ± .43
7	559 ± 88.6	5.31 ± 1.30
8	504 ± 42.7	4.15 ± .70
10	311 ± 58.4	2.76 ± .94

[a] Each value represents the mean of 4–8 determinations ± one standard error of the mean.

these data. It was as a result of technical difficulties encountered in obtaining satisfactorily reproducible duplicate DNA-P determinations with this technique that the proposed method was evaluated and adopted. For each animal studied the total leukocyte specific activity, expressed as counts/min/mg of leukocyte nitrogen, and the specific activity of the DNA-P were determined.

DISCUSSION

The results of this study are in agreement with the findings of others (15, 30) in demonstrating the suitability of modifications of the Schmidt-Thannhauser procedure for obtaining DNA in a sufficiently uncontaminated state for use in techniques involving labeling of DNA with inorganic radiophosphorus. The prime requisite of a method for DNA isolation for the determination of the specific activity of the DNA-P is that it provide a sufficient quantity of DNA not contaminated by significant amounts of phosphorus of any sort and not that it necessarily yield quantitative recovery of DNA from the source. Accordingly, the quantitative yield of DNA by the technique described was not evaluated and no statement can be made regarding it. Lundin (15) repeated segments of Davidson's (14) work and found insignificant contamination of DNA with inorganic P^{32}. He suggested that Davidson's findings of DNA contamination might be explained by the presence of colloidal polyphosphates and/or pyrophosphates in the inorganic P^{32} preparation. Daoust (30) has demonstrated no decrease in the specific activity of DNA-P when additional RNA has been added prior to alkaline hydrolysis.

Considering that in man 15–30 μc P^{32}/kg represents a therapeutic dose, it is possible that the rather massive "tracer" doses employed by some

may create special problems in contamination and biologic effects. The present study supports the value of this method at lower concentrations of P^{32} *in vivo.*

The digestion procedure described effects complete hydrolysis of the deoxyribose phosphate esters in DNA (Table 1). Organic matter remaining after this rather mild treatment does not appear to interfere with the determination of orthophosphate in the method used.

Several points are noteworthy in Table 3. The rather striking difference between total cell activity as related to nitrogen content and DNA-P activity during the first 3 days post injection further supports the reliability of the method concerning the removal of non-DNA-P from the DNA fraction. The significant increase in total cell activity which occurred between days 1 and 2 was not accompanied by a concomitant increase in DNA-P activity. This perhaps represents the conversion of inorganic P^{32} to some saline insoluble form other than DNA-P as the procedure used for isolating the leukocytes probably extracts saline-soluble phosphorus from the cells. DNA-P specific activity peaked sharply on day 4 and thence declined virtually exponentially. No statistically significant difference in the slopes of the declining portions of the two curves was present.

SUMMARY

A modification of the Schmidt-Thannhauser procedure for isolating DNA coupled with a new method for DNA digestion and an adaptation of the Berenblum and Chain method for phosphorus determination have been shown to be suitable for use in studying the incorporation of inorganic P^{32} into leukocyte DNA. With these techniques tracer doses as small as 2 μc/kg are permitted.

Peak tagging of rabbit leukocytes was found to occur on the fourth day following injection. Disappearance of the label through the tenth day was virtually exponential.

ACKNOWLEDGMENT

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REFERENCES

1. Andreasen, E. and Ottesen, J., *Acta Physiol. Scand.* **10**, 258–70 (1945).
2. Osgood, E. E., Li, J. G., Tivey, H., Duerst, M. L., and Seaman, A. J., *Science* **114**, 95–8 (1951).
3. Howard, A., and Pelc, S. R., *Exptl. Cell Research* **2**, 178–87 (1951).
4. Osgood, E. E., Tivey, H., Davison, K. B., Seaman, A. J., and Li, J. G., *Cancer* **5**, 331–5 (1952).

5. KLINE, D. L., AND CLIFTON, E. E., *Science* **115,** 9–11 (1952).
6. LAJTHA, L. G., OLIVER, R., AND ELLIS, F., *Brit. J. Cancer* **8,** 367–79 (1954).
7. OTTESEN, J., *Acta Physiol. Scand.* **32,** 75 (1954).
8. CRADDOCK, C. G., PERRY, S., AND LAWRENCE, J. S., *J. Clin. Invest.* **35,** 285–296 (1956).
9. COASSIN, N., AND KLINE, D. L., *Am. J. Physiol.* **190,** 147–51 (1957).
10. PERRY, S., CRADDOCK, C. G., AND LAWRENCE, J. S., *J. Lab. Clin. Med.* **51,** 501–15 (1958).
11. KLINE, D. L., *in* "The Kinetics of Cellular Proliferation" (F. Stohlman, Jr., ed.), p. 142–50. Grune and Stratton, New York, 1959.
12. PERRY, S., CRADDOCK, C. G., VENTZKE, L., CREPALDIT, G., AND LAWRENCE, J. S., *Blood* **14,** 50–9 (1959).
13. MCCARTER, J. A., AND STELJES, E. L., *Can. J. Research* **26,** *Sec. E–F* 333–46 (1948).
14. DAVIDSON, J. N., FRAZER, S. C., AND HUTCHISON, W. C., *Biochem. J.* **49,** 311–21 (1951).
15. LUNDIN, P. M., *Acta Endocrinol.* **28,** *Suppl.* 40 (1958).
16. BRUES, A. M., TRACY, M. M., AND COHN, W. E., *J. Biol. Chem.* **155,** 619–33 (1944).
17. MARSHAK, A., *J. Gen. Physiol.* **25,** 275–91 (1941).
18. HULL, W., AND KIRK, P. L., *J. Gen. Physiol.* **33,** 335–42 (1949).
19. TAYLOR, J. H., *Exptl. Cell Research* **4,** 164–73 (1954).
20. FURST, S. S., ROLL, P. M., AND BROWN, G. B., *J. Biol. Chem.* **183,** 251–66 (1950).
21. HUGHES, W. L., *in* "The Kinetics of Cellular Proliferation" (F. Stohlman, Jr., ed.), pp. 83–93. Grune and Stratton, New York, 1959.
22. SCHMIDT, G., AND THANNHAUSER, S. J., *J. Biol. Chem.* **161,** 83–9 (1945).
23. DRASHER, M. L., *Science* **118,** 181–3 (1953).
24. KING, E. J., *Biochem. J.* **26,** 292–7 (1932).
25. ALLEN, R. J. L., *Biochem. J.* **34,** 858–65 (1940).
26. JONES, A. S., LEE, W. A., AND PEACOCK, A. R., *J. Chem. Soc.* **1951,** 623–4.
27. ATHENS, J. W., MAUER, A. M., ASHENBRUCKER, H., CARTWRIGHT, G. E., AND WINTROBE, M. M., *Blood* **14,** 303–33 (1959).
28. BERENBLUM, I., AND CHAIN, E., *Biochem. J.* **32,** 295–8 (1938).
29. SCHNEIDER, W. C., *J. Biol. Chem.* **163,** 293–303 (1945).
30. DAOUST, R., AND HOOPER, C. E. S., *Can. J. Biochem. and Physiol.* **35,** 721–8 (1957).

ANALYTICAL BIOCHEMISTRY 1, 391–396 (1960)

Preparation and Properties of Acrylamide Gel for Use in Electrophoresis[1]

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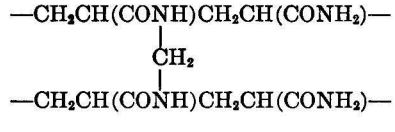
Received September 15, 1960

The introduction of a new gel medium for electrophoresis requires some justification in view of the general use and satisfactory resolutions of the Smithies (4) starch gel. This report presents reasons for considering acrylamide as a replacement for starch and describes the preparation and properties of acrylamide gel.

Acrylamide is completely soluble in buffer solutions and polymerizes in solution to form a suitable supporting medium for gel electrophoresis (3). When cross-linked with a small proportion of methylenebisacrylamide, the gel is completely insoluble and stable against temperature or pH changes. This gel possesses chemical composition and physical structure which are entirely different from starch and other gels heretofore used. Its unique characteristics may result in useful differences in ability to effect separations of different classes of compounds. In addition, we find that acrylamide gel is superior to starch in physical properties, is prepared more easily and more reproducibly, and produces protein patterns with improved definition. On the other hand, it is easier to recover protein components from starch gel by the freezing technique, which is not applicable to acrylamide gel since the latter is unaffected by freezing.

The chemical structure of the gel is a linear polymer of acrylamide, cross-linked at intervals by means of methylene bridges, viz:



This structure, which extends in three dimensions, consists of long hydrocarbon chains made hydrophilic by the amide groups attached at regular intervals. Except possibly at the terminations of the chains, there are no

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hydroxyl groups or acidic groups in the molecule. Thus this gel differs markedly from gels of starch or agar, which consist of long polysaccharide chains, having numerous hydroxyl and acidic groups. Acrylamide gel also differs from proteins because of the absence of free phenolic, carboxylic, sulfhydryl and amino groups.

EXPERIMENTAL

Preparation of the Gel

Dissolve 5.0 gm of acrylamide monomer (Cyanogum-41, American Cyanamid Co.) in 100 ml of the desired buffer. Add 1.0 ml of a freshly made 10% solution of dimethylaminopropionitrile (DMAPN, American Cyanamid Co.) in the same buffer and 1.0 ml of a freshly made 10% solution of ammonium persulfate. Pour into a suitable mold and allow to stand 3 hr or until gelled.

The gel mold (Fig. 1) is a rectangular box of Plexiglass, 33 × 10 × 5

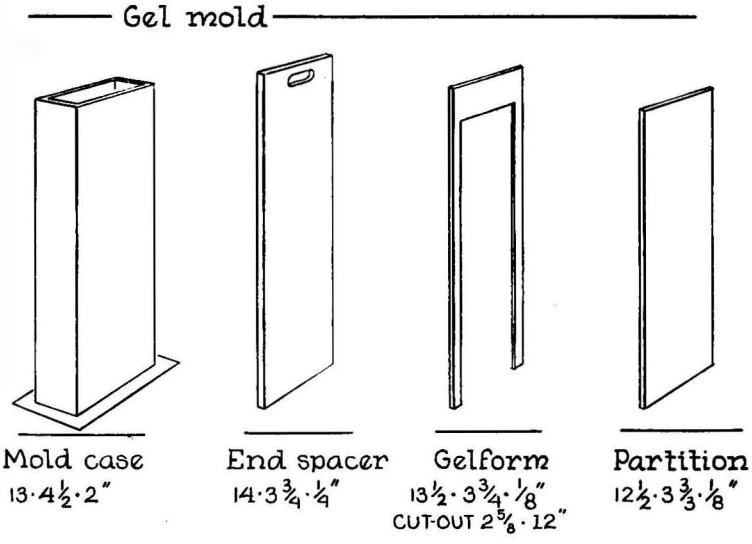

FIG. 1. Gel mold for preparation of acrylamide gel strips 6.5 × 30 cm. Material: Plexiglass.

cm. Fitting inside it are removable partitions and spacers to form three slabs of gel each 3 mm thick. By appropriate rearrangement of the partitions, gel slabs up to 9 mm thick can be prepared. Air is excluded during the gelling period by filling the mold with solution to a level above the horizontal part of the gelform.

Notes on the Preparation

The commercially available monomer mixture usually contains less than 50 mg of insoluble material per gram, and is used without repurification. The insolubles can be removed by filtration or decantation if desired. Buffers used were formate, acetate, phosphate, barbiturate, EDTA, Tris, and borate, and also dilute sulfuric acid, with a pH ranging from 1.0 to 11.0. The gel appeared unaffected by the buffer used. The catalyst solutions must be freshly made. The catalyst furnishes free radicals for initiation of the polymerization reaction but does not enter into the structure of the gel to any significant extent. When the monomer–catalyst solution is exposed to air, an upper layer to a depth of 1 cm does not polymerize. The gel mold must be designed with the necessity in mind of protecting the upper layer of solution.

The formulation given produces a gel which is insoluble in all common buffers and organic solvents and is unaffected by boiling or freezing. On dehydration it shrinks to a thin transparent film; this change is fully reversible. The flexibility and mechanical strength are comparable to that of wet filter paper, except in compression.

Horizontal Electrophoresis Cell

Because of the low resistance and high water content of acrylamide gel, it is essential to use an apparatus in which the gel is in contact with water-cooled plates on both upper and lower surfaces. Failure to observe this requirement will result in serious distortion of the gel cause by local evaporation and overheating. An apparatus with vapor space, even if hermetically sealed and cooled externally so that the vapor space is saturated with water vapor, does not prevent distillation from the surface of the gel to the walls of the gel enclosure (2).

The apparatus, Fig. 2, used for horizontal gel electrophoresis consists of a migration chamber 30 × 22 cm with attached electrode compartments, each of about 3 l capacity. The floor of the chamber on which the gel slabs rest is cooled by tap water circulating through channels in the base plate. The cover of the migration chamber, also water-cooled, extends to cover the electrode compartments and is moveable vertically with respect to the floor. Thus the space between cover and floor adjusts itself to the exact thickness of the gel used. Electrical contact between the ends of the gel slabs and the electrode compartments is made by means of sponges soaked in buffer. The space beneath the cover is entirely filled with gel, sponge, or buffer, so that no evaporation can take place anywhere.

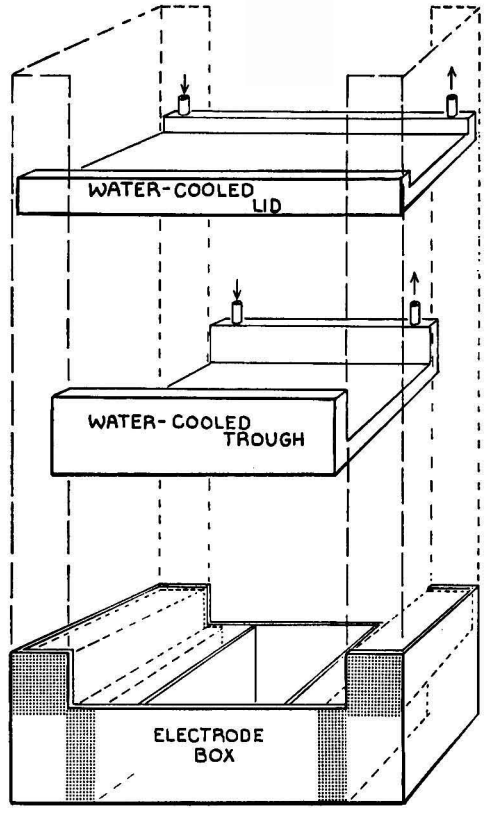

FIG. 2. Water-cooled electrophoresis chamber, for completely enclosed gel electrophoresis. Material: Plexiglass. Over-all dimensions 40 × 15 × 25 cm.

Application of Specimen

It is not necessary to precondition or equilibrate the gel in the apparatus before applying the specimen. The filter paper technique (4) is used. Serum specimens from clotted blood were used without further processing. The razor blade used was broken off to be 15 mm wide, allowing three specimens to be placed side by side across each gel strip. Place one set of specimens 4 cm from the negative end and one set 12 cm from the negative end. This allows six specimens per strip.

Running Conditions

After application of the specimen, cover the gel surface with a sheet of plastic (Saran-Wrap) eliminating all air bubbles. Place the water-cooled

lid in contact with the surface of the covered gel. With cooling water running at 20–25°C, use 300 v at about 180 ma. When analyzing hemoglobins, allow the main hemoglobin component to migrate 5–6 cm from its origin (about 2½ hr). This is sufficient to give well-defined separations. When analyzing serum proteins, the addition of a small crystal of bromophenol blue (which colors the albumin component without affecting the electrophoretic mobility), allows the progress of the run to be observed visually. To avoid excessive trailing in serum protein patterns, the current should be turned off after 5 min (when all the specimen will have moved off the wick onto the gel strip) and the wick removed from the gel. After closing up the slit, restart the run.

Dyeing the Patterns

After completion of the electrophoresis run, write identifying data on the gel with a toothpick dipped in a 5% protein solution. Immerse the gel in dye solution (e.g., 1% amidoblack 10B Bayer in a mixed solvent containing methanol–water–acetic acid 5:5:1 v/v) with agitation for 1 min. Drain and wash in five changes of the mixed solvent until the background is clear. Finally wash with water until transparency is restored.

The transparency of acrylamide gel is advantageous. A thickness of 3 mm has been adopted as standard in our laboratory. In this thickness, the optical clarity of the gel throughout the visible range allows stained patterns to be measured by transmission densitometry, with certain advantages over reflection densitometry as proposed by Pert *et al.* (1). There is no significant light scattering either from the interior of the gel or from the surfaces of the gel as molded, so that slicing off the outer layers as recommended by Smithies (4) contributes nothing to the definition of the pattern and in fact depreciates it.

Because acrylamide is a synthetic, water-soluble monomer of which the physical and chemical properties are carefully controlled during manufacture, gels prepared from it are uniform, homogeneous, and reproducible. Concentration changes during heating or deaeration are avoided by the elimination of these steps in the preparation of the gel. No more than 5% monomer is needed to form a gel optimum strength and clarity. With this smaller concentration of gel, rates of migration are correspondingly increased, as shows in Fig. 3.

The gel as prepared contains a small proportion of catalyst, which consists of a mixture of ammonium persulfate and a tertiary aminonitrile. Most of the catalyst reacts with the gel in initiating the polymerization, but there is a small residue free within the gel structure. Although we were concerned about a possible effect of the residual catalyst on the electrophoretic patterns, experiments in which the catalyst was removed by dialysis failed to demonstrate any qualitative or quantitative effect

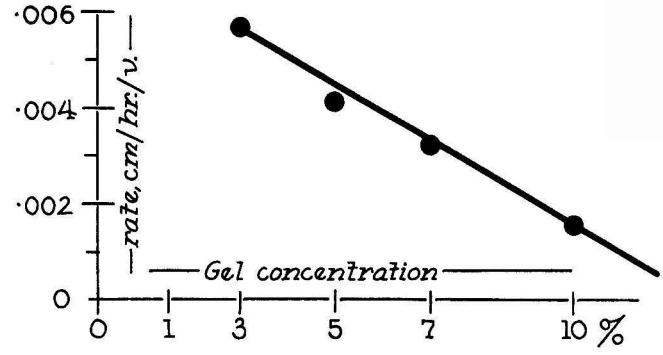

FIG. 3. Migration of normal adult hemoglobin in Tris–EDTA buffer, pH 9, containing different concentrations of acrylamide gel. Temperature 25°, field strength 15 v/cm.

attributable to the presence of catalyst residue. In several experiments, two strips of gel were prepared from the same catalyzed monomer solution. One was exhaustively dialyzed against the buffer used in preparing the gel, to remove any free catalyst residue. During the dialysis the gel strip swelled about 20% in all dimensions but appeared otherwise unchanged. Samples of hemoglobin and of serum were applied to both dialyzed and the nondialyzed strips which were run simultaneously in the electrophoresis chamber, after trimming the dialyzed strips to the original dimensions in length and width. No effect of dialysis could be seen in the resulting patterns except that migration rates in the dialyzed gel were slightly greater. This may be attributed to the swelling of the gel during dialysis, which opens up the pores in the gel structure, allowing more free space for migration.

SUMMARY

Acrylamide is a water-soluble monomer which dissolves in buffers over a wide range of pH. On polymerization it forms transparent flexible stable insoluble gels suitable for gel electrophoresis. The gel must be adequately protected from evaporation during electrophoresis. Suitable apparatus for preparing the gel and for carrying out the electrophoresis is described.

REFERENCES

1. PERT, J. H., ENGLE, R. E., WOODS, K. R., SLEISENGER, M. H., *J. Lab. Clin. Med.* **54**, 572–84 (1959).
2. RAYMOND, S., "Manual of Electrophoresis." E. C. Apparatus Co., Swarthmore, 1959.
3. RAYMOND, S., AND WEINTRAUB, L., *Science* **130**, 711 (1959).
4. SMITHIES, O., *Biochem. J.* **61**, 629–41 (1955).

ANALYTICAL BIOCHEMISTRY 1, 397–401 (1960)

A Micromethod for the Determination of Hydrogen Sulfide

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INTRODUCTION

Sulfide can be determined quantitatively by oxidation to sulfate followed by the precipitation of barium sulfate (1). It can also be precipitated as cadmium sulfide and then titrated by the iodine–thiosulfate method after acidification (2). Neither of these methods can be adapted easily to microtechniques, and both are time consuming. An accurate and sensitive colorimetric determination was developed by Fogo and Popowsky (3) based upon the formation of methylene blue when zinc sulfide is treated with an acid solution of *p*-aminodimethylaniline followed by the addition of ferric chloride. This procedure, a refinement of a method described earlier (4–6), is capable of accurate determinations in the range of 0.1–1.0 μg of sulfide sulfur. It is, perhaps, the method of choice where high sensitivity is required. In our study of the cysteine desulfhydrase system of *Escherichia coli,* we required accuracy only over the range 1–100 μg, but a method of greater manipulative simplicity which could be applied to the simultaneous determination of sulfide in large numbers of samples. The method employed was developed from the colloidal lead sulfide method originally described by Winkler (7). The modified method was described in its briefest principles (8), but many important details were not reported leaving the interested analyst with the task of developing the standardization procedure as well as numerous other details of application already in common practice in our laboratory. It is the purpose of this report to relate in necessary detail the manner in which the method can be given absolute standardization, and some of our experiences with it. Winkler's method (7) as refined by ter Meulen and co-worker (9, 10) involved the precipitation of colloidal lead sulfide and its quantification by colorimetric procedures. In the conduct of the test by the latter workers, the sulfide in the sample is precipitated as lead sulfide by the addition of alkaline potassium or sodium plumbite. As conducted originally by Winkler, lead acetate was used as the precipitating reagent. Our modification of the Winkler method involves the use of

gum arabic as a stabilizing agent, the adaptation of the method to the modern photoelectric colorimeter, and the use of a secondary standard to obviate the usage of the unsatisfactory metallic sulfides as primary standards. Our data show that accurate and convenient standardization can be accomplished, and that the method, by virtue of its extreme simplicity, can be a very convenient and useful analytical tool.

MATERIALS AND METHODS

Reagents for the standardization of the test and its conduct include 0.1 *N* NaOH, 2%$Na_2S \cdot 9H_2O$, glacial acetic acid, standard solutions of the trihydrated crystalline lead acetate (or lead nitrate), and an "assay reagent" composed of 1 gm of crystalline lead acetate, 5.0 ml of glacial acetic acid, and 2.5 gm of gum arabic/l of aqueous solution.

In the test procedure aliquots of the solution to be analyzed, containing 10–100 μg hydrogen sulfide in a volume up to 4.0 ml in amount, are added directly to 10–ml colorimeter tubes each containing 2.0 ml of 0.1 *N* NaOH. Water is added, if necessary, to a volume of 6.0 ml. The nonorganic sulfide present is precipitated as colloidal lead sulfide by the addition of 4.0 ml of the assay reagent to each tube. An immediate precipitate of colloidal lead sulfide is formed, which is stable for at least 1 hr, and which can be read in the colorimeter. Sulfide can be calculated from the data obtained by direct reference to a standard curve prepared by the use of crystalline lead acetate (or lead nitrate) as a secondary standard and the solution of sodium sulfide as the precipitating agent (see Fig. 1).

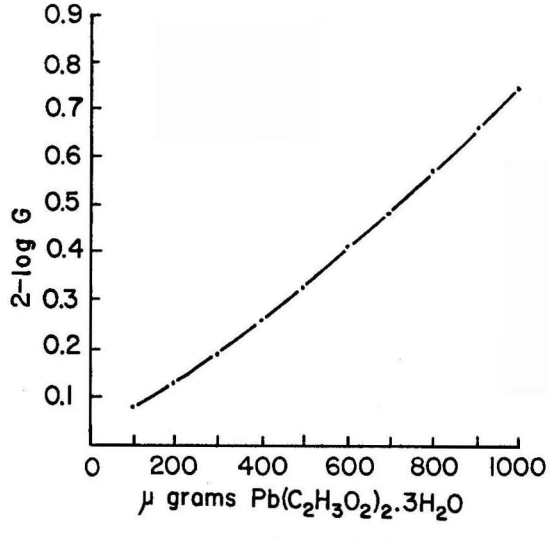

FIG. 1. Standard curve for sulfide determination.

STANDARDIZATION PROCEDURE

In the preparation of the standard curve a secondary standard was employed because of the poor characteristics of the soluble metallic sulfides as primary standards. Standard solutions of lead acetate trihydrate (1.115 gm/l) or lead nitrate (0.974 gm/l) with an equivalence of 100 μg H_2S/ml were chosen for convenience. In the preparation of the standard lead acetate solution a few drops of glacial acetic acid are included to eliminate the slight turbidity resulting from hydrolysis of the lead acetate. Carefully pipetted volumes of increasing amounts of the lead standard are transferred to a series of matched colorimeter tubes. Water is added to a volume of 5.0 ml followed by the addition of 4.0 ml of assay reagent from which the lead acetate or lead nitrate *has been omitted for purposes of the standardization procedure.* A yellow to brown colloidal precipitate of lead sulfide is formed by the addition of 1.0 ml of the 2% Na_2S solution. Forceful and agitating pipetting seems to be contributory to greater reproducibility. A reagent blank containing no lead is prepared, the tubes are placed in the colorimeter, and optical density is read at light wavelengths in the visible range. Greater absorption (and scattering) occurs with the shorter wavelengths, with perhaps too great a sensitivity in the blue portion of the spectrum. We have chosen 500 mμ as a desirable compromise between too great and too little sensitivity.

RESULTS

In the Bausch and Lomb "Spectronic 20" using 12.5-mm tubes, we obtain (see Fig. 1) an optical density of 0.078 with 100 μg lead acetate (equivalence of approximately 9 μg H_2S) and an optical density of 0.75 with 1000 μg lead acetate. Practical and near-theoretical linearity exists at concentrations between these somewhat arbitrary limits. In the range of 1–100 μg, the test is erratic, probably because of sulfide oxidation. The first point on the standard curve (100 μg lead acetate) is consistently high if one attempts to draw a straight line through the subsequent points and back into the origin. There is a decrease in this departure from linearity with increasing concentration followed again by an increasing trend up to the range of 700–800 μg lead acetate. The result is a slight concavity of the curve in the range of 10–80 μg H_2S equivalence. The careful preparation and use of a standard curve is thus dictated where very precise analyses are desired.

DISCUSSION

Our experimental system involved the enzymic desulfhydration of cysteine to hydrogen sulfide, ammonia, and pyruvic acid by cell-free

extracts of *Escherichia coli* in phosphate buffer. The incubation mixture could be assayed directly with no apparent interference, so it may be generalized that dilute phosphate, cysteine (sulfhydryl groups), and soluble protein do not interfere with the test. In cases where lead phosphate or lead sulfate turbities are developed along with the colloidal lead sulfide, these interfering white precipitates may be easily sedimented and removed by low-force centrifugation. In the course of our studies we likewise found no interference with the B vitamins when present in microgram concentrations, or with substrate concentrations of semicarbazide, phosphoglyceric acid, and α-keto acids. Oxidizing agents destroy the sulfide, but since oxidizing conditions are inimical to the presence of hydrogen sulfide, this interference is of little practical consequence. One probable exception concerns primarily the enzymologist. Trichloroacetic acid, commonly employed to terminate enzyme experiments, interfered. We were successful in stopping our experiments with alkali, thus stabilizing the sulfide, and at the same time solubilizing much of the protein, the residue being removable by centrifugation. Analysis of the enzyme controls further minimized any error.

We have found it feasible also to transfer the H_2S to a solution of 0.1 *N* NaOH by means of a current of N_2. With the relatively low concentration of lead in the assay reagent, and in the presence of excess acetate, lead hydroxide does not precipitate; the lead remains in solution as basic lead acetate. Excessive alkalinity might interfere, but this can be circumvented to a considerable degree by the inclusion of a higher concentration of acetic acid in the assay reagent.

The question arises as to whether the development of the colloidal lead sulfide in the presence of an excess of sulfide ion, as in the standardization procedure, might produce a colloidal suspension of different optical properties than when the colloid is formed in the presence of an excess of lead ion, which is the case in the actual analytical procedure. In this regard it should be noted that ter Meulen (9) reported a difference in the color of the colloidal solution when the colloidal lead sulfide was developed in the presence of excess sulfide than when the colloid was produced under the opposite conditions of concentration. If the colloidal suspensions were different in their absorptive and scattering properties, the standardization procedure would be invalid in terms of absolute accuracy. By comparison with an independent method, we have satisfied ourselves that the test procedure based on the standard curve does yield reasonably valid results for a micromethod. A solution of sodium sulfide of approximately 0.1 molar concentration was titrated by the iodometric method. When diluted and assayed by the optical procedure, excellent agreement was obtained with an error not exceeding 2%. Herein, perhaps,

lies the best reason for the addition of gum arabic to the assay reagent. The original thought was to use it to stabilize the colloid long enough to permit accurate instrumentation. It seems likely, however, that the gum plays an important role in controlling particle size.

SUMMARY

An analytical procedure suitable for the determination of soluble sulfide in the range of 10–100 μg of sulfide sulfur is described. The method is based upon procedures involving the precipitation of colloidal lead sulfide, stabilization and particle size control with gum arabic, and quantification in the photoelectric colorimeter. The method is simple, convenient, and rapid, and it has excellent precision in intermediary concentration ranges. It is useful where large numbers of samples must be analyzed simultaneously.

ACKNOWLEDGMENT

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REFERENCES

1. Allen, W. S., and Bishop, H. B., *Intern. Congr. Appl. Chem., 8th Congr.* **48,** 1–2 (I–II) (1912).
2. Shaw, J. A., *Anal. Chem.* **12,** 668 (1940).
3. Fogo, J. K., and Popowsky, M., *Anal. Chem.* **21,** 732 (1949).
4. Almy, J., *J. Am. Chem. Soc.* **47,** 1381 (1925).
5. Mecklenberger, W., and Rosenkranzer, F., *Z. anorg. Chem.* **86,** 143 (1914).
6. Sheppard, S. E., and Hudson, J. H., *Anal. Chem.* **2,** 73 (1930).
7. Winkler, L. W., *Z. anal. Chem.* **52,** 641 (1913).
8. Delwiche, E. A., *J. Bacteriol.* **62,** 717 (1951).
9. Meulen, H. ter, *Rec. trav. chim.* **41,** 112 (1922).
10. Meulen, H. ter, and Heslinga, J., "Neuen methoden der organisch-chemischen analyse," p. 29. Akadamische Verlagsgesellschaft, Leipsig, 1927.

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Absorption Spectra of Δ^5-3β -Hydroxy Steroids in Several Sulfuric Acid Reagents

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Sulfuric acid, both concentrated and variously diluted with ethanol and water, has been widely employed in steroid analysis. Three types of reactions with neutral steroids are: (*a*) development of characteristic absorption spectra at room temperature after addition to a dried sample of steroid (1–3), (*b*) development of a characteristic major absorption peak in the visible range after heating with the reagent and diluting (4), and (*c*) development of fluorescence (5–8).

The blue color given by dehydroepiandrosterone (DHA) after heating with the Allen reagent and diluting with ethanol is well known (4). Little attention has, however, been paid to the absorption spectrum developed by this and other steroids in the reagent itself, without heating or diluting. Our attention was directed to this phenomenon by the observation of a deep blue color from Δ^5-pregnene-3β,17α,20α-triol (5-PT) at room temperature. Other Δ^5-3β-OH steroids were then found to give distinctive spectra with this "cold" Allen reagent. Meanwhile Oertel and Eik-Nes (3) showed that several Δ^5-3β-OH steroids gave an intense yellow color in a more dilute ethanolic sulfuric acid reagent.

The present communication compares absorption spectra of a number of Δ^5-3β-OH steroids in the Allen and Oertel reagents and in concentrated sulfuric acid. A group of other steroids has been studied with the Allen reagent alone. It is shown that spectra in the diluted reagents are useful (*a*) for identification purposes, (*b*) for quantitative determination of several steroids for which no simple method existed, and (*c*) as spot tests on paper chromatograms, or for testing column eluates.

MATERIALS AND METHODS

Reagents and Equipment

Concentrated Sulfuric Acid. Baker and Adamson Co., reagent grade.

Ethanol. U. S. Industrial Chemical Co. "U.S.I. Absolute pure ethyl alcohol" U.S.P.–N.F. reagent quality. Negligible absorption in the visible and ultraviolet wavelengths was found when 5 ml of this ethanol was evaporated to dryness and the residue treated with sulfuric acid.

Ethanol, 90% and 95%. Prepared from absolute ethanol by adding distilled water.

Allen Reagent (4). Eighty milliliters of conc. sulfuric acid was added to 20 ml of 90% ethanol, with cooling.

Oertel Reagent (3). Sixty-seven milliliters of conc. sulfuric acid was added to 33 ml of 95% ethanol, with cooling.

Spectrophotometer. Cary model 14 recording spectrophotometer. Applied Physics Corp., Pasadena, Cal.

Spectrophotometer Cuvettes. One milliliter capacity, 10 mm light path. Pyrocell Mfg. Co., 207 East 84th St., New York 28, N. Y. Other brands tried have been unsuitable for use with sulfuric acid.

Steroids. The pure steroid compounds used in this study were purchased from (*a*) Mann Research Laboratories, 136 Liberty St., New York 6, N. Y.; (*b*) Nutritional Biochemicals Corp., Cleveland 26, Ohio; (*c*) California Foundation for Biochemical Research, 3408 Fowler St., Los Angeles 63, Calif.

Generous gifts of steroids not available commercially were received from Merck, Sharp & Dohme Co., the Upjohn Co., Ciba Pharmaceutical Products, Inc., Dr. Max N. Huffman, Dr. C. D. Kochakian, and Dr. J. J. Schneider.

Procedures

Absorption Spectra. Aliquots of ethanolic solutions of pure steroids, containing between 5 and 40 μg, were measured into 12 $\times$ 75 mm test tubes and dried in a vacuum oven. Precautions necessary for the determination of sulfuric acid chromogen curves were observed (9). One milliliter of sulfuric acid or diluted reagent was added to each tube and the time noted. After thorough agitation to dissolve the dried steroid, the solution was transferred to the spectrophotometer cuvette, and the spectrum was immediately determined. A scanning speed of 300 mμ/min was usually employed, covering the region from 200 to 700 mμ. Spectra were again determined at longer intervals.

"Hot" Allen Reaction. The original procedure of Allen *et al.* (4) was modified only by reducing the volume of reagents. Each steroid was dissolved in 0.5 ml reagent in a 10 $\times$ 75 mm tube, then heated to 56° in a water bath for 12 min. After cooling, 0.75 ml of 95% ethanol was added, and the contents were mixed with a footed glass rod.

Preparation of Δ^5-Pregnene-3β,17α,20β-triol (20β-5PT)

Twenty milligrams of Δ^5-17-OH-pregnenolone (5-17PG) was dissolved in 10 ml of absolute ethanol with warming. To this solution was added 50 mg potassium borohydride in 10 ml of 60% ethanol. After standing 18 hr, the mixture was neutralized with glacial acetic acid and extracted with ethyl acetate, and the ethyl acetate was washed with water and evaporated barely to dryness. The product dissolved with difficulty in 25 ml of absolute ethanol. When chromatographed on paper it appeared to contain about 10% of the 20α-OH isomer. Completeness of reduction was shown by the absence of any test for 5-17PG either in fractions from partition columns or on paper chromatograms. [On paper chromatograms employing Bush (10) systems, 5-17PG did not move from the origin and it streaked badly in the toluene–propylene glycol system of Burton *et al.* (11).] A 10-mg portion of the reduction product was chromatographed on a partition column, and eluates containing white crystalline material of the appropriate mobility were combined. The melting point was 214–218° (Kofler micro hot stage) as compared to 224–228° quoted in the literature (13). On oxidation with periodic acid, the product was exclusively dehydroepiandrosterone, as proved by mobility on paper chromatograms and color reactions.

Preparation of Δ^5-Pregnene-3β-17α-20α-triol (5-PT) from Urine Extracts

Total neutral fractions of urine extracts from three patients with adrenal carcinoma were chromatographed on a modified partition column (12). The eluate containing 5-PT was treated with digitonin, and the recovered 3β-OH fraction was chromatographed on paper in the Bush B1 system (10). The 5-PT band was located by the yellow–green color given when a test strip was overrun with the Allen reagent. This band was more polar than the 20β-OH isomer run on a parallel strip, and well separated from it, indicating the urinary component to be the 20α-OH isomer. After elution with ethanol, the material in this band was reapplied to another partition column. Fractions in the appropriate region were white and semicrystalline. These were combined, and the compound was crystallized from absolute methanol in the cold. The melting point was 213.5–218° compared to 221–224° as quoted in the literature (13). The absorption spectra of the crystalline product in concentrated sulfuric acid and in the Allen reagent were identical with that of the 20β-OH isomer after similar purification on a partition column (Fig. 1). On oxidation with periodic acid, dehydroepiandrosterone was the only product, as indicated by paper chromatography and color tests.

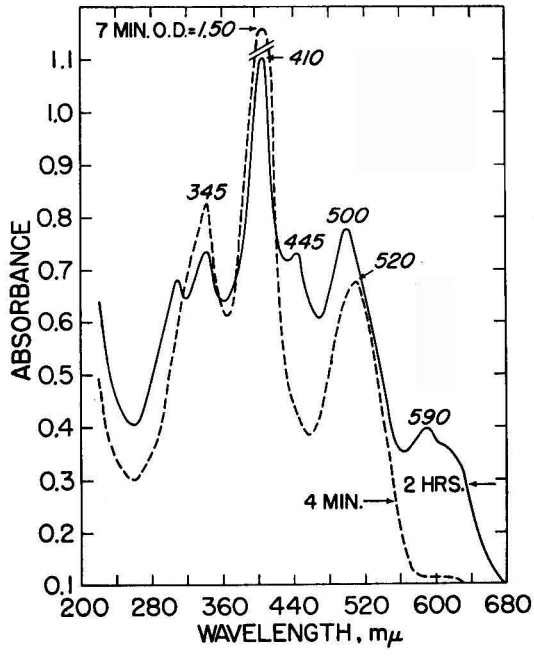

FIG. 1. Sulfuric acid chromogen absorption spectrum of crystalline Δ^5-pregnene-3β,17α,20α-triol prepared from urine.

RESULTS

Major Absorption Peak Near 405–408 mμ

As is seen in Figs. 2 and 3 and in Table 1, the strong absorption near 405 mμ first observed by Oertel and Eik-Nes (3) is indeed characteristic of the majority of these Δ^5-3β-OH steroids in both the Allen and Oertel reagents. Notable exceptions are 7-ketodehydroepiandrosterone (7-KD) and Δ^5-androstene-3β,16α,17β-triol (5-AT) which had no peaks in this region. Moreover this band was not strongly dominant in either Δ^5-pregnene-3β-20α-diol diacetate (5-PDL) or 5-PT.

The precise maximum wavelength varied from 405 to 412 mμ. DHA showed a peak at 405 mμ with the Oertel reagent rather than at 400 mμ as reported (3).

Absorption Maxima at Other Wavelengths

With the Allen and Oertel reagents, most Δ^5-3β-OH steroids also showed a characteristic pattern of lesser peaks (Figs. 2 and 3 and Table 1). Frequently the complete spectra yielded by these reagents are more

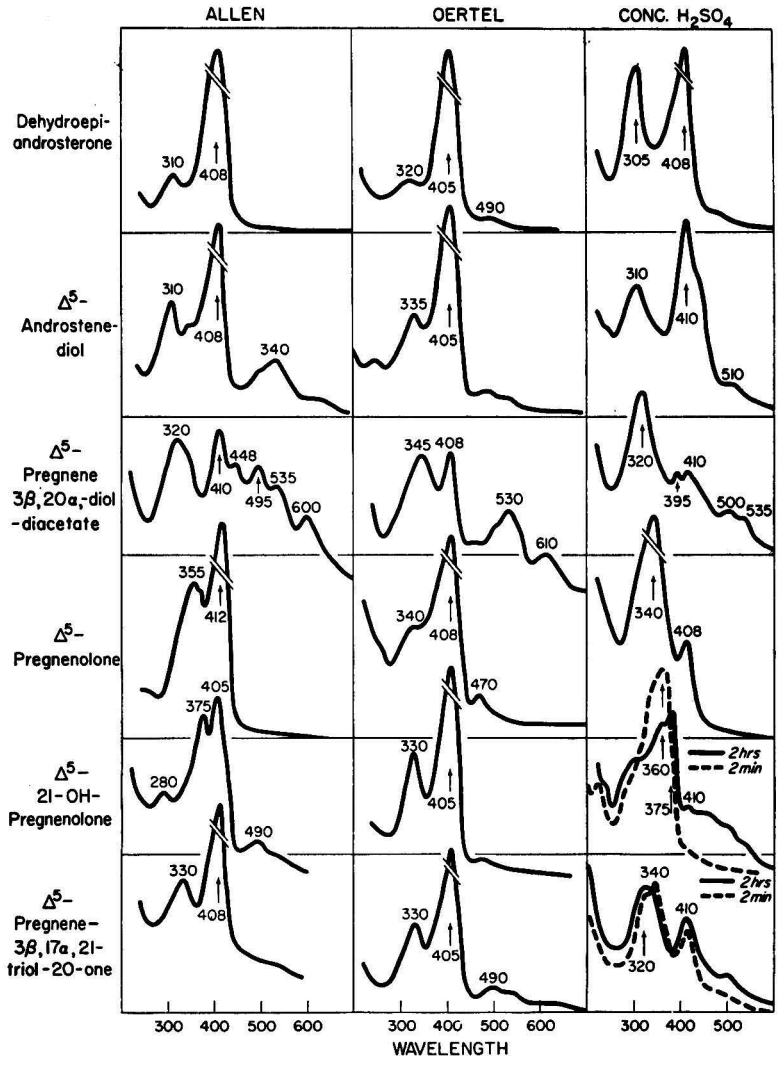

FIG. 2. Absorption spectra of Δ^5-3β-OH steroids at room temperature in the Allen and Oertel reagents and in concentrated sulfuric acid.

The curves are drawn essentially to scale, except that some very high peaks are foreshortened. Absorbance values at the peaks can be calculated from the reaction constants given in Table 1.

Reading times are listed in Table 1.

The Δ^5-pregnene-3β,17α,20α-triol was prepared from urine. (See text.) The Allen reagent added to this steroid contained 77.5% rather than 80% H_2SO_4.

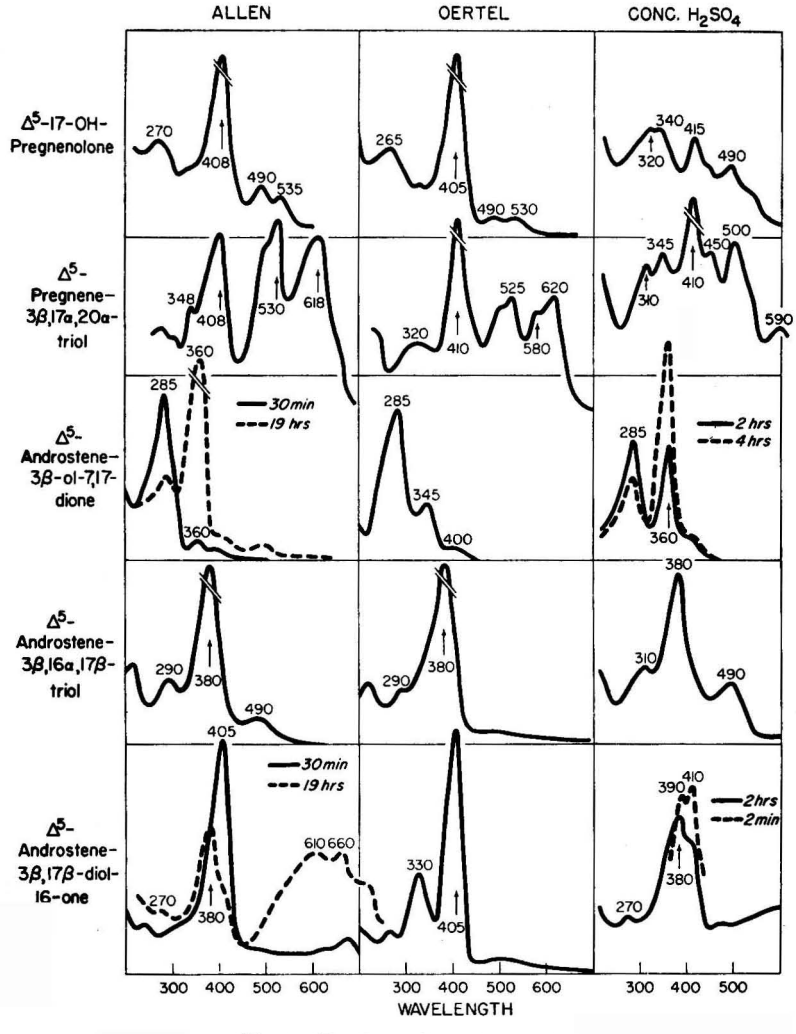

FIG. 3. For legend, see Fig. 2.

valuable for identification purposes than the sulfuric acid chromogen spectra, as they tend to have a larger number of clearly defined maxima.

Characteristic Spectra of Δ^5-Pregnene-3β,17α,20α-triol with the Allen Reagent

The reaction of this compound and its 20β-hydroxy isomer at room temperature ("cold Allen") has been particularly useful in its estima-

TABLE 1

MAJOR AND MINOR ABSORPTION PEAKS OF Δ^5-3β-HYDROXY STEROIDS, AND CHANGES WITH TIME[a]

Steroid	Code name	Allen reagent						Oertel reagent						Concentrated H_2SO_4					
		Major peak				Minor peaks		Major peak				Minor peaks		Major peak				Minor peaks	
		Time (min)	mμ	Change	K[b]	mμ	Change	Time (min)	mμ	Change	K	mμ	Change	Time (hr)	mμ	Change	K	mμ	Change
Dehydroepi-androsterone	DHA	30	408	S	65	310	I	30	405	S	73	320 490	S S	2	408	F	80	305	I
Δ^5-Androstene-3β,17β,-diol	5-ADL	30	408	S	60	310 540	I I	30	405	S	85	335	S	2	410	I	42	310	S
Δ^5-Pregnene-3β,20α-diol-diacetate	5-PDL	30	410	S	23	320 448 495 535 600	I I I I I	10	408	F	17	345 530 610	F I I	2	320	I	34	395 410 500	I I I
Δ^5-Pregnene-3β-ol-20-one	5-PG	30	412	F	70	355	S	10	408	S	95	330 470	S I	2	340	S	68	408	F
Δ^5-Pregnene-3β,21-diol-20-one	21-OH-5-PG	20	405	S	39	280 375 490	I I I	30	405	S	80	330	S	2	375	S	36	410	I
Δ^5-Pregnene-3β-17α,21-triol-20-one	5-TS	30	408	F	50	330	I	30	405	S	55	330 490	I I	2	330	S	28	410	S
Δ^5-Pregnene-3β-17α-diol-20-one	5-17PG	30	408	F	65	270 490 535	S I I	30	405	S	70	265 490 530	I I I	2	320	F	24	340 415 490	F S S
Δ^5-Pregnene-3β,17α,20α-triol[c]	5-PT	30	530	I	52	348 408 618	F F I	30	410	F	55	320 525 580 620	S I I I	2	410	F	55	310 345 450 500 590	I F I I I
Δ^5-Androstene-3β-ol-7,17-dione-acetate	7KD	30	285	S	36	360	I	30	285	F	31	345 400	I S	2	285	F	26	360	I
Δ^5-Androstene-3β,16α,17β-triol	5-AT	30	380	S	63	290 490	S I	30	380	I	70	290	S	2	380	S	39	310 490	I I
Δ^5-Androstene-3β,17β-diol-16-one	5-ADLO	30	405	S	50	—	—	30	405	S	51	330	S	2	390	S	34	270 660	S I

[a] The direction of change in absorption between 2 min and the quoted reading time is indicated after each peak wavelength: F = fades, I = Increases, S = stable.
[b] K = optical density reading/mg steroid reacting; 20 μg steroid was used throughout.
[c] Δ^5-Pregnene-3β,17α,20α-triol prepared from urine (see text). The Allen reagent used for this steroid contained 77.5% H_2SO_4 and 22.5% of 90% ethanol.

tion in urine extracts. When the reagent was added to a dried sample of either pure steroid, a strong but fleeting yellow color appeared, quickly changing to pink, deepening to purple, and finally becoming violet–blue after about 30–40 min. Figure 4 compares a spectrum obtained between

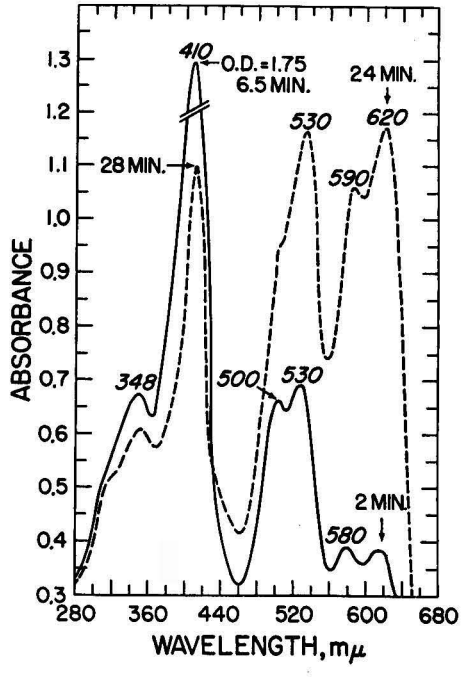

FIG. 4. The effect of time of standing at room temperature on the absorption spectrum of Δ^5-pregnene-3β,17α,20α-triol in the Allen reagent. The initially strong yellow color absorbing at 410 mμ faded rapidly, whereas the red and blue colors (530 and 620 mμ) increased.

2 and 10 min with one near 30 min. It is seen that the peak at 410 mμ (yellow color) faded rapidly, while those at 530 mμ (red) and 620 mμ (blue) increased. On diluting the developed violet color with 1.5 vol of 95% ethanol, a clear blue solution ensues. Figure 5 shows that this color is due to the disappearance of all absorption bands except that at 620 mμ. Chromatographic fractions of urine extracts containing 5-PT often give clear blue colors with this procedure even though they are far from pure.

The relationship among the three major peaks (Figs. 4 and 5) is dependent not only on the time of color development, but also on the composition of the reagent. Thus the high peak seen at 620 mμ could not be reproduced with another batch of reagent, but reappeared when the

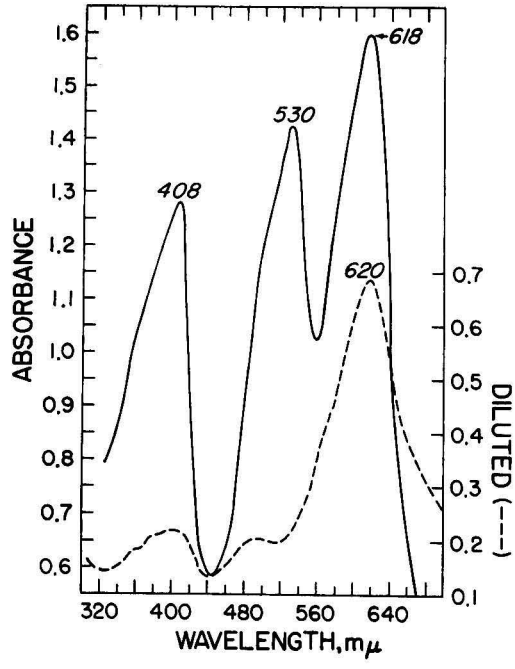

FIG. 5. The effect of dilution with 1.5 vol of 95% ethanol on the absorption spectrum of Δ^5-pregnene-3β,17α,20α-triol in the Allen reagent after 30 min at room temperature. Only the peak at 620 mμ remained.

sulfuric acid concentration was reduced to 77.5%. Moreover, contamination with other materials in the extracts, or impurities acquired from paper chromatograms, may inhibit the blue chromophore. This effect is more pronounced with the regular 80% reagent than with a lesser sulfuric acid concentration. Hence, a reagent containing about 77–78% sulfuric acid is more reliable when testing qualitatively for 5-PT. Maximum absorbance is then reached after about 1 hr.

The Oertel reagent gives a green color with 5-PT on standing at room temperature.

Spectral Changes as a Function of H_2SO_4 Concentration

The change from 80% H_2SO_4 (Allen) to 67% (Oertel) resulted in marked spectral changes with five steroids, while six gave very similar curves (Figs. 2 and 3). However, all but one of the curves in concentrated sulfuric acid differed markedly from those in the ethanolic reagents. The most notable difference was the diminution of the major peak near 408 mμ, with the strongest bands shifting to 320–390 mμ. Exceptions to this

rule were DHA, Δ^5-androstene-3β,17β-diol (5-ADL), 5-17PG, and 5-PT, which retained strong bands at 408 and 410 mμ.

Comparison of Extinction Coefficients

Table 1 lists the K values (K = optical density/mg steroid) obtained with 20 μg steroid in the several reagents. The great sensitivity of the Allen and Oertel reagents in measuring Δ^5-3β-OH steroids absorbing strongly near 408 mμ is indicated by K values from 50 to 90. Thus 10 μg DHA had an optical density of about 0.700 in 1 ml of either reagent. In concentrated sulfuric acid (except for DHA), the extinction coefficients were lower or the strongest bands had shifted to another wavelength.

Changes in Spectra with Time

The heights of the absorption maxima usually varied greatly with time of standing in the reagent, though some compounds were fairly stable up to about 2 hr. Table 1 shows the direction of the changes observed between the initial scanning after 2 min and that at the quoted reading time. In general, the absorption near 408 mμ was either stable or diminishing during 30 min with either ethanolic reagent, while the lesser peaks tended to increase in height. Thus in many instances the visual color changed from yellow to reddish orange or purple. These changes are characteristic in themselves and can be used as an aid in identification. Frequently the curves at later times were less dominated by a single peak and showed additional lesser bands, making the entire spectrum more specific for a given steroid.

Proportionality of Absorbance to the Amount of Steroid; Cold Allen Reagent, Undiluted

Table 2 shows that five Δ^5-3β-OH steroids give reasonably proportional corrected readings, as shown by constant K values. Absorption was measured at reaction times when the peak value was maximum and fairly stable, and when characteristic lesser bands had also appeared.

Figure 6 illustrates how the corrected maximum absorbancies were determined from the spectrophotometer recordings. A straight line was drawn across near the base of the peak; in the case of DHA, between 360 and 440 mμ. The absorption where this line crosses 408 mμ was subtracted from the total 408-mμ reading. A similar procedure can be followed if the "hot Allen" procedure is used (Fig. 6). If point readings must be relied upon, the correction can readily be calculated or obtained graphically from readings at three appropriate wavelengths. This correction, proposed by Allen (15), makes allowance for the asymmetry of many peaks.

TABLE 2

PROPORTIONALITY BETWEEN ABSORBANCE AND AMOUNT OF STEROID; COLD ALLEN REAGENT, UNDILUTED

	Time (min)	μg	K[a] observed		K corrected[b]	
			408 mμ		408 mμ	
Dehydroepiandrosterone	30	5	85		70	
		10	75		64	
		20	72		64	
		30	70		63	
Δ⁵-Androstenediol	20	5	69		51	
		10	62		49	
		20	62		48	
Δ⁵-Pregnenediol	60	5	31		6.0	
		10	23		6.0	
		20	19		5.5	
			530 mμ	620 mμ	530 mμ	620 mμ
Δ⁵-Pregnene-3β,17α,20β-triol[c]	60	10	46.5	39.5	23.5	16.3
		20	40.0	39.0	19.0	15.0
		30	42.3	37.3	22.3	16.0
		40	40.5	39.9	20.2	16.1
Δ⁵-Pregnene-3β,17α,20α-triol from urine[d]	60	10.4	49.4	56.6	17.6	18.1
		20.8	47.4	61.0	15.0	20.2

[a] K = optical density reading/mg steroid.

[b] Corrected optical density readings were determined graphically by drawing a straight line across the base of the peak, as shown for DHA in Fig. 6. The absorbancy at its intersection with the peak wavelength was then subtracted from the observed reading.

[c] Prepared by reduction of Δ⁵-17-hydroxypregnenolone.

[d] A solution of a slightly impure fraction from urine, standardized by measuring the dehydroepiandrosterone formed after oxidation with periodic acid.

In the case of 5-PT, insufficient crystalline 20α-OH isomer was available for this experiment, and the residue from evaporation of the final mother liquors was used. This preparation was standardized by oxidation with periodic acid and determination of the resulting DHA by the micro-Zimmermann reaction (14). Hence differences between the two isomers (Table 2) may be more apparent than real. The band at 530 mμ is the most stable and least affected by extraneous materials, hence best for quantitation. The blue color absorbing at 620 mμ, which remains after dilution, also gives proportional readings with purified samples of 5-PT.

The △⁵-3β-OH steroids shown in Table 2 must be of a high order of purity before quantitative recoveries can be obtained with this reaction. Absorption may be severely depressed by traces of contaminants, even when the spectral curve appears to be that of a pure compound. Inhibition

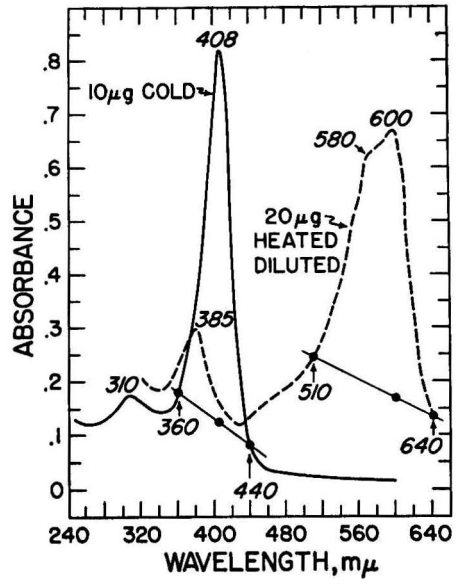

FIG. 6. Comparison of the absorption spectra of dehydroepiandrosterone in the "cold" Allen reagent (yellow color, peak at 408 mμ) and after heating and diluting (blue color, peak at 600 mμ).

The graphic method of determining the corrected peak absorbancies is illustrated. A straight line is drawn across near the base of each peak, and the absorbance at its intersection with the maximum wavelength is subtracted from the total observed absorbance.

can be detected by determining the increase in absorption on adding a known amount of pure compound.

Subject to this qualification, the reaction provides a simple quantitative method for determining these steroids, and probably others as well.

Color Reactions in the Allen and Oertel Reagents after Heating and Diluting

Table 3 lists the colors observed with a series of Δ^5-3β-OH and other steroids when the heating and diluting procedure of Allen *et al.* (4) was carried out. The only really strong colors were given by DHA and 5-ADL, but other Δ^5-3β-OH compounds gave varying degrees of weak colors.

The reactions of several Δ^4-3-ketones are of interest. Testosterone exhibited an intense yellow–green fluorescence in the "hot Allen" reaction. A blue color with a visually red fluorescence was given by 17-OH-progresterone and by DOC, while compound S gave a pale blue green color.

On standing with the Allen reagent at room temperature, compound S

TABLE 3

COLORS GIVEN BY VARIOUS Δ^5-3β-OH- AND OTHER STEROIDS[a] AFTER HEATING WITH THE ALLEN AND OERTEL REAGENTS, FOLLOWED BY DILUTION WITH ETHANOL[b]

Steroid	Allen	Oertel
DHA	Blue	Pale lavender
5-ADL	Blue	Blue
5-PDL	Very pale blue	Pale gray–blue
5-PG	—	Pink
5-TS	Pale pink	Pale violet
5-17-PG	Pale Pink	Pale purple
5-PT	Pale blue	Very pale blue
17-OH-Progesterone	Blue with red fluorescence	Blue with red fluorescence
DOC	Blue with red fluorescence	—
Cpd. S	Pale blue–green	—
Testosterone	Strong yellow with green fluorescence	—

[a] Chemical names of the Δ^5-3β-OH steroids are given in Table 1. DOC is 11-deoxycorticosterone, Cpd. S is Δ^4-pregnene-17α,21-diol-3,20-dione.

[b] Samples of dried steroids (about 20 μg) were heated at 56° for 12 min with 0.5 ml of reagent, cooled, and diluted with 0.75 ml of 95% ethanol.

turned a purplish red, which changed to clear blue on diluting with ethanol. This reaction hence resembles that given by 5-PT.

Absorption Spectra of Steroids without the Δ^5-3β-OH Group in the Allen Reagent

Spectra of a small series of these compounds are shown in Fig. 7. Pregnanetriol, cortisol, compound S, THF, and THS gave curves not unlike those seen with concentrated H_2SO_4. Pregnanediol showed a very high absorption at 350 mμ, and corticosterone had a new band at 590 mμ. Etiocholanolone differed from androsterone in having a maximum at 500 mμ. These spectra, as well as those of other steroids, would prove useful adjuncts to identification.

Use of the Allen Reagent in Spot Tests on Paper Chromatograms

Many of the Δ^5-3β-OH steroids shown in Table 3 give strong instant yellow colors when the Allen reagent is run over a narrow strip of a paper chromatogram. A weak orange color turning to brown, is given by 5-17PG. In many cases the yellow soon turns to orange or gray, and on dropping 95% ethanol over the strip after 10–20 min, a blue color is seen. A fleeting yellow color from 5-PT, usually turns green on standing, though occasionally it may become reddish orange, then purple. This, therefore, is an easy way to test for these compounds. It is not specific for the Δ^5-3β-OH group, however, as pregnanetriol, Δ^4-pregnene-17α,20β,20-triol-3-

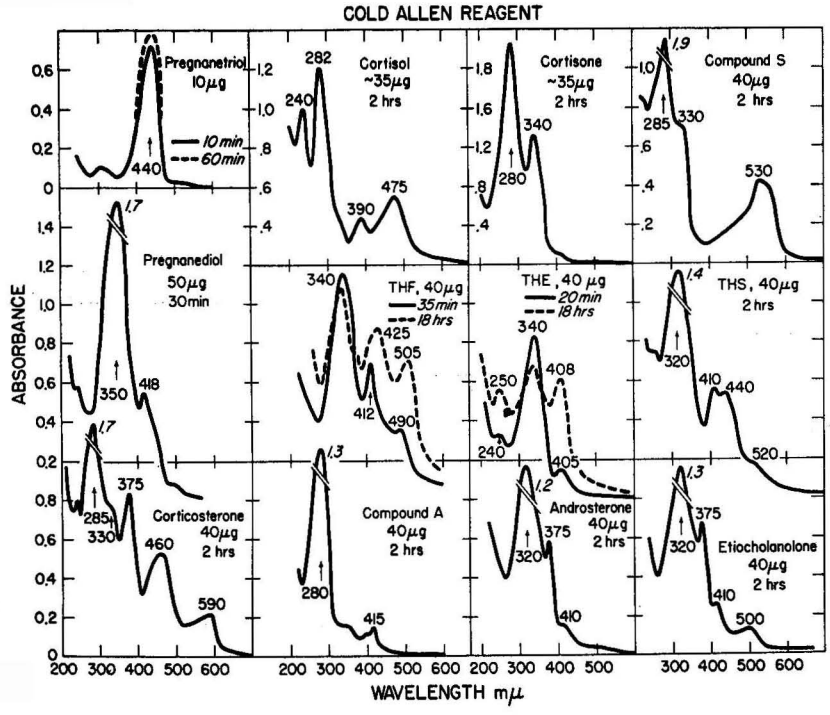

FIG. 7. Absorption spectra in the Allen reagent of various steroids without the Δ^5-3β-OH group. Maximum absorbancies of foreshortened peaks are given by the nearby numbers.

Cortisol is Δ^4-pregnene-11β,17α,21-triol-3,20-dione (compound F, hydrocortisone); THF is pregnane-3α,11β,17α,21-tetrol-20-one (tetrahydro F); THS is Δ^4-pregnene-17α,21-diol-3,20-dione (tetrahydro S).

one, and probably other steroids also give varying degrees of yellow color. Similarly, the reagent can be used to locate reactive steroids in dried eluates from chromatographic columns.

DISCUSSION

The ethanolic sulfuric acid reagents here described are clearly valuable aids in the analysis of Δ^5-3β-OH steroids. When the steroids are adequately purified, their absorption spectra can often be used both for identification and quantitation. A number of compounds, such as 17-OH-pregnenolone and pregnenediol, for which no practicable method existed, can be colorimetrically assayed.

Comparison of the Allen and Oertel reagents shows that there is often little to choose between them. In general, the Allen reagent gives more distinctive spectra.

It should be emphasized that not all Δ^5-3β-OH steroids give the strong absorption near 405 mμ (yellow color) reported by Oertel and Eik-Nes (3). However, those compounds in our series which lacked this band, had other characteristic maxima which could serve the same purpose. The conjugated Δ^5-7-ketone of 7-ketodehydroepiandrosterone resembles conjugated Δ^4-3-ketones in that a strong band at 285 mμ dominates the spectrum initially.

The ethanolic reagents also give typical spectra with a number of other steroids. Their use in identification requires less rigid standards of cleanliness in glassware and reagents than are needed with concentrated sulfuric acid.

SUMMARY

Absorption spectra between 200 and 700 mμ of eleven Δ^5-3β-OH steroids have been determined at room temperature in the Allen and Oertel ethanolic sulfuric acid reagents, and in concentrated sulfuric acid.

All but three of these steroids gave typical high peaks near 408 mμ with either ethanolic reagent. Those three had other characteristic peaks.

Δ^5-Pregnene-3β,17α,20α-triol showed an unusual play of colors with the Allen reagent, turning deep violet–blue in 30–40 min.

The ethanolic reagents, particularly that of Allen, provide means for the simultaneous identification and determination of these Δ^5-3β-OH steroids. Distinctive curves were given by a number of other steroids as well. The reagents are useful for locating reactive compounds on paper chromatograms and in column eluates.

REFERENCES

1. Zaffaroni, A., *J. Am. Chem. Soc.* **72,** 3828 (1950).
2. Bernstein, S., and Lenhard, R. H., *J. Org. Chem.* **18,** 1146 (1953).
3. Oertel, G. W., and Eik-Nes, K., *Anal. Chem.* **31,** 98 (1959).
4. Allen, W. M., Hayward, S. J., and Pinto, A., *J. Clin. Endocrinol.* **10,** 54 (1950).
5. Kalant, H., *Biochem. J.* **69,** 79 (1958).
6. Sweat, M. L., *Anal. Chem.* **26,** 773 (1954).
7. Peterson, R. E., *J. Biol. Chem.* **225,** 25 (1957).
8. Kalant, H., *Biochem. J.* **69,** 93 (1958).
9. Wilson, H., Fairbanks, R., Scialabba, D., McEwen, C., and Ziff, M., *J. Clin. Endocrinol. and Metabolism* **16,** 86 (1956).
10. Bush, I. E., *Biochem. J.* **50,** 370 (1952).
11. Burton, R. B., Zaffaroni, A., and Keutmann, E. H., *J. Biol. Chem.* **188,** 763 (1951).
12. Wilson, H., Lipsett, M. B., and Butler, L. C., *J. Clin. Endocrinol. and Metabolism* **20,** 534 (1960).
13. Hirschmann, H., and Hirschmann, F. B., *J. Biol. Chem.* **187,** 137 (1950).
14. Wilson, H., *Arch. Biochem. Biophys.* **52,** 217 (1954).
15. Allen, W. M., *J. Clin. Endocrinol.* **10,** 71 (1950).

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A Method for Studying the Properties of Intracellular and Liberated Xanthine Oxidase[1]

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Enzyme determinations are usually carried out in tissue homogenates or extracts at optimum pH, substrate, and cofactor levels in order to obtain maximum enzymic activity and linear kinetics (1). Such ideal conditions may not necessarily exist at the cellular level. In order to understand better the role enzymes play within the cell, a comparison was made of the properties of a single enzymic reaction in its normal intracellular milieu and when liberated into a synthetic medium following cellular destruction (2).

A method is herein described which permits quantification of the catalytic activity and elucidation of the kinetic properties of xanthine oxidase in both intact and "sonicated" lymphocytes. The enzyme assay is based on the conversion of xanthine-6-C^{14} by xanthine oxidase to uric acid, which is then further metabolized to allantoin by uricase, releasing the C-6 carbon atom as $C^{14}O_2$. Addition of an excess of exogenous purified uricase to the reaction mixture renders xanthine oxidase the rate-limiting enzyme in the over-all process. Measurement of the rate of $C^{14}O_2$ evolution in this system provides an index of xanthine oxidase activity. The use of this procedure permits the detection of the oxidation of 10^{-12} mole xanthine, thus making it possible to estimate xanthine oxidase with high sensitivity and at tracer levels of substrate.

METHODS

Spleens removed from DBA/2 strain mice were immediately suspended in ice-cold saline, minced, and then gently squashed between watch

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[2] Investigator, Health Research Council of the City of New York (under contracts #I-109 and #I-104, respectively).

glasses. The preparations were then passed through gauze filters. Examination by phase-contrast microscopy indicated a suspension of single unagglutinated cells with intact cell membranes as evidenced by impermeability to nigrosin (3). Contaminating erythrocytes were removed by a brief osmotic shock employing hypotonic saline, as previously described (4). The isolated splenic lymphocytes were then suspended in a salts–0.2 *M* sodium phosphate buffer at pH 7.2. Cells were counted employing conventional hemocytometer techniques. When required, disruption of cell membranes was accomplished by placing an aliquot of the buffered cell suspension in a Raytheon ultrasonic oscillator, model No. 9 KC with setting No. 80 at 0°C for 5 min. Phase microscopic examination confirmed the absence of intact cells after ultrasonic oscillation. Enzymic activities of preparations subjected to ultrasonic oscillation are expressed in terms of the number of cells present in the original cell suspensions.

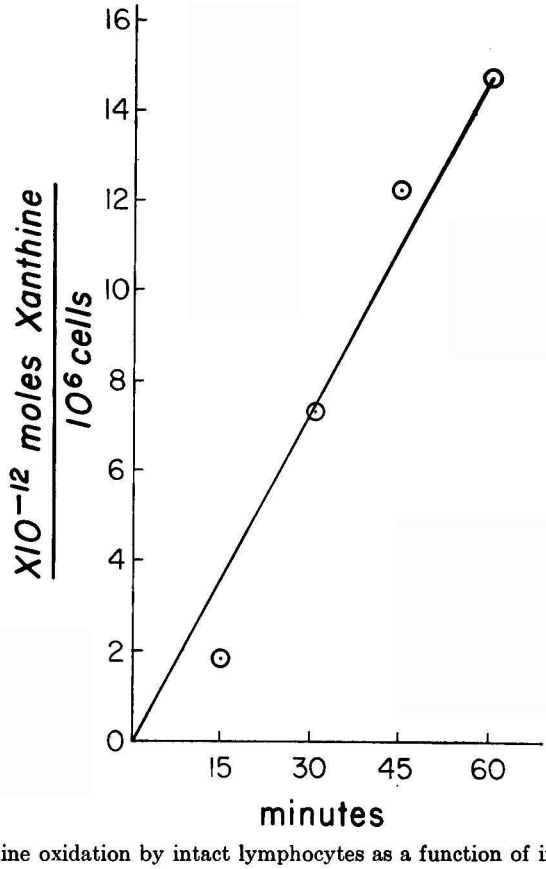

FIG. 1. Xanthine oxidation by intact lymphocytes as a function of incubation time.

For the estimation of xanthine oxidase activities, the following procedure was employed: 2 ml of the indicated cell suspension was placed in the main compartment of Warburg vessels kept in chipped ice and containing 12,500 counts/min xanthine-6-C^{14} (Isotopes Specialties Co., specific activity 0.4 mc/mmole). Two and one-half enzyme units of uricase (as assayed by the method of Worthington Biochemical Co.) were added to the reaction mixture, rendering xanthine oxidase rate-limiting. When desired, nonradioactive xanthine (Nutritional Biochemical Co.) was added as indicated in the text and tables. Unless otherwise stated, the enzymic processes in the reaction mixtures were initiated by incubating the Warburg vessels at 38°C. Introduction of 0.3 ml of 5 *N* sulfuric acid from the side arm into the reaction mixture terminated the incubation period and liberated the enzymically formed CO_2. Absorption of the released $C^{14}O_2$ into a mixture of 0.2 ml hyamine hydroxide (Castor Oil Co., New York) and 0.2 ml toluene present in the center wells of the flasks was accomplished by shaking the flasks for an additional hour at 38°C after the introduction of the sulfuric acid (5). The hyamine–toluene mixture in the center well was then quantitatively transferred into a 5-dram counting bottle using 5.0 ml toluene containing 0.3% of 2,5-diphenyloxazole (Arapaho Co.) and 0.03% of 1,4-di[2-(phenyloxazole)]-benzene (Arapaho Co.) and 1.0 ml of absolute ethanol. The samples were counted in an automatic Tricarb Liquid Scintillation Counter (6). Sufficient counts were accumulated to reduce the probable counting error of each sample to less than ±5%.

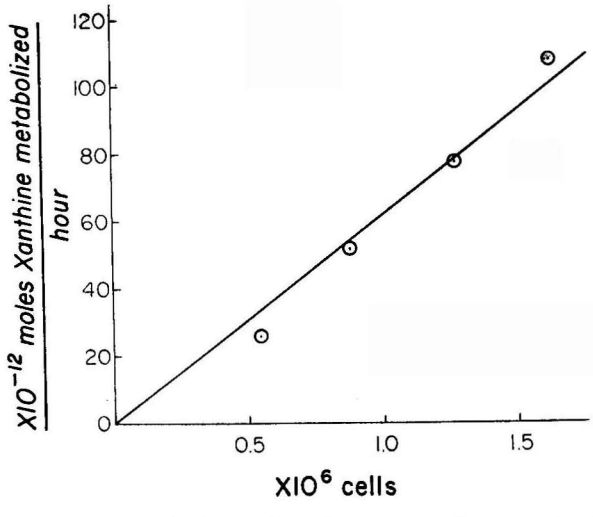

FIG. 2. Xanthine oxidation by intact lymphocytes as a function of cell number.

RESULTS AND DISCUSSION

An ideal enzyme assay should satisfy two criteria: The measured activity should be linear with respect to the concentration of the enzyme and the activity should be linear with respect to time. Figures 1 and 2 demonstrate that this assay system applied to whole-cell suspensions

TABLE 1

CALCULATIONS FOR THE ESTIMATION OF THE MICHAELIS-MENTEN CONSTANT FOR XANTHINE OXIDASE OF INTACT SPLEEN CELLS[a]

Molarity of xanthine	Xanthine (counts/min/μ mole)	Observed $C^{14}O_2$ (counts/min/10^6 cells)	$C^{14}O_2$ (μ mole/hr/10^6 cells)	$\frac{1}{S}$	$\frac{1}{v}$
$\times 10^{-5}$			$\times 10^{-5}$	$\times 10^5$	$\times 10^5$
1.06	470,000	7.32	1.56	0.940	0.642
1.57	318,000	7.08	2.23	0.635	0.449
2.08	240,000	5.54	2.31	0.480	0.433
3.10	161,000	5.21	3.23	0.322	0.309
5.14	97,000	3.30	3.40	0.194	0.295
7.18	70,000	2.32	3.33	0.139	0.300
21.46	23,000	0.80	3.43	0.047	0.291

[a] The Warburg flasks contained 12,500 counts/min xanthine-6-C^{14}.

meets both criteria, linearity being observed throughout the experimental period employed and the cell concentration studied.

The Michaelis-Menten constants (K_m) of the intracellular and liber-

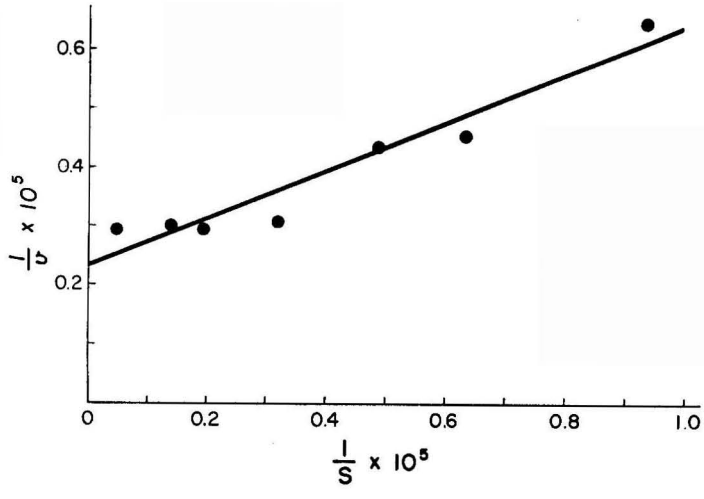

FIG. 3. The Michaelis-Menten relationship for the xanthine oxidase of intact lymphocytes (●–●).

ated spleen xanthine oxidases were determined. Increasing amounts of unlabeled xanthine were added to Warburg flasks containing a constant amount of xanthine-6-C^{14}. Assuming the endogenous xanthine pool is negligible with respect to the added xanthine, the specific activity of the substrate pool available to the enzyme may be calculated. The results of a typical experiment using intact spleen lymphocytes are shown in Table 1. Enzyme activity as a function of substrate concentration was thus determined and is represented as a Lineweaver-Burk plot (7) in Fig. 3 from which the K_m is derived. The mean K_m's, from duplicate experiments, for the xanthine oxidase of intact cells and preparations subjected to ultrasonic oscillation were found to be 1.8×10^{-5} and 2.0×10^{-5} M, respectively.

Energies of activation of the xanthine oxidase reaction in intact and

TABLE 2

XANTHINE OXIDASE ACTIVITIES[a] OF INTACT AND "SONICATED" CELL PREPARATIONS AT VARIOUS TEMPERATURES

T(°K)		Moles of xanthine metabolized $\times 10^{-12}$	
		Intact	"Sonicated"
293.5		2.33	3.55
		2.61	4.04
		3.26	3.26
	Mean:	2.73	3.62
298.6		5.02	6.73
		5.96	6.53
		5.79	5.39
	Mean:	5.59	6.22
303.0		10.4	7.75
		10.5	9.71
		11.8	8.49
	Mean:	10.9	8.65
310.7		20.6	11.5
		15.3	14.9
		17.4	12.5
	Mean:	17.8	13.0

[a] The Warburg flasks contained 12,500 counts/min xanthine-6-C^{14} and 5.1×10^{-6} mole of unlabeled xanthine.

"sonicated" spleen lymphocytes were compared in order to study the influence on the catalytic properties of an enzyme when it is functioning outside the normal cytoplasmic environment and to determine whether, in the intact cells, diffusion of the substrate, xanthine, across the cell membrane is a rate-limiting process in the over-all reaction sequence. If the physical process of substrate diffusion across the lymphocyte membrane is a rate-limiting step, the order of magnitude of the activation energy would be considerably lower than that observed for enzyme-catalyzed reactions. Table 2 depicts the results of triplicate experiments, in which the changes in xanthine oxidase activity as a function of temperature are compared in intact and "sonicated" spleen cell suspensions. It is evident that the large dilution of the intracellular enzyme upon liberation into the incubation medium has not significantly altered the

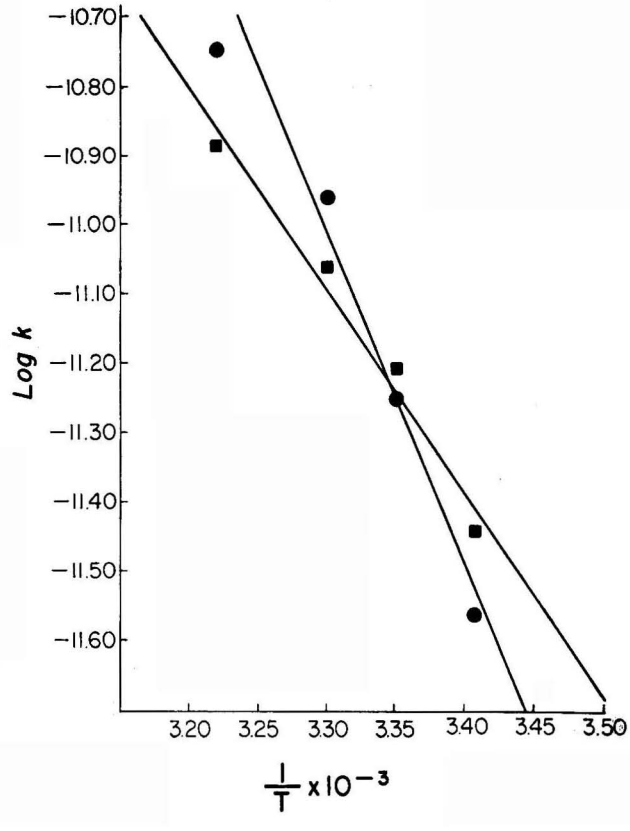

FIG. 4. Arrhenius plot of xanthine oxidase activity as a function of temperature (●–●, intact lymphocytes; ■–■, "sonicated" lymphocytes).

rate of xanthine oxidation at 38°C, thus indicating the absence of intracellular xanthine oxidase inhibitors or activators. These experiments further demonstrate a typical Arrhenius relationship in which the reaction rate increases exponentially with the temperature. In Fig. 4, the mean data of these triplicate experiments are plotted as an Arrhenius plot. The activation energies derived by the method of least squares for xanthine oxidase for intact and "sonicated" cells are 20,000 and 13,300 cal, respectively. The activation energy for intact cells is of the same order of magnitude as for disrupted cells and is characteristic of enzyme-catalyzed reactions, thus eliminating the possibility that passive transport of xanthine across the cell membranes is rate-limiting.

SUMMARY

A radioisotopic method is described which permits quantitation of the catalytic activities and kinetic properties of intracellular and extracellular xanthine oxidases. No intracellular activator or inhibitor of xanthine oxidase is present. Differences exist in the energies of activation for the intracellular and liberated spleen xanthine oxidases. Passive diffusion of xanthine into the intact cell is not rate-limiting.

REFERENCES

1. Weber, G., *Rev. can. biol.* **18,** 245 (1959).
2. Pardee, A. B., *in* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), p. 682. Academic Press, New York, 1959.
3. Kaltenbach, J. P., Kaltenbach, M. H., and Lyons, W. B., *Exptl. Cell Research* **15,** 112 (1958).
4. Feigelson, P., Ultmann, J. E., Harris, S., and Dashman, T., *Cancer Research* **19,** 1230 (1959).
5. Passman, J. M., Radin, N. S., and Cooper, J. A. D., *Anal Chem.* **28,** 484 (1956).
6. Davidson, J. D., and Feigelson, P., *Intern. J. Appl. Radiation and Isotopes* **2,** 1 (1957).
7. Wilson, P. W., *in* "Respiratory Enzymes" (Lardy, H., ed.), p. 20. Burgess Publ. Co., Minneapolis, Minn., 1949.

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Sequential One-Dimensional Chromatography: Analysis of Free Amino Acids in the Brain

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Since the studies by Roberts and Frankel (1) on γ-aminobutyric acid, two-dimensional paper chromatography has been commonly used for estimating levels of free amino acids in the brain. The chief limitation of this method, apart from the problems created by the often unpredictable behavior of phenol, results from the necessity of applying the starting material as a single spot. This limits the amount of material that can be applied to the paper without overloading it with the major components. The concentration of a major brain amino acid such as glutamic has been shown by Tallan, Moore, and Stein (2) to be about 100 times greater than that of a minor component such as leucine or phenylalanine. This big difference makes it almost impossible, by means of two-dimensional chromatography, to get valid estimates of the minor components without losing resolution of the major components through overloading.

The Moore and Stein (3) procedure, though probably unequaled when an accurate analysis of a single mixture of amino acids is required, becomes impractical when numerous samples must be analyzed simultaneously. Special techniques have been developed for the estimation of a few brain components such as glutathione, glutamic acid, glutamine, and γ-aminobutyric acid (4); but when information on levels of a larger number of components is required, paper chromatography still remains the method of choice.

Chromatography in one dimension offers the advantage that the material can be applied as a streak rather than as a spot and that larger amounts of solution can thus be applied without overloading the paper. This makes possible the estimation of levels of both major and minor components with reasonable accuracy. The limitation of one-dimensional chromatography is its low resolving power. It has been shown by Vrba (5) that repeated chromatography in one dimension, the sheet being dried after each development, greatly improves the separation of the free amino acids in the brain. Vrba's method, however, is exceedingly time consum-

ing, involving 10–12 consecutive runs taking as long as 2 weeks. Moreover, in our hands, it failed to give adequate separation of four important components glutamine, taurine, glycine, and aspartic acid.

The problem of separating the ten components in brain extracts with which this work was concerned, namely, glutamic acid, aspartic acid, γ-aminobutyric acid (GABA), alanine, glycine, taurine, glutamine, ethanolamine, phosphoethanolamine, and glutathione, was finally solved by the consecutive use of two different solvent systems run in the same direction, the first being employed twice, the second once. Theoretically, there is no limit either to the number of systems that can be used or to the frequency with which they can be applied. Practically, a limit is imposed by the length of the paper and the width of the individual bands. It is suggested that sequential one-dimensional chromatography is a useful term to describe this technique.

MATERIALS AND METHODS

Preparation of Brain Extracts

Wistar rats (250-gm males) were fasted 24 hr and killed by decapitation. Their brains were removed immediately and frozen with powdered Dry Ice, the operation taking about 40 sec. Two methods of extraction were employed as follows:

1. Five brains (average wet weight 1.927 gm each) were pooled and homogenized in 7.5 times their weight of ice-cold distilled water in a Potter-Elvehjem homogenizer. Sixty-five milliliters of the homogenate (equivalent to 8.15 gm tissue) was mixed with an equal volume of saturated picric acid. The homogenate was stirred with 5 gm acid-washed Celite (diatomaceous earth), and the precipitate was removed by filtration. The filter cake was washed with 50 ml water, and the filtrate was mixed with 25 gm of moist Dowex 2-X8 (chloride form) 50–100 mesh resin. After stirring for 30 min at room temperature, the mixture was poured on a coarse sintered-glass filter, and the resin was washed three times with 20 ml of 0.02 *M* acetic acid. Filtrate and washings combined were shell-frozen and lyophilized. The dry residue was redissolved in 4 ml of distilled water giving a solution containing the equivalent of 2.0375 gm brain tissue (wet weight) per milliliter.

2. The five pooled brains were homogenized in three times their total weight of ice-cold 80% methanol (by volume). Thirty milliliters of the resulting homogenate was centrifuged at 2000 × *g* for 15 min. Twenty-two milliliters of the clear supernatant was adjusted to pH 4.0 with 1 *N* HCl and concentrated *in vacuo* to 5 ml. After standing in the chill room

overnight, a precipitate formed in the extracts which was removed by further centrifugation.

Application of Sample

Sheets of Whatman No. 1 paper (18¼ × 22½ in.) were ruled into five sections 11.5 cm wide, and the material was applied as a streak 7 cm long at a distance of 8 cm from the upper (long) edge of the paper. Even resolution was dependent on evenness of application of the material. This was obtained by the use of a specially designed pipet with a drawn out tip bent at an angle of 45° enabling the pipet to be held almost horizontally during streaking. During application the paper was rested on a plastic box in which a slit 7 cm long had been cut; the interior of the box was kept under negative pressure, a flow of air being drawn through the paper to hasten drying. A thin sheet of plastic with a slit corresponding to the one in the box was placed over the paper during streaking, and a straight edge was used to guide the pipet. With practice it was found possible to apply the material very evenly at a rate of 10 μl/7 cm. The amount applied in most of these experiments was 20 or 40 μl.

Development

Two solvent systems were used. System I contained *n*-butanol–acetic acid–water (5:1:4); system II contained methyl ethyl ketone–*tert*–butanol–water–pyridine (40:40:20:1). System I alone was used to determine glutathione, phosphoethanolamine, glutamic acid, alanine, ethanolamine, and GABA. Sheets developed in this system were run three times consecutively for 16 hr and dried for 8 hr between runs (sequence I). Sheets in system II were run twice consecutively for 16 hr and dried for 8 hr between runs. A guide strip was then developed with ninhydrin to determine the position of the alanine. The sheets were then developed with system I until the solvent front just reached the point where the alanine should be as indicated by the guide strip (sequence II).

After removal from the chromatography jar, the sheets were allowed to dry by hanging in a moving stream of air for 2 hr. They were sprayed with pyridine and heated for 15 min at 60°C, then passed rapidly through an acetone solution of ninhydrin (0.25%) and heated again for 25 min at 60°C.

Elution

The developed chromatograms were cut into strips corresponding to the separate amino acids. Each strip was cut up into pieces of suitable size and placed in a 250-ml conical flask. A quantity of eluting fluid (75% acetone adjusted to pH 8.5 with 1 ml/l of *M* phosphate buffer) was added

to the flask, the amount being determined by the intensity of the ninhydrin color. Four, 8, or 12 ml was the amount generally used. Flasks were stoppered and shaken periodically. The eluting fluid was decanted and its optical density evaluated by means of a Beckman DU spectrophotometer at a wavelength of 570 mμ. Chromatograms streaked with amounts of known amino acids comparable to those in brain were run at the same time to provide standards. Recoveries of amino acids by the extraction systems used were determined by the addition of known amounts of amino acids to the brain before extraction.

RESULTS

Sequence I gave satisfactory separation of glutathione, phosphoethanolamine, glutamic acid, alanine, ethanolamine, and GABA. Glutamine and taurine remained unseparated. Aspartic acid and glycine were partially separated but not well enough to make possible their accurate determination.

The progressive separation of compounds by sequence II after the first, second, and third run, is shown in Fig. 1. Both taurine and ethanolamine

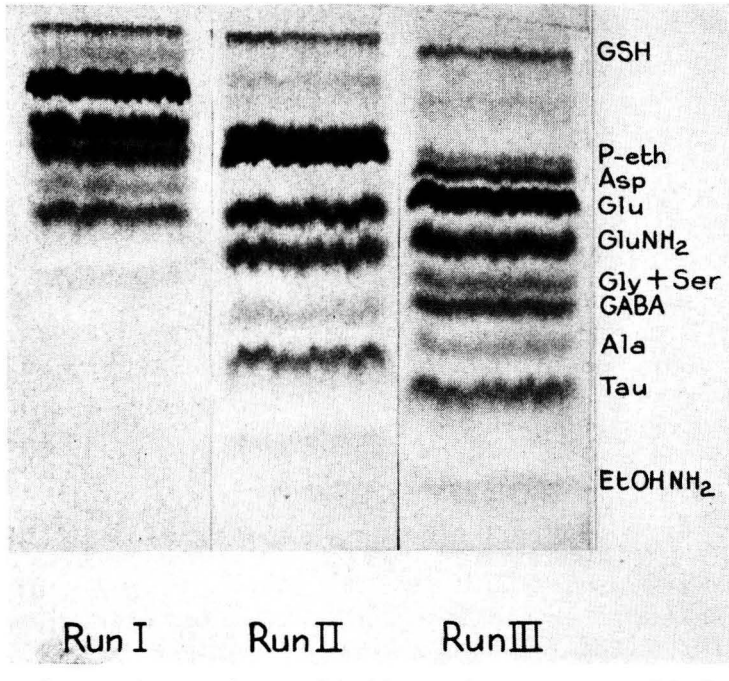

FIG. 1. Degree of separation of ninhydrin reacting components of brain extract obtained by sequence II.

moved rapidly in solvent system II, and glutamine was separated from glutamic acid. The final development in solvent system I moved the glutamic acid ahead of the aspartic acid and separated the aspartic acid from the phosphoethanolamine. It also separated GABA from glycine without mixing it with the alanine. A general idea of the quantitative distribution of the brain components as separated in sequence II can be obtained from Fig. 2, which represents the tracing obtained on passing a

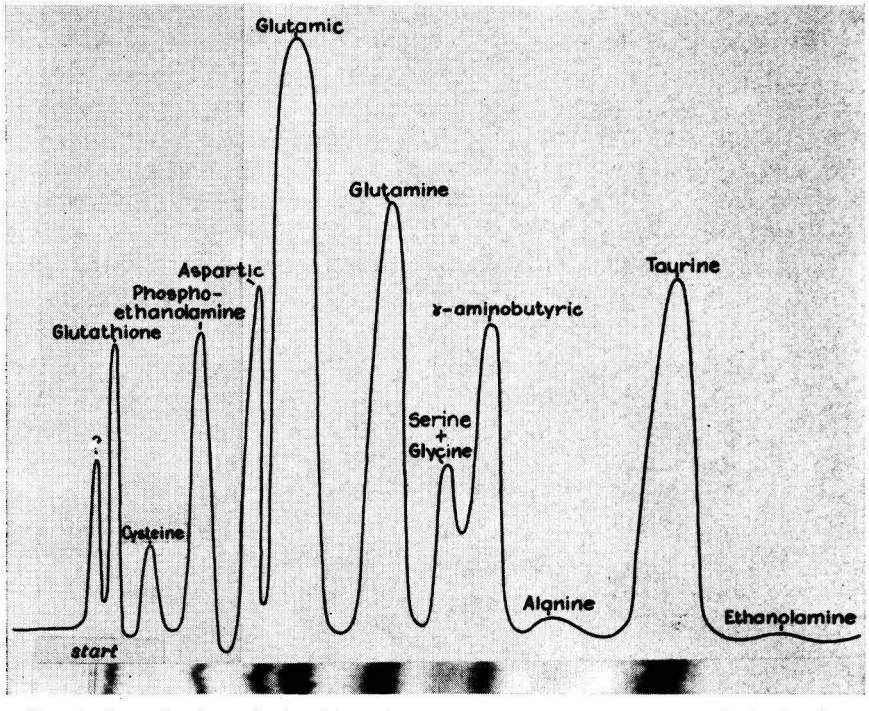

FIG. 2. Quantitative relationships of ninhydrin reacting components in brain tissue as separated by sequence II.

negative photograph of the paper strip through an Analatrol analyzer. In this sequence serine remained mixed with glycine and the two were determined together.

A standard solution of amino acids at levels corresponding to those in the brain added to the brains before extraction gave values for recoveries by the two extraction methods. Recoveries by the picric acid method averaged 95%. Those by the alcohol method ranged from 80 to 90%.

Table 1 shows the average levels in the brain of the fasted rat of ten brain components. The figures are derived from ten separate experiments

TABLE 1

AVERAGE LEVELS OF TEN COMPONENTS IN PICRIC ACID AND ALCOHOL EXTRACTS OF BRAINS OF FASTED RATS (MEANS OF FIVE ESTIMATES) EXPRESSED AS MICROGRAMS OR MICROMOLES PER GRAM WET WEIGHT

Compound	Picric acid		Alcohol	
	μg/gm	μmoles/gm	μg/gm	μmoles/gm
1. Glutathione	522 ∓ 61	1.70	280 ∓ 31	0.91
2. Phosphoethanolamine	410 ∓ 21	2.88	370 ∓ 28	2.61
3. Aspartic acid	340 ∓ 16	2.55	310 ∓ 17	2.33
4. Glutamic acid	1360 ∓ 56	9.25	1280 ∓ 48	8.70
5. Glutamine	690 ∓ 27	4.79	600 ∓ 24	4.16
6. Glycine + serine	180 ∓ 7	2.40	160 ∓ 6	1.77
7. Alanine	20 ∓ 3	0.22	25 ∓ 3	0.28
8. GABA	210 ∓ 7	2.04	180 ∓ 6	1.74
9. Taurine	470 ∓ 17	3.76	480 ∓ 19	3.84
10. Ethanolamine	10 ∓ 2	0.16	14 ∓ 2	0.22

in each of which five brains were pooled and extracted. In five experiments the picric acid procedure was used; in the other five, the alcohol procedure. Corrections have been made for recoveries.

Alcohol gave low values for glutathione and phosphoethanolamine. Otherwise the values obtained with alcohol agreed fairly closely with those obtained by the more time-consuming picric acid procedure. The following observations on the chromatographic behavior of individual components were made.

Glutathione

This substance was observed by Lindan and Work (6) to give three spots on chromatography in phenol followed by collidine–lutidine. In the systems described here it also broke up into three components. The slowest component, identified as glutathione in Figs. 1 and 2, had the same R_f as cysteine. The other components had the same R_f as phosphoethanolamine and glutamic acid (sequence II) and glycine and glutamic acid (sequence I). This instability of glutathione may result in artificially high values for glutamic acid. Thus 80 μg glutathione yielded the equvalent of 20 μg glutamic acid after chromatography in sequence I. The 522 μg/gm glutathione estimated to be present in the brain would, if it broke down to the same extent, contribute about 130 μg/gm to the level of glutamic acid, necessitating a correction of the glutamic acid value. It is almost impossible to tell whether glutathione in brain breaks down in the same way as it does when pure glutathione is chromatographed. For

this reason, it is difficult to decide whether or not this correction should be applied.

Paper chromatography does not seem a very reliable method of estimating the level of glutathione. The method of Berl and Waelsch (4), which depends on the reaction of glutathione with *p*-chloromercuriphenyl sulfonic acid, probably gives more accurate values. These workers determined glutathione in eight parts of the brain and their average value (1.86 μmoles/gm) is only slightly higher than the value obtained by chromatography.

Phosphoethanolamine

This substance was stable in both systems, but its accurate determination was made difficult by the fact that a breakdown product of glutathione has the same R_f value in sequence I. Estimates were, therefore, based on values obtained by sequence II. This component was poorly extracted with methanol. The amount extracted with picric acid (410 μg/gm) corresponds closely to that found by Tallan, Moore, and Stein (2) in the cat's brain (419 μg/gm).

Aspartic Acid

This component was rather inadequately extracted by methanol. The amount extracted with picric acid (340 μg/gm) is somewhat higher than that found by Tallan, Moore, and Stein (2) in the cat's brain (297 μg/gm).

Glutamic Acid

This major amino acid was also extracted in somewhat larger amount by picric acid than by 80% methanol. If a correction is made for glutamic contributed by glutathione, the actual amount in the whole brain would be about 9.25 μmoles/gm. The value is in close agreement with that found by a different method by Berl and Waelsch (4) (9.10 μmoles/gm). The glutamic acid level in cat brain given by Tallan, Moore, and Stein (2) was 8.70 μmoles/gm.

Glutamine

This component was well resolved in sequence II, and the value obtained by picric acid extraction was very close to that obtained by a different method by Berl and Waelsch (4) (4.5 μg/gm).

Glycine, Serine

The lack of separation of these substances by sequence II made it impossible to do more than estimate their total level. In the cat brain the

level of these two components was glycine 101 μg/gm, serine 76 μg/gm (4), a total of 177 μg/gm compared with 180 μg/gm obtained by paper chromatography of extracts of rat brain.

Alanine

This amino acid was present only in small amounts in the rat brain, and a rather large error was involved in its estimation. The level of alanine found in the cat brain by Tallan, Moore, and Stein (2) (84 μg/gm) was considerably higher than that found in the rat brain.

γ-Aminobutyric Acid

The level of this amino acid could be determined either by sequence I or sequence II. Sequence I gave more consistent values. The value obtained was close to that found by a different method by Berl and Waelsch (4) (2.1 μmoles/gm) and fairly close to that found by Tallan, Moore, and Stein (2) for the cat brain (234 μg/gm).

Taurine

This was one of the major amino acids in the rat brain. The value found by paper chromatography (470 μg/gm) was higher than that found in cat brain by ion-exchange chromatography (4) (240 μg/gm).

Ethanolamine

The level of this compound could be determined either by sequence I or by sequence II, though in sequence II the color developed with ninhydrin was weak. It was present in the smallest amount of all the compounds studied (10 μg/gm). The value for this compound given for the cat brain by Tallan, Moore, and Stein (2) is very much higher (207 μg/gm). We have observed that in old brain extracts, even though stored at — 5°C, great increases in the level of ethanolamine occurred, suggesting that it may be liberated from some bound form present in the extract.

Leucine, Methionine, and Phenylalanine

These three components were resolved by sequence I and could be seen below GABA on the chromatograms, but their level in fresh brain extract was too low to be determined accurately.

N-Acetyl Aspartic Acid

By spraying the paper strips with a solution of bromocresol green at pH 8.0, a well-defined acidic area could be detected having an R_f of 0.66 in system I. This corresponded to *N*-acetyl aspartic acid, the presence of which in the brain of the cat was demonstrated by Tallan, Moore, and

Stein (7). The dye method was not sufficiently sensitive to make possible quantitative estimations of the level of this component from paper strips. Acid hydrolysis of the *N*-acetyl aspartic fraction obtained from the brain by partition chromatography gave a value for this compound of 550 μg/gm in the fasted rat.

SUMMARY

1. One-dimensional sequential chromatography was used to separate amino acids and related components in extracts of rat brain.

2. One per cent picric acid and 80% alcohol were compared as extracting agents. Higher values for several components were obtained with picric acid.

3. System I, *n*-butanol–acetic acid–water (5:1:4), run three times descending for 16 hr each run, separated glutathione, phosphoethanolamine, glutamic acid, alanine, ethanolamine, and *N*-acetyl aspartic acid. It failed to resolve taurine, glutamine, glycine, and serine.

4. System II, *tert*-butanol–methyl ethyl ketone–water–pyridine (40:40:20:1) run twice for 16 hr descending followed by system I run as far as the alanine, resolved all components with the exception of glycine and serine.

5. The method made possible application of 20 μl of brain extract without overloading.

6. With a few exceptions, levels of brain components determined by this method were in good agreement with those obtained by other investigators by other means.

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REFERENCES

1. ROBERTS, E., AND FRANKEL, S., *J. Biol. Chem.* **187**, 55 (1950).
2. TALLAN, H. H., MOORE, S., AND STEIN, W. H., *J. Biol. Chem.* **211**, 927 (1954).
3. MOORE, S., AND STEIN, W. H., *J. Biol. Chem.* **211**, 893 (1954).
4. BERL, S., AND WAELSCH, H., *J. Neurochem.* **3**, 161 (1958).
5. VRBA, R., *Nature* **176**, 1258 (1955).
6. LINDAN, O., AND WORK, E., *Biochem. J.* **48**, 337 (1951).
7. TALLAN, H. H., MOORE, S., AND STEIN, W. H., *J. Biol. Chem.* **219**, 257 (1956).

ANALYTICAL BIOCHEMISTRY 1, 433–442 (1960)

SHORT COMMUNICATIONS

A New Vitamin A Color Reaction[1]

Recently a new method for cholesterol estimation has been introduced which produces a color having a maximum absorbence at 490 mμ (1). Subsequent investigation disclosed that vitamin A in chloroform produced a different chromogen with the same reagents. The reaction mixture was prepared by diluting 50 μg of pure crystalline vitamin A dissolved in 0.5 ml chloroform with 6.0 ml of glacial acetic acid saturated with ferrous sulfate.[2] Addition of 2.0 ml of concentrated sulfuric acid with stirring

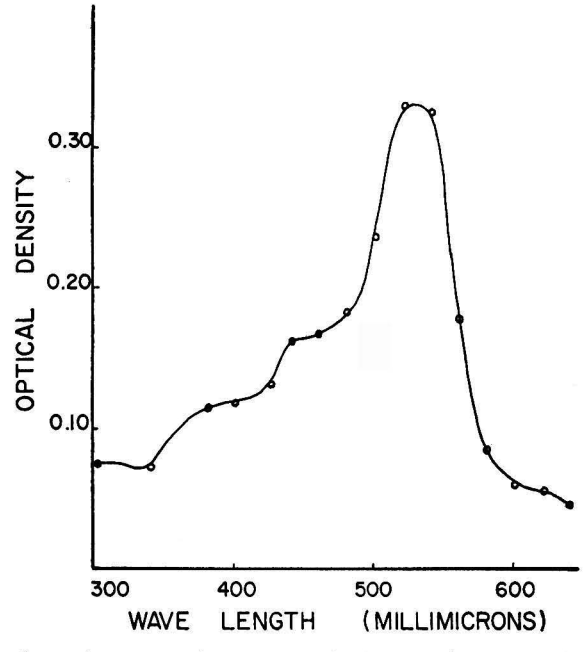

FIG. 1. The absorption curve of 50 μg vitamin A in a mixture of chloroform, glacial acetic acid, ferrous sulfate, and sulfuric acid.

[1] This work was supported in part by a grant from the American Osteopathic Association, Project T-1602 and the Attending Staff Association of the Los Angeles County Osteopathic Hospital.

[2] This reagent is prepared by shaking several grams of analytical reagent-grade $FeSO_4 \cdot 7H_2O$ with 1 l of glacial acetic acid. After allowing to stand overnight, a clear solution is obtained by decantation.

caused formation of a blue color which almost instantly changed to bright pink. The absorption spectrum of this solution was determined on a Beckman DU spectrophotometer using a Corex cuvette with a 10-mm light path. Maximum absorbence occurred at 520 mμ with two peaks of lesser intensity near 380 and 440 mμ (Fig. 1). Using ferric chloride and HCl, Brüggemann and co-workers (2, 3) obtained a chromogen with vitamin A which had absorption maxima at 570 and 620 mμ.

The influence of time upon chromogen formation was studied in reaction mixtures containing 50 μg vitamin A. Colorimetric measurements were performed in round cuvettes measuring 19 × 105 mm on a Coleman spectrophotometer at 520 mμ at intervals ranging from 1 to 30 min. After the addition of sulfuric acid, development of the pink color was rapid and reached maximum intensity within 2 min (Fig. 2). Thereafter, no

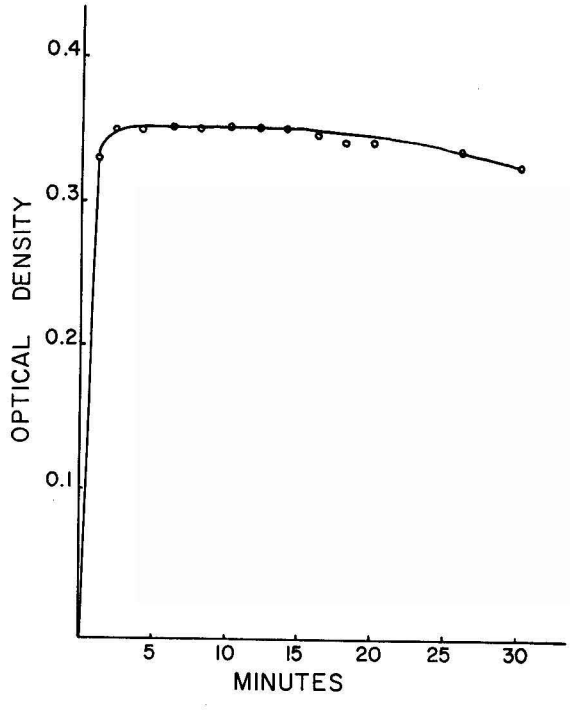

FIG. 2. The effect of time upon color formation in reaction mixtures containing 50 μg vitamin A.

significant change in the optical density could be detected during the next 12 min. After ½ hr decreases in color intensity of less than 10% were observed. Although the Carr-Price color (4) for vitamin A is produced in

only 5–15 sec, appreciable fading occurs during the next 45 sec. This makes accurate quantitation difficult unless the Carr-Price reagent is added to colorimeter tubes which have been previously placed in the instrument. Biffoli (5) on the other hand has reported that an ethanolic solution of vitamin A in 75% sulfuric acid produces a stable chromogen which has an absorption peak at 525 mμ. However, a 2-hr incubation period is required for maximum color development.

The effect of acid concentration upon chromogen formation was determined by preparing reaction mixtures containing 50 μg vitamin A so that the final sulfuric acid levels were 12.5, 25, 37.5, 50, and 75%. Maximum color development was obtained in solutions containing 25% sulfuric acid. Increasing the acid concentration above this level diminished color intensity. Likewise, color was reduced with acid concentrations of 12.5%.

Color was developed using amounts of vitamin A ranging from 20 to 100 μg dissolved in 0.5 ml chloroform. After 8 min the optical densities of the reaction mixtures were measured on the Coleman spectrophotometer at 520 mμ and the Klett colorimeter using the No. 54 green filter (510–570 mμ). Figure 3 demonstrates that on the two instruments Beer's law was obeyed at the concentrations of vitamin A employed up to 100 μg.

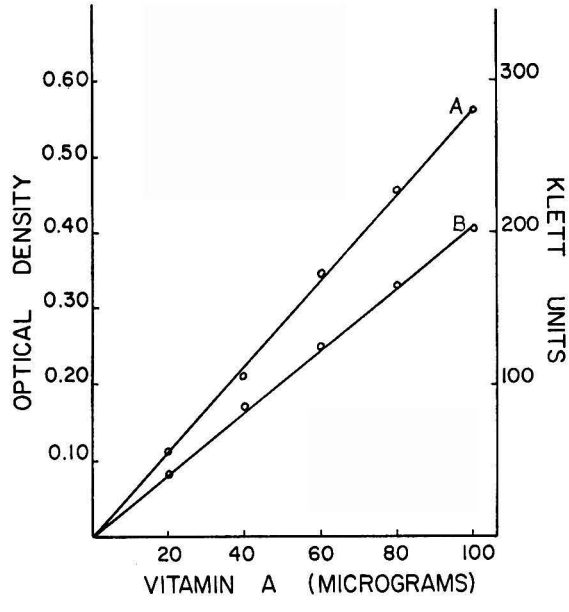

FIG. 3. Standard curves obtained with increasing amounts of vitamin A as measured on (*A*) the Coleman spectrophotometer at 520 mμ and (*B*) the Klett colorimeter using filter No. 54.

The proposed method does not attain the sensitivity of the Carr-Price test which has an $E^{1\%}_{1cm}$ of 4400. Using the ferrous sulfate color reagent, the $E^{1\%}_{1cm}$ for vitamin A has been calculated to be 550.

The reaction described with vitamin A may be advantageous since formation of color is rapid and sufficiently stable for accurate measurement. In contrast to the Carr-Price reaction, β-carotene and vitamin A do not produce the same color with the ferrous sulfate color reagent. Crystalline β-carotene was observed to peak at 740 mμ on the Beckman DU spectrophotometer. Application of these color reactions for measuring both vitamin A and β-carotene in mixtures is currently under investigation in our laboratories.

REFERENCES

1. SEARCY, R. L., AND BERGQUIST, L. M., *Clin. Chim. Acta* **5**, 192 (1960).
2. BRÜGGEMANN, J., KRAUSS, W., AND TIEWS, J., *Chem. Ber.* **85**, 315 (1952).
3. BRÜGGEMANN, J., KRAUSS, W., AND TIEWS, J., *Z. anal. Chem.* **141**, 161 (1954).
4. CARR, F. H., AND PRICE, E. A., *Biochem. J.* **20**, 497 (1926).
5. BIFFOLI, R., *Boll. lab. chim. provinciali* (Bologna) **10**, 380 (1959).

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A Simple Arrangement for the Concentration of Protein Solutions

When protein solutions are fractionated by means of zone electrophoresis, chromatography, or the newly developed gel filtration technique (1), relatively dilute solutions of the biochemical substance under study is often obtained. When it is undesirable to use precipitation or lyophilization before subsequent purification steps, concentration of the pooled fractions is necessary. Commercially available collodion membranes are useful for this purpose but require rather elaborate equipment

for large volumes to be concentrated in a minimum of time. Direct pervaporation from a dialysis bag filled with solution and placed in front of a fan has been widely used; and Palmstierna has recently introduced the use of carboxymethylcellulose for increasing the rate of pervaporation through dialysis tubings (2).

The technique to be described involves the use of dialysis tubing surrounded by a solution which is connected to a vacuum pump. The authors have found that attention to some details in the practical arrangements will improve the efficiency and usefulness of the procedure. In principle, the technique is not new, and a large number of publications could be cited where various modifications of it has been used. As an example, Laurell (3) has used this type of ultrafiltration for the quantitative concentration of serum fractions obtained after zone electrophoresis.

The accompanying Fig. 1 illustrates an apparatus which can be used for the concentration of 100–200 ml of solution down to a few milliliters. The dialysis tubing, *A* must be of uniform quality, and Visking cellulose tubing obtained as a 10-mm wide inflated band has been found to fulfill this requirement. A piece of appropriate length of the tubing is soaked in water, and the ends are then put through short pieces of polyvinyl chloride (PVC) tubing on to the ends of the funnel *B* and the glass rod *C*. The dialysis tubing should fit tightly to the glass, and the pieces of PVC tubing are then put around it as seals. With the rod *C* serving as a weight, the dialysis tubing is lowered through the outer glass tube *D*. The upper stopper is put in its position, and by adjusting the rod *C* (passed through the hole in the lower stopper), the dialysis tubing can be stretched out to the desired extent. Water, or a buffer solution of suitable composition, is introduced through the hole *E*, and a glass tube connected to the flask *F* is then put into the hole.

Before the dialysis tubing is filled with solution, the vacuum pump is started, which will cause the tubing to expand. Introduction of the solution to be concentrated is facilitated by the use of a syringe, and the funnel *B* may then serve as a reservoir. As shown on the figure, larger volumes can be siphoned over from the flask *G*, by means of a PVC tubing introduced to the bottom of the bag. When the concentration process is finished, it is then easy to suck out the solution and wash the inside of the bag with the aid of this tubing.

The rate of ultrafiltration is proportional to the length of the dialysis tubing used. Usually about 1 ml will filter per 5 cm of tubing per hour, and the ionic composition of the solution will remain relatively constant during the procedure. The same dialysis tubing can be used repeatedly after washing, but if it should be damaged and break during the con-

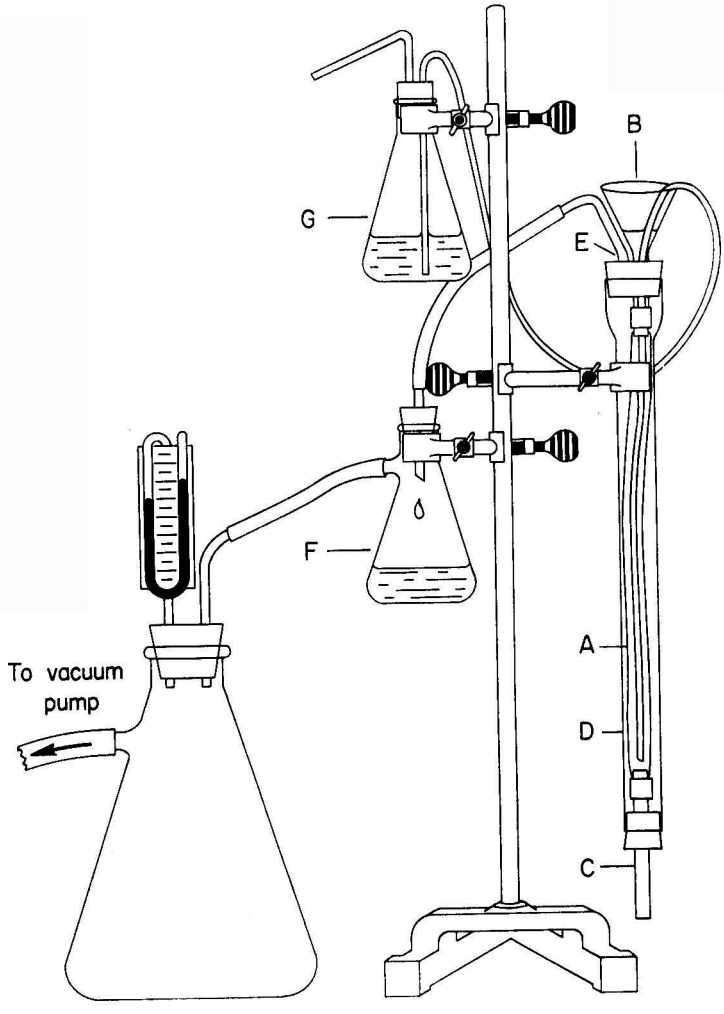

FIG. 1. Apparatus for the concentration of protein solutions.

centration the flask *F* will serve as a safety trap. To avoid the risk of carrying the concentration too far, the glass rod *C* may be replaced by a glass tube attached to a stopcock, through which the solution can also be emptied. Very labile enzyme solutions are best transferred to a similar but smaller apparatus when the level gets below the funnel *B*, if further concentration is desirable.

The concentration is usually best carried out in a cold room, but the whole apparatus can also be immersed in an ice bath. It is not neces-

sary to have the vacuum pump on at all times during the concentration, provided one has a large evacuated flask connected to the flask *F*. The best vacuum that can be obtained is about 10 mm Hg, but the rate of filtration is only about 10% less at 50 mm Hg.

If very large volumes are to be concentrated, it is possible to circulate the solution through several dialysis tubings by means of a peristaltic pump. The authors have also used this arrangement for obtaining the dialyzable fraction of peptone intended for the production of extracellular toxins by bacteria.

As an example of the usefulness of the technique described, 1000 ml of a bacterial extract containing the enzyme β-galactosidase was concentrated to 125 ml in the course of 30 hr in a 150-cm long dialysis tubing with 98% recovery of the enzyme activity. A much smaller unit has been used for the concentration of 10-ml fractions of protein solutions prior to paper electrophoresis. Further examples of the applications of the technique are given in a paper on the purifications of an acid phosphatase from *Escherichia coli* (4).

REFERENCES

1. PORATH, J., *Biochim. et Biophys. Acta* **39**, 193 (1960).
2. PALMSTIERNA, H., *Biochim. et Biophys. Research Communs.* **2**, 53 (1960).
3. LAURELL, H., "Studies on Preparative Vertical Zone Electrophoresis." Dissertation. Almqvist and Wiksell, Uppsala, 1958.
4. HOFSTEN, B. V., AND PORATH, J., manuscript in preparation.

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A Device for the Homogenization and Extraction of Proteins from *Neurospora crassa*[1,2]

Many investigators who have utilized *Neurospora crassa* in biochemical investigation have been faced with the problem of the efficient extraction of proteins in their native state from kilogram quantities of the mycelium of *Neurospora crassa*. We have reported in a prior publication (1) that this could be readily accomplished on gram quantities of mycelium by the use of the Ten Brock homogenizer[3] using a buffered sodium chloride solution. In this same publication, we also showed that the recovery of cobalt-60 incorporated by the mycelium during growth provided a simple index for the efficiency of the homogenization process. By the use of modern well-type scintillation counters, one can therefore readily estimate quantitatively the degree of extraction of soluble proteins from the mold mycelium, thus allowing quantitative studies of enzyme levels to be easily performed. However, when this procedure is applied to 100–1000 gm of wet mycelium, fatigue of the operator, the long time required to process this bulk of material, and the small size of aliquot accommodated by the Ten Brock homogenizer tends to reduce the efficiency of the extraction process.

A simple modification of the Ten Brock homogenizer is depicted in Fig. 1. We have utilized this device to process upwards of a kilogram of wet *Neurospora* mycelium at 0° in a matter of 1–2 hr and have achieved with it an efficiency of extraction of 70% (Table 1). In Table 1 we have included data utilizing the cobalt extraction as reference for the efficiency of extraction of proteins by several other techniques. Extraction using the device described can be recommended over extraction by these methods on the basis of (*a*) speed, (*b*) efficiency, (*c*) low cost, and (*d*) prevention of the type of destruction of subcellular structures associated with freezing and drying.

The mold mycelium, after being harvested and suitably washed, is chilled in 10 vol (w/v) of 1% sodium chloride containing 0.067 *M*

[1] This work was performed under Contract AT(30-1)-933 between the U. S. Atomic Energy Commission and the Johns Hopkins University. The isotopes used were obtained from the Oak Ridge National Laboratories of the U. S. Atomic Energy Commission.

[2] Contribution No. 306 from the McCollum-Pratt Institute.

[3] The Ten Brock homogenizer is available as item No. J-2324 from the Scientific Glass Apparatus Company, Bloomfield, N. J.

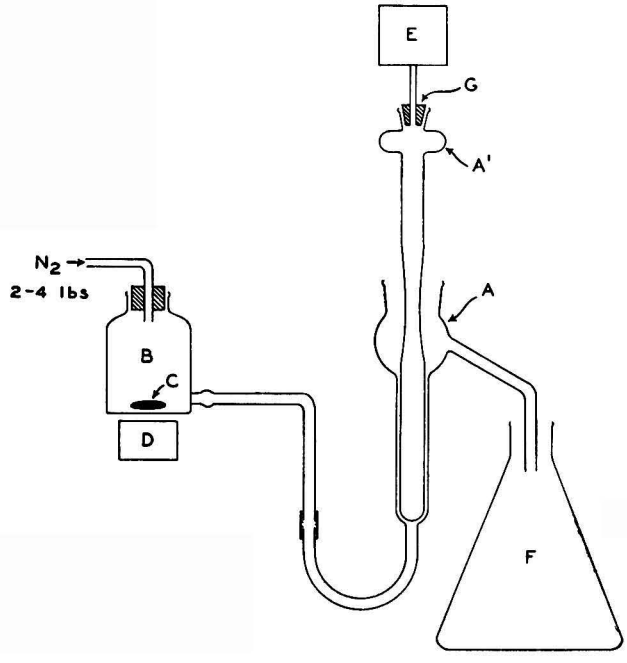

FIG. 1. Device for homogenizing large amounts of *Neurospora crassa*.
A. Modified outer portion of the Ten Brock homogenizer.
A'. Pestle of Ten Brock homogenizer.
B. Container for blenderized mycelial suspension.
C. Teflon-covered magnetic stirring bar.
D. Magnetic stirrer.
E. Variable, low-speed motor (Mix-Master).
F. Receiver for homogenate.
G. Rubber-stopper coupling.

(*M*/15) phosphate buffer, pH 7.2 at 0°C. The mold is reduced to a finely divided state by blendorizing for 30 sec in a Waring blendor, or better an Eppenbach colloid mill.[4] During this time the homogenization device is assembled and precooled in an ice bath. It is important that all the ground surfaces of the homogenizer proper (*A*, *A'*) be wet with the homogenization solution. The chilled, dispersed suspension of mycelium is then transferred to the reservoir (*B*), the motor (*E*) started, to give a rotational speed of approximately 100–300 rpm. The suspension is then fed into the homogenizer proper (*A*) by applying 2–5 lb nitrogen pressure to the reservoir. The homogenate is collected in the chilled reservoir (*F*). A

[4] The Eppenbach micro mill is available from Gifford-Wood Company, Eppenbach Division, 420 Lexington Avenue, New York 17, N. Y.

TABLE 1

EXTRACTION OF PROTEINS FROM *Neurospora crassa* BY VARIOUS PROCEDURES[a]

Procedure	Wet Mycelium Processed (gm)	Dry weight (%)	Protein (mg/100 mg dry weight)	Efficiency homogenization (%)
A	122	9.1	23.2	91.1
	40	16.2	27.1	74.0
	672	6.8	19.8	66.3
B	155	13.3	28.2	73.0
	100	20.8	19.2	77.0
C	5	—	0.0	0.0
D	214	16.7	4.0	ca 16
E	498	14.3	—	74.4
	420	7.8	22.4	87.5
	450	—	—	71.4

[a] *A.* Hand-homogenized with a Ten Brock homogenizer. *B.* Frozen with liquid nitrogen, thawed, and dispersed with a Ten Brock homogenizer. *C.* Blendorized in a Waring blendor for 30–120 sec. *D.* Homogenized in the Eppenbach micro mill. *E.* Homogenized in the device described in this paper.

single pass through this device is adequate to achieve 70–80% release of the soluble protein. Occasionally, a large chunk of mycelium may cause a momentary jam of the homogenizer and consequently it is advisable to use a rubber coupling (*G*) between the motor (*E*) and the pestle (*A*). These jams can be most readily avoided by being certain that the pestle is being freely rotated by the motor before applying pressure to the reservoir.

REFERENCE

1. BALLENTINE, R., AND STEPHENS, D. G., *J. Cellular Comp. Physiol.* **37**, 369 (1951).

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ACTIVATION ANALYSIS HANDBOOK

by **R. C. KOCH**

Nuclear Science and Engineering Corporation
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October 1960, 8¼ x 10½" 219 pp., illus., $8.00

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The University, Exeter, England *U. K. Atomic Energy Authority, Capenhurst, England*

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Volume 1 *1958, 398 pp., illus., $12.00*
Volume 2 *1959, 387 pp., illus., $12.00*

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ANALYTICAL BIOCHEMISTRY 1, 443–451 (1960)

Some Observations on Fluorometric Determinations

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Received August 12, 1960

INTRODUCTION

The use of fluorometry as a qualitative and quantitative technique offers many advantages. First, advances in instrumentation now permit the measurement of fluorescence with a high degree of sensitivity, and therefore fluorometric methods are attractive in cases where the compounds to be determined or the material from which they are derived are not available in large quantities. Even when larger amounts are accessible, it may be an advantage to use smaller quantities of material. Furthermore, the fact that two characteristics of a compound are utilized (absorption of light of one wavelength and emission at another) rather than one only, as in absorptiometric determinations, may contribute to the specificity of such a measurement. The availability of spectrophotofluorometers during recent years has facilitated the application of fluorometric methods, and the present paper reports a detailed examination of the Aminco-Bowman instrument. This was undertaken with the aim of using it for the determination of steroids in human blood (1).

METHODS

The Aminco-Bowman spectrophotofluorometer (American Instrument Co.) was used with a 1P21 photomultiplier (Radio Corporation of America). The quartz cells (external dimensions: 1.2 cm square $\times$ 4.8 cm high; internal volume: 4.8 ml) were supplied with the instrument. The apparatus has high sensitivity which can be varied by a factor of 1000. The full-scale meter deflection is 100 divisions, but readings above 40 divisions are unstable and can be avoided by using a less sensitive range. The slit system consists of three metal strips with slits of various widths, which are placed in both the primary and secondary light paths. The center slits determine the band widths, and therefore the resolution of the incident and fluorescent light beams; slightly wider slits placed either side of these reduce stray light. The increase in sensitivity obtained with wider slits is accompanied by decreased resolution and more scatter. The

system is rather crude, and a set of easily and finely adjustable slits would offer considerable advantage. A further slit, placed in front of the photomultiplier also affects resolution and sensitivity. The secondary slit system determines the size of the image falling on the secondary grating, while the photomultiplier slit affects the band width reaching the detector.

To measure the variation of light intensity reaching the test solution in the Aminco instrument, a selenium photocell (14 mm diameter, type B, metal mount with terminals at the back, from Megatron Ltd., London) of known wavelength response was placed in the cell compartment and connected to suitable resistances and a galvanometer. The fluorescence monochromator was calibrated as recommended by the manufacturers, using a Pen-ray mercury lamp (Ultra-violet Products Inc., San Gabriel, California).

It has been pointed out by Hercules (2), that certain instrumental factors can affect fluorescence spectra. In the present work, spectra were determined photometrically to avoid errors due to recorder-pen response; those due to reabsorption of emitted light were not likely to occur, since adequately purified material was used at low concentrations and the fluorescence efficiencies were relatively high.

The methods used for obtaining fluorescent solutions are described in another paper (3).

RESULTS

Effect of Slit Size on Spectral Resolution

To study the effect of slit size on the resolution of the instrument, the Pen-ray lamp was placed in the cell compartment and the spectrum of the 365-mμ mercury line was recorded. Figure 1 shows the results obtained by varying the secondary slit width. The effect of the photomultiplier slit was studied at three wavelengths: 313, 365, and 577 mμ. These spectra are shown in Fig. 2; the results are essentially the same at these three wavelengths, and slits wider than 1/32 in. cause a decrease in resolution and some distortion. Results with a 1/64-in. slit (not shown in Fig. 2) were almost identical with those for the 1/32-in. slit. Figure 3 shows the effect of the photomultiplier slit size on the resolution of the fluorescence spectrum of corticosterone in ethanolic sulfuric acid. The primary and secondary slits were 1/32-in. (0.795 mm) wide, and 1/16-in. (1.59 mm) slits were placed on each side of these. While affecting the sensitivity of these measurements and the scatter recorded at the shorter wavelengths, the width of the photomultiplier slit did not alter the spectrum obtained. This indicates that in this case the spectrum is wide and no loss in resolution occurs even with the 3/16-in. photomultiplier slit.

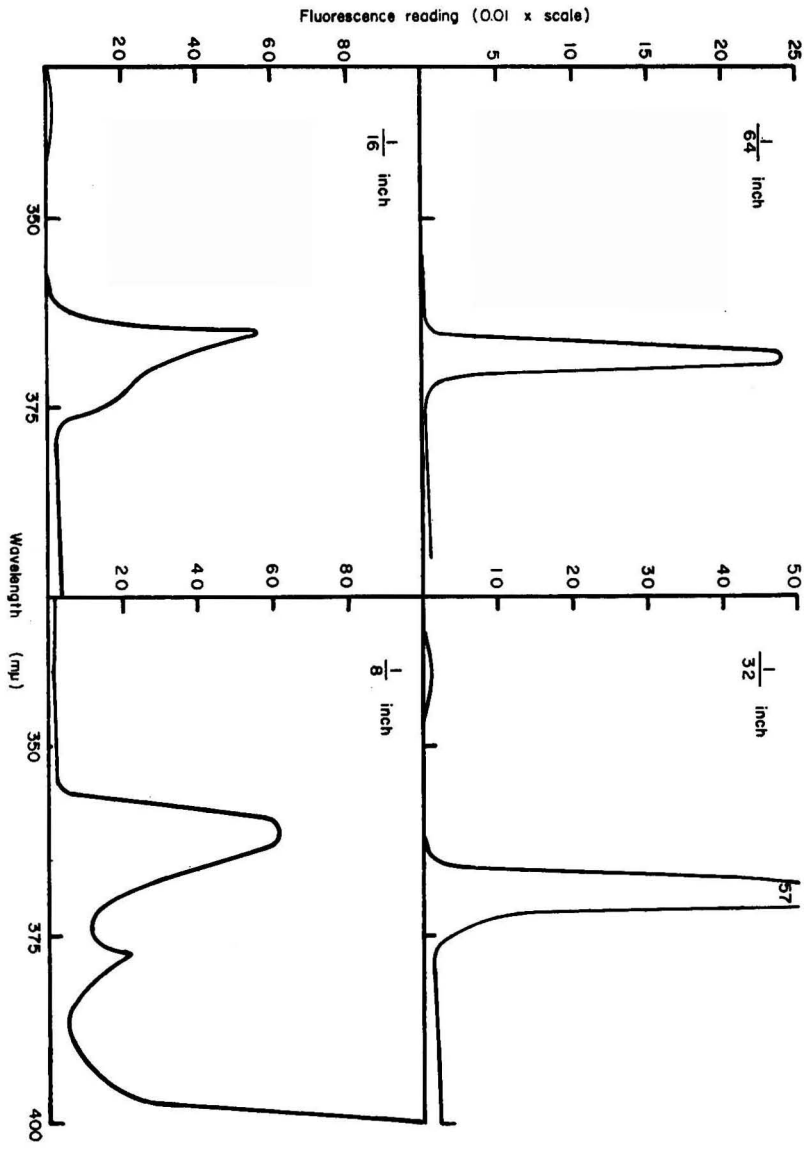

FIG. 1. Effect of secondary slit width on spectral resolution. Mercury line at 365 mμ from a Pen-ray lamp placed in the cell compartment. The next wider slit was placed between the lamp and the secondary slit to reduce scatter. A 1/64-in. photomultiplier slit was used throughout.

Wavelength Calibrations

Calibration of the secondary monochromator with the Pen-ray lamp revealed errors on the wavelength disk of 0–8 mμ in the range 235–730 mμ. To test the monochromator calibrations with respect to one another, the scatter from a quartz cell was used. The primary wavelength was

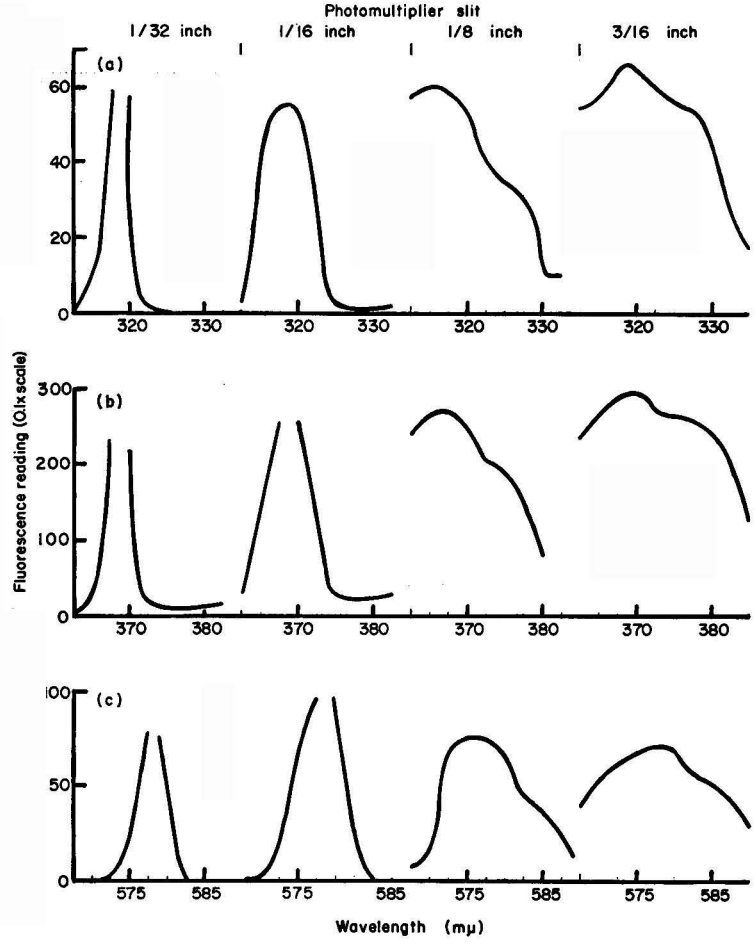

FIG. 2. Effect of width of photomultiplier slit on the spectral resolution of mercury lines at (*a*) 313 mμ, (*b*) 365 mμ, and (*c*) 577 mμ. A secondary slit of 1/64 in. was used.

fixed at the desired value, and the secondary grating was moved until a maximum galvanometer reading was obtained. Errors of up to 10 mμ were found in the range 250–550 mμ, and attempts to adjust the monochromators were not successful. Correction of some spectra was, therefore, necessary [cf. (2)].

The calibration experiments also revealed considerable variation of the complete optical system of the instrument with wavelength. Figure 4 shows the variation of maximum scatter readings with wavelength. Three factors are likely to contribute to this: (*a*) variation in lamp output with

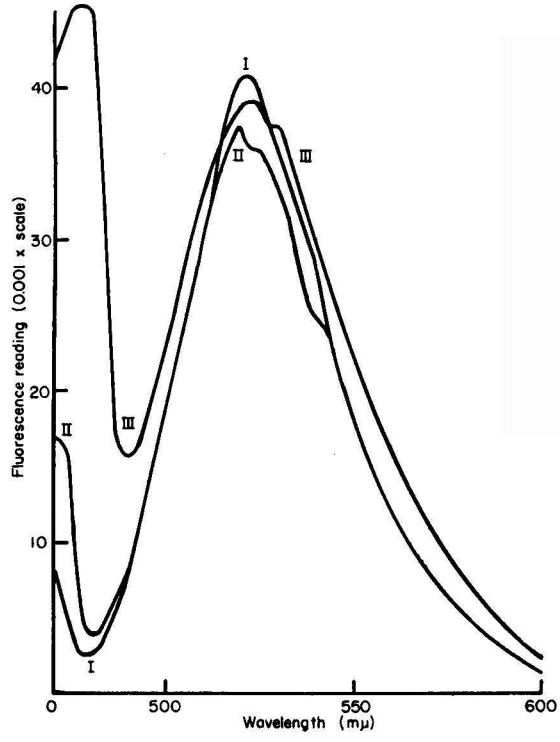

FIG. 3. Fluorescence spectra of corticosterone in 70% (v/v) ethanolic sulfuric acid recorded with different photomultiplier slits.

I. 1/64-in. slit. 0.63 μg corticosterone/ml.
II. 1/32-in. slit. 0.32 μg corticosterone/ml.
III. 3/16-in. slit. 0.08 μg corticosterone/ml.

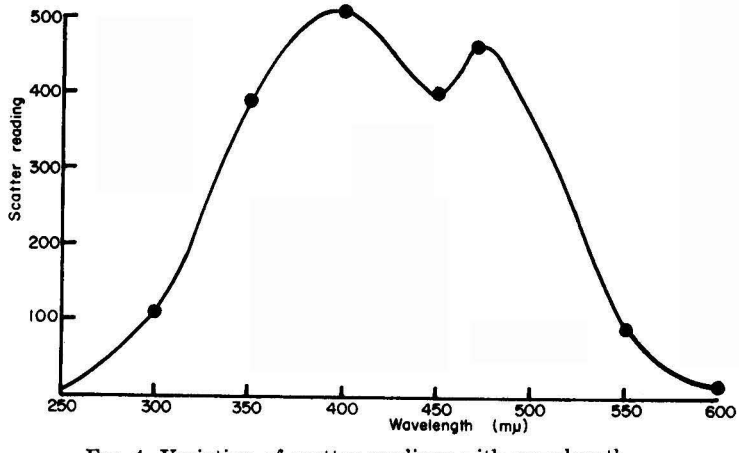

FIG. 4. Variation of scatter readings with wavelength.

wavelength; (*b*) variation in the recording system with wavelength; and (*c*) possible faults in the gratings. An attempt was therefore made to investigate this further.

Experiments to Investigate Instrumental Variation with Wavelength

It was apparent from scatter readings (Fig. 4) that the over-all sensitivity of the instrument varied considerably with wavelength. The lamp output (through the primary grating) over the range of wavelengths generally used in the present work was measured with a selenium cell as described under *Methods*. The galvanometer readings obtained were corrected for variations in the photocell response with wavelength, and the results are plotted in Curve I (Fig. 5). The lamp output thus appeared to vary slightly with wavelength in the range investigated.

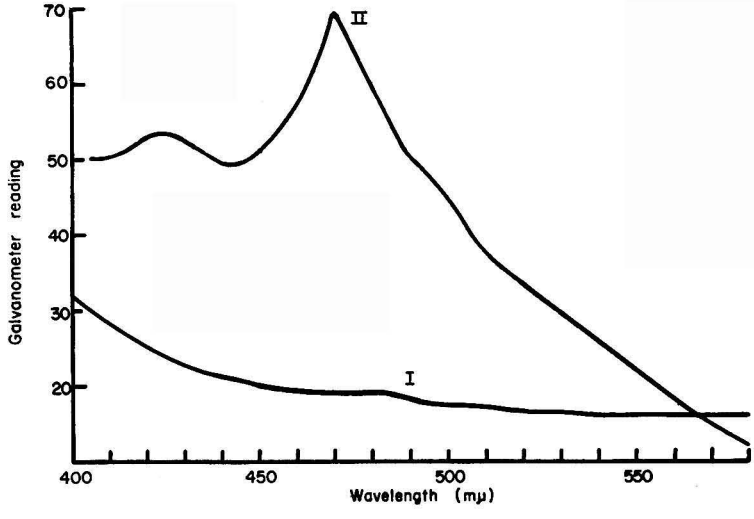

FIG. 5. Variations in the Aminco spectrofluorometer with wavelength.
I. Variation of light intensity reaching the solution, as recorded by a selenium cell.
II. Variation of sensitivity of fluorescence measurements with wavelength, not corrected for fluctuations in the primary light beam.

To examine the over-all instrumental response, a front-silvered plane mirror was placed in the cell compartment at 45° to the incident light beam. To cut down the light reaching the photomultiplier, 1/64-in. slits were placed into the primary and reflected light beams. With the primary wavelength setting at a given value, the secondary grating was adjusted to give a maximum reading (this was necessary because the two gratings were slightly out of alignment), and the readings are plotted in Curve II (Fig. 5). In the range studied, there is a sharp increase in sensitivity with

a peak at 470 mμ, falling off rapidly with increasing wavelength. These experiments appeared to indicate that the instrumental variation was confined to the secondary grating or the recorder system, or both. However, at this time, White, Ho, and Weimer (4) published the results of a similar investigation, and since their data conflicted with those obtained here, the primary system was further examined by an indirect method.

Since the intensity of fluorescence is proportional to the number of absorbed light quanta, and the ratio E_f/E_a—fluorescent light energy to energy absorbed—is practically independent of exciting wavelength from 300 to 480 mμ for fluorescein [cf. (5)], a quantity proportional to this ratio was calculated from the measured absorption and fluorescence activation spectra for this dye and plotted against wavelength. It was found that there was a marked peak for this ratio in the region 460–470 mμ. Similar peaks were found for the fluorescent solutions of steroids in sulfuric acid. These findings are considered in the *Discussion*.

Stability

The instrumental stability was tested by taking sets of 20 replicate readings on standard solutions of cortisol in sulfuric acid. For the three most sensitive settings of the recording equipment, readings between 10 and 40 divisions gave standard deviations of 2% of the mean readings. This is a wider variation than that reported by Goldzieher *et al.* (6), but these workers did not state at which sensitivity they examined the precision.

DISCUSSION

A number of satisfactory fluorometers have been described in the literature (6–10) for measurement of fluorescence in solution, and that of Ayres, Simpson, and Tait (10) can also be used for sections cut from paper chromatograms. More recently, instruments have become available commercially,[1] and for those employing optical filters[2] the best combina-

[1] Filter instruments are manufactured by Farrand Optical Co., New York; Neteler and Hinz, Hamburg, Germany; Kipp and Zonen, Delft, Holland; Fisher Scientific Co., Pittsburgh, Pa.; Locarte Co., London; Hilger and Watts, London; Central Scientific Co., Chicago, Ill.; Photovolt Corp., New York; Klett Manufacturing Co., New York. Spectrofluorometers are available from Farrand Optical Co., New York American Instrument Co., Silver Spring, Maryland; Perkin-Elmer Corp., Norwalk, Conn.; Carl Zeiss, Oberkochen, Württbg., Germany.

[2] Glass filters are available from Corning Glass Works, Corning, N. Y.; Chance-Pilkington Optical Works, St. Asaph, Flintshire, England; and Schott, Mainz, Germany; gelatin filters from Kodak, England and United States; and Ilford Ltd., Ilford England; interference filters from Geraetebau-Anstalt, Balzers, Liechtenstein; Farrand Optical Co., New York; Schott, Mainz, Germany; Bausch and Lomb Optical Co., Rochester, N. Y., and Barr and Stroud, Ltd., Glasgow, Scotland.

tion often has to be found empirically. The interference filters with high transmissions and narrow band widths are perhaps preferable to other types, but their higher order transmissions may result in high "blanks" if scatter from cells or reagent cannot be eliminated.

More versatility is offered by spectrophotofluorometers, which ultilize gratings or prisms in incident and fluorescent light paths, and the establishment of optimum conditions for fluorescence production and measurement of a given compound is greatly facilitated. The Aminco instrument is of this type, and employs a xenon source and two gratings, together with a simple slit system. The resolution of the instrument depends upon the slit system employed, and the effect of variations in the arrangement of slits is shown in Figs. 1 and 2, which record spectra of certain mercury lines. It can be seen that the optimum resolution is not high, as was observed by Goldzieher *et al.* (6), but, on the other hand, the high sensitivity available would almost certainly be decreased by improved resolution. It is also apparent that the use of larger slits introduces considerable spectral distortion, and care should thus be exercised in the choice of slits if information is required on spectral characteristics of compounds under study.

Spectral distortion may also be due to variation in the energy output of the light source with wavelength, and attempts were made to investigate this. Direct measurement of light reaching the cell compartment, using a selenium cell, indicated only a small variation with wavelength (Fig. 5); when measurements were made through the whole optical system using a mirror or scatter (Figs. 4 and 5), a peak was found at about 470 mμ. It would thus appear that the increased sensitivity in this region was due to variations in the secondary grating or the recording system. However, White *et al.* (4) had also investigated the variation of the primary system by using a photomultiplier tube of known characteristics, and observed an increased light intensity in the 470-mμ region. Their method is basically similar to the one employed here with a selenium photocell, and in view of the discrepancy between the results, an attempt was made to investigate further the primary system by an indirect method. This gave the variation of E_f/E_a ratios with wavelength, and since these ratios were obtained from the activation spectrum with no alteration in the secondary or recording systems, the increase observed at about 460–470 mμ must be due to variations in the primary system. This finding would substantiate that reported by White *et al.* (4) for their instrument, but is not in agreement with the observations made directly using a selenium cell in the cell compartment. It is possible that the data supplied by the makers for the spectral response of the cell were inaccurate, and hence the corrections for its variation which were applied to

the experimental data may have been in error. Further work is required to elucidate the cause of the variation of response with wavelength in the Aminco spectrofluorometer.

Results on the variation of incident radiation and sensitivity of the recording unit with wavelength, and on the stability, sensitivity, and resolution of other instruments, such as the Farrand, would be a welcome addition to the literature on fluorometric analysis. Indeed, publication of activation and fluorescence spectra without such data limits their usefulness. It appears that further work on instrumentation is also desirable if fluorometric techniques are to be firmly established in analysis.

SUMMARY

A detailed study has been made of the Aminco-Bowman spectrophotofluorometer with reference to its variation of sensitivity with wavelength, spectral resolution, and stability.

ACKNOWLEDGMENTS

We are grateful to Dr. A. Rosen for calibrating the Aminco instrument, and to Dr. Vezzi of Megatron Limited, who very kindly made available the selenium cell. It is a pleasure to acknowledge the advice and loan of equipment from Dr. S. Rowlands. One of us (H.B.) is indebted to St. Mary's Hospital and Medical School for grants. We should like to thank Professor A. Neuberger for his interest and help.

REFERENCES

1. Braunsberg, H., and James, V. H. T., *J. Endocrinol.* **21,** 333 (1960).
2. Hercules, D. M., *Science* **125,** 1242 (1957).
3. Braunsberg, H., and James, V. H. T., *Anal. Biochem.* **1,** 452 (1960).
4. White, C. E., Ho, M., and Weimer, E. Q., *Anal. Chem.* **32,** 438 (1960).
5. Pringsheim, P., *in* "Fluorescence and Phosphorescence," p. 307. Interscience, New York and London, 1949.
6. Goldzieher, J. W., Bauld, W. S., Engel, L. L., and Givner, M. L., *Can. J. Biochem.* **38,** 233 (1960).
7. McAnally, J. S., *Anal. Chem.* **26,** 1526 (1954).
8. Braunsberg, H., Osborn, S. B., and Stern, M. I., *J. Endocrinol.* **11,** 177 (1954).
9. Freeman, D. C., PhD. Thesis, University of Maryland, June, 1955.
10. Ayres, P. J., Simpson, S. A., and Tait, J. F., *Biochem. J.* **65,** 647 (1957).

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The Fluorometric Determination of Adrenocortical Steroids

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INTRODUCTION

The present investigation was carried out with the aim of determining cortisol[1] and corticosterone in human peripheral blood, and an extensive review on the analytical problems involved is being published elsewhere (1). The fluorescence of these steroids in sulfuric acid had already formed the basis of an analytical method for this purpose (2), and the availability of a sensitive spectrophotofluorometer prompted a more detailed study. While this work was in progress, Peterson (3) reported data on the conditions of fluorescence development and measurement for corticosterone, and his results have been confirmed and extended.

METHODS

The Aminco-Bowman spectrophotofluorometer (American Instrument Co.) with a 1P21 photomultiplier (Radio Corporation of America) was used for all measurements of activation and fluorescence spectra and fluorometric determinations. The quartz cells (outside dimensions 1.2 cm square $\times$ 4.8 cm high; internal volume 4.8 ml), supplied with the instrument, are available in the United Kingdom from Thermal Syndicate Ltd., Wallsend, Northumberland.

Optical Filters: Ilford 109 and 110, 1½ in. (3.7 cm) square were suitable for reducing scattered incident light.

Small thin glass test tubes (7.5 cm long, 0.8 cm internal diameter) were used for developing fluorescence. For accurate quantitative work, at raised temperatures, it was important to control temperatures to better than $\pm 1°C$. A large thermostatic bath was used for this purpose.

All glassware was cleaned initially with chromic acid, hot tap water, and glass-distilled water, and was segregated for use for this work only. It was then cleaned with hot and glass-distilled water only [cf. (4)].

[1]The following trivial names are used: cortisol (11β,17α,21-trihydroxypregn-4-en-3,20-dione); cortisone (17α,21-dihydroxypregn-4-en-3,11,20-trione); corticosterone (11β,21-dihydroxypregn-4-en-3,20-dione); 11β-hydroxyandrostenedione (11β-hydroxyandrost-4-en-3,17-dione); aldosterone (11β,21-dihydroxypregn-4-en-18-al-3,20-dione).

Absorption spectra were measured in a Unicam SP 500 spectrophotometer, using quartz cells with 1-cm light path.

Two brands of sulfuric acid (May and Baker, grade R, and British Drug Houses, AnalaR) gave similar results. The May and Baker reagent was used routinely.

Ethanol was refluxed for 2 hr with 5 gm/l dinitrophenylhydrazine and 10 ml/l of conc. hydrochloric acid and distilled twice through a column.

Methanol was redistilled through a column.

Cortisol and corticosterone were purified by partition chromatography (5).

Steroid standards were prepared by dissolving 0.5–1.0 mg (weighed on an accurate microbalance) in 10 ml redistilled methanol. These were diluted with redistilled methanol or purified ethanol to give solutions of the required concentrations. The standards, used for cortisol and corticosterone determinations in plasma (5), contained 0.2 μg corticosterone or 0.75 μg cortisol/ml and were stable at 4°C for at least 2 months. Aliquots of 0.1 to 0.5 ml of these were used as standard for calibration curves. Steroid standards were taken to dryness at <50°C under nitrogen jets before addition of acid. The aqueous or ethanolic sulfuric acid reagents were prepared (v + v) by slowly adding ice-cold conc. sulfuric acid to ice-cold water or ethanol and cooling the mixture in ice. There was a tendency for reagent blanks to be slightly higher if the mixtures were allowed to become too warm. For development of fluorescence, 1.5 ml reagent was added to the dry steroid from a 10-ml buret.

RESULTS

Slit Systems and Sensitivity

The effect of slit widths on the sensitivity and resolution of the Aminco-Bowman instrument has been discussed in another paper (6). For the present investigations, 1/32-in. (0.795 mm) slits were used in the incident and fluorescent beams with 1/16-in. (1.59 mm) slits on each side to reduce scatter. A 3/16-in. (4.77 mm) slit, placed in front of the photomultiplier, allowed sufficient sensitivity without distortion of fluorescence spectra (6). The most sensitive ranges of the instrument were generally used, and all readings were corrected to the most sensitive (0.001×) scale. By adjusting the sensitivity to the appropriate range, all readings taken were below 40 divisions on the meter.

Wavelength Calibrations

Investigation of the wavelength disks of the instrument revealed some errors in the calibration (6). However, over the range of fluorescence

spectra reported in the present paper, errors were no greater than 2 mμ. The activation spectra shown require a correction of +5 mμ in the entire range studied.

Excitation and Fluorescence Spectra of Steroids in Sulfuric Acid

Reagent Blanks. Concentrated sulfuric acid alone or diluted with ethanol gave rise to considerable blanks which could not readily be decreased by filtration through sintered glass. Goldzieher, Bodenchuk, and Nolan (7) advocated distillation of the acid to reduce blanks; however, it was found that these could be reduced by means of secondary filters which transmitted most of the fluorescent light. Table 1 shows the results of an

TABLE 1
SCATTER FROM CONCENTRATED SULFURIC ACID WITH AND WITHOUT PHOTOMULTIPLIER FILTERS. ACTIVATED AT 470 mμ

Read at (mμ)	No filter	Ilford 109	Ilford 110
450	0	0	0
460	40.5	0	0
470	130.5	0	0
475	150	0	0
480	162	0	0
485	120	0	0

experiment without and with filters placed in front of the photomultiplier. Considerable scatter of the primary beam was recorded when no filter was used, even 10–15 mμ from the exciting wavelength. Both filters absorbed this scatter sufficiently to give zero readings on the same (most sensitive) setting of the instrument. Table 2 shows readings in the region of the fluorescence peak for a solution of cortisol (0.48 μg/ml) in con-

TABLE 2
EFFECT OF ILFORD FILTERS 109 AND 110 ON FLUORESCENCE READINGS FOR CORTISOL (0.48 μG/ML) IN CONCENTRATED SULFURIC ACID. ACTIVATED AT 470 mμ

Read at (mμ)	No filter	Ilford 109	Ilford 110
510	84	27	3
515	87	42	7.5
520	88.5	54	18
525	88.5	60	27
530	88.5	72	42
535	87	73.5	51
540	82.5	72	58.5

centrated sulfuric acid. The Ilford filter 109 transmits about 85% of the fluorescence at the peak wavelength.

In the present investigation the activation and fluorescence peaks were 50–60 mμ apart and it was not considered necessary to eliminate scatter of the incident light with filters which might distort the emission spectra. (This is not true for all steroids; for example, estradiol-17β has an activation peak much closer to its fluorescence peak.)

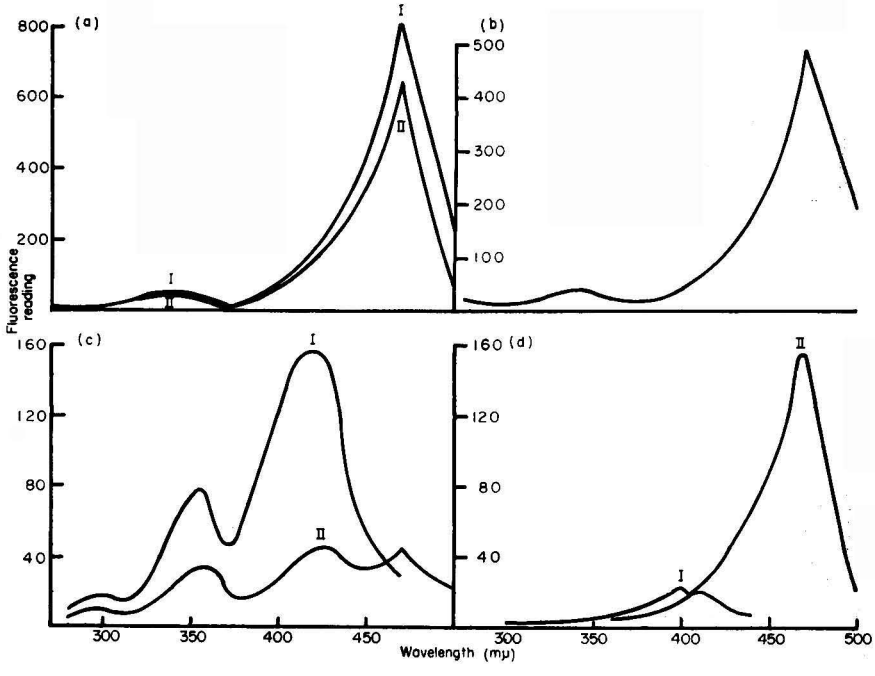

FIG. 1. Activation spectra of (*a*) corticosterone, 0.5 μg/ml in 60% (v + v) ethanolic sulfuric acid (I) and 70% (v + v) ethanolic sulfuric acid (II), fluorescence read at 530 mμ; (*b*) cortisol, 0.425 μg/ml in 70% (v + v) ethanolic sulfuric acid, fluorescence read at 530 mμ; (*c*) cortisone, 7 μg/ml in concentrated sulfuric acid, fluorescence read at 460 mμ (I) and 550 mμ (II); (*d*) 11β-hydroxyandrostenedione, 2.4 μg/ml in concentrated sulfuric acid, fluorescence read at 460 mμ (I) and 550 mμ (II).

Corticosterone. Peterson (3) measured the activation and fluorescence spectra of corticosterone in ethanolic sulfuric acid using a similar instrument.

Figures 1(*a*) and 2(*a*) show activation and fluorescence spectra respectively for corticosterone. The activation spectra in 60% and 70% ethanolic sulfuric acid show good agreement with Peterson's results. While the concentration of the acid affected the intensity of fluorescence, it had

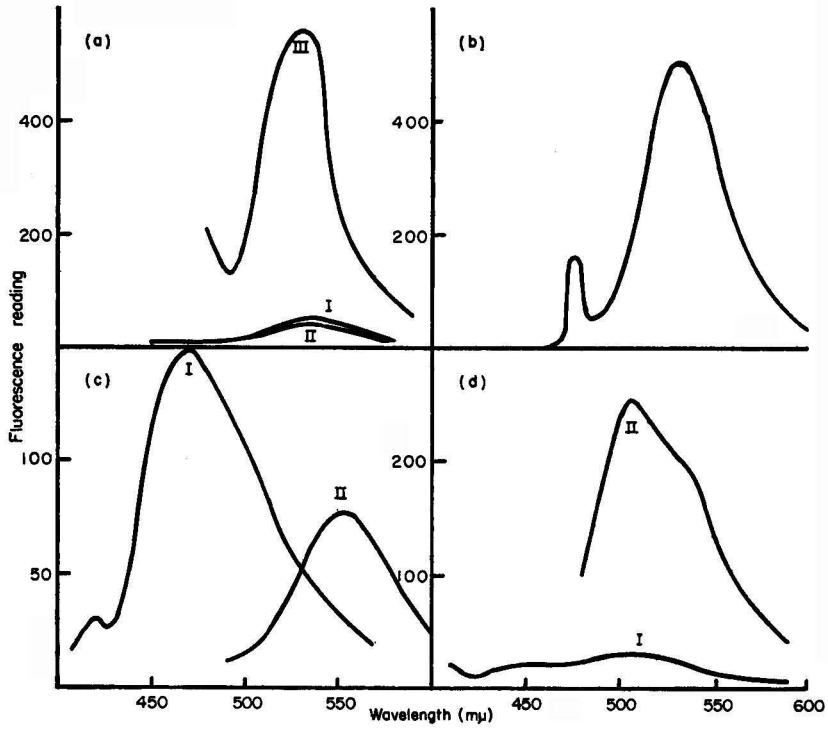

FIG. 2. Fluorescence spectra of (*a*) corticosterone, 0.5 μg/ml in 60% (v + v) ethanolic sulfuric acid, activated at 335 mμ (I) and in 70% (v + v) ethanolic sulfuric acid activated at 335 mμ (II) and 470 mμ (III); (*b*) cortisol, 0.425 μg/ml in 70% (v + v) ethanolic sulfuric acid, activated at 470 mμ; (*c*) cortisone, 7 μg/ml in concentrated sulfuric acid, activated at 415 mμ (I) and 470 mμ (II); (*d*) 11β-hydroxyandrostenedione, 2.4 μg/ml in concentrated sulfuric acid, activated at 400 mμ (I) and 470 mμ (II).

little effect on the activation or fluorescence spectra, and this was also found to be the case for the other steroids investigated. In addition to the intense activation peak at 470 mμ, corticosterone had a weak activation peak between 330 and 350 mμ. Activation at 335 mμ produces a weak but measurable fluorescence with a peak in the same position as that produced by optimal activation at 470 mμ. In the latter case, however, there is some distortion due to scatter up to 495 mμ. Figure 3(*a*) shows the absorption spectrum of corticosterone in 60% ethanolic sulfuric acid. The short wavelength absorption peak at about 285 mμ does not appear to produce any measurable fluorescence, but the other intense peak at 475 mμ corresponds well with the fluorescence activation peak in Fig. 5. The slight fluorescence activation peak at 330 mμ may correspond to the

slight shoulder at 340 mμ in the absorption spectrum. On the other hand, the shoulder at 390–400 mμ had no corresponding detectable peak in the fluorescence activation spectrum.

Cortisol. Figure 1(*b*) shows the activation spectrum for cortisol in 70% ethanolic sulfuric acid. It is very similar to that for corticosterone, but the latter gives a considerably stronger fluorescence under the same conditions

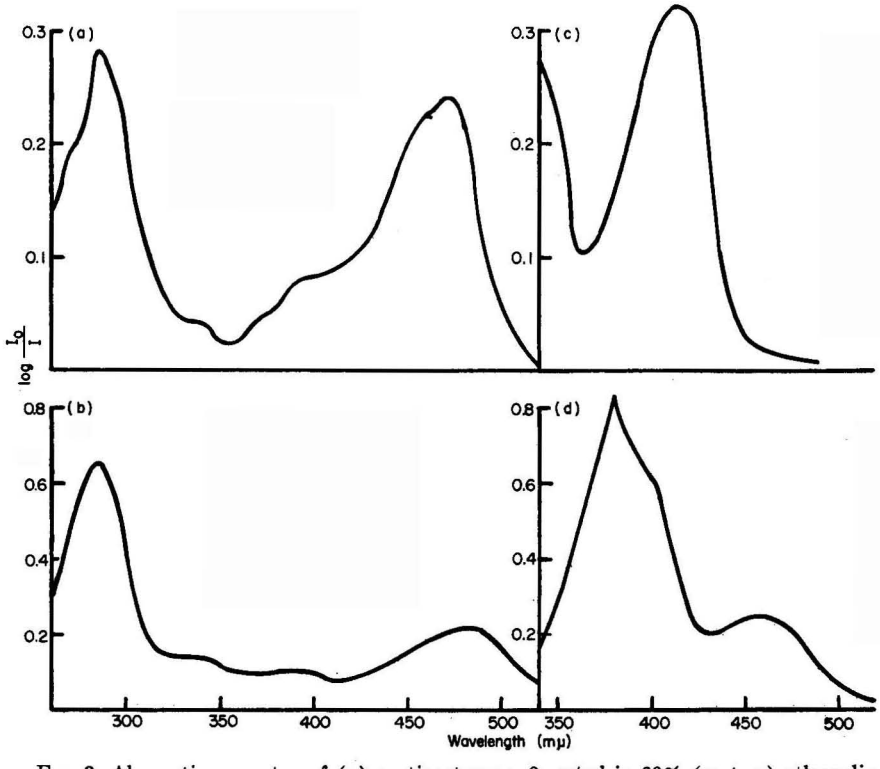

FIG. 3. Absorption spectra of (*a*) corticosterone, 9 μg/ml in 60% (v + v) ethanolic sulfuric acid, 1 hr at 22°C; (*b*) cortisol, 9.6 μg/ml in 70% (v + v) ethanolic sulfuric acid, 1 hr at 22°C; (*c*) cortisone, 10 μg/ml in concentrated sulfuric acid, 20 min at 22°C; (*d*) 11β-hydroxyandrostenedione, 9.5 μg/ml in concentrated sulfuric acid, 1 hr at 45°C.

[cf. Tamm and Starlinger (8)]. Comparison of the two absorption spectra, Figs. 3(*a*) and (*b*), shows that at 475 mμ corticosterone has an extinction 1.2 times that of cortisol (on a weight for weight basis). However, the fluorescence produced by corticosterone is two to three times that produced by cortisol. It appears that corticosterone produced solutions of considerably greater fluorescence yield than cortisol. The intense absorption peak at 290 mμ, Fig. 3(*b*), again produces no detectable fluorescence

at 530 mμ, and there is a shoulder at about 340 mμ which may correspond to the slight fluorescence activation peak near this wavelength. Figure 2(*b*) shows the fluorescence spectrum for cortisol in 70% ethanolic sulfuric acid activated at 470 mμ which closely resembles that for corticosterone. A scatter peak is seen near 470 mμ.

Cortisone. Figure 1(*c*) shows activation spectra for cortisone measured at two different emission wavelengths. This compound shows considerable differences in its behavior in sulfuric acid from cortisol, the activation spectrum being more complex and the fluorescence yield very low. Figure 2(*c*) shows the emission spectra for cortisone with activation at 415 and 470 mμ, respectively. The difference in emissions would suggest the existence of two different processes involved in the production of fluorescence, depending on the light energy absorbed. Inspection of the absorption spectrum of cortisone in concentrated sulfuric acid, Fig. 3(*c*), shows that each of the absorption peaks corresponds to an activation peak in Fig. 1 (*c*). However, absorption at 475 mμ is very weak, and for this wavelength the fluorescence efficiency appears to be greater than for light absorbed at 415 mμ. Nevertheless, activation at 415 mμ and measurement of emission at 460 mμ produce greater sensitivity for fluorometric determination.

11β-Hydroxyandrostenedione. Activation spectra for this compound in concentrated sulfuric acid, recorded for emissions at 460 and 550 mμ, respectively, are shown in Fig. 1(*d*). The slight activation peak at 400 mμ was not detected when emission was recorded at 550 mμ. The activation peak at 470 mμ resembles those for corticosterone and cortisol. Figure 2(*d*) shows the emission spectra for 11β-hydroxyandrostenedione in concentrated sulfuric acid produced by activation at 400 and 470 mμ, respectively. The emission peak is at a slightly shorter wavelength (505 mμ) than are those for corticosterone and cortisol. Inspection of the absorption spectrum for 11β-hydroxyandrostenedione in concentrated sulfuric acid, Fig. 3(*d*), shows a strong complex peak at 380 mμ which is probably responsible for the weak activation peak in this region, Fig. 1 (*d*). The absorption at 460 mμ, on the other hand, gives rise to high fluorescence efficiency, as revealed by a strong activation peak.

Conditions of Fluorescence Development

Corticosterone. As shown by Peterson (3), the rate of fluorescence development for corticosterone in ethanolic sulfuric acid varies with the acid concentration. Figure 4 shows the effect of time on the fluorescence of solutions in different dilutions of the acid with ethanol. It appears from these results that several processes are taking place in these solutions:

concentration of the acid, and (*b*) destruction of the fluorescent species, the rate of which also increases with increasing concentration of the acid. In the 70 and 80% acids, destruction of the fluorescence presumably is so

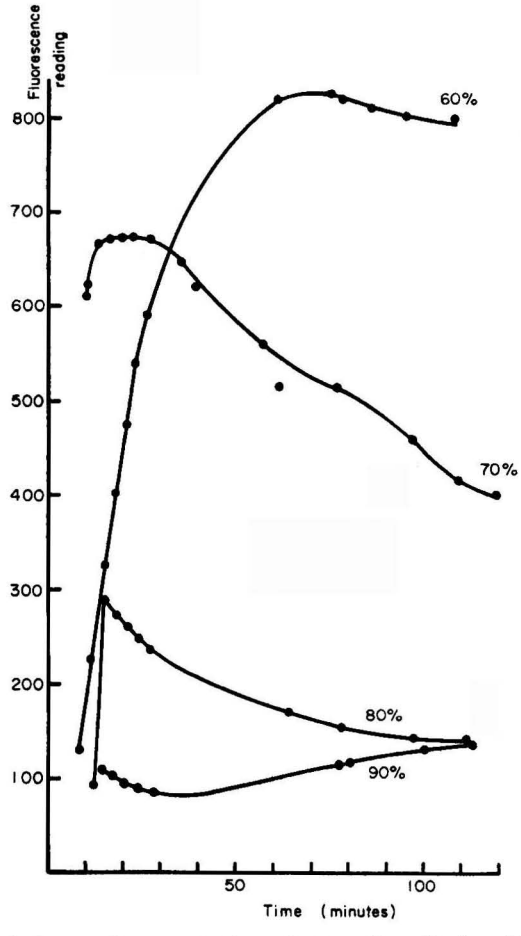

FIG. 4. Effect of time and concentration of ethanolic sulfuric acid on the development of fluorescence of corticosterone. 0.5 μg/ml. Activation 470 mμ, read at 530 mμ. 22°C.

Readings for reagent blanks at 170 ±3 min were: 60% acid: 15.5; 70%: 14; 80% 12.5; 90%: 11.

rapid that the maximum reading obtained in 60% acid can never be reached. The fluorescence in 90% acid is already decreasing at 15 min, but there is a further slow increase which may be due to the formation of another compound. Maximum fluorescence is thus obtained in 60% acid

after 1 hr at 22°C. However, the process can be speeded up by warming, and 20 min at 45 ±1.5°C has been found to give satisfactory results.

Figure 5 shows the effect of time and concentration of aqueous sulfuric acid on the fluorescence produced by corticosterone. The results resemble those for ethanolic solutions (Fig. 4), but both the production and de-

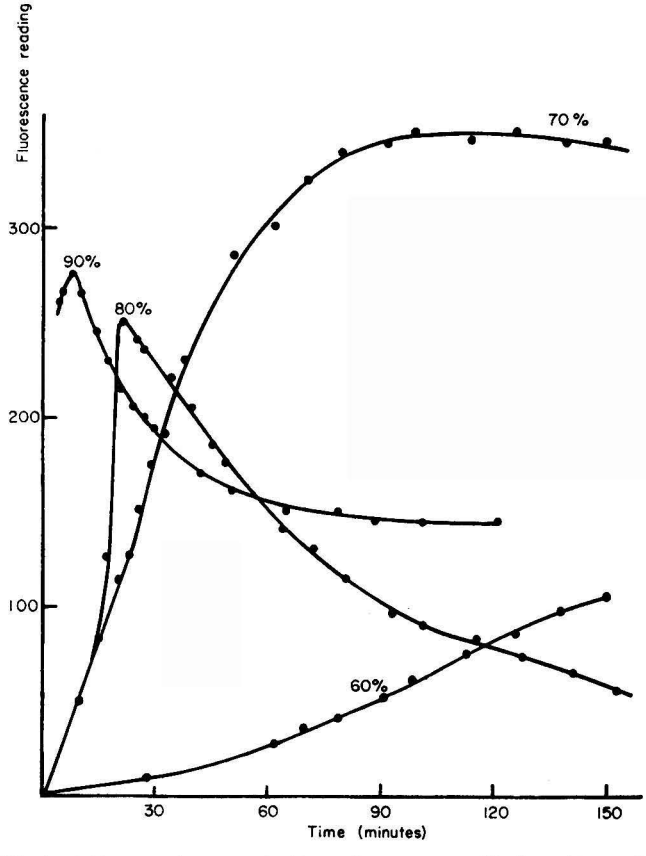

FIG. 5. Effect of time and concentration of aqueous sulfuric acid on the development of fluorescence of corticosterone. 1.58 μg/ml. Activation 470 mμ, read at 530 mμ. 20°C.

struction of fluorescence are slower and less fluorescence is produced. The solution in 70% aqueous acid was diluted with water after 1½ hr to give a 60% solution, and the fluorescence was measured after mixing. The reduction in fluorescence corresponded to the dilution factor, indicating that water does not quench the fluorescence developed.

Cortisol. Figure 6 shows the effect of time and ethanolic acid concen-

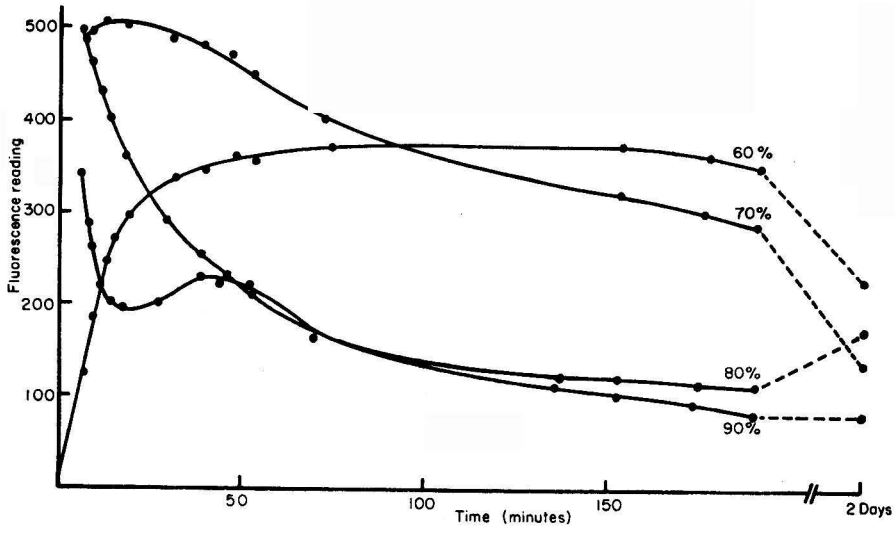

FIG. 6. Effect of time and concentration of ethanolic sulfuric acid on the development of fluorescence of cortisol. 0.475 μg/ml. Activation 470 mμ, read at 530 mμ. 22°C. Readings for reagent blanks are shown in the tabulation.

	Concentration of acid (v + v)			
Time	60%	70%	80%	90%
0	8	8.5	8	7.5
190 min	10	12	15	13.5
2 days			28.5	27

tration on the fluorescence produced by cortisol. The 70% acid gave more fluorescence during the first 1½ hr than does the 60% acid. However, the fluorescence in 70% acid is not sufficiently stable to permit accurate readings, and the 60% acid was preferred for quantitative determination in spite of the lower sensitivity. The destructive process in 80 and 90% acid seems to be more rapid than that for corticosterone, and this may account partly for the lower fluorescence produced by cortisol. The 90% acid, as with corticosterone, produced a second increase of fluorescence, possibly due to formation of another compound. In 60% acid a maximum stable fluorescence is produced after 80 min at 22°C. The process can be speeded up by warming, and 30 min at 45°C has been found to give consistent results.

Cortisone. No conditions have yet been found under which cortisone produces as much fluorescence as corticosterone and cortisol. Figure 7 shows the effect of time and ethanolic acid concentration on fluorescence

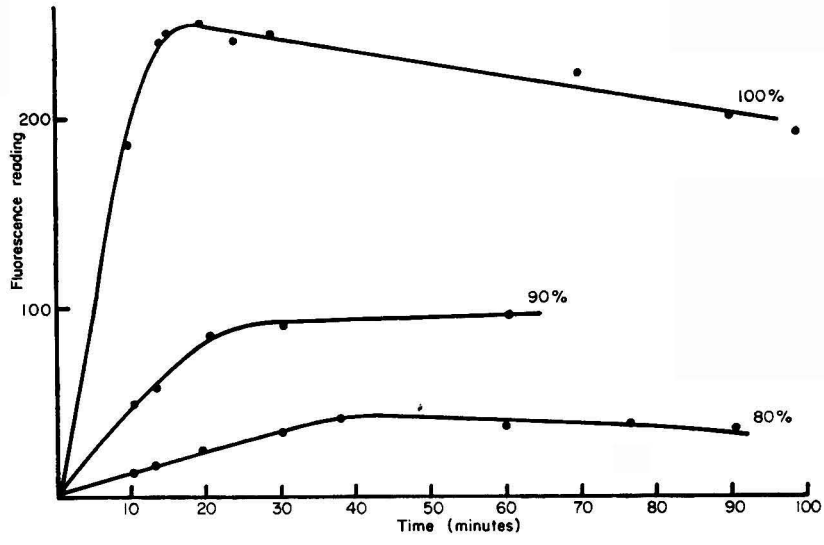

FIG. 7. Effect of time and concentration of ethanolic sulfuric acid on the development of fluorescence of cortisone. 6.67 μg/ml. Activation 415 mμ, read at 460 mμ. 20°C.

development. Fluorescence is produced rapidly in concentrated acid, and the destructive process is much slower than that for corticosterone or cortisol. Very little fluorescence developed in the weaker acid solutions.

11β-Hydroxyandrostenedione. Figure 8 shows the effect of time and ethanolic acid concentration on the production of fluorescence by this compound. Here both the fluorescence-producing and the destructive processes appear to be much slower than those for corticosterone and cortisol, and the more concentrated the acid the more fluorescence is produced. Thus in concentrated sulfuric acid the rate of the destructive process does not exceed that of the fluorescence-producing process even at 2 hr, in contrast to cortisol and corticosterone with which fluorescence decreases rapidly at this time even in 70% acid. At 45°C maximum fluorescence was reached in 1 hr.

Quantitative Fluorometric Determination

Reagent Blanks. With excitation at 470 mμ and recording at 530 mμ the concentrated sulfuric acid and ethanol–sulfuric acid reagents give small but detectable blank readings, due partly to scatter and partly to actual fluorescence in the solutions. Peterson (3) claimed that his 65% ethanolic sulfuric acid reagent was stable for many months, but it was found that blank readings increased with time even at 4°C, and the use of

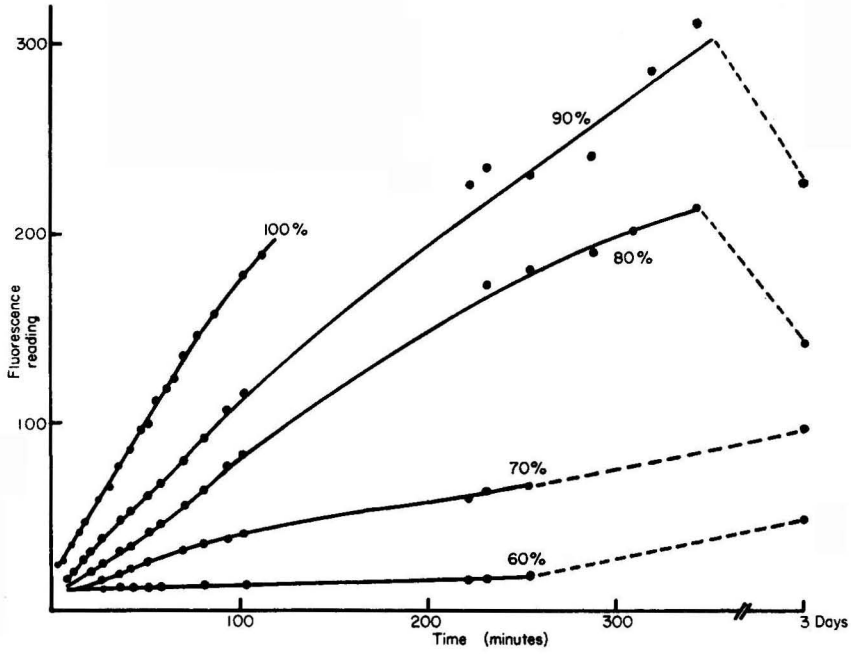

FIG. 8. Effect of time and concentration of ethanolic sulfuric acid on the development of fluorescence of 11β-hydroxyandrostenedione. 0.95 μg/ml. Activation 470 mμ, read at 510 mμ. 22°C.

fresh reagent is preferable. Blank readings varied between 3 and 9 divisions on the most sensitive scale.

Precision of Standards. The precision of the fluorometric determination on standard amounts of steroids was examined by estimating the standard deviations from the results of duplicate experiments (9). Table 3 shows a summary of results. The estimate of the standard deviation, s, is given by $\sqrt{\Sigma d^2/2N}$, where d is the difference between a pair of duplicates and N

TABLE 3

ESTIMATES OF THE STANDARD DEVIATIONS (s) OF FLUOROMETER READINGS FOR STEROID STANDARDS FROM THEIR MEANS (BASED ON N DUPLICATE DETERMINATIONS)

	Corticosterone			Cortisol		
	0.16 μg	0.32 μg	0.48 μg	0.12 μg	0.36 μg	0.60 μg
N	20	14	15	20	20	20
Mean of all readings	109	224	331	30	82.5	133
Range	80–138	180–290	267–426	20–40	56–126	93–189
s	5.7	9	13.5	1.5	3.8	4.8

the number of duplicates, obtained by running standard curves in duplicate over a period of several weeks. The slope of these standard curves varies from time to time; this accounts for the large range of readings at

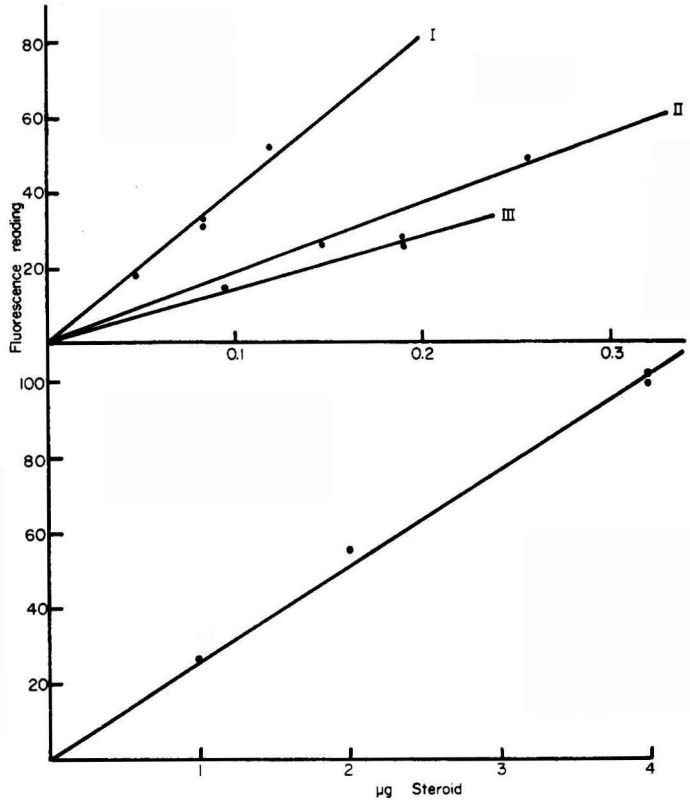

FIG. 9. Standard calibration curves. Abscissae: μg steroid/1.5 ml. Ordinates: Fluorometer readings.

I. Corticosterone in 60% (v + v) ethanolic sulfuric acid. 20 min at 45°C. Activation 470 mμ, read at 530 mμ.

II. Cortisol in 60% (v + v) ethanolic sulfuric acid. 30 min at 45°C. Activation 470 mμ, read at 530 mμ.

III. 11β-Hydroxyandrostenedione in concentrated sulfuric acid. 1 hr at 45°C. Activation 470 mμ, read at 510 mμ.

IV. Cortisone in concentrated sulfuric acid. 20 min at 20°C. Activation 415 mμ, read at 460 mμ.

each steroid level. A similar variation in slope was noted in earlier work on estrogens (10) and appears to be inherent in fluorometric determinations. The estimate of the standard deviation, s, increases with increasing

quantity of steroid, bearing a fairly constant relation to the mean fluorescence reading. This would indicate that the variation is not due to pipet and volume errors, the proportion of which would tend to decrease with increasing amount. Furthermore, the errors indicated by the values of s are larger than those obtained in this laboratory with, for example, absorptiometric determinations. They may be due to instrumental factors, effects resulting from the use of a fluorometric method (e.g., quenching by impurities present in spite of careful cleaning of glassware) or to difficulties encountered in the use of extremely small quantities (e.g., escape of traces during removal of solvents, adsorption on glass surfaces). It must be concluded that the precision of an analysis based on this fluorometric method will be somewhat lower than one based on other methods using larger quantities of steroids.

Sensitivity. Figure 9 shows calibration curves for corticosterone, cortisol, 11β-hydroxyandrostenedione, and cortisone. For the first two steroids, the sensitivity was sufficiently high to allow their determination in 5–10 ml plasma. Amounts of 0.025 μg corticosterone and 0.1 μg cortisol can be determined with an accuracy of $\pm 10\%$. For 11β-hydroxyandrostenedione, 0.15 μg would be required and for cortisone 0.8 μg.

DISCUSSION

The fluorescence of C_{21} steroids in sulfuric acid has been studied for quantitative use by several workers (3, 7, 8, 11–13), and Bernstein and Lenhard (14) have published data on the absorption spectra in concentrated sulfuric acid. Of the steroids studied, only corticosterone, cortisol, their 11-epimers, and 4-pregnen-11β,17α,20β,21-tetrol-3-one fluoresce strongly (7, 11, 15). Tamm and Starlinger (8) found that under their conditions [presumably those of Sweat (11)], 5,16-pregnadien-3β-ol-20-one gave appreciable fluorescence. We have found that 11β-hydroxyandrostenedione also produces fluorescence of intensity comparable to that given by cortisol, but were unable to obtain fluorescence with aldosterone. The C-18 aldehydic group thus appears to prevent formation or facilitate destruction of a fluorescent molecule. The strong fluorescence produced by estrogens in sulfuric acid (16, 17) is well known, and numerous other compounds fluoresce under similar conditions (18–20). Fluorescence produced by extracts from washed chromatography paper presents some difficulties when fluorometry is applied after paper chromatographic purification.

In spite of attempts to elucidate the mechanism of these reactions in strongly acid media (21, 22), any explanations must at present be considered highly speculative. The best conditions for developing fluorescence must therefore be arrived at empirically, and no general agreement has

so far been reached. Other workers (3, 7, 11, 13) have used conditions differing from those recommended in the present paper.

The rates of the fluorescence-producing and destroying processes both increase with increasing acid concentration. Two possible sequences may be involved in these reactions:

$$A \rightleftharpoons B \rightleftharpoons C \quad \text{or} \quad A \rightleftharpoons B \rightleftharpoons C \rightarrow \text{products}$$

where B is strongly fluorescent, C is either weakly or nonfluorescent and produces further nonfluorescent compounds, and some of these reactions may be catalyzed by acid. The possibility of more than one fluorescent compound being formed cannot be excluded (22).

The effect of ethanol on the reaction is difficult to explain. In the case of 11β-hydroxyandrostenedione, ethanol never enhanced the fluorescence and the concentrated acid produced optimal results. Possibly, the fluorescence destruction process was so slow, even in concentrated acid, as never to exceed the fluorescence-producing process, and dilution of the acid merely slowed down fluorescence development. In the cases of cortisol and corticosterone, however, the destructive process is faster and can be slowed down by dilution with ethanol. Aqueous acid produced much less fluorescence than ethanolic acid. This may be due to (*a*) a solubility effect, the ethanol facilitating solution of the steroid residue, and thereby affecting the fluorescence-developing process; (*b*) an independent retarding influence of ethanol on the fluorescence destruction. The solubility effect (*a*) is not consistent with the results on 11β-hydroxyandrostenedione for which the fluorescence development is sufficiently slow to show that its rate is directly proportional to the acid concentration. Also, in the case of cortisol the 80% acid produced more fluorescence than the 70% acid during the first 6 min, but subsequent destruction was rapid, leading to lower readings. There are two further possibilities which may explain the difference between aqueous and ethanolic acid. The ethanol may take some part in the reaction in addition to diluting the acid, or water may quench the fluorescence more than ethanol, although this could not be demonstrated experimentally.

The fluorescence destruction process is more rapid for corticosterone than for cortisol. Thus the fluorescence in 70% acid decreases more rapidly with the former than with the latter compound, for which the 70% acid produces most fluorescence during the first 1½ hr.

Sweat (11) found his method to be reproducible at concentrations down to 0.05 μg/ml, while Peterson (3) claims a sensitivity of 0.01 μg/ml of corticosterone under his conditions. While the sensitivities of the fluorometric method may thus be adequate for the determination of corticosterone and cortisol in human peripheral blood, amounts of 11β-hydroxy-

androstenedione and cortisone detectable by this technique are not likely to be present. Unfortunately, attempts to render the method more sensitive for these substances were not successful. The preparation of fluorescent compounds by condensing certain steroids with salicyloyl hydrazide (23), although promising, produced no useful results in our hands.

In spite of exact standardization of methods for the development of fluorescence, the slope of calibration curves can vary considerably from one experiment to another. Furthermore, these methods so far seem to be less precise than those using measurement of light absorption, but this may be due to the minute quantities involved. For accurate analysis at least three duplicate standards are included in each determination.

No distinction is possible between the activation and fluorescence peaks of cortisol and corticosterone. Whether this is due to their identity or to insufficient resolution of the instrument remains to be ascertained. Conditions for the determination of these compounds in mixtures have been suggested (13), but preliminary separation and purification would seem to be preferable.

The only other method of comparable sensitivity is that of isotopic "double labeling" (24). While such a technique is capable of circumventing many of the problems encountered in the determination of steroids in biological material, its application to human peripheral blood requires development and investigation. The fluorometric method has been applied to fractions from peripheral plasma, and the results of this work are reported elsewhere (5).

SUMMARY

Data are presented on the activation, fluorescence, and absorption spectra and conditions for fluorescence development for corticosterone, cortisol, cortisone, and 11β-hydroxyandrostenedione, and on the sensitivity and precision attained in determinations. Some aspects of these reactions and problems of instrumentation are discussed.

ACKNOWLEDGMENTS

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REFERENCES

1. Braunsberg, H., and James, V. H. T., *J. Clin. Endocrinol.* (submitted for publication).
2. Sweat, M. L., Abbott, W. E., Jefferies, W. M., and Bliss, E. L., *Federation Proc.* **12**, 141 (1953).

3. PETERSON, R. E., *J. Biol. Chem.* **225,** 25 (1957).
4. LEWBART, M. L., AND MATTOX, V. R., *Nature* **183,** 820 (1959).
5. BRAUNSBERG, H., AND JAMES, V. H. T., *J. Endocrinol.* **21,** 333 (1960).
6. BRAUNSBERG, H., AND JAMES, V. H. T., *Anal. Biochem.* **1,** 443 (1960).
7. GOLDZIEHER, J. W., BODENCHUK, J. M., AND NOLAN, P., *Anal. Chem.* **26,** 853 (1954).
8. TAMM, J., AND STARLINGER, H., *4th Symposium Deut. Ges. f. Endokrinol.*, p. 309. Springer, Berlin, 1957.
9. SNEDECOR, G. W., *Biometrics* **8,** 85 (1952).
10. BRAUNSBERG, H., OSBORN, S. B., AND STERN, M. I., *J. Endocrinol.* **11,** 177 (1954).
11. SWEAT, M. L., *Anal. Chem.* **26,** 773 (1954).
12. BOMPIANI, A., *Boll. soc. ital. biol. sper.* **30,** 868 (1954).
13. GOLDZIEHER, J. W., AND BESCH, P. K., *Anal. Chem.* **30,** 962 (1958).
14. BERNSTEIN, S., AND LENHARD, R. H., *J. Org. Chem.* **38,** 1146 (1953).
15. REICHSTEIN, T., AND SHOPPEE, C. W., *Vitamins and Hormones* **1,** 345 (1943).
16. BATES, R. W., AND COHEN, H., *Federation Proc.* **6,** 236 (1947).
17. JAILER, J. W., *Endocrinology* **41,** 198 (1947).
18. BANDOW, F., *Biochem. Z.* **298,** 81 (1938).
19. BANDOW, F., *Biochem. Z.* **299,** 199 (1938).
20. BANDOW, F., *Biochem. Z.* **301,** 37 (1939).
21. LINFORD, J. H., *Can. J. Biochem.* **35,** 299 (1957).
22. KALANT, H., *Biochem. J.* **69,** 79 (1958).
23. CAMBER, B., *Clin. Chim. Acta* **2,** 188 (1957).
24. AVIVI, P., SIMPSON, S. A., TAIT, J. F., AND WHITEHEAD, J. K., *Proc. 2nd Radioisotope Conf., Oxford, 1954,* Vol. 1, p. 313.

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Spectrophotometric Determination of Germanium in Biological Materials

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INTRODUCTION

There has been a renewal of interest in the physiological significance and properties of germanium because of its discovery in fission products (1), its rapidly growing industrial use, and the possibility of medical application of its radioisotopes. The purpose of the present investigation was the development of a simple, accurate, and sensitive method which could be employed in biochemical and pharmacological studies of germanium compounds for determining microgram quantities in tissues, tissue fluids, urine, and feces. None of the previously published ones, which have been adequately reviewed elsewhere (2) and involve gravimetric, volumetric, colorimetric, polarographic, or spectrographic procedures, has been adapted specifically for the assay of germanium in biological materials.

The present method depends on the reaction of germanium with molybdate (3) in acid solution to form germanomolybdic acid ($H_2GeO_3 \cdot 12MoO_3 \cdot 3H_2O$) and the subsequent reduction of this heteropoly acid by the ferrous ion (4) to a soluble blue complex. The effect of time, pH, reagent concentrations, and possible interfering substances on the intensity of the blue color was studied spectrophotometrically. Based on the data obtained, an analytical procedure was developed which permits the detection of as little as 2 μg in biological materials and is not interfered with by other substances normally present. The procedure was applied to studies of the normal occurrence of germanium in the rat and its distribution among various organs after parenteral administration.

[1] The opinions or assertions contained herein are the private ones of the author and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

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MATERIALS AND METHODS

Digestion of Material

The sample of biological material is placed into a 125-ml Erlenmeyer flask, and to it are added 15 ml of conc. HNO_3, 10 ml of conc. H_2SO_4, and three glass beads. The contents of the flask are heated and digested on a hot plate under a hood. Oxidizing conditions are maintained in the flask at all times during the digestion in order to prevent charring; if the solution darkens, the flask is removed and cooled and 5-ml portions of conc. HNO_3 are added until digestion is complete and a light-colored solution results. The heating is then continued until the water and excess HNO_3 are boiled off and fumes of SO_3 are evolved. The final solution is either colorless or light-straw colored. In the present studies, samples as large as 5 gm were conveniently digested for analysis.

The contents (approx. 10 ml) of the Erlenmeyer flask are transferred to a distillation flask (*C* of Fig. 1) through a funnel which extends almost to the bottom, and two 4-ml water rinses of the Erlenmeyer are added. The diluted digest is then heated with a Bunsen burner until white fumes of SO_3 appear. This is done in order to remove the water as well as to expel any last traces of nitrogen oxides, which seriously interfere with the color development to be described. The contents of the flask are cooled to room temperature, and the side arm is flushed with water to remove any traces of acid in it by inserting a curved pipet through the neck of the flask and holding the flask at a 45° angle during the delivery.

Apparatus and Distillation of $GeCl_4$

The all-glass apparatus (Fig. 1), a modification of one proposed by Scherrer (5), is used to distill the $GeCl_4$ (b.p. 86°C) formed upon the addition of HCl. *A* is a 35-ml bulb for holding the acid; *B*, a delivery tube with a side arm to which a stream of air is connected in order to force all the acid into the contents of the flask after the delivery-tube stopcock is closed, as well as to drive over the volatilized $GeCl_4$. *C* is a 125-ml distilling flask with a neck 2.2 cm in diameter and a side arm 0.5 cm in diameter. The latter is connected by means of a ground-glass joint to *D*, a water-cooled condenser, 30 cm in length with an adapter tip, 0.4 cm in diameter and 19 cm long, which extends almost to the bottom of the receiver, *E*. The receiver is a tube tapered so that a volume of 8 ml placed in it will provide a level of liquid about 8.5 cm from the bottom.

Five milliliters of 1:3 HCl is placed in the bulb of the delivery tube, which is fitted tightly to the flask containing the digest. The flask is placed in an electrically heated mantle (the temperature of which is controlled by a rheostat), and the side arm is attached to the condenser.

The adapter tip of the condenser tube extends almost to the bottom of the receiver, resting in a Thermos cylinder filled with cracked ice. The receiver contains a cooled mixture of 4 ml of distilled water and 4 ml of a sodium acetate solution, prepared by dissolving 100 gm $NaC_2H_3O_2 \cdot 3H_2O$ in 100 ml water, and is stored in a paraffined bottle. As soon as $GeCl_4$

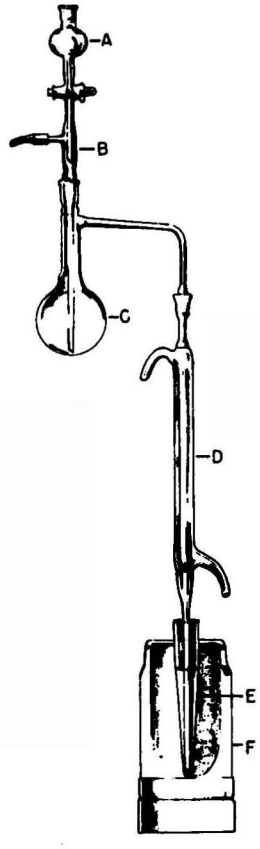

FIG. 1. Apparatus for the distillation of germanium tetrachloride.

dissolves in water, it hydrolyzes to form the oxide and HCl, according to the following equation: $GeCl_4 + 2H_2O \rightarrow GeO_2 + 4HCl$. The sodium acetate solution in the receiver accelerates the hydrolysis reaction by neutralizing the HCl as it forms, while the cold bath serves to absorb any heat evolved, thereby preventing a revolatilization of the $GeCl_4$.

The stopcock of the delivery tube is opened, and as soon as all the HCl has drained out of the bulb, it is closed and the side arm is connected to a moderate air current. The solution in the flask is distilled for 10 min while

air is passed through at a rate of about 10 bubbles/min. The temperature of the flask contents at the end of the distillation should not exceed 120°C in order to avoid the evolution of H_2SO_4 fumes. Three rinses of the condenser tube with distilled water totalling 13 ml are collected directly into the receiver. From 2 to 500 μg germanium has been distilled in this way with recoveries of 93% or more.

Color Development and Measurement

Two milliliters of an ammonium molybdate solution (5.31 gm $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ dissolved in water to make 100 ml) is added to the receiver tube and the contents are shaken. Then 1 ml ferrous ammonium sulfate (prepared by dissolving 21.00 gm $FeSO_4\cdot(NH_4)_2SO_4\cdot 6H_2O$ in water containing 20 ml of 1 *N* H_2SO_4 and making up to a liter)[3] is added, and the whole shaken again and diluted to 25 ml with water. The resulting blue color is read after 30 min in a Beckman spectrophotometer at 880 mμ.

Preparation of Standards and Reference Curve

A standard germanium stock solution containing 1000 μg/ml is prepared by dissolving 1.4408 gm of pure GeO_2 (Eagle-Picher Co., Joplin, Mo.) in 500 ml of distilled water containing 25 ml of 1 *N* NaOH. This solution is neutralized with dilute HCl and made up to 1 l. Working standards of 1 and 10 μg/ml are made when needed from suitable dilutions of the stock solution. The standard solution, kept in a paraffin-coated bottle to prevent reaction with the glass, was found to be stable for months.

A reference curve was obtained by adding known amounts of germanium to 25-ml tubes containing 4.0 ml sodium acetate and 1.75 ml of 1:1 HCl. These solutions were diluted with water to 21 ml and developed as previously described. A blank was similarly prepared, the only difference being the omission of the germanium solution. When analyzing any particular biological material, the blank is obtained by adding the acid digestion reagents to an equivalent sample of the same kind of material from a control animal not treated with germanium,[4] and carrying the sample through the entire procedure, including digestion, distillation, and color development. The blank prepared in this way serves as a control for any amounts of germanium or interfering substances introduced through the use of the various reagents and vessels. In the determination of a series of unknowns, it is also advisable to run simultaneously two standard samples to which known amounts of germanium have

[3] Stored in a dark bottle.

[4] In the present studies with adult albino rat tissues, germanium was not detected as a normal microconstituent.

been added to the control biological material. In this way, the accuracy of the recovery procedure can be evaluated for any particular series.

EXPERIMENTAL

A plot of optical density vs. micrograms of germanium per 25 ml solution shows that the color intensity of the "molybdenum blue" chromogen obeys Beer's law between 2 and 50 μg. It does not at higher concentrations under the experimental conditions. Consequently, samples of biological materials should be selected to yield amounts within the linear portion of the reference curve. However, solutions already developed and containing more than 50 μg can still be employed for accurate assays if they are diluted with a solution prepared exactly as the reference-curve blank. In this way, the concentrations of all the reagents remain the same, and only the germanium concentration is altered by the dilution. Since the

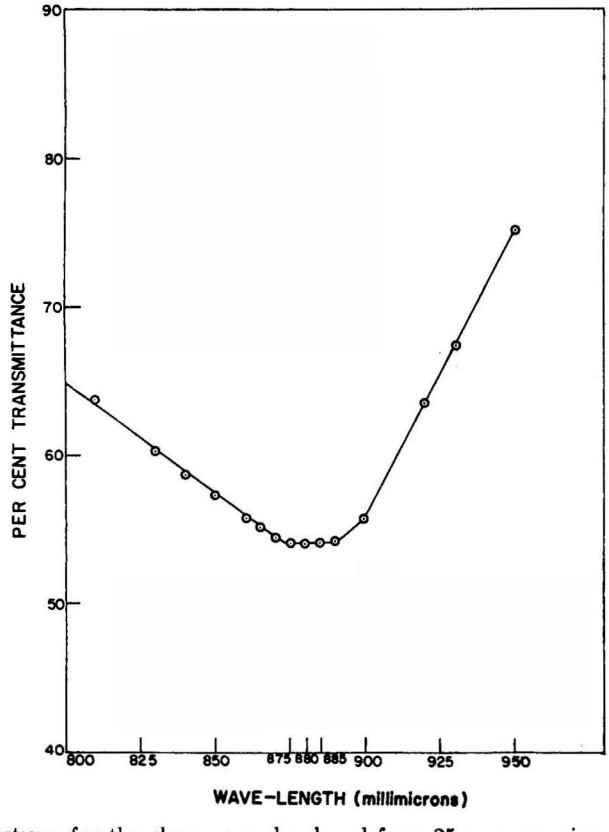

FIG. 2. Spectrum for the chromogen developed from 25 μg germanium. Curve prepared with a Beckman spectrophotometer.

distillation procedure is applicable to amounts of germanium up to approx. 500 μg, this method can be used over a range of from 2 to 500 μg if the proper dilutions are made.

The color intensity is essentially independent of the ammonium molybdate and ferrous ammonium sulfate provided their concentrations are at least 0.2 and 0.002 gm/100 ml, respectively. The maximum color intensity develops at room temperature within 30 min, remains constant for 30 min or longer, and then diminishes slowly. The wavelength of maximum absorption by the chromogen formed is 880 mμ (Fig. 2). At 880 mμ, the per cent transmittance of the chromogen developed with 30 μg of ger-

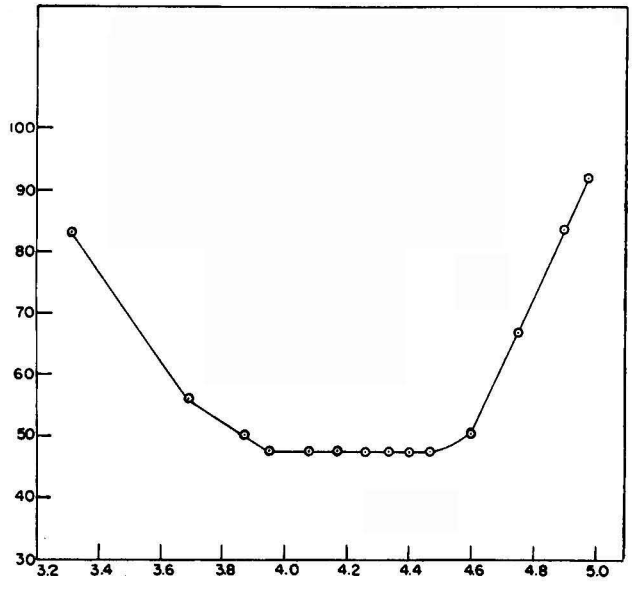

FIG. 3. Effect of pH on the per cent transmittance of the chromogen developed from 30 μg germanium.

manium varies with the pH as shown in Fig. 3. The amounts of HCl used for distillation and of sodium acetate placed in the receiver to trap the distilled $GeCl_4$ were selected to provide automatically a final buffered pH which lies within the optimum pH range (3.95–4.45) for maximum color development under the experimental conditions.

Arsenic, silicic, and phosphoric acids behave similarly to germanic acid, reacting with molybdates in acid solution to form arsenomolybdic, silicomolybdic, and phosphomolybdic acids, respectively. These complex heteropoly molybdic acids also have an enhanced oxidizing power toward ferrous salts, and can be reduced to yield a blue color. To determine the

possibility of interference by these three substances, 1000 μg arsenate, silicate, or phosphate was added separately to triplicate 3-gm samples of tissue, and the samples were carried through the entire procedure. Samples of tissue alone and tissue with 40 μg of added germanium were analyzed simultaneously. The results, shown in Table 1, demonstrate that the present analytical procedure eliminates the possibility of obtaining falsely high values for germanium when arsenate, silicate, or phosphate is present in the sample analyzed. This noninterference is particularly

TABLE 1
EFFECT OF PHOSPHATE, ARSENATE AND SILICATE ON GERMANIUM ASSAY

Substance added to tissue	Amount added (μg)[a]	Germanium found (μg)
Sodium phosphate	1000	0
Sodium arsenate	1000	0
Sodium silicate	1000	0
Germanium dioxide	40	38.3 ± 1.3[b]

[a] Micrograms of P, As, Si, or Ge.
[b] Mean recovery of 38.3 μg with a maximum deviation of 1.3 in three determinations.

significant with respect to phosphates, which are usually present in large amounts in most biological materials.

RESULTS

Table 2 summarizes the recovery of germanium when known amounts are added to 3 gm of muscle tissue or 5 ml urine before digestion and the samples are analyzed as described. The recovery varies from 85% for 2

TABLE 2
RECOVERY OF GERMANIUM FROM BIOLOGICAL MATERIALS

	Tissues[a]			Urine[a]		
Germanium added (μg)	Amount found (μg)	Maximum deviation	Ge recovered (%)	Amount found (μg)	Maximum deviation	Ge recovered (%)
0	0			0		
2	1.7	±0.5	85	1.8	±0.4	90
5	4.4	±1.1	88	4.7	±0.9	94
10	9.7	±1.3	97	9.2	±1.4	92
20	19.0[b]	±1.8	95	18.0	±3.0	95
30	29.6[c]	±2.4	98	27.9	±3.5	93
40	38.7[b]	±2.9	97	37.2	±4.3	93
50	47.5[c]	±3.9	95	45.5	±5.2	91

[a] Mean of five determinations unless otherwise indicated.
[b] Mean of 22 samples run as standards.
[c] Mean of ten determinations run as standards.

μg to 97% for 40 μg. In the range from 2 to 10 μg the maximum deviation was approx. ± 1 μg, and from 30 to 40 μg, about ± 3 μg. For 50 μg, the maximum deviation was ± 3.9 μg for ten determinations.

Table 3 shows the concentration of germanium in μg/gm of wet tissue

TABLE 3

DISTRIBUTION OF GERMANIUM IN RATS 24 HOURS AFTER INTRAPERITONEAL INJECTION OF 300 MG/KG OF GeO_2[a]

Micrograms of germanium per gram of wet tissue weight[b]								
							Bone	
Liver	Kidney	Spleen	Lungs	Brain	Muscle	Skin	Trabecular	Cortical
12 (10–16)	10 (8–11)	15 (13–20)	6 (3–8)	4[c]	7 (5–8)	9 (6–10)	8[c]	5[c]

[a] Neutralized to pH 7.3.

[b] Mean values (and the range) for the tissues of five animals.

[c] Pooled samples.

found in various organs of the adult albino rat 24 hr after the intraperitoneal injection of 300 mg/kg of neutralized GeO_2 (pH 7.3). These preliminary data suggest that germanium is not deposited selectively in any particular sites of concentration but is widely distributed to all the organs of the rat. The small amounts found in the tissues 24 hr after injection indicate a rapid elimination from the body. The latter conclusion is in accord with an analytical survey of 15 representative tissues of untreated rats which failed to show the presence of germanium as a normal microconstituent.[5]

SUMMARY

A simple, reliable, and sensitive method for the determination of from 2 to 500 μg germanium in biological materials is presented. The germanium is distilled as $GeCl_4$ from a sample digested with nitric and sulfuric acids. The addition of ammonium molybdate and ferrous ammonium sulfate to the buffered distillate yields a blue color, the intensity of which is a linear function of the concentration from 2 to 50 μg. Amounts up to 500 μg can be determined with the use of a proper diluent. The effect of time, pH, reagent concentrations, and possible interfering substances on the colorimetric reaction is described. The analytical procedure was applied to studies of the normal occurrence of germanium in the rat and its distribution among various organs after parenteral administration.

[5] If germanium occurs at all in these mammalian tissues, it would constitute less than 0.4 ppm.

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REFERENCES

1. "Plutonium Project: Nuclei Formed in Fission" *J. Am. Chem. Soc.* **68,** 2411 (1946).
2. KRAUSE, H. H., AND JOHNSON, O. H., *Anal. Chem.* **25,** 134 (1953).
3. GROSSCUP, C. G., *J. Am. Chem. Soc.* **52,** 5154 (1930).
4. POLUEKTOV, N. S., *Z. anal. Chem.* **105,** 23 (1936).
5. SCHERRER, J. A., *J. Research Natl. Bur. Standards* **16,** 255 (1936).

ANALYTICAL BIOCHEMISTRY 1, 478–485 (1960)

An Improved Microelectrophoresis Apparatus and Technique for Studying Biological Cell Surfaces

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At the present time the biological cell surface is the subject of extensive investigation. Physical, rather than chemical, methods are preferred for characterizing the nature of components at such surfaces, since the manipulation of the cells and hence cellular disorganization is minimized. Microelectrophoresis is an important technique with diagnostic (1, 2) as well as research uses.

Many different types of microelectrophoresis apparatus and techniques for using them have been described (3–5); all suffer from one or more of the following disadvantages: failure to conform to the theoretical requirements, inadequacy of temperature control, difficulty of construction and manipulation. In the apparatus described here based essentially on previous types of apparatus (6, 7), these disadvantages, and in consequence errors arising from them, have been eliminated. The only drawback remaining, which is common to all fused rectangular observation chambers, is that the apparatus requires calibration with particles of known electrophoretic mobility; this disadvantage is offset by ease of use.

The apparatus has been extensively tested by statistically designed experiments, and a standard procedure has been established. Using these conditions, the results obtained day to day over a period of weeks are reproducible to $\pm 3\%$.

METHODS

Apparatus

(*a*) *Cell and Electrode Compartments.* The cell (Fig. 1), used in the horizontal position (8), is constructed by fusing two optically flat Hysil glass plates ($40 \times 25 \times 0.5$ mm thick) round the edges using spacers to give a cell depth of 0.5mm. Spherical joints sealed to the cell arms and the electrode compartments simplify assembly and mounting. The elec-

trode compartments are constructed by sealing side arms, carrying taps of vacuum quality, onto a 10-mm Pyrex tube containing the sintered-glass disk (No. 2) as close as possible to the disks. This prevents the accumulation of pockets of liquid which cannot be flushed out. (The glassware can be obtained from Linskey Bros., 72, Holloway Road, London N.7).

(*b*) *Electrodes.* These are made of pure silver foil ($8 \times 3 \times 0.015$ cm) rolled into a cylinder and welded to 3 cm of 0.05 cm diameter platinum wire. The wire is sealed into a length of bent glass tubing, filled with

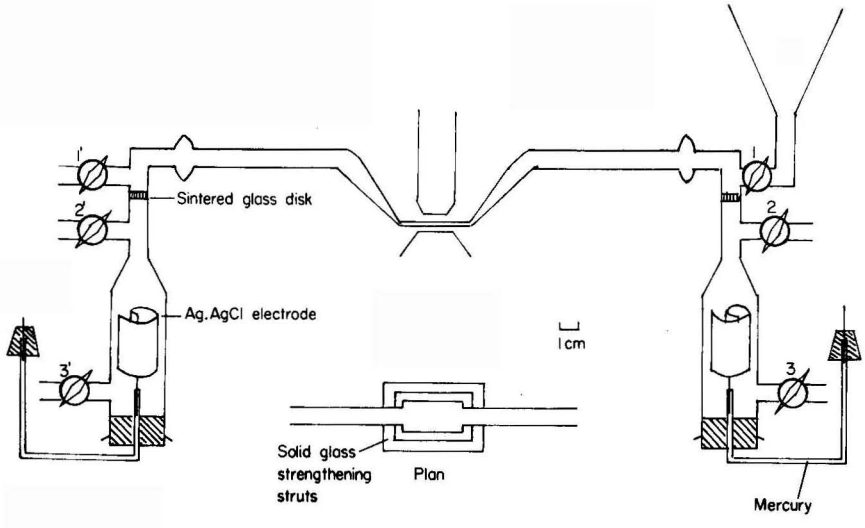

FIG. 1. The microelectrophoresis apparatus.

mercury for electrical connection, which carries a rubber bung to fit the electrode compartment (Fig. 1). The electrodes and platinum wire are cleaned with acid, silver plated, and then anodized at a current density of 1 ma/sq cm for 1 hr. The electrodes are always kept in potassium chloride solution (3.5 *M*).

(*c*) *Electric Circuit.* The best one is that proposed by Lerche (9) (Fig. 2), supplied by two 90-v H. T. batteries. r_1 and r_2 are wire-wound potentiometers each of 50,000 ohms dimensioned for 10 w. The current passing through the microelectrophoresis cell is set with r_2, and with r_1 it can be kept constant during observation. The current is measured with a multirange milliameter (e.g., Sangamo-Weston). S_1 is a shorting switch for the electrodes, S_2 the battery switch, and S_3 a reversing switch.

(*d*) *Microscope and Stage.* Any microscope in which the focusing is by movement of the objective is suitable. It should be fixed securely to a firm bench. A $\times$ 40 phase-contrast objective (N.A. 0.73) and an angled

eyepiece of 1.5 magnification carrying a × 10 focusing eyepiece containing a cross-hatch graticule are suitable for observing bacteria. The phase-contrast condenser and objective lenses are waterproofed. A high-intensity microscope lamp provides the illumination.

The normal microscope stage is replaced by a thick brass plate (*a*, Figs. 3 and 4) permitting the substage condenser *b* to pass up into the thermostat tank *c*. Adjustable mounting clamps *d*, made from two brass plates separated by four adjustable bolts, are bolted to the stage for holding the cell arms. These are clamped to the top plates of each using a Perspex strip and foam rubber pads to protect the glass. The electrode compartments are held on to this assembly by clips *e* and suitably supported on the bench *f*. The condenser pushes up into the thermostat *c* through a tight-fitting hole in a rubber sheet fixed to the bottom of the tank.

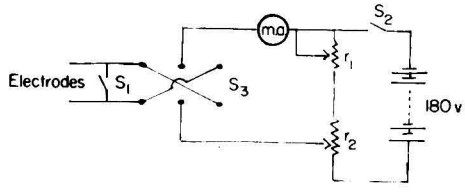

FIG. 2. The electric circuit.

(*e*) *Thermostat Tank.* The depth of the large tank *g* constructed from 1.5 cm thick Perspex, depends upon the level of the microscope stage. The tank, *c*, constructed from 0.75 cm thick Perspex, is joined at one corner to the tank *g* and connected by a Polythene tube to the circulating pump *h* of the temperature control unit (e.g., Shandon's Circotherm II Constant Temperature Unit). Thus a constant flow of water throughout the apparatus maintains any required temperature within ±0.02°C. Both tanks are covered and the reading thermometer is inserted through the lid of *c* close to the cell.

(*f*) *Additional Apparatus.* A conductance bridge (e.g., Wayne-Kerr Magic Eye bridge) and conductivity cell of known constant are required to measure the conductance of the suspension for the calculation of the field strength. For multitemperature work the conductivity cell must be calibrated at the working temperature. Results indicate that the cell constant may not vary linearly with temperature as widely believed.

(*g*) *Assembly and Preparation.* The graduated fine-adjustment screw of the microscope is calibrated over its complete range with a coverslip of known thickness; the most constant region is selected for subsequent use. To aid cell depth determinations, bacteria are deposited on the upper and lower inside surfaces of the cell by introducing a dilute alcoholic bacterial suspension and drying out.

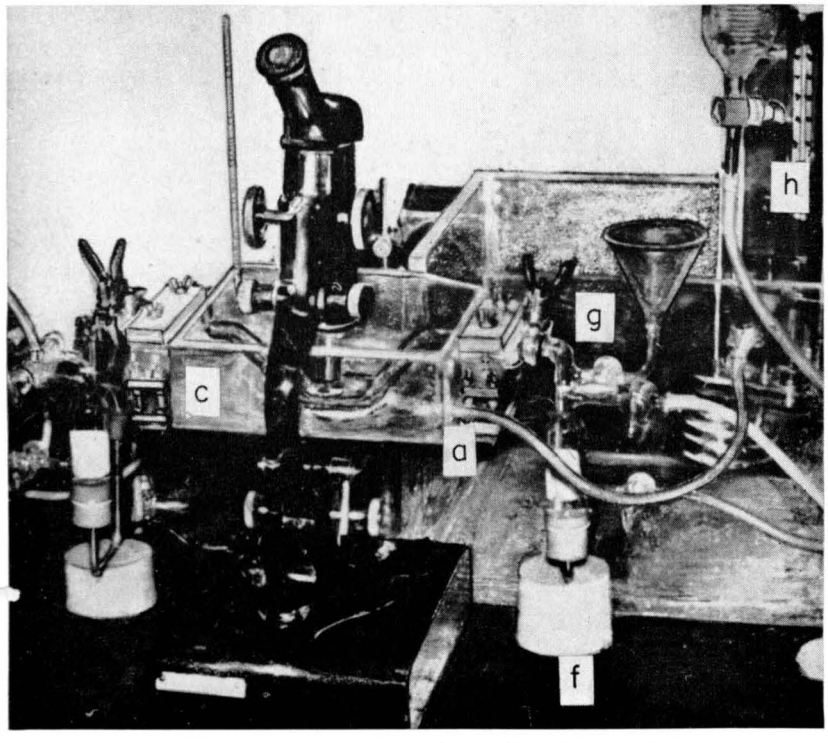

FIG. 3. The complete microelectrophoresis assembly.

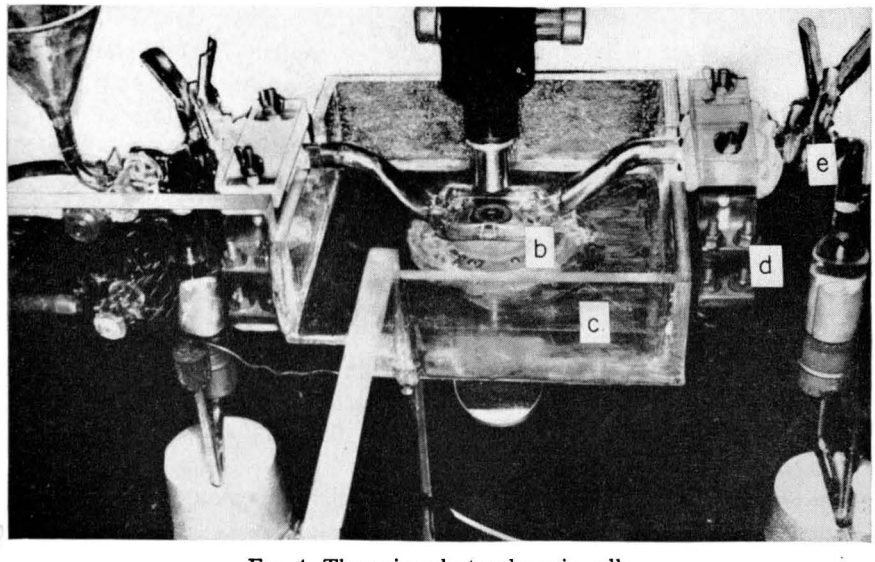

FIG. 4. The microelectrophoresis cell.

When all the joints have been greased, the apparatus is assembled and mounted in the tank. The electrode compartments are washed and finally filled with potassium chloride solution, and the cell with distilled water.

The condenser is raised as close to the cell as possible and, with the objective focused into the cell, the phase contrast is adjusted.

Operational Procedure

This procedure, established after consideration of results to be described, should be followed for each electrophoretic measurement on a bacterial suspension to ensure reproducibility of results.

1. Check the temperature of the thermostat and adjust the regulator if necessary.

2. Close S_1 for 1 min to eliminate electrode polarization.

3. Flush through each electrode compartment with 50 ml of 3.5 *M* potassium chloride solution. With taps 1, 3, 3′ open and taps 1′, 2, 2′ closed, allow a small amount of potassium chloride solution to pass through the sintered disks into the cell compartment. Finally pass another 25 ml potassium chloride solution through the electrode compartments and close all taps.

4. Thoroughly wash the observation cell and connections with glass-distilled water until all electrolytes have been removed.

5. Flush the experimental suspension (previously thermostatted at the working temperature) through the apparatus, and close taps 1, 1′, thereby trapping a sample.

6. Determine the cell depth in arbitary units (using the fine adjustment over the region selected) by focusing on bacteria adhering to the top and bottom inside surfaces of the observation chamber. Always come up to focus from below to avoid backlash in the fine adjustment screw. Hence focus on the upper stationary level (4).

7. Check that the bacteria do not move systematically due to bad-fitting joints or incomplete temperature equilibrium. Switch on the current and adjust it to give a suitable excursion time (e.g., 5 sec) of the bacteria. Select a cell in focus and record the time across a fixed distance in the eyepiece graticule, using a stopwatch accurate to 0.02 sec. Reverse the polarity and time a bacterium in the opposite direction. Repeat for 30 cells. On no account should the current be left flowing in one direction for more than 30 sec. The value of the current in either direction and throughout a set of readings should be kept constant with r_1.

8. Wash out the cell and leave filled with distilled water with tap 1 open when not in use.

9. Determine the conductivity of the cell suspension at the working

Calculation

The electrophoretic mobility $\bar{v}_\theta$ (cm/sec/v/cm) i.e., velocity per unit field strength, at temperature θ can be shown (9) to be:

$$\bar{v}_\theta = \frac{l \times q \times K_\theta}{t \times I}$$

where t (sec) is the time to cover l (cm), q (sq cm) cross-sectional area of the cell, K_θ (mhos/cm) the conductivity of the suspension and I (amp) the current.

It is difficult to determine the cross-sectional area of fused rectangular cells; thus l and q are combined as a constant A and determined using standard particles, such as human erythrocytes in $M/15$ phosphate buffer solution at pH 7.35 when $\bar{v}_{25} = 1.31 \pm 0.02$ μ/sec/v/cm (10).

Thus

$$\bar{v}_\theta = \frac{A \times K_\theta}{t \times I}$$

RESULTS AND DISCUSSION

All results were obtained on overnight culture of *Aerobacter aerogenes* grown in synthetic medium (11). Cells were harvested, washed three times, and resuspended in phosphate buffer solution, pH 7.0, $I = 0.0123$. Measurements were made at 25°C.

Velocity Depth Curves

The velocity of bacteria was determined at different depths in the cell at constant field strength. The equation to the velocity depth curve through the experimental points was calculated by the method of least squares, and the true electrophoretic velocity of the bacteria was determined by integration of this equation. The position of the stationary levels was obtained by substituting the velocity into the original equation and solving. This method is applicable to cells in which the ratio of width to depth is greater than 20 when the theoretical positions of the stationary levels are at 0.21 and 0.79 of the total cell depth (12).

The positions of the stationary levels determined over a period of 6 months, on one cell, are listed as fractional depths from the lower inside surface of the cell (Table 1).

TABLE 1

THE POSITION OF THE STATIONARY LEVELS

Lower:	0.240	0.220	0.246	0.280	0.254	0.214	0.234	0.241	0.247	0.252	0.227	0.255
Upper:	0.790	0.790	0.792	0.787	0.790	0.789	0.790	0.791	0.792	0.790	0.791	0.792

If the cell was geometrically asymmetrical, it would be expected that the upper and lower stationary levels, while not necessarily agreeing with the theoretical values, would remain fixed. The position of the upper stationary level is constant and in excellent agreement with theory. The lower stationary level, however, shifts erratically even over such a short period of time as 30 min; this is most probably due to a change in the nature of the lower cell surface and the concomitant change of the electroosmotic velocity (13, 14). This can be accounted for by bacteria and bacterial debris depositing and adhering to this surface preferentially, the nature of which will thus depend on the extent of washing. Cleaning the cell with acid results in a marked shift of the lower stationary level, but such a method cannot be used routinely as the debris rapidly reaccumulates.

All subsequent measurements were made at the upper stationary level only.

Statistical Analysis of Variables

Statistically designed experiments were carried out to observe any differences between mobility values of a suspension when determined by two observers over different distances, in both directions at the two stationary levels for different applied field strengths. The following are the conclusions:

1. There is a significant difference in measurements made between observers; thus all measurements should be taken by one observer.
2. The mobility value was independent of the distance over which timings were made.
3. There was a significant difference in one experiment between results obtained in both directions. This was traced to electrode polarization; after the introduction and use of the shorting switch S_1 no significant difference was found.
4. There was a significant difference between results obtained at the two theoretical stationary levels in confirmation of the previous results.
5. No significant difference between values at different field strengths, i.e., currents, was observed.

Reproducibility of Results

A typical analysis of a group of 30 timings gives a mean of 6.04 sec, standard deviation of 0.430 sec and a standard error of 0.079 sec. Analysis of timings obtained on the same and different days showed that the confidence limit for a single mean (15) at $p = 0.05$ is $\pm 3\%$.

SUMMARY

A modified form of microelectrophoresis apparatus and technique for studying biological cell surfaces is described. The design of apparatus has

been simplified thereby increasing the ease of assembly and manipulation. This is the first description of a microelectrophoresis cell using a rectangular observation chamber, conforming to the theoretical requirements in which the temperature can be accurately controlled. It thus makes an advance in design for it permits experimental verification of the theoretical mobility temperature relation.

The apparatus has been extensively tested by statistically designed experiments. These revealed the main sources of errors, which have been erradicated by modifications and the establishment of a standard experimental procedure. Using this procedure, results obtained day to day and over a period of weeks are reproducible to $\pm 3\%$.

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REFERENCES

1. DAVIES, D. F., *Clin. Sci.* **17,** 563 (1958).
2. GORDON, M. J., *Genetics* **43,** 913 (1957).
3. THOMPSON, M. R., *Trans. Electrochem. Soc.* **81,** 147 (1942).
4. JAMES, A. M., *Progress in Biophys. and Biophys. Chem.* **8,** 96 (1957).
5. BRINTON, C. C., AND LAUFFER, M. A., *in* "Electrophoresis" (Bier, ed.), p. 427. Academic Press, New York, 1959.
6. MOYER, L. S., *J. Bacteriol.* **31,** 531 (1936).
7. JAMES, A. M., AND LOVEDAY, D. E. E., *Chem. Prods.* **21,** 237 (1958).
8. HARTMAN, R. S., BATEMAN, J. B., AND LAUFFER, M. A., *Arch. Biochem. Biophys.* **39,** 56 (1952).
9. LERCHE, C., *Acta. Pathol. Microbiol. Scand. Suppl* **98,** 727 (1953).
10. HEARD, D. H., AND SEAMAN, G. V. F., *J. Gen. Physiol.* **43,** 635 (1960).
11. LOWICK, J. H. B., AND JAMES, A. M., *Biochim. et Biophys. Acta* **17,** 424 (1955).
12. ABRAMSON, H. A., MOYER, L. S., AND GORIN, M. H., "Electrophoresis of Proteins," pp. 48–52. Rheinhold Publ. Corp., New York, 1942.
13. WHITE, P., *Phil. Mag.* **23,** 811 (1937).
14. LANE, T. B., WHITE, P., *Phil. Mag.* **23,** 824 (1937).
15. BROWNLEE, K. A., "Industrial Experimentation," p. 33. Her Majesty's Stationery Office, London, 1957.

ANALYTICAL BIOCHEMISTRY 1, 486–490 (1960)

Determination of Neuraminic Acid in Cerebral Tissues[1]

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INTRODUCTION

Neuraminic acid[2] is a normal constituent of brain (1, 2). The same compound has been identified in spinal fluid (3). Following its identification, a procedure was developed for the determination of neuraminic acid in spinal fluid (4). Application of the procedure to the analysis of spinal fluid samples from patients with various neurological disorders revealed an elevation in Parkinsonism (5). Since this condition is associated with degeneration of the basal ganglia areas of the brain, rich in neuraminic acid-containing gangliosides, an investigation was undertaken for the determination of neuraminic acid in the gray (cortex and basal ganglia) and white matter of human brain.

Determinations of neuraminic acid in lipid extracts from human brains for the estimation of gangliosides and glycolipids were reported by Svennerholm on senile human brains (6), by Cumings *et al.* at various ages (7), and by Tingey on certain types of lipidoses (8). In this study, neuraminic acid was determined in the lipid extract as well as in the lipid-free residue for the estimation of total neuraminic acid in various anatomical areas of human brain.

EXPERIMENTAL

White matter from corpus callosum and gray matter from the cortex and the basal ganglia areas were carefully dissected from fresh human brains obtained at autopsy, 24 hr after death. One gram from each area was weighed and homogenized with 19 ml chloroform–methanol 2:1 v/v. The homogenate was filtered through a preweighed, fat-free filter paper, and the residue was dried at 50°C to constant weight. A 2-ml aliquot from

[1] This work was supported in part by grants from the National Multiple Sclerosis Society and the U. S. Public Health Service.

[2] Neuraminic acid is the unsubstituted compound whose derivatives are known as sialic acids.

the filtrate was placed in a weighing pan, evaporated to dryness, and weighed for the estimation of the dry weight of the total lipid extract. This dry weight of the total lipid extract, plus the dry weight of the residue, represented the total dry weight of each tissue sample. A 10-ml aliquot from the filtrate was partitioned into two phases by the procedure of Folch (9). The two phases were separated carefully, and the solvents were evaporated by a stream of nitrogen. Five milliliters of 0.1 N H_2SO_4 was added to each residue of the two phases as well as to the original lipid-extracted residue. The three fractions were then hydrolyzed at 80°C for 1 hr and chromatographed on ion-exchange columns by the procedure of Svennerholm (10). Aliquots from the eluates from each of the three fractions were used for the identification of neuraminic acid by paper chromatography and preparation of absorption curves as reported previously (3). One- and 2-ml aliquots from the eluates were used for the determination of neuraminic acid by the orcinol test. (4).

RESULTS AND DISCUSSION

Post-mortem brains with no evident neuropathology were used in this study for the establishment of normal values. The samples from each anatomical area of the brain were separated into three fractions: a lipid-free residue, an aqueous methanol upper, and a chloroform-rich lower phase of the filtrate. Paper chromatographic analysis of each fraction gave a single spot corresponding to standard N-acetylneuraminic acid for the residue and the upper phase, but not for the lower phase. Only the eluates from the residue and the upper phase produced, with the orcinol test, the violet color with an absorption maximum at 570 mμ, characteristic of neuraminic acid. Low molecular weight substances present in the upper phase interfere with the orcinol test (6). Preparation of absorption curves showed that these substances produce minor peaks at 450 and 650 mμ. The column chromatography eliminated this interference. These findings indicate that neuraminic acid in the brain is associated with proteins and water-soluble glycolipids but not with water-insoluble lipids, in accordance with the reports of other investigators (6, 11). For this reason, only the upper phase and the residue were analyzed, and the total neuraminic acid in each tissue was estimated by summation of the two fractions.

While this work was in progress, a method was reported (12) using thiobarbituric acid reagent for the determination of neuraminic acid in body fluids and tissues. Analysis of eluates from the upper phase and the residue with the orcinol and the thiobarbituric acid tests gave similar results. Direct application of the thiobarbituric acid method on lipid extracts from the brain, however, gave higher values. Examination of the

absorption curves showed interference by other substances with absorption peaks at 450 and 532 mμ.

Table 1 shows the concentration of neuraminic acid in various anatomi-

TABLE 1
NEURAMINIC ACID OF CEREBRAL TISSUES[a]

Brain	Anatomical area		Upper phase	Residue	Total
69-year-old male died of pneumonia	Anterior temporal lobe	(1)	3.0	2.9	5.9
	Insula	(2)	2.7	2.9	5.6
	Caudate nucleus	(3)	2.8	2.7	5.5
	Putamen	(4)	2.5	2.8	5.3
	Globus pallidus	(5)	1.0	1.9	2.9
	Corpus callosum	(6)	.46	1.0	1.5
61-year-old female, died of pulmonary embolism		(1)	3.6	2.5	6.1
		(2)	3.1	2.7	5.8
		(3)	3.1	2.9	6.0
		(4)	2.0	2.3	4.3
		(5)	1.2	2.3	3.5
		(6)	.47	.9	1.4
59-year-old male, died of pneumonia		(1)	3.2	2.8	6.0
		(2)	3.3	2.6	5.9
		(3)	3.0	2.8	5.8
		(4)	2.8	2.5	5.3
		(5)	1.7	2.4	4.1
		(6)	.37	1.1	1.5
44-year-old female, died of jaundice		(1)	2.9	4.0	6.9
		(2)	3.1	4.2	7.3
		(3)	2.5	3.7	6.2
		(4)	2.1	3.2	5.3
		(5)	1.0	2.7	3.7
		(6)	.44	1.1	1.5
40-year-old female, died of cancer		(1)	3.3	3.2	6.5
		(6)	.41	1.0	1.4
32-year-old female, died of uremia		(1)	3.0	2.8	5.8
		(6)	.30	.8	1.1
25-year-old male, died of hypertensive nephrosis		(1)	3.6	3.1	6.7
		(6)	.50	1.0	1.5
82-year-old male, died of coronary thrombosis		(1)	3.0	2.9	5.9
		(6)	.40	.9	1.3
Average		(1)	3.2	3.0	6.2
		(6)	.40	1.0	1.4

[a] Values in mg/gm dry weight.

cal areas of eight human brains. In this table areas (1) and (2) represent cortical gray matter; areas (3), (4), and (5), gray matter from the basal ganglia areas; and area (6), pure white matter. The upper phase represents a water-soluble lipid fraction, and the residue, a lipid-free fraction.

The values listed were obtained by single determinations. It was found by preliminary control analyses of several determinations, in aliquots from the same samples, that the variation was ±4%. The values of the two areas of the cortical gray matter show very little difference. The same results were reported by Svennerholm (6) in studies of gray matter from several areas of the cortex (but not of the basal ganglia). There are, however, significant differences in the concentration of neuraminic acid, not only between gray and white matter, but also differences between the cortical gray, on the one hand, and the gray matter of the basal ganglia on the other. Structurally the two types of gray matter investigated, (cortex and basal ganglia) display a differential characteristic cytoarchitecture. This structural difference is reflected in the differential chemical constitution of these two types of gray matter. The lower concentration of neuraminic acid in the globus pallidus, as compared with the other two areas of the basal ganglia, caudate nucleus, and putamen, reflects the histological difference of these areas. While possible errors associated with technical difficulties of dissection cannot be totally excluded, it is, nevertheless, a fact that globus pallidus is richer in white matter than caudate nucleus and putamen. Examination of the average values shows that the concentration of neuraminic acid in the upper phase of the white matter was ⅛ of that in the cortical gray. The small concentration of neuraminic acid in the white matter, together with the large concentration of interfering substances, as noted by Svennerholm (6), is one of the reasons that previous investigators had difficulties in determining it accurately. In this study, partition into two phases, followed by column chromatography eliminated interfering substances and gave lower values. The concentration of the residue of the white matter was one third of that in the cortical gray. The values of the upper phase of the cortical gray matter were somewhat higher than those of the residue. In the white matter, however, the values of the residue were about twice as high as those in the upper phase. The level of neuraminic acid did not show any change with age in the eight cases analyzed. The values in Table 1 were calculated on a dry-weight basis, and the average water content of the gray matter was 82% and of the white 68.5%. The same values for total neuraminic acid, when calculated on a wet-weight basis, gave an average of 1.1 and 0.45 mg/gm for cortical gray and white matter, respectively.

Svennerholm determined neuraminic acid in lipid extracts from cortical gray and from white matter for the estimation of gangliosides (6). In the same report, he stated some limitations of elimination of interfering substances by using either the partition or the chromatographic techniques. In the present investigation, more recent methods of partition and chromatography (9, 10) were used sequentially, which eliminated interfering

substances and resulted in lower values in the upper phase than those reported by Svennerholm.

These results establish control values for neuraminic acid in protein and lipid fractions of various cerebral tissues of non-neurologic cases. They will form the basis of comparison with similar studies, now in progress, of analysis of brain tissues from degenerative and demyelinating cases, particularly those involving the basal ganglia areas, such as Parkinsonism.

SUMMARY

The concentration of neuraminic acid was determined in the gray matter of the insular and temporal cortex as well as of the basal ganglia, and in the white matter of normal autopsy human brains. Samples from the cerebral tissues were separated into two fractions: a water-soluble lipid and a lipid-free residue. Neuraminic acid was determined in both fractions with the orcinol test after elimination of interfering substances by column chromatography. Only tissues from non-neurological cases were analyzed in this study in order to obtain control values for comparison with samples from areas with neurological disorders involving neuraminic acid.

REFERENCES

1. KLENK, E., *Z. physiol. Chem.* **268,** 50 (1941).
2. SVENNERHOLM, L., *Acta Chem. Scand.* **10,** 694 (1956).
3. PAPADOPOULOS, N. M., AND HESS, W. C., *Georgetown Bull. No.* **14,** 15 (1960).
4. PAPADOPOULOS, N. M., AND HESS, W. C., *Arch. Biochem. Biophys.* **88,** 167 (1960).
5. PAPADOPOULOS, N. M., MCLANE, J. E., O'DOHERTY, D., AND HESS, W. C., *J. Nervous Mental Disease* **128,** 450 (1959).
6. SVENNERHOLM, L., *Acta Soc. Med. Upsaliensis* **62,** 1 (1957).
7. CUMINGS, J. N., GOODWIN, H., AND CURZON, G., *J. Neurochem.* **4,** 234 (1959).
8. TINGEY, A., *J. Neurochem.* **3,** 230 (1959).
9. FOLCH, J., LEES, M., AND STANLEY, S. G. H., *J. Biol. Chem.* **226,** 497 (1957)
10. SVENNERHOLM, L., *Acta Chem. Scand.* **12,** 547 (1958).
11. LONG, C., AND STAPLES, D. A., *Biochem. J.* **73,** 385 (1959).
12. WARREN, L., *J. Biol. Chem.* **234,** 1971 (1959).

ANALYTICAL BIOCHEMISTRY 1, 491–497 (1960)

The Determination of Arsenic and Antimony in Biologic Material[1]

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INTRODUCTION

The criminal use of arsenic and antimony is today infrequent. Nevertheless, both metals are of toxicologic importance because of their presence in many insecticides, rodenticides, antiprotozoal preparations, and other drugs. The laboratories of the Office of the Chief Medical Examiner of New York City receive annually about 500 requests for arsenic, antimony, and mercury determinations. A large number of these analyses is performed on blood and urine in cases of suspected metal intoxication. A method for arsenic and antimony which is at once rapid, sensitive, and specific is thus desirable. The Reinsch test is neither sensitive nor specific. The classic Gutzeit method is not wholly satisfactory because of the variability of paper staining due to nonuniform evolution of the arsine or stibine. The molybdenum blue method is lengthy and requires careful control of final acidity and reducing conditions lest the reagent itself be reduced.

In 1952, Vasak and Sedivek (1) reported a method for the estimation of arsenic in biologic material. It involves the absorption of arsine by a pyridine solution of the silver salt of diethyldithiocarbamate. The resulting red color is measured photometrically. Their technique lacks the necessary sensitivity. More recently, several investigators (2–4) have employed the reaction for the determination of arsenic in various petroleum products. Excellent sensitivity and precision are achieved using specially designed absorbing columns.

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The present report describes a simple, sensitive method for the estimation of arsenic and antimony in biologic matter using the reaction of arsine and stibine with silver diethyldithiocarbamate. An improved absorber is described which possesses the advantages of ease of operation and subsequent cleaning.

EXPERIMENTAL

Apparatus

Wet Digestion Apparatus. Bethge's (5) modification of the Klein extractor is used for wet destruction of organic matter (Fig. 1).

Absorbing Apparatus (Fig. 2). This column was designed by Umberger (6) for use originally as a scrubber in an alkoxyl apparatus. The absorber

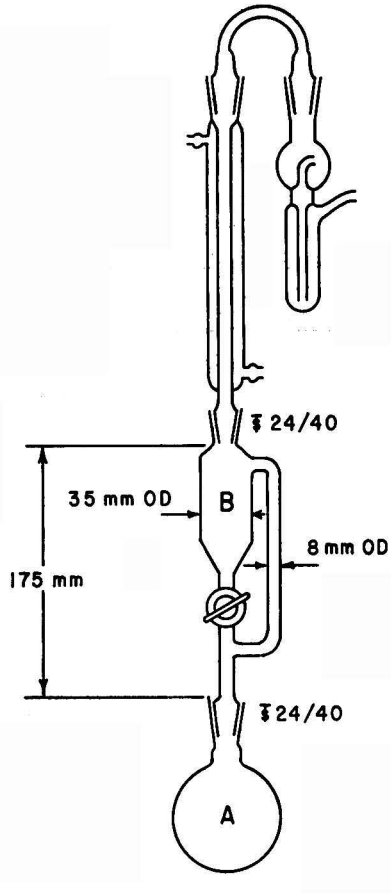

FIG. 1. Wet digestion apparatus.

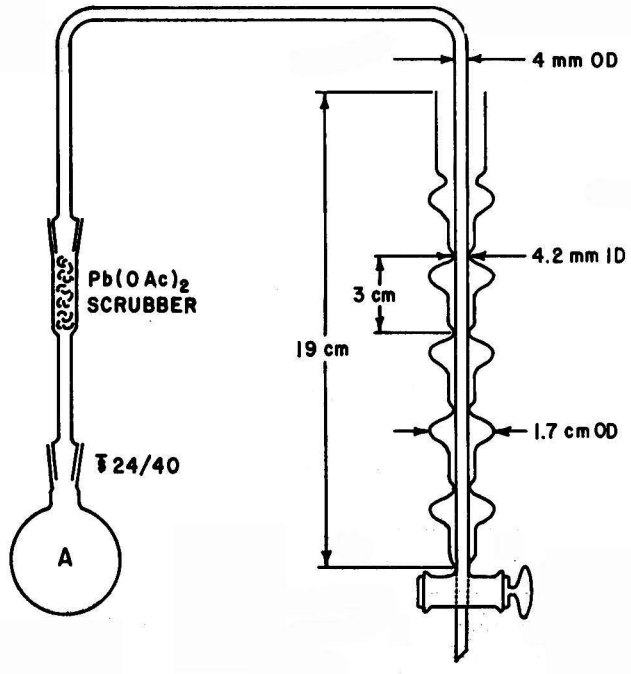

FIG. 2. Arsine (stibine) absorber.

consists of five bulbs shaped from a 25-cm length of 2 cm O.D. tubing. Between each bulb there is a constricted portion of 4.2 mm I.D. These constrictions permit insertion of a 25-cm length of 4 mm O.D. tubing throughout the length of the absorber. The 0.1-mm clearance at the constrictions insures complete and intimate contact between evolved gas and reagent during the percolation process. A No. 1 stopcock fused to the bottom of the column facilitates drainage and cleaning.

The gas delivery tube is connected to a scrubber containing a pledget of glass wool and powdered lead acetate to remove evolved hydrogen sulfide.

Reagents

Standard Arsenic and Antimony Solutions. Solutions covering the concentration range 0–100 μg/ml are prepared from the oxides of arsenic and antimony.

Zinc Metal. Reagent grade (low in arsenic), 20 mesh.

Silver Diethyldithiocarbamate. Prepare as follows: Solution A: 1.7 gm silver nitrate in 100 ml water. Solution B: 2.3 gm sodium diethyldithiocarbamate in 100 ml water.

Slowly and with stirring add soln. A to soln. B. Filter off the yellow

precipitate and wash with water. Dry in a vacuum desiccator. The salt is stable for months at room temperature.

Working Solution of Silver Diethyldithiocarbamate. Prepare a solution containing 5 mg silver diethyldithiocarbamate/ml of anhydrous, reagent-grade pyridine. The working solution is stable for at least 8 months if kept refrigerated.

Cadmium Sulfate Solution. Prepare a 5% aqueous solution of reagent-grade cadmium sulfate.

Lead Acetate. Powdered.

Concentrated Sulfuric and Nitric Acids. Reagent-grade nitric acid is redistilled. Reagent-grade sulfuric acid is used without further purification.

Ion-Free Water. Prepare by passing singly distilled water through a mixed-bed deionizing column.

Procedures

Urine. The first step in the digestion process for urine is the removal of excess water. To a suitable aliquot of sample (100 ml) in the digestion flask *A* (Fig. 1), are added 5 ml of conc. sulfuric acid and 5 ml of conc. nitric acid. Heat is applied to the boiling point. The first 80–90 ml of distillate collected in the receiver vessel *B* (Fig. 1) is discarded. The digestion flask is cooled and 20 ml of conc. nitric acid added. The contents of the flask are heated just to the appearance of sulfuric acid fumes with care to avoid excessive charring. Usually, the digest is not sufficiently clear at this point. The flask is cooled, and the contents of the receiver are reintroduced by means of the stopcock. Digestion is resumed just to the evolution of sulfuric acid fumes. If the digest is still not clear, the process is repeated. Two such steps are usually sufficient to furnish a clear, colorless digest. Particularly stubborn samples may be treated with 0.5 ml of 30% hydrogen peroxide or 0.5 ml of 70% perchloric acid. In any event, care must be exercised to remove all traces of oxidants for they have a deleterious effect on the subsequent production of the color. This is readily accomplished by the addition of 10 ml water and 0.5 gm reagent-grade oxalic acid to the clear digest. Heat is applied just to the appearance of sulfuric acid fumes.

The flask is permitted to cool and is detached from the digestion unit. The following are added in the order noted: 0.5 ml of 5% cadmium sulfate solution, 50 ml of 25% sulfuric acid, and 10 $\pm$ 0.1 gm zinc metal. The lead acetate scrubber and the absorbing column, charged with 2 ml silver diethyldithiocarbamate–pyridine reagent, are attached immediately. Bubbling is continued for approximately 1 hr. The colored end product is transferred to a 5-ml volumetric flask. Two milliliters pyridine is used to

rinse down the absorber and the gas delivery tube. The volume is made up to 5 ml with pyridine. Color intensity is read at 540 mμ for arsenic and 510 mμ for antimony. Pyridine is used as a blank. Concentrations are established by referring to previously constructed calibration curves.

Blood. The usual sample size is 10–20 ml of whole blood. To this are added 5 ml of conc. sulfuric acid and 5 ml. of conc. nitric acid. The first 15–25 ml of distillate collected is discarded. Then 10 ml of conc. nitric acid is added, and the digestion is continued to the appearance of sulfuric acid fumes. From this point, the procedure for blood is similar to that for urine.

Tissue and Hair. Tissue and hair samples are treated in a manner similar to that for urine and blood except that the minced tissue (10–20 gm) or hair is permitted to stand at room temperature in 10–50 ml of conc. nitric acid for about 3–4 hr. This preliminary step minimizes frothing in the digestion process.

RESULTS

Recoveries of arsenic and antimony added to tissue and body fluids are shown in Table 1. In all cases recovery is good. Precision of the method is stated as standard deviation.

TABLE 1
RECOVERY OF ARSENIC AND ANITMONY ADDED TO VARIOUS BIOLOGIC MATERIAL

Sample	Metal added	Recovery (μg) and standard deviation
Urine, 100 ml	5 μg As	4.7 ± 0.5
Urine, 100 ml	10 μg As	9.5 ± 0.4
Blood, 10 ml	5 μg As	4.3 ± 0.3
Blood, 10 ml	10 μg As	9.2 ± 0.4
Liver, 20 gm	10 μg As	9.0 ± 0.2
Urine, 100 ml	5 μg Sb	4.8 ± 0.3
Urine, 100 ml	10 μg Sb	9.7 ± 0.6
Blood, 10 ml	5 μg Sb	4.5 ± 0.5
Liver, 20 gm	10 μg Sb	9.1 ± 0.3

Analysis of urine from 30 presumably normal individuals furnished values ranging from 0 to 11 μg/l with an average of about 4 μg/l.

The results appearing in Table 2 represent arsenic levels in several cases of suspected arsenic intoxication. Particular attention is invited to the elevated levels found during the administration of BAL.

DISCUSSION

The colored complex resulting from the reaction of arsine with silver diethyldithiocarbamate in pyridine exhibits a maximum absorption at

540 mμ; the absorption maximum for the stibine analog occurs at 510 mμ. Both adhere closely to the Beer-Lambert law over the concentration range 0–15 μg. The slopes for both, using this method, are approximately 0.05 O.D. unit/μg metal. This can be improved by making the final dilution less than 5 ml. The colors are stable for long periods, both showing only a 1% increase in % T over 8 hr.

TABLE 2
ARSENIC FOUND IN CASES OF SUSPECTED INTOXICATION

Case No.	Sample	Arsenic found[a] (μg%)
ME 14/59HW	Urine, 100 ml before BAL	2.0
ME 14/59HW	Urine, 100 ml during BAL	5.6
ME 14/59HW	Urine, 100 ml after BAL	1.3
ME 30/59MJ	Urine, 100 ml before BAL	Negative
ME 30/59MJ	Urine, 100 ml during BAL	2.8
Me 46/59AS	Hair, 0.310 gm	3.3
ME 46/59AS	Urine, 100 ml	13.2

[a] Average of duplicate analyses (except for hair).

The reagent itself possesses a maximum at 420 mμ but shows negligible absorbance (98% T) at 540 and 510 mμ.

The interference of oxidizing agents has already been mentioned. Hydrogen sulfide seriously affects the color but is removed by the lead acetate scrubber. Excessive moisture also interferes; thus the absorbing column must be thoroughly dry prior to use.

Sandell (7) reports that the presence of various metals inhibits the evolution of arsine and stibine. In the present study, no interferences due to copper, iron, cobalt, nickel, etc. normally present in tissue urine and blood were noted. The effects of platinum have been well described (2), but the probability of encountering platinum in biologic media in large enough concentrations is remote.

If antimony and arsenic are present together in comparable concentrations, they may be determined simultaneously by obtaining readings at both 540 and 510 mμ. Two equations are set up and are solved simultaneously. However, if one metal is present in much greater quantities, a preliminary separation by established techniques (7) becomes necessary.

Deposits on Reinsch wires may be washed off using the methods of Gettler and Kaye (8) and subjected to this procedure. In this manner, both qualitative and quantitative information on the nature of the deposit is available.

Experiments are in progress to ascertain the feasibility of using the absorbing column for the determination of other volatile substances of toxicologic importance.

SUMMARY

A method for the estimation of arsenic and antimony in biologic material is presented. It involves the evolution of arsine and stibine into a pyridine solution of silver diethyldithiocarbamate. The resulting color(s) are read spectrophotometrically. A new absorbing column is described which increases sensitivity and facilitates cleaning. Results are tabulated showing the excellent sensitivity and precision attained with the technique. Results of actual Medical Examiner cases are also presented.

ACKNOWLEDGMENTS

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REFERENCES

1. VASAK, V., AND SEDIVEK, V., *Chem. listy* **46,** 341 (1952).
2. LIEDERMAN, D., BOWEN, J. E., AND MILNER, O. I., *Anal. Chem.* **31,** 2052 (1959).
3. POWERS, G. W., JR., MARTIN, R. L., PIEHL, F. J., AND GRIFFIN, J. M., *Anal. Chem.* **31,** 1589 (1959).
4. ALBERT, D. K., AND GRANATELLI, L., *Anal. Chem.* **31,** 1593 (1959).
5. BETHGE, P. O., *Anal Chim. Acta* **10,** 317 (1954).
6. UMBERGER, C. J., Ph.D. Thesis, New York University, 1939.
7. SANDELL, E. B., "Colorimetric Determination of Traces of Metals," 3rd Ed. Interscience, New York, 1959.
8. GETTLER, A. O., AND KAYE, S., *J. Lab. Clin. Med.* **35,** 146 (1950).

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A Rapid Procedure for the Determination of Adrenal Ascorbic Acid. Application of the Sullivan and Clarke Method to Tissues

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The estimation of ascorbic acid in the adrenal glands has been of particular value in endocrinological studies, since a decrease in content of the acid is a sensitive response of the pituitary–adrenal system to stressful stimuli (1). In addition, the effect on the level of adrenal ascorbic acid in hypophysectomized animals is the basis of a method for the estimation of adrenocorticotropic hormone (ACTH) (2). The usual methods for estimating the content of tissue ascorbic acid are titration with 2,6-dichloroindophenol (3) or oxidation to dehydroascorbic acid followed by reaction of the latter compound with 2,4-dinitrophenylhydrazine to form a derivative that yields a color when treated with 85% H_2SO_4 (4).

In 1955, Sullivan and Clarke (5) described a method for assaying ascorbic acid in urine which depends on the reduction of Fe^{+++} by ascorbic acid and estimation of the Fe^{++} as the red–orange, α,α'-dipyridyl complex; other reducing material is inhibited by the presence of orthophosphate and a high degree of acidity, pH 1–2. The present communication describes the adaptation of this reaction to the determination of ascorbic acid in the adrenal glands. The method is much simpler than other methods and makes it possible to estimate a large number of samples in 1–2 hr.

REAGENTS

Trichloroacetic acid, reagent grade, 5% aqueous solution, made fresh each week, protected from light, and kept at 4°C.

Orthophosphoric acid, 85% H_3PO_4, reagent grade.

Ferric chloride, reagent grade. A 1% aqueous solution is made up each week.

α,α'-Dipyridyl, 0.5% aqueous solution, is made by dissolving 5.0 gm of crystalline α,α'-dipyridyl in 500 ml of hot water and diluting to 1000 ml.

PROCEDURE

Adrenals, trimmed of fat, are homogenized immediately. Alternatively, the whole adrenals may be frozen and stored at —4°C for at least 4 weeks without loss of ascorbic acid. Adrenal tissue (10–100 mg) is homogenized in 200 vol of cold 5% trichloroacetic acid, in an all-glass homogenizer, and centrifuged at 4°C for 5 min at 9000 × g. One milliliter of the supernatant (containing 10–40/μg ascorbic acid) is transferred to a colorimeter tube. To this solution is added 0.3 ml of 85% H_3PO_4, 5.0 ml of 0.5% aqueous α,α'-dipyridyl, and 1.0 ml of 1% aqueous $FeCl_3$, mixing after each addition. The tubes are allowed to stand at room temperature for 10 min. The optical density of the red–orange Fe^{++}–α,α'-dipyridyl complex is then measured at 525 mμ, using water as a zero reference. A standard solution, containing 25 μg ascorbic acid/ml, in 5% trichloroacetic acid, is prepared daily. When 1.0 ml of the standard is run through the procedure, an optical density of 500–550 is obtained, using 15-mm diameter colorimeter tubes in the Coleman Jr. spectrophotometer. This value corresponds to a molar extinction coefficient of 1.65×10^4. The optical density following Beer's law over the range of 5.0–75.0 μg ascorbic acid per 7.3 ml final volume (0.68–10.3 μg/ml).

TABLE 1

RECOVERY OF VARYING AMOUNTS OF ASCORBIC ACID ADDED TO HOMOGENATES OF ADRENAL TISSUE[a]

	Ascorbic acid			
Tube No.	Adrenal content (μg)	Added (μg)	Found (μg)	Recovery (% of added ascorbic acid)
1	241	—	—	—
1′	241	50	287	92.0
1″	241	50	289	96.0
2	276	—	—	—
2′	276	100	370	94.0
2″	276	100	374	98.0
3	288	—	—	—
3′	288	200	481	96.5
3″	288	200	478	95.0
4	257	—	—	—
4′	257	400	631	93.6
4″	257	400	641	96.0
			Avg. ± S.D. = 95.1 ± 1.9%	

[a] For each set of samples, one pair of adrenals was homogenized in 3.0 ml water. The homogenate was divided into two equal portions, and 1.0 ml water was added to one part and the desired amount of ascorbic acid in 1.0 ml of water was added to the other portion. Each portion was then treated with 2.5 ml of cold 10% trichloroacetic acid and carried through the described procedure.

RESULTS AND DISCUSSION

Adrenals were removed from normal animals and homogenized in water. The homogenates were divided into two parts and assayed with and without the addition of known amounts of ascorbic acid. The results in Table 1 show an average recovery of ascorbic acid of 95.1% over the range of 50–400 μg.

TABLE 2

COMPARISON OF THE ASSAY OF ADRENAL ASCORBIC ACID BY VARIOUS METHODS

Method used	Sample No.			
	1 (mg%)	2 (mg%)	3 (mg%)	Average ± S.D. (mg%)
Fe^{++}–α,α'-dipyridyl	507	504	502	509 ± 5.5
	517	512	509	
2,6-Dichloroindophenol (3)	500	496	493	503 ± 7.9
	508	513	509	
2,4-Dinitrophenylhydrazine (4)	520	517	511	522 ± 8.7
	531	534	520	

Results with this method were compared with those obtained using the Mindlin and Butler (3) and Roe and Kuether (4) methods. Twelve pairs of adrenals from normal rats were pooled and homogenized in 60 ml water. Three samples were removed for duplicate assay by each method.

TABLE 3

DETERMINATION OF ASCORBIC ACID CONCENTRATIONS OF VARIOUS TISSUES[a]

Tissue	Ascorbic acid found (mg%)
Adrenal	494
Spleen	33
Liver	31
Brain	30
Heart	25
Lung	24
Kidney	19
Whole blood	1.2
Blood plasma	1.4

[a] Tissues from four adult male rats were pooled, homogenized in 9 vol of cold 5% trichloracetic acid and carried through the procedure.

The results in Table 2 indicate good agreement between the methods. The slightly higher values obtained by the Roe and Kuether method (4) may be due to dehydroascorbic acid, which would not be measured by the other methods.

The content of ascorbic acid in the adrenal glands of rats and guinea

pigs is 10–20 times higher than in other tissues (6). This method has also been used to determine the concentration of ascorbic acid in whole blood, blood plasma, and various tissues of rats. The results shown in Table 3 are in agreement with values obtained by other methods (3, 4).

The advantages of the method described in this communication lie chiefly in its simplicity and speed. It would seem to be of particular value in the studies of pituitary–adrenal stimulation or in the bioassay of ACTH in the Sayers' (2) method, where a large number of determinations of adrenal ascorbic acid must be made.

SUMMARY

A simple and rapid method for the determination of ascorbic acid in adrenal glands and other tissues is described. Tissues are homogenized in trichloroacetic acid, and ascorbic acid is assayed in the filtrate by measuring the reduction of ferric ions at pH 1–2 in the presence of excess orthophosphate, coupling the Fe^{++} produced with α,α'-dipyridyl. The red–orange complex is estimated spectrophotometrically.

REFERENCES

1. Long, C. N. H., *Recent Progn. in Hormone Research* **1,** 99 (1947).
2. Sayers, M. A., Sayers, G., and Woodbury, L. A., *Endocrinology* **42,** 379 (1948).
3. Mindlin, R. L., and Butler, A. M., *J. Biol. Chem.* **122,** 673 (1938).
4. Roe, J. H., and Kuether, C. A., *J. Biol. Chem.* **147,** 399 (1943).
5. Sullivan, M. X., and Clarke, H. C. N., *J. Assoc. Offic. Agr. Chemists* **38,** 514 (1955).
6. Bourne, G. H., *Protoplasmatologia* **II,** B2bd (1957).

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Resolution of Some Protein Mixtures by Gradient Elution Paper Chromatography

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INTRODUCTION

Various types of paper chromatography for resolution of proteins have been the subject of a great number of publications in the past 10 years (1). Homogenous liquids of constant composition and mixtures of organic solvents or salt solutions have been used as eluants (2–6).

Paper chromatography of proteins has not, however, achieved the same success as paper chromatography applied to other classes of organic compounds.

The purpose of adsorption chromatography on paper strips is the resolution of a protein mixture so that separate protein components are obtained as visible, discrete, and reproducible spots. We have found that in some cases this aim can be accomplished using paper fibers as adsorbent and a series of salt solutions in increasing concentrations as eluants.

We have succeeded in resolving proteins from *Vip. ammodytes* venom on paper strips applying salt eluants in progressive concentrations (7). The same method was used for the separation of protamine (*Mugil cephalus*), bovine serum albumin, and γ-globulin from an artificial mixture (8). Similarly, we have resolved ribonuclease from insulin, and crystalline pancreas ribonuclease itself into two enzymically active components (9, 10).

The purpose of this communication is to demonstrate new possibilities in the paper chromatography of proteins by forming a controlled concentrations gradient of salt solutions along the paper strip, as applied to the protein mixtures of insulin–protamine and egg albumin–lysozyme. Some experimental results on the influence of cations and anions on the elution of proteins in paper chromatography are presented.

METHODS

Development of Chromatograms

The original apparatus for paper strip chromatography and elution procedure has already been described in detail (10). Minor changes are given in the text. All chemicals used for chromatography were of highest grade chemical purity.

The chromatograms were always developed by ten-step serial dilutions of a salt or buffer stock solution, so that the total dilution range was 1:1000. Dilution factor $f = (C_{10}/C_1)^{1/9} = 2.15$.

In all the experiments ascending chromatography was applied. The paper chromatograms, when developed, were dried at 105°C for 5 min and stained.

The most diluted solution of the set was allowed to move to the starting line first; then it was changed to a more concentrated one, and so on for each centimeter of front advance.

The mixtures of lysozyme and egg albumin were chromatographed using serial dilutions of 4 and 2 *M* sodium chloride, and mixtures of insulin and protamine using 4 *M* sodium chloride only.

In order to acquire some evidence with respect to the influence of various cations and anions, elution experiments were performed with various salts, such as LiCl, NaCl, KCl, RbCl, CsCl, LiI, $LiNO_3$, and $LiSO_4$. The stock solutions of these salts were 2 *M*, and serial dilutions were prepared in the same manner as previously cited.

Paper Strips

For many of the chromatographic experiments, Whatman No. 1. filter paper strips 25 × 200 mm were used. Other filter papers used were Schleicher Schüll Nos. 2024a, 2043a, 2043 bMgl, 602 hP, 598 L; several sorts with directed paper fibers in the sheet: 2040 bM, 2045 aM, 2045 bM, and 2045 bgl were employed for demonstration of the effect of paper quality in chromatographic procedures.

Materials

All protein preparations used for paper chromatography experiments were tested for homogeneity by paper electrophoresis in sodium Veronal buffer pH 8.6, 0.067 *M* (*M*/15) and sodium phosphate buffer pH 6.0, *M*/15. Minor impurities in beef serum albumin and protamine preparations were taken into consideration.

Protamine preparations showed some absorption of ultraviolet light with a maximum at 2670 A. This very probably indicates the presence of small amounts of nucleic acids in the preparation. All the protein prepara-

tions were dialyzed against distilled water in a refrigerator (+ 2°C) for 48 hr and, when needed, freeze-dried.

The most satisfactory concentrations of protein solutions for chromatography were determined by trial. The lower limit of the quantity of the substance is given by the staining capacity. Excessive material drifted toward the front and caused strong tailing.

The aqueous protein solutions were used in the following concentrations: Beef serum albumin, fraction V Armour & Co., 10 mg/ml; human serum albumin, 20% solution, Institute for blood Transfusion, Belgrade, 10 mg/ml; egg albumin, three-times recrystallized sample, prepared by the method of Keckwick-Cannan (11), 25 mg/ml; beef pancreas ribonuclease, four-times recrystallized product, 115 K.U./mg, prepared by the method of Kunitz (12), modified by McDonald (13), 25 mg/ml; human γ-globulin, 16% solution, Institute for Blood Transfusion, Belgrade, 40 mg/ml (0.250 ml solution + 0.750 ml water); protamine (*Mugil cephalus*) prepared by the method of Kossel (14), 5 mg/ml; insulin, prepared from fresh beef pancreas, "Pliva" Zagreb, 22–23 I.U. and then four-times recrystallized, 15 mg/ml 0.01 *N* HCl; original protein chemicals produced by Armour & Co. were used: chymotrypsin Lot 283, 12 mg/ml; lysozyme, Lot D-638040, 7 mg/ml; chymotrypsinogen, Lot 128–214, 12 mg/ml; trypsin, Lot 181, 10 mg/ml.

STAINING AND EVALUATION OF CHROMATOGRAMS

After drying, paper chromatograms were stained for 20 min in 1% ethanolic solution of bromophenol blue. The ethanol (96%) used was saturated with mercuric chloride prior to use.

The stained paper chromatograms were washed by three changes of 0.2% mercuric chloride aqueous solution, the first containing 20 ml *M*/15 Na_2HPO_4/l.

When dried (5 min at 105°C), the stained chromatograms were made translucent by a mixture of paraffin oil and Cedax (Behring Werke, Bayer) or cedar oil, diluted with petroleum ether.

Light transmittance of paper chromatograms was determined at intervals of 1 mm with a modified Fisher electrophotometer and green filter B 525. No better results were obtained either with Amidoschwartz 10 B or with Azocarmin G.

RESULTS

The technique of ascending chromatography was used in all our experiments because of the relatively simple construction of the original apparatus (10), which permitted the changing of solvents during the elution procedure without opening the chromatograph chamber. The

desorption of human and bovine albumin from the paper fibers was accomplished at the lowest concentration (2×10^{-3} *M*) of chlorides irrespective of cations Li^+, Na^+, K^+, Rb^+, Cs^+ used. The lowest concentrated chloride solution (2×10^{-3} M) of Na^+ or K^+ cations for elution of egg albumin was necessary, whereas the next higher concentration (4.30×10^{-3}) chloride solution for Li^+, Rb^+, and Cs^+ cations was needed.

Human γ-globulin could be desorbed at a concentration of 4.3×10^{-2} *M* chlorides of all the cations cited except RbCl which eluted at 9.3×10^{-2} *M*.

The elution of insulin could be achieved at different concentrations of chlorides, the concentrations increasing in succession for cations K^+, Li^+, Rb^+, Cs^+, Na^+, the lowest being for K^+.

Protamine (*Mugil cephalus*), lysozyme, trypsin, and ribonuclease were always eluted by certain molar concentrations of chlorides, characteristic of each protein, which were the same for all alkali-group cations investigated.

The lithium salts of various acids were employed in order to obtain some evidence of the anion influence on the desorption of proteins. The results of these investigations are presented in Table 1.

A series of purified proteins, constituents of blood plasma: albumins, γ-globulin; purified enzyme preparations: chymotrypsin, chymotrypsinogen, ribonuclease, lysozyme; and some other proteins: protamine, insulin, egg albumin; which show different physical and chemical properties, were chromatographed by means of ten-step serial dilution of 2 *M* sodium chloride. The photometric evaluations of bromophenol blue-stained chromatograms are presented in Fig. 1.

Since lysozyme and egg albumin are present in the native protein mixture of egg white, it was of interest to determine the experimental conditions for their separation on the paper strip.

The mixture of protamine and insulin is commonly used in the therapy of diabetes mellitus, and chromatographic resolution of this mixture might be of some practical value.

For chromatography 250 μg egg albumin and 70 μg lysozyme were dissolved in 0.01 ml water, placed on each paper strip (Whatman No. 1. filter paper), and immediately developed without drying, so as to avoid denaturation. The chromatograms were developed by a series of ten solutions in a progressive scale of concentrations, prepared by the serial dilution method (dilution factor = 2.15) from 4 *M* sodium chloride, so that the total dilution range was 1 : 1000. The shape and slope of salt gradient along the paper strip at points apart was measured by means of a pair of footlike platinium electrodes and Mullard conductometer. It may be assumed that by diffusion a nearly continuous gradient of salt

TABLE 1

CHARACTERISTIC MOLAR CONCENTRATIONS OF THE SALTS ELUTING PROTEINS FROM THE PAPER FIBERS[a]

	LiCl	LiI	$LiNO_3$	Li_2SO_4	NaCl	KCl	RbCl	CsCl
Bovine serum albumin	2.0×10^{-3c}	2.0×10^{-3c}	2.0×10^{-3c}	2.0×10^{-3c}	2.0×10^{-3c}	2.0×10^{-3c}	2.0×10^{-3c}	2.0×10^{-3c}
Egg albumin	4.3×10^{-3}	4.3×10^{-3}	4.3×10^{-3}	4.3×10^{-3}	2.0×10^{-3c}	2.0×10^{-3c}	4.3×10^{-3}	4.3×10^{-3}
Human serum albumin	2.0×10^{-3c}	2.0×10^{-3c}	2.3×10^{-3c}	2.0×10^{-3c}	2.0×10^{-3c}	2.0×10^{-3c}	2.0×10^{-3c}	2.0×10^{-3c}
γ-Globulin	4.3×10^{-2}	4.3×10^{-2}	9.3×10^{-2}	2.0×10^{-2}	4.3×10^{-2}	4.3×10^{-2}	9.3×10^{-2}	4.3×10^{-2}
Insulin	2.0×10^{-1}	2.0×10^{-1}	9.3×10^{-2}	4.3×10^{-1}	2.0[b]	4.3×10^{-2}	4.3×10^{-1}	9.3×10^{-1}
Protamine	4.3×10^{-1}	2.0[b]	9.3×10^{-2}	9.3×10^{-2}	4.3×10^{-1}	4.3×10^{-1}	4.3×10^{-1}	4.3×10^{-1}
Lysozyme	2.0×10^{-1}	2.0×10^{-1}	2.0×10^{-1}	9.3×10^{-2}	2.0×10^{-1}	2.0×10^{-1}	2.0×10^{-1}	2.0×10^{-1}
Trypsin	9.3×10^{-2}	9.3×10^{-2}	9.3×10^{-2}	4.3×10^{-2}	9.3×10^{-2}	9.3×10^{-2}	9.3×10^{-2}	9.3×10^{-2}
Chymotrypsin	2.0×10^{-2}	9.3×10^{-2}	4.3×10^{-2}	2.0×10^{-2}	4.3×10^{-2}	4.3×10^{-2}	4.3×10^{-2}	4.3×10^{-2}
Chymotrypsinogen	4.3×10^{-2}	2.0×10^{-2}	4.3×10^{-2}	2.0×10^{-2}	4.3×10^{-2}	2.0×10^{-2}	4.3×10^{-2}	4.3×10^{-2}
Ribonuclease	2.0×10^{-2}	9.3×10^{-3}	2.0×10^{-2}	9.3×10^{-3}	2.0×10^{-2}	2.0×10^{-2}	2.0×10^{-2}	2.0×10^{-2}

[a] All chromatograms were developed with salt solutions of different concentrations ranging from 2.00 to 2.00×10^{-3} M; dilution factor $f = 2.15$.

[b] This denotes that the protein remained on the spot of application.

[c] Protein moved to the front of the eluant.

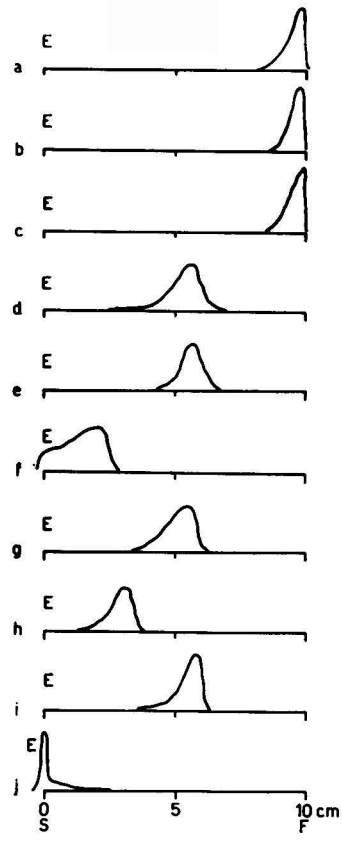

FIG. 1. Photometric evaluations of the chromatograms developed with a 1 : 1000 sodium chloride gradient 2.00 × 10^{-3} *M* to 2.00 *M*. (*a*) 100 μg human serum albumin, (*b*) 100 μg bovine serum albumin, (*c*) 250 μg recryst. egg albumin, (*d*) 400 μg human γ-globulin, (*e*) 250 μg recryst. beef pancreas ribonuclease, (*f*) 50 μg protamine, (*g*) 120 μg chymotrypsin, (*h*) 70 μg lysozyme, (*i*) 120 μg chymotrypsinogen, (*j*) 150 μg recryst. bovine insulin.

concentrations was formed on the paper strip. The photometric evaluation of chromatograms is presented in Fig. 2.

It is evident that the mixture of lysozyme and egg albumin can be well separated chromatographically using paper fibers as adsorbent and neutral salt solutions as eluants. The same characteristic concentration of sodium chloride solution for resolution of these protein components was established as found on guide chromatograms, run in a parallel manner, for a single protein. No further attempts were made to resolve pure lysozyme into its components (15).

In the next chromatographic experiment an artificial mixture, contain-

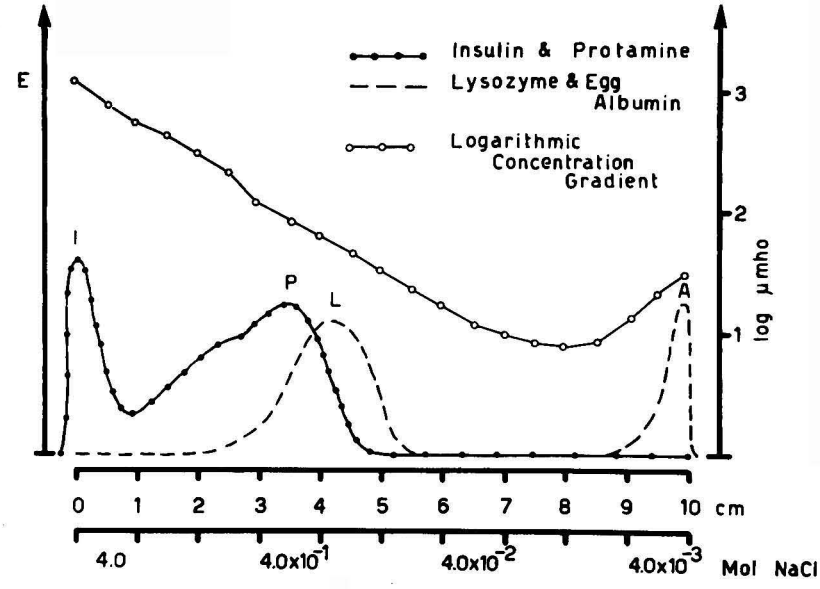

FIG. 2. Photometric evaluations of the chromatograms developed with a sodium chloride gradient 4.00 × 10^{-3} to 4.00 *M*: mixtures of egg albumin (250 μg) + lysozyme (70 μg), and recryst. insulin (150 μg) + protamine (50 μg). Diagram of the logarithmic concentration gradient of sodium chloride on the paper strip, as determined by the conductivity measurement superimposed on the diagrams of photometric evaluations of the paper chromatograms.

ing 50 μg protamine (*Mugil cephalus*) and 150 μg four-times recrystallized insulin dissolved in 0.01 ml of 0.01 *N* HCl was used. The chromatograms were developed with sodium chloride solution using the same experimental technique as described above.

We succeeded in chromatographically separating protamine from insulin by elution with neutral salt solutions only, and no organic solvent or acid (butanol–acetic acid) as suggested by Robinson and Fehr (4) was needed. Insulin was not eluted with any concentration used and remained on the spot of application. It is interesting to note that the electrophoretically detected slight impurity of protamine preparation, obtained by the method of Kossel (14), could be clearly confirmed by this chromatographic method. These impurities, very probably nucleoprotamines, producing a light-absorption maximum at 2640 A, could be removed by filtration of an aqueous protamine solution through sodium hydroxide-treated Amberlite IRA-410.

DISCUSSION

The general characteristics of the proposed paper chromatography technique are the application of paper fibers as adsorbent and consecu-

tive elution of proteins only with a high number of salt solutions of increasing ionic strength thus avoiding organic solvents which may cause denaturation. Since the proteins make up a large group of organic compounds showing different physical and chemical properties, there seems to be but little prospect of finding a general chromatographic method for resolving all protein mixtures. The only prospect for a certain type of paper chromatography is probably in separating a specific protein component of interest, or resolving some particular proteins from a complex mixture containing many more.

The experimental results presented in this communication clearly demonstrate that a protein mixture such as egg albumin and lysozyme or insulin and protamine can be resolved on paper strips by elution with neutral salt solutions. We have observed that the characteristic constant for elution of each protein depends on the salt concentration rather than on the pH of the eluant (8, 10, 16).

In testing the cations belonging to the first group of the periodic system of elements, and lithium halides for influence in paper chromatography, we were not able to establish any regularities.

A fairly uniform salt concentration gradient was formed along the paper strip up almost to the front, where the concentration suddenly increased. This fact persisted after repeated washing of the paper with hydrochloric acid and redistilled water and could be attributed not alone to the salt impurities but also to exchangeable cations held by the carboxyl groups on the cellulose fibers.

The results of our experiments presented in this communication and in previous papers (7–10) can be considered as a real basis for further development of this type of paper chromatography.

SUMMARY

Chromatographic resolution of protein mixtures: egg albumin and lysozyme, and another containing insulin and protamine (Mugil cephalus) was achieved on paper strips using a several step elution with neutral salt solutions of increasing concentrations.

Experimental data concerning the influence of cations and anions on chromatographic separation of proteins on paper strips are presented.

ACKNOWLEDGMENTS

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REFERENCES

1. Porter, R. R., *Brit. Med. Bull.* **10,** 237 (1954).
2. Franklin, A. E., and Quastel, J. H., *Science* **110,** 447 (1949); *Proc. Soc. Exptl. Biol. Med* **74,** 803 (1950); *ibid.* **77,** 783 (1951).

3. JONES, J. I. M., AND MICHAEL, S. E., *Nature* **165,** 685 (1950).
4. ROBINSON, F. A., AND FEHR, K. L. A., *Biochem. J.* **51,** 295 (1952).
5. TAUBER, H., AND PETIT, E. L., *Proc. Soc. Exptl. Biol. Med.* **80,** 143 (1952).
6. BOMAN, H. G., *Nature* **170,** 703 (1952).
7. PIANTANIDA, M., AND MUIĆ, N., *Arch. Biochem. Biophys.* **46,** 110 (1953).
8. PIANTANIDA, M., MENIGA, A., AND MUIĆ, N., *Arch. Biochem. Biophys.* **57,** 334 (1955).
9. MENIGA, A., MUIĆ, N., AND FLEŠ, M., *Scand. J. Clin. Lab. Invest.* **10,** *Suppl.* **31,** 291 (1957).
10. MUIĆ, N., MENIGA, A., AND MELITA FLEŠ, *Arch. Biochem. Biophys.* **77,** 20 (1958).
11. KECKWICK, R. A., AND CANNAN, R. K., *Biochem. J.* **30,** 227 (1936).
12. KUNITZ, M., *J. Biol. Chem.* **164,** 563 (1946).
13. MCDONALD, M. R., *J. Gen. Physiol.* **32,** 39 (1948).
14. KOSSEL, A., *Sitzber. preuss. Akad. Wiss.* **18,** 403 (1896).
15. TALLAN, H. H., AND STEIN, W. H., *J. Biol. Chem.* **200,** 507 (1953).
16. TISELIUS, A., HJERTEN, S., AND LEVIN, Ö., *Arch. Biochem. Biophys.* **65,** 132 (1956).

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A Modified Manometric Vessel for Special Studies on Tissue Metabolism

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INTRODUCTION

When carbon-14-labeled substrates are employed in the study of tissue metabolism, it is often desirable to be able to measure oxygen uptake and $C^{14}O_2$ evolution simultaneously. The $C^{14}O_2$ evolved is most conveniently trapped in a separate compartment of the reaction vessel containing the organic alkali Hyamine with subsequent transfer of the $C^{14}O_2$-containing alkali into a toluene phosphor solution for liquid scintillation counting (2). In conventional manometry a double side-bulb vessel is necessary—one side-bulb containing the C^{14}-labeled substrate to be tipped into the main reaction mixture at the start of incubation, and the other containing acid to be tipped in to end the reaction period and liberate any carbon dioxide dissolved in the incubation medium. The disadvantages inherent in the use of double side-bulb vessels and multiple tippings, as well as the problem of ensuring quantitative removal of viscous organic alkalies from conventional center-well compartments of such vessels, have led us to design a special flask for this type of study.

DESCRIPTION OF FLASK

The flask is a conventional, Warburg-type single side-bulb vessel of Pyrex glass with the following innovations:

(*a*) A port has been added opposite the side bulb (Figs. 1 and 2). The port projects approximately 15 mm from the vessel wall with an inside diameter of approximately 13 mm. The port can be reproducibly sealed with disposable rubber stoppers, held in place by a crimped-on aluminum sealing cap (Figs. 1 and 3). The rim of the port has an outside diameter of approximately 20 mm, making it possible to use commercially available sealing equipment. (The vessel port as designed takes rubber stoppers

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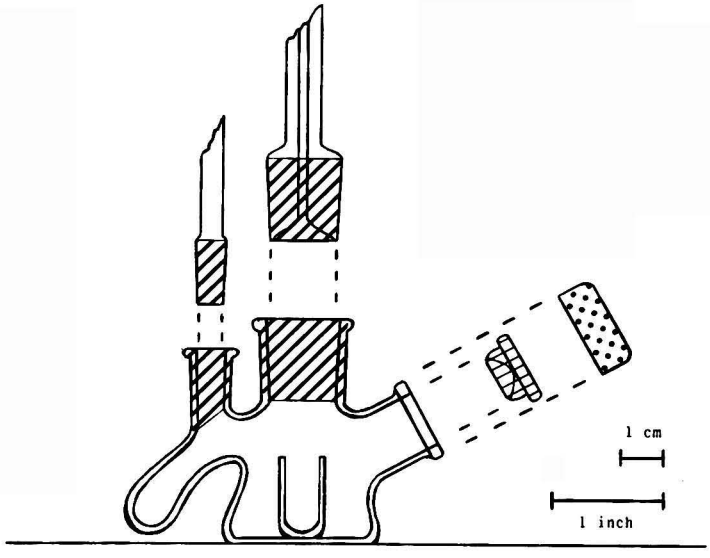

FIG. 1. Scale drawing (side view) of modified flask, showing rounded center well and port opposite the side bulb with exploded view of rubber cap (lightly hatched) and aluminum sealer (stippled). Side-bulb plug and manometer stem are shown above, both being standard taper joints as supplied commercially.

#S-46, 12Y and aluminum seals #20-10TL supplied by T. C. Wheaton Co., Millville, N. J. Crimping on of the latter is done with a Fermpress #H207 supplied by West Co., Phoenix, Penna.)

(*b*) The center well of the vessel has been designed with a gently

FIG. 2. Photograph of actual vessels (side and oblique views) with rubber caps and aluminum sealers (foreground). There is some optical distortion of the regular contour of the center wells (see Fig. 3).

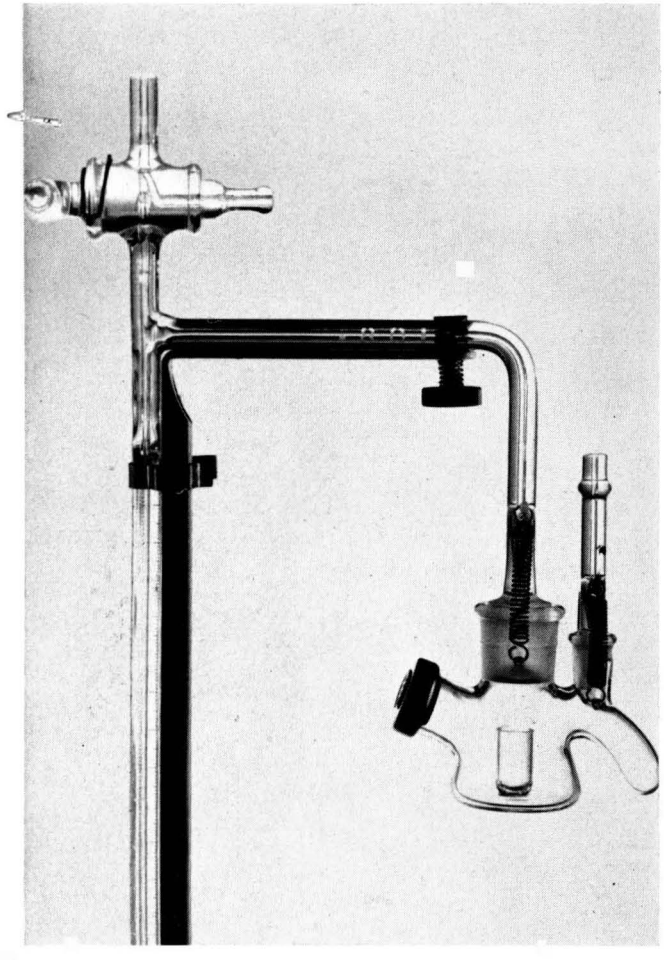

FIG. 3. Photograph of capped vessel in place on its manometer.

rounded bottom (Figs. 1 and 2) to permit easy visualization and removal of viscous fluids by hypodermic syringe needles.

The assembled flask in place on its manometer is illustrated in Fig. 3. The accessibility of all parts of the flask interior—main vessel, side bulb, and center well—to hypodermic needles inserted through the rubber stopper (Fig. 4) is ensured by the wide mouth of the port. The port projects far enough from the vessel wall to permit application of the crimping instrument with adequate clearance.

The total volume of the flask is approximately 20 ml. Standardization data for three different flasks each sealed with three different rubber

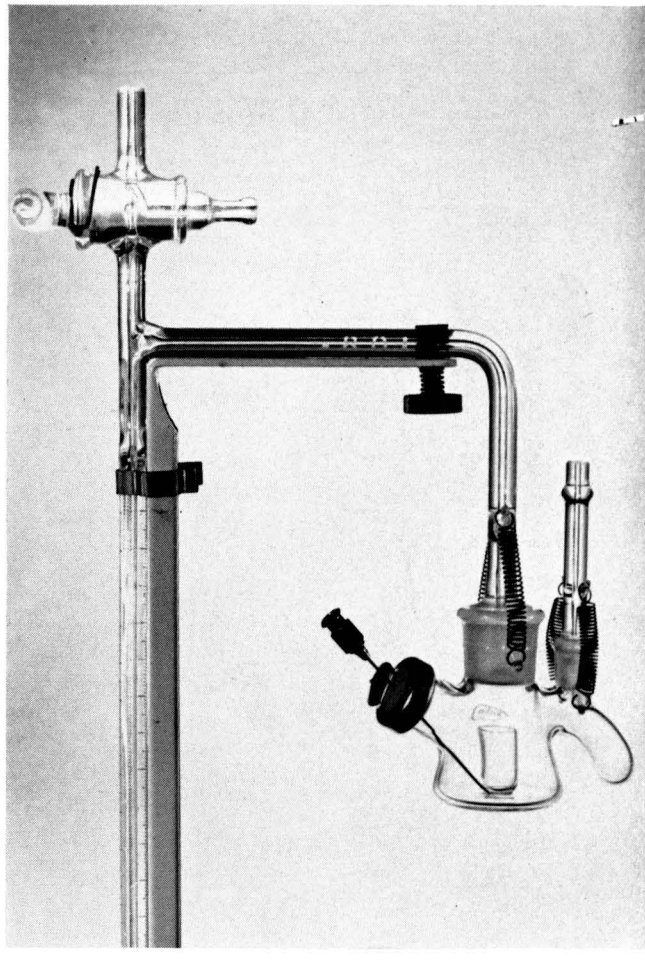

FIG. 4. Photograph illustrating accessibility of vessel interior to hypodermic needle inserted through the exposed, but still crimped-on rubber stopper of the vessel port. Some optical distortion is present in the photograph.

stoppers and aluminum sealers are given in Table 1. Reproducibility is well within limits required for manometric analyses (3).

Cleaning of flasks and removal of residual C^{14} contamination with a hot sulfuric–nitric acid bath have proved entirely satisfactory.

DISCUSSION

The design of the flask retains the features which make it applicable to usual manometric incubation techniques while adding versatility by modification of the center well and introduction of the injection port.

TABLE 1
MERCURY CALIBRATION OF FLASK VOLUMES[a]

	Flask No. A	B	C
	Manometer No. 1	2	3
Cap No. 1	255.4	263.5	259.5
Cap No. 2	255.4	263.5	259.5
Cap No. 3	255.7	263.3	259.5
Mean	255.7	263.4	259.5
S.D.	±0.17	±0.12	±0

[a] Net weight in grams of Hg.

These innovations permit the following criteria for use of $C^{14}O_2$ as an accurate measure of a metabolic process to be met: (*a*) reproducible addition of C^{14}-labeled substrate; (*b*) no contamination of center-well alkali with C^{14}-containing incubation medium; (*c*) addition of acid to stop metabolic reactions and liberate dissolved $C^{14}O_2$; and (*d*) quantitative transfer of $C^{14}O_2$-containing alkali without loss or contamination. The addition through the rubber-sealed port of alkali to collect $C^{14}O_2$, of additional substrates, of acid to stop metabolism, and of osmium tetroxide fixative for electron microscopy of tissue have all been satisfactorily carried out with this vessel.

Some typical results from experiments where oxygen uptake and pyruvic acid-1-C^{14} utilization (as sole substrate) by cat brain mitochondria were simultaneously determined with these vessels are given in Table 2. The standardization data (Table 1) indicate that gas changes

TABLE 2
SIMULTANEOUS DETERMINATION OF OXYGEN UPTAKE AND $C^{14}O_2$ EVOLUTION BY CAT BRAIN MITOCHONDRIA WITH PYRUVATE-1-C^{14} AS SOLE SUBSTRATE[a,b]

Expt.	$-O_2/3$	−Pyruvate (as $C^{14}O_2$)
1	0.55	0.54
2	1.57	1.61
3	0.40	0.39
4	0.93	0.90

[a] Micromoles/flask/30 min.
[b] From unpublished data of G. M. McKhann, and D. B. Tower.

determined manometrically would not be "damped" by the rubber seal. Additional support for this view has been obtained by incubating identical samples of brain tissue in regular flasks and the modified flasks described here and observing identical oxygen uptakes.

The possibilities of using flasks of this design for a variety of other purposes can be anticipated. Where histological examination of incubated tissue samples is subsequently desired, fixatives can be added through the rubber seal of the port and/or the sample can be readily floated out through the opened port without tissue distortion or fragmentation. We have used this method for obtaining electron micrographs of incubated brain slices.[2] The insertion of various types of electrodes through the port should make it possible to adapt these vessels for the type of incubation studies which utilize electrically stimulated tissue samples (1). Finally, various gas measuring electrodes now being developed may be adaptable to *in vitro* metabolic studies with a vessel of this type providing the requisite access for such electrodes.

SUMMARY

A modified Warburg-type manometric vessel designed for simultaneous measurement of oxygen uptake and $C^{14}O_2$ evolution by incubated tissue samples is described. The bottom of the center well of the vessel has been rounded to permit quantitative removal of viscous alkalies used to trap CO_2, and a wide-mouthed port has been added which is sealed by a rubber stopper and aluminum crimped sealer and through which various additions to or removals from the various interior compartments of the vessel can be made. Calibration data and various potential uses are described.

ACKNOWLEDGMENT

We are indebted to the American Instrument Co., Silver Spring, Md., for construction of prototype vessels, based on our design.

REFERENCES

1. McIlwain, H., "Biochemistry and the Central Nervous System," 2nd Ed., p. 45. Little Brown and Co., Boston, 1959.
2. Passman, J. M., Radin, N. S., and Cooper, J. A. D., *Anal. Chem.* **28**, 484 (1956).
3. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., "Manometric Techniques," 3rd ed., p. 46. Burgess Publ. Co., Minneapolis, 1959.

[2] Wanko, T., Gavin, M. A., and Tower, D. B., unpublished data.

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