Summary. A simple enzymic-diffusion reactor flask is described for the enzymic assay of amino acid amidases from tissues grown *in vitro*. The procedure is rapid, precise, and applicable for measuring enzymic reactions which involve the determination of ammonia.

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Technics for Separation of Plasma Cholesterol Esters for Determination of Iodine Value, and of Cholesterol.* (24197)

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To investigate the effects of ingestion of such preparations as ethyl linoleate and ethyl oleate upon fatty acid composition of the plasma cholesterol esters, new methodology was required. Such methods are here reported, as well as a modification of the Schoenheimer-Sperry technic for plasma cholesterol determination which substitutes relatively stable colorimetry for the Liebermann-Burchard reaction.

I. *Separation of cholesterol esters from other plasma lipids.* A number of adsorbents were tried in conjunction with various solvents or mixtures of solvents. Activated and non-activated silicic acid was used during the phase of standardization. In our hands, equal parts by weight of celite and "non-activated" silicic acid have produced the best separation. Many investigators using silicic acid for separation of the different lipid components advise certain mesh size and also "activation" of the silicic acid by heat or by using dehydrating solvents. Such procedures increase the efficiency of the column considerably where maximum efficiency is desired, but these additional steps are unnecessary and time-consuming when one is separating small quantities of lipids. Silicic acid, as received from the manufacturer,† contains about 22% water. The column consists of a 25 ml burette with self-lubricating teflon plug valve assembly,‡ and a 125 ml flask fused to the top of the burette as a reservoir. A glass wool plug is introduced, and the celite-silicic acid mixture is added, with tamping, to a height of 10 cm. An aliquot of the plasma filtrate (in acetone alcohol) containing approximately 20 to 30 mg of total lipid is evaporated just to dryness under partial vacuum at approximately 50°C, and the lipids dissolved immediately in a small amount of petroleum ether (5 ml). This is poured upon the *dry* column. (In a recent report, Hirsch also recommends addition of the dissolved lipids to the column in the dry state)(1). The test tube containing the lipids is washed 3 times with 2 ml portions of petroleum ether. These are added to the column and allowed to flow in without pressure until the liquid has been completely absorbed. The cholesterol esters are eluted with a total volume of 100 ml of petroleum ether under slight positive pressure to maintain a flow rate of approximately 1 ml per

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† J. T. Baker Chemical Co., Phillipsburg, N. J.

‡ "Ultramax", Fischer and Porter Co.

minute. Efficiency of the separation is checked by comparing values for free and total cholesterol determined on the eluate with those obtained by direct analysis of the original acetone-alcohol filtrate and with values for total fat determined by Bragdon's method (2) on the eluate. Values for cholesterol in a series of 29 unselected eluates differed from direct determination for esterified cholesterol on the same filtrates by an average of 7.3 ± 5.1§ mg %, which was 4.8 ± 3.7% of the direct values. No free cholesterol was present in the eluates, nor were other lipid fractions eluted with the cholesterol esters. This last fact was confirmed by the good agreement shown between values for total fat determined directly on aliquots of eluates and total fat values calculated from the ester cholesterol using the factors given by Bragdon(2) for cholesterol esterified with a C_{18} fatty acid. In the same series of 29 samples as above, the mean difference between total fat determined directly and calculated was 9.00 ± 3.00§ mg % or 6.2 ± 1.12%§ of the direct value. The cholesterol esters constitute approximately 35% of the total lipids in normal plasma. However, in some patients with xanthomatosis, in spite of the marked absolute increase in cholesterol, the cholesterol esters may represent only 10%, the bulk of the lipid being neutral fat. Satisfactory separation has been obtained even in such plasma.

II. *Colorimetric determination of the iodine value of fats.* The pyridine sulfate dibromide reagent of Rosenmund-Kuhnhenn as modified by Yasuda(3) has been approved by Bloor (4) for determination of the iodine value of blood lipids. Using this procedure, the theoretical iodine value is obtained for cholesterol and a number of other blood lipids. Conversion of this titrimetric procedure to a simple colorimetric method enables one to use smaller amounts of material for analysis and increases sensitivity as well as accuracy of the technic. A known amount of pyridine sulfate dibromide is added to the lipid which has been dissolved in chloroform. As the fatty acids are saturated with bromine, the yellow color disappears. This extinction is measured colorimetrically. Oleic acid in amounts ranging from 1 to 6 mg is likewise treated with the reagent. The standard curve thus prepared is used to calculate iodine value of the lipid, using 89.9 for the iodine number of oleic acid.

Reagents. 1. 0.10 N pyridine sulfate dibromide. Measure 16.5 ml (16 g) of purified pyridine and 10.9 ml (20 g) of concentrated sulfuric acid into separate flasks each containing about 40 ml glacial acetic acid. Add these reagents slowly, with cooling, then combine. Into a third flask containing 40 ml glacial acetic acid, add 5 ml (16 g) bromine. Add this to the first mixture and bring the entire volume to 2000 ml with glacial acetic acid. Store in a dark bottle. This reagent is good for one year. The working solution should be prepared daily by adding 3 parts (by volume) of glacial acetic acid to 1 part of the stock pyridine sulfate dibromide reagent. 2. Chloroform (reagent grade). 3. Glacial acetic acid.

Procedure. Dissolve fat to be analyzed (containing preferably between 2 and 4 mg lipids) in 5 ml chloroform. Add 3 ml working dibromide reagent, mix, filter rapidly through No. 40 Whatman filter paper into test tubes. Cap tightly with parafilm and place in the dark. (Avoid contact with parafilm). At the same time prepare standards and 2 blanks, one containing 5 ml chloroform and 3 ml pyridine sulfate-dibromide solution and a second blank containing 5 ml chloroform and 3 ml glacial acetic acid. Read after 30 minutes in a photoelectric colorimeter using No. 42 (blue) filter. Set the colorimeter to the zero mark with the chloroform-acetic acid blank. Then read the reagent blank, standards, and the unknowns. Do not expose the tubes to sunlight or to fluorescent light. Fig. 1 shows a typical standard curve for oleic acid.

Calculations. If the quantity of fat used for iodine value determination is not known, an aliquot should be taken for total fat analysis. Bragdon's method yields satisfactory results. Example: Fat content of sample

§ Standard error of mean.

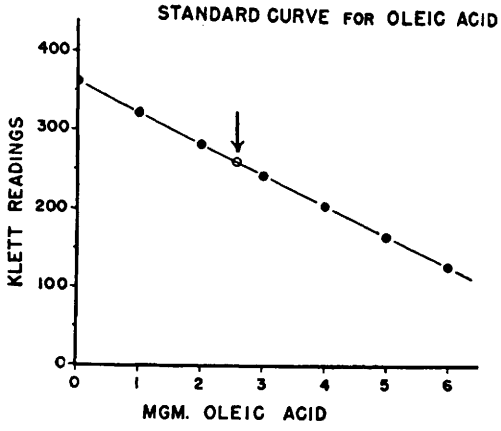

FIG. 1.

3.50 mg. Klett reading 260 = 2.58 mg as oleic acid from the curve, $\frac{2.58}{3.50} \times 89.9 = 66.3$ iodine number of fat.

$$\frac{\text{Reading from curve as mg of oleic acid}}{\text{Total fat}} \times 89.9 = \text{iodine number of unknown.}$$

Using this simple color extinction procedure we have been able to obtain the theoretical iodine value for cholesterol and the reproducibility in duplicate analysis is excellent compared with the more difficult iodine titration using starch as the indicator. In a series of 50 samples, the average variation between duplicate analyses was ± 0.4%.

III. *Modified Schoenheimer-Sperry cholesterol method.* The cholesterol method here reported differs from that described by Schoenheimer and Sperry(5) chiefly in regard to the color development and the solvent wash. In the Schoenheimer and Sperry method, after precipitation and washing of the cholesterol as the digitonide, the Liebermann-Burchard reaction is used, with development of a transient color. This reaction occurs only with unsaturated sterols. Orcinol reacts with digitonin rather than with unsaturated sterols and yields a color which is stable for more than 2 hours (see below). In development of the Schoenheimer-Sperry technic, the micro-colorimetric method was shown to be in agreement with the gravimetric cholesterol digitonide method of Windaus. The present method yields values which agree well with determinations by the Schoenheimer-Sperry method. Representative comparative determinations are shown in Fig. 2. In a series of 12 determinations by both the Schoenheimer-Sperry and orcinol methods, the mean difference was 5.8 ± 1.12§ mg % or 2.6 ± 0.49%§ of the Schoenheimer-Sperry values for total cholesterol, and 4.7 ± 0.88 mg %§ or 7.4 ± 1.51%§ for free cholesterol.

Reagents. 1. *Orcinol.* 3 g orcinol is added to 1.00 liter of glacial acetic acid (reagent grade). Then 0.4 ml superoxol is added and mixed. This reagent is good for many months but should stand for 4 weeks before being used. 2. *Isopropyl wash.* Add 50 ml water to 450 ml of 99% isopropyl alcohol. A pinch of cholesterol digitonide is then added, and the solution is shaken well. The amount used should be filtered before use. 3. *Ferric chloride.* Add 1 ml of 10% ferric chloride to approximately 500 ml distilled water, then add 500 ml concentrated hydrochloric acid (reagent grade) and mix.

Color development using orcinol reagent. After precipitation of the cholesterol with digitonin,‖ centrifugation and pouring of the solvent containing the excess digitonin, the tubes are inverted and placed on clean filter paper for complete drainage. The precipitate and the sides of the tube are then washed with 3 ml of the isopropyl wash. The tubes are

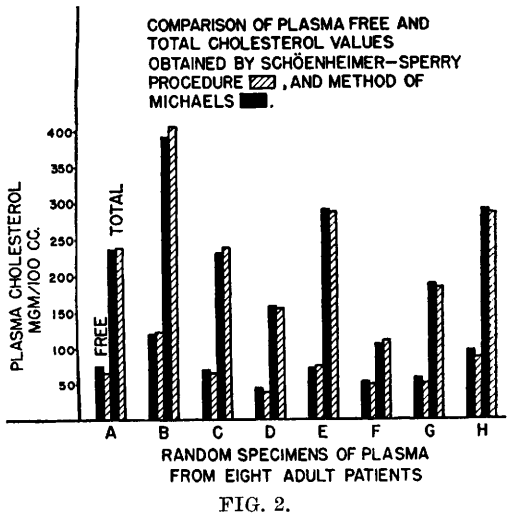

FIG. 2.

‖ Reagent Digitonin, Merck and Co., Inc., Rahway, N. J.

again centrifuged, decanted, inverted, and allowed to drain for at least 5 minutes. To the washed digitonide precipitate add 5 ml of the orcinol reagent and 2 ml of the acid ferric chloride reagent. Cap the top of the tube with parafilm and mix thoroughly. It is best to set up at least 2 standards and run them through the entire procedure. We use a stock standard containing 2.50 mg of cholesterol per ml, and a working standard containing 0.10 mg of cholesterol. Both of these standards are made up in acetone-alcohol and are stable indefinitely. Two blanks are prepared, one containing orcinol and ferric chloride, and the second, in addition, containing digitonin (1 ml of a 1% solution). The latter is included to check for completeness of removal of excess digitonin by the isopropyl wash. The two blanks should yield identical readings, within the limits of accuracy of the colorimeter. All the tubes are heated in a boiling water bath for 20 minutes and cooled. It is necessary for the water bath to be evenly heated throughout. Differing temperatures in different sections of the water bath will give poor results. The colorimetric readings are made at 540 mμ wave length. Some change in color will occur if the tubes are left in direct sunlight; otherwise, no demonstrable change occurs if the readings are made within 2 hours after cooling. The number of duplicate determinations of free and total cholesterol carried out in this laboratory by this method exceeds ten thousand. Duplicates vary by less than ± 3%.

Summary. 1. A method for quantitative chromatographic separation of cholesterol esters from other blood lipids is presented. 2. A colorimetric micromethod for determination of the iodine number of plasma lipid is described. 3. A more stable reagent and a more stable color reaction than the Liebermann-Burchard reaction for the determination of plasma cholesterol is presented. 4. Employment of the above methods, together with determination of total lipids by the method of Bragdon, permits determinations of net unsaturation of the fatty acids esterified with cholesterol in the plasma.

Grateful acknowledgement is made to Merck, Sharp and Dohme for generous supplies of Digitonin.

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Plasma Cholesterol Ester Fatty Acid Composition in Relation to Diet.* (24198)

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In our previous paper it was noted that in a young normal adult male subject during the intake of 80 g of ethyl linoleate daily, the level of plasma cholesterol fell in a remarkable fashion and the iodine value of the cholesterol ester fatty acids at times exceeded 250(1). The present report includes data dealing with fatty acid composition of the plasma cholesterol esters in 4 male and one female subjects during the course of controlled studies.

Methods. Cholesterol esters were fractionated by column chromatography as described by Michaels, *et al.*(2) and unsaturated fatty acids determined by the Michaels modi-

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