# Automated Techniques in Serum Lipid Analysis[1]

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**Abstract**

Semi- and partially-automated procedures for the analysis of serum lipids directly on the initial solvent extract which have been developed recently by the author and other investigators are reviewed. Techniques are discussed which involve either colorimetric or fluorimetric estimation of the concentration of the individual lipid components of serum such as total cholesterol, acyl esters, phospholipids, triglycerides and free fatty acids. The results obtained with the automated methods are presented and evaluated by comparison to established manual methods now routinely used in clinical laboratories, and the advantage as well as the limitations of the automated procedures are indicated.

DURING THE PAST DECADE considerable changes have been made in the techniques of analysis used in analytical and clinical laboratories. With the increased demand for rapid and reproducible methods of analysis of considerable numbers of specimens, attention has been directed away from the many excellent but time-consuming manual methods requiring highly skilled technical assistance. The advent of automated analytical equipment (Technicon International Ltd., Chauncey, N.Y.) based on the principle of reaction in a flowing stream as opposed to classical methods requiring the reaction of discrete quantities of reactants in a test tube, has led to the development of new methods, or the modification of existing methods, which have not only satisfied the requirement of speed of analysis of large numbers of specimens but have also reduced the risks of human error and considerably improved the reproducibility of the estimations.

While it has not been possible to completely automate the analysis of serum lipids directly on specimens of serum, methods have been developed which require the initial manual preparation in various solvent systems of serum lipid extracts which may then be submitted to automated analytical procedures using the AutoAnalyzer (Technicon, Ltd.)

In the present paper semi- and partially-automated procedures developed by the author and other investigators are reviewed and evaluated against established manual methods. Techniques are discussed for the colorimetric and fluorometric estimation of the concentration of serum components such as total cholesterol, acyl esters, phospholipids, triglycerides and free fatty acids.

## Serum Cholesterol Concentration

### Colorimetric Estimation

The colorimetric estimation of cholesterol was the first of the serum lipid analyses to be subjected to automation, and a method developed by the Technicon Corporation (1), as reported by Levine and Zak (2), has been widely used in clinical laboratories. This method which was developed from the manual technique of Zlatkis, Zak and Boyle (3) is based on the reaction between concentrated sulfuric acid and ferric chloride in acetic acid with steroids such as cholesterol, which have a 5-ene, 3$\beta$-ol grouping. In the automated procedure (1,2), an isopropanol extract of serum lipids (1:10) is caused to react with a premixed reagent at 95C, and the extinction of the resulting solution measured in a flow cell at 520 m$\mu$. This method lacks a high enough degree of precision and reproducibility, and very often correlation is poor, when results are compared with those obtained by the manual techniques of Sperry and Webb (4), and Abell, Levy, Brodie and Kendall (5). Block, Jarret and Levine (6) have recently modified the automated procedure by employing a completely anhydrous ferric chloride-sulfuric acid-acetic acid reagent which is preheated before reacting with a 1:20 isopropanol extract of serum lipids, and the extinction of the resulting solution is measured at 550 m$\mu$. These modifications have considerably improved the precision and reproducibility of the procedure; however, correlation of results with those obtained by the above manual methods (4,5) is not yet sufficiently satisfactory, and lower values are usually obtained with the automated procedure. The lack of correlation between the two methods is mainly due to the different color yields given by the reaction between the ferric chloride reagent and free cholesterol or its esters. In my own laboratory I have found that cholesteryl esters such as oleate and linoleate produce 90–95% of the response given by free cholesterol. In the manual methods (4,5) the esters are initially saponified, and the anhydrous color reagent reacts either with free cholesterol or its digitonide.

Attempts to date to include saponification in the automated procedure have been unsuccessful owing to the precipitation of salts in the anhydrous color reagent. This problem has been circumvented in my own laboratory by employing a manual saponification with 0.5 N alkali followed by removal of proteins, pigments and a major proportion of the salts with silicic acid. The isopropanol extract is then subjected to the modified automated procedure (6) and results have shown excellent correlation with method of the Abell et al. (5), as shown in Table I.

The saponified extracts are obtained by the following procedure: 0.5 ml of serum is added to 4.5 ml isopropanol containing 3% KOH (0.5 N) and the mixture is saponified at 60C for half an hour. 5 ml of isopropanol containing 6% glacial acetic acid (1 N) are added, well mixed on a vibromixer, and allowed to stand for five minutes. 1.5 g of silicic acid (Mallinckrodt, 100 mesh for chromatography) is added, again well mixed as before, and the mixture allowed to stand for 10 minutes before being centrifuged at 1000 rpm for 10 minutes. The clear supernatant is decanted into sample cups for analysis, or stored at 4C until sufficient samples are obtained. Cholesterol standards containing 10 to 40 mg cholesterol per 100 ml are prepared in isopropanol containing 6% (w/v) glacial acetic acid, and are analyzed in a similar manner: 0.5 ml isotonic saline, 4.5 ml of alkaline isopropanol, and 5 ml of each standard are mixed; 1.5 g of silicic acid added, and the mixture well shaken and centrifuged as before.

[1] Presented at the Symposium, "Automation in Lipid Research," AOCS Meeting, Los Angeles, April 1966.

TABLE I

Comparison of Serum Cholesterol Levels Estimated by Manual Procedure of Abell et al. (5) and by Automated Procedure of Block et al. (6), with and without Manual Saponification

| Specimen No. | Manual[a] (Abell et al.) | Antoanalyzer[a] With sap. | Δ | Without sap. | Δ | % Recovery without sap. |
|---|---|---|---|---|---|---|
| 1 | 228 | 220 | − 8 | 196 | −24 | 89 |
| 2 | 275 | 260 | −15 | 224 | −36 | 86 |
| 3 | 211 | 214 | + 3 | 194 | −20 | 90 |
| 4 | 219 | 225 | + 6 | 188 | −37 | 84 |
| 5 | 258 | 268 | +10 | 237 | −31 | 89 |
| 6 | 188 | 186 | − 2 | 161 | −25 | 87 |
| 7 | 190 | 186 | − 4 | 163 | −23 | 88 |
| 8 | 350 | 369 | +19 | 329 | −40 | 89 |
| 9 | 367 | 365 | − 2 | 318 | −47 | 87 |
| 10 | 203 | 196 | − 7 | 172 | −24 | 88 |
| Mean | 248.9 | 248.9 | 0 | 218.2 | −30.7 | 87.7 |

S.D. of single estimation $\frac{\Sigma \Delta^2}{2N} = \pm 6.6$

On 80 specimens; range 156 to 369 mg cholesterol per 100 ml serum.
Mean by automated procedure (6) with saponification: 243.7 mg.
Mean by manual procedure of Abell et al. (5): 242.4 mg.
Mean difference + 1.4 mg. Standard deviation of single estimation ± 7.55 mg.

[a] Means of duplicate estimations; concentrations in mg per 100 ml serum.

The series of standards used correspond to values of 100 to 400 mg cholesterol per 100 ml of serum when analyzed by the above procedure, and a linear relationship between concentration and extinction coefficient is obtained throughout the range.

Operating conditions are as described by Block et al. (6), except that 95% aqueous isopropanol is used as a blank wash in the sampler 2 reservoir, and 30 specimens are analyzed per hour using a 30, 1:1 cam. Specimens and wash are sampled alternately for 60-second periods. This is sufficient to allow peaks to reach a steady state, and to ensure efficient wash between specimens.

### Fluorometric Estimation

Cholesterol has also been estimated by an automated fluorometric method (7) which is extremely sensitive and may be used for the analysis of serum lipoprotein fractions having low cholesterol concentrations, of the order of 10 to 20 mg per 100 ml serum. The automated procedure (7) is based on the method of Albers and Lowry (8), as modified by Carpenter, Gotsis and Hegsted (9), in which cholesterol after treatment with sulfuric acid in acetic anhydride and 1:1:2 trichloroethane is irradiated with light of 546 mμ, and an orange-red fluorescence is produced with a maximum at 590 mμ. The reaction suffers from the same defect as the colorimetric method, since cholesteryl esters such as oleate produce only 80% of the fluorescence response given by free cholesterol, and manual saponification is required before automated analysis of extracts. Any of the recognized serum lipid extraction techniques may be used for obtaining the lipid extract which, after saponification with alkali of not less than 0.5 N ionic strength, is neutralized, taken to dryness and reconstituted in 1:1:2 trichloroethane. The evaporation stage is essential since the estimation is highly sensitive to traces of alcohol or water which cause quenching of the fluorescence. The extraction and saponification procedure used for the colorimetric method described in the previous section has proved suitable for the fluorometric method; and an aliquot of the saponified and neutralized isopropyl alcohol extract is blown to dryness and reconstituted in the same volume of 1:1:2 trichloroethane. The small residue of salts which are insoluble in the solvent is removed by filtration or centrifugation.

The fluorometric procedure, which is approximately ten times as sensitive as the colorimetric method, had also proved useful for the analysis of fractions obtained from silicic acid chromatography of lipids. In this case saponification is not essential since cholesteryl oleate standards may be used for ester fractions, and cholesterol standards for fractions containing only free cholesterol. The flow diagram shown in Fig. 1 has been used for the automated stage of the analysis with the following reagent: 1,1,2 trichloroethane (*A*) 100 vol; acetic anhydride (*B*) 20 vol, and concentrated $H_2SO_4$(C) 5 vol. *A* and *B* are mixed, cooled to −15C, and *C* is added shortly before use. The reagent is stored in an amber glass container fitted with a drying tube ($CaCl_2$).

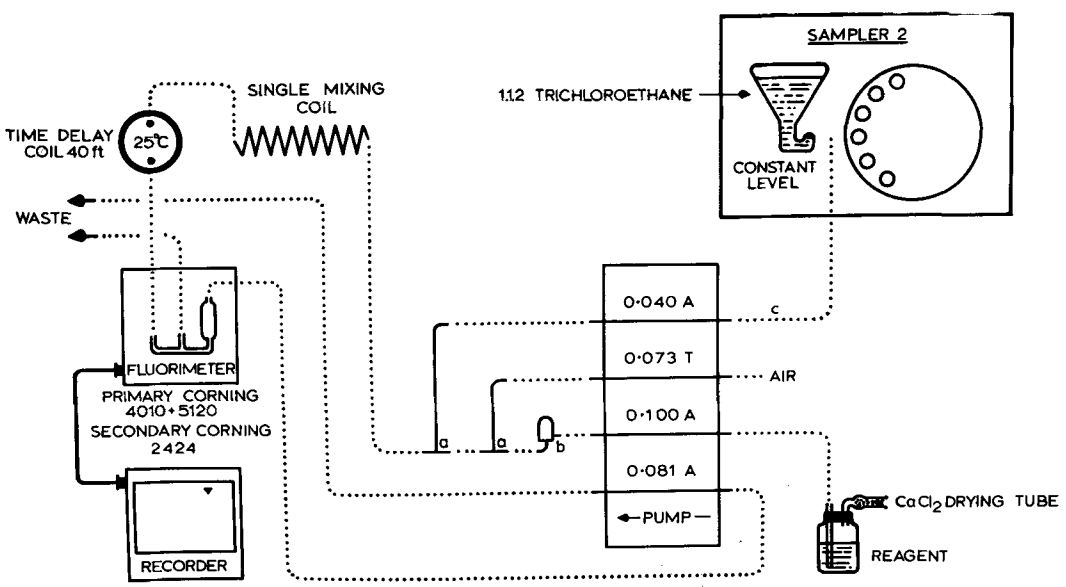


FIG. 1. Flow diagram for fluorometric cholesterol determination. *T*, Tygon and *A*, Acidflex pump tubing; bore as indicated (inches). Additional abbreviation: *a*, glass h-piece, capillary side arm; *b*, glass pulse suppressor; *c*, polythene tubing, 0.040 in. I.D.; a 0.005 in. I.D. tubing pulse suppressor is included in the air line (not shown). All other delivery tubing: polythene 0.060 in. I.D., 0.025 in. wall thickness.

Acidflex tubing is used for all lines in the pump, except that supplying air, with internal diameters as shown in the diagram. The proportion of reagent (2.75 ml/min) to sample (0.60 ml/min) must be held within fairly constant limits for reproducible analytical conditions to be maintained. Since considerable variablility in delivery rate has been found in different batches of acidflex tubing of a given internal diameter, care must be taken to test the tubing before use.

Polyethylene tubing (0.060 in. I.D.) is used throughout for delivering reagent of 1:1:2 trichloroethane from the pump. A constant level source of blank solvent wash (1:1:2 trichloroethane) is provided in an inverted flask instead of the normal reservoir of the sampler 2, and the coverplate normally supplied is fitted with a polyethylene disc taped to its undersurface. Glass or polyethylene sample cups (0.5 in. internal diameter, 1 in. internal depth, and 3 ml capacity) are used instead of the usual polystyrene cups supplied with the Auto-Analyzer.

N. B.: Due to the toxicity of 1:1:2 trichloroethane, it is essential to lead all waste lines directly into a sink which is continuously flushed with running water, and, where possible, the equipment should be set up in a well-ventilated fume hood.

Specimens are sampled at the rate of 30/hr using a 30, 1:1 cam, and operating conditions correspond to 60-sec sampling of the specimen (approximately 0.6 ml of a 1:20 plasma extract) followed by 60 sec of blank.

For measurement of fluorescence any sensitive fluorometer adapted for recording may be used. The E.I.L. Model 27 A (Electronic Instruments Ltd., Richmond, Surrey, England) fitted with a flow cell and adapted for recording may be used at maximum sensitivity (Scale 1) for the concentration range 5 to 25 mg cholesterol per liter of extract; at approximately one third of this sensitivity for concentrations of up to 75 mg cholesterol per liter (Scale 2); and at a tenth of the original sensitivity for cholesterol concentrations of up to 250 mg per liter (Scale 3). For 1:20 serum lipid extracts these ranges correspond to 10 to 50; up to 150; and up to 500 mg per 100 ml of serum, respectively. The primary filter used is a combination of Corning 4010 and 5120 filters, and the secondary filter is a Corning 2424.

The relationship between fluorescence and cholesterol concentration is linear for concentrations of not more than 150 mg cholesterol per liter of extract (corresponding to 300 mg/100 ml serum); with higher concentrations the divergence from linearity becomes considerable. Results obtained from saponified extracts by the colorimetric (6) or fluorometric (7) procedures have shown excellent correlation, as shown in Table II.

TABLE II

Comparison of Serum Cholesterol Levels Estimated on the Same Saponified Extracts by the Automated Colorimetric (6) and Fluorometric (7) Procedures

| Specimen No. | Colorimetric (mg/100 ml) | Fluorometric (mg/100 ml) | Difference (Δ) |
|---|---|---|---|
| 1 | 63 | 61 | −2 |
| 2 | 55 | 57 | +2 |
| 3 | 73 | 70 | −3 |
| 4 | 63 | 64 | +1 |
| 5 | 57 | 59 | +2 |
| 6 | 64 | 61 | −3 |
| 7 | 68 | 69 | +1 |
| 8 | 132 | 136 | +4 |
| 9 | 113 | 111 | −2 |
| 10 | 104 | 108 | +4 |
| 11 | 112 | 114 | +2 |
| 12 | 102 | 100 | −2 |
| 13 | 127 | 132 | +5 |
| 14 | 148 | 143 | −5 |
| 15 | 226 | 218 | −8 |
| 16 | 198 | 204 | +6 |
| 17 | 207 | 211 | +4 |
| 18 | 279 | 270 | −9 |
| 19 | 365 | 373 | +8 |
| 20 | 390 | 381 | −9 |
| Mean | 147.3 | 147.1 | −0.2 |

S.D. of a single estimation $\frac{\Sigma \Delta^2}{2N} = \pm 3.4$

All values are the means of duplicate estimations.

Specimens 1–7, and 8–14 were obtained from pigs on soya oil and tallow diets, respectively; 15–20 were from human subjects.

## Serum Acyl Ester Concentration

An automated method for the colorimetric determination of acyl esters in serum has recently been reported by Antonis, Platt and Thorp (10). The method requires the preliminary extraction of serum lipids with isopropanol and separation of the precipitated proteins. The lipid extract is then analyzed automatically by a colorimetric procedure based on the reaction between acyl esters and hydroxylamine in alkaline solution, forming hydroxamic acids which produce highly colored ferric-chelate complexes with ferric ions in acid solution. Since its original introduction as a spot test by Feigl et al. (11), the reaction has been widely applied in numerous manual methods as an indirect method of assessing serum triglyceride concentrations in specimens in which the cholesteryl ester and phospholipid concentrations have previously been determined. Owing to the number of assumptions which have to be made for this calculation, direct methods for the estimation of serum triglyceride levels have been preferred; however, the estimation of the total esters provides a useful means of studying the overall changes in serum total lipid concentrations brought about in metabolic studies by dietary and other manipulation.

In the semiautomated procedure (10) the protein-free lipid extract, air and an alkaline hydroxylamine reagent are pumped together through a time-delay coil at 25C for a period of approximately 20 min. After leaving the coil the mixture encounters an acid solution of ferric perchlorate and finally passes through the flow-cell of a colorimeter, where the extinction of the solution is measured at 520 mμ and recorded. Details of the operating procedure, reagents and standards have been documented by the authors (10), who have also discussed the influence of a number of factors on the precision of the method.

The standard error of the semiautomated method was 0.36 meq over the range of acyl ester concentration of 7 to 20 meq/liter of serum. Correlation with a manual procedure (12) was good, with an average difference between the two methods of 0.24 meq, and a standard error of differences between paired items of 0.076 meq.

## Serum Phospholipid Concentration

Whitley and Alburn (13) have recently reported a semiautomated method for the determination of serum phospholipids in which the manual technique of Zilversmit and Davies (14) has been modified and adapted for use with the Technicon AutoAnalyzer. Proteins and lipoproteins to which the phospholipids are bound, are precipitated from plasma (or serum) by the addition of trichloroacetic acid. The suspension is centrifuged, the supernatant decanted, and

the precipitate dissolved in dilute alkali. The solution is sampled and digested in a continuous flow system with sulfuric acid, using perchloric acid and vanadium pentoxide as catalysts for the oxidation. Inorganic phosphate is determined automatically on the digest by the molybdate procedure of Lundgren (15) using hydrazine sulfate as the reductant and heating at 95C; the extinction being measured at 815 mμ. The automated procedure which is sensitive, accurate and less time-consuming than manual procedures, has been used for the analysis at the rate of 10 or 20 samples per hour.

## Preparation of Phospholipid-Free Extracts

Semiautomated procedures have recently been described for the determination of plasma free fatty acid (FFA) (16) and serum triglyceride concentrations (17,18). These methods are dependent on the preliminary manual preparation of phospholipid-free plasma or serum lipid extracts, which are then analyzed automatically. While serum lipids may be separated from each other by well-established methods of silicic acid column or thin-layer chromatography, these methods are too time-consuming for application to the routine investigation of serum lipids in clinical laboratories. Alternative methods have been developed, based on these chromatographic principles, in which plasma or serum, silicic acid or zeolite, and specific solvents are mixed together. The precipitated solids are usually removed by centrifugation, and the clear supernatant phospholipid-free lipid extract decanted and either subjected to further treatment or analyzed directly.

Van Handel and Zilversmit (19) used zeolite and chloroform to prepare a phospholipid-free chloroform extract; Mendelsohn and Antonis (20) have similarly recommended the use of silicic acid and diisopropyl ether; while Lofland (17) and Kessler and Lederer (18) have used zeolite and isopropanol.

Treatment of plasma (or serum) with diisopropyl ether and silicic acid (16,20) has provided a simple and efficient method for the extraction of neutral lipids and free fatty acids (FFA), free from other plasma components including phospholipids, glycerol, sugars and intermediary metabolites such as lactic acid, etc. Silicic acid powder finer than 100 mesh (Baker) was originally recommended (20) for efficient extraction; however, the addition of 0.1% acetic acid to diisopropyl ether has enabled slightly coarser grades (Mallinckrodt; containing approximately 25% of 60–100 mesh) to provide equally efficient extraction of lipids from human plasma. Studies with pig plasma in my laboratory, on the other hand, have shown that the lipids appear to be more strongly bound to protein than in human plasma, and a particle size of finer than 100 mesh is necessary for efficient lipid extraction even in the presence of 0.1% acetic acid. Similar findings have been reported by Cheng and Zilversmit (21) in their studies with rat plasma, using zeolite and chloroform.

In the silicic acid/diisopropyl ether extraction procedure (16, 20), silicic acid (4 g) is slurried with diisopropyl ether containing 0.1% acetic acid (25 ml) in a stoppered test tube containing a few large glass beads. Serum (1 ml) is added, and the mixture is well shaken on a vibromixer until free from lumps. The precipitate is separated by centrifugation, and the supernatant taken off into a separate tube. Aliquots are either taken to dryness or reconstituted in a suitable solvent before subsequent analysis.

Phospholipid-free lipid extracts prepared with zeolite (or silicic acid) and chloroform (19,21) are free from the nonlipid components described before, and are equally suitable for the determination of neutral lipids in plasma or serum. For the determination of FFA concentration, however, this method of extraction has proved unsuitable, yielding low and variable recoveries of FFA from plasma (16).

In the isopropanol-zeolite extraction procedure of Lofland (17), 1 ml of serum is extracted with 11 ml of isopropanol in the presence of 1 g of zeolite as the phospholipid adsorbent. While essentially all of the phospholipid (±96%) is removed by adsorption to zeolite, the presence of approximately 8% of water in the mixture does not prevent the simultaneous extraction of other serum components such as glycerol, sugars, lactic acid and other intermediary metabolites. These interfere in the estimation of glyceride-glycerol, yielding results which are considerably higher than those obtained by extraction with chloroform or diisopropyl ether. Kessler and Lederer (18) have improved the procedure by treating 1:20 serum/isopropanol extracts (i.e., containing 5% water) with a zeolite/Lloyd reagent (fuller's earth) for complete removal of phospholipids, and sugars are removed by a copper-lime treatment. Free glycerol, lactic acid and other metabolites, however, are still present in the isopropanol extract, and duplicate determinations must be carried out with and without saponification of the glycerides. Results obtained by difference are essentially similar to those found using diisopropyl ether or chloroform extracts.

## Serum Triglyceride Concentration

Methods currently used for the direct determination of serum triglyceride concentration are based on the chemical or enzymatic estimation of glyceride-glycerol obtained by saponification of phospholipid-free serum lipid extracts.

### Chemical Methods

*Colorimetric.* The colorimetric determination of glyceride-glycerol is based on its oxidation to formaldehyde with periodate according to the procedure of McFadyen (22) as modified by Lambert and Neish (23). Excess periodate and iodate are reduced to iodide with arsenite, and formaldehyde is estimated colorimetrically after reaction with chromotropic acid. The method was applied manually for the estimation of serum triglyceride concentration by Van Handel and Zilversmit (19) and Carlson and Wadström (24), and has been successfully automated by Lofland (17) who has documented full details of reagents, standards and conditions of operation. In my own laboratory I have successfully used the above automated procedure after manual saponification of the silicic acid-diisopropyl ether extract obtained as described in the previous section. Minor modifications have also been introduced in order to decrease the standard error of replicate analyses. These are indicated in the flow diagram shown in Fig. 2, which is essentially similar to that described by Lofland (17). It differs mainly in that after reaction with periodate and arsenite the reaction mixture is redrawn through the pump before reacting with the chromotropic acid reagent, and air which was eliminated as the debubbler is reintroduced. This modification prevents errors introduced by pressure buildup in the time delay coil.

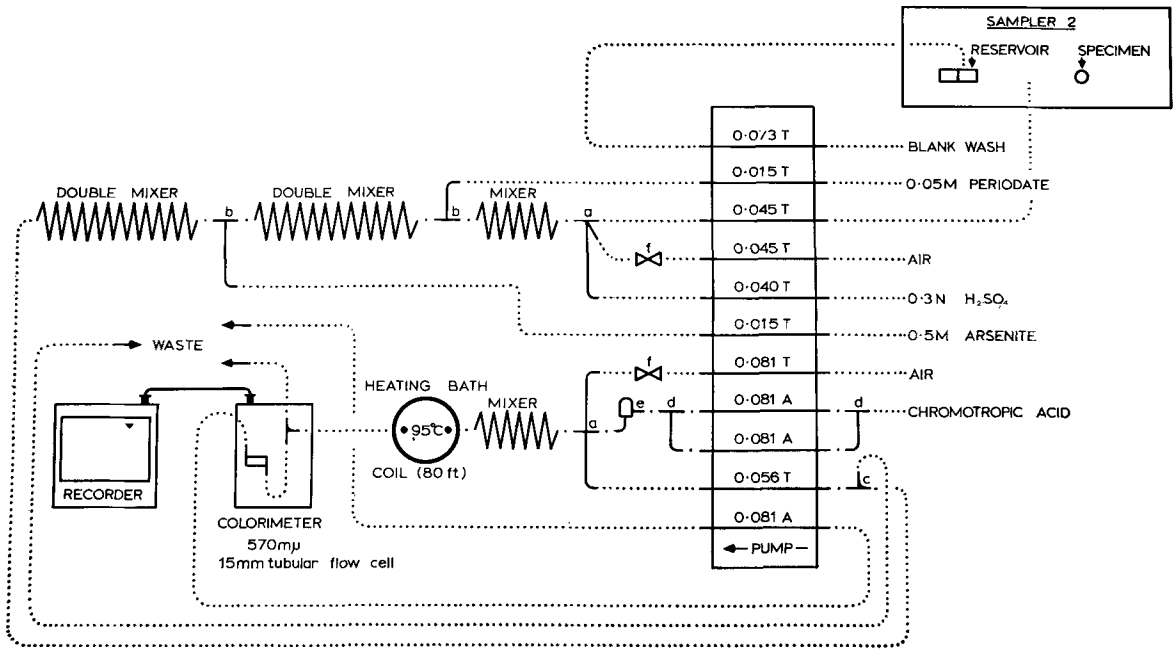


FIG. 2. Flow diagram for colorimetric glycerol determination (modification of system of Lofland [17]). *T*, Tygon and *A*, Acidflex pump tubing; bore as indicated (inches). Additional abbreviation: *a*, glass cactus, capillary side arms; *b*, glass h-piece, capillary side arm; *c*, glass debubbler T-piece; *d*, glass pulse suppressor; *f*, 0.005 in. I.D. tubing pulse suppressor. All other delivery tubing: polythene 0.060 in. I.D., 0.025 in. wall thickness.

Five-milliliter aliquots of the 1:25 serum/diisopropyl ether extracts, and of triolein standards in the same solvent (containing 2 to 12 mg per 100 ml), are blown to dryness. The standards correspond to the range 50 to 300 mg of triglyceride (as triolein) per 100 ml serum when carried through the extraction procedure. After being taken to dryness, specimens and standards are saponified with 0.1 N ethanolic KOH (0.5 ml) for half an hour at 60C, then neutralized with 0.2 N ethanolic acetic acid (0.5 ml), and taken to dryness on a boiling water bath. Blank tubes are also taken through the saponification stage. 2.5 ml of 0.3 N $H_2SO_4$ are added to each tube, and the solutions obtained are analyzed automatically at the rate of 30 specimens per hour with a 30, 1:1 cam. A blank wash of 0.3 N $H_2SO_4$ is provided for the sampler reservoir.

Long chain fatty acids and sterols liberated by hydrolysis do not interfere in the procedure, and need not be removed with petroleum ether as recommended by Carlson and Wadström (24).

*Fluorometric.* The automated fluorometric procedure developed by Kessler and Lederer (18) for the determination of serum triglycerides is based on the Hantzsch condensation reaction between an amine, β-diketone and an aldehyde. Triglycerides are saponified to glycerol, and its oxidation with periodate produces formaldehyde which reacts with ammonia and acetylacetone to form 3,5-diacetyl-1,4-dihydrolutidine. The latter fluoresces with a maximum at 530 mμ when activated with light of 405 mμ.

In the procedure described by Kessler and Lederer (18), manually prepared 1:20 serum-isopropanol extracts are freed from phospholipid and glucose with a mixed zeolite-fuller's earth-copper-lime reagent and analyzed automatically according to the flow diagram shown in Fig. 3 (reproduced by kind permission of the authors [18]).

The isopropanolic extract is added to an air-regimented stream of isopropanolic KOH, and complete saponification to glycerol is achieved by passing through a 50C heating bath. Following saponification both periodic acid and an acetylacetone reagent are added to the reaction mixture. The stream passes through a 50C heating bath where glycerol is oxidized to formaldehyde and simultaneously the fluorescent condensation product is formed. The solution then passes through the flow-cell of a fluorometer where it

TABLE III

Comparison of Serum Triglyceride Levels Estmiated by Manual and Semiautomated Procedures

| Specimen No. | Manual Chemical Colorimetric[a] | Semiautomated | | |
|---|---|---|---|---|
| | | Chemical | | Enzymatic Fluorometric[d] |
| | | Colorimetric[b] | Fluorometric[c] | |
| 1 | 16.0 | 15.0 | 15.3 | 15.5 |
| 2 | 17.5 | 16.5 | 17.0 | 16.5 |
| 3 | 16.5 | 16.5 | 17.8 | 17.0 |
| 4 | 20.0 | 20.0 | 19.8 | 20.5 |
| 5 | 24.0 | 23.5 | 23.8 | 22.0 |
| 6 | 30.5 | 31.5 | 31.9 | 30.5 |
| 7 | 44.5 | 45.5 | 46.0 | 46.5 |
| 8 | 48.0 | 47.0 | 47.5 | 46.5 |
| 9 | 68.5 | 66.0 | 66.2 | 65.5 |
| 10 | 68.5 | 71.0 | 70.8 | 70.5 |
| 11 | 75.0 | 72.5 | 72.5 | 72.0 |
| 12 | 74.0 | 77.5 | 78.5 | 77.0 |
| 13 | 87.5 | 87.0 | 88.0 | 87.5 |
| 14 | 111.5 | 106.5 | 107.5 | 109.5 |
| 15 | 144.0 | 142.0 | 144.5 | 143.0 |
| 16 | 164.0 | 155.5 | 159.0 | 158.5 |
| 17 | 186.0 | 185.0 | 182.0 | 181.0 |
| 18 | 197.5 | 201.0 | 202.0 | 200.5 |
| 19 | 211.5 | 215.5 | 218.0 | 213.5 |
| 20 | 287.0 | 281.5 | 284.5 | 288.5 |
| Mean | 94.6 | 93.8 | 94.6 | 94.1 |
| Standard error of duplicates | ±7.2 | ±3.2 | ±0.96 | ±2.5 |

All values (mg/100 ml serum) are means of duplicate estimations carried out on aliquots of the same diisopropyl-ether extract of each serum specimen.

[a] Manual method of Van Handel and Zilversmit (19).
[b] Modification of method of Lofland (17).
[c] Method of Kessler and Lederer (18).
[d] Enzymatic method described in text.

In colorimetric[a], colorimetric[b] and colorimetric[d] aliquots taken to dryness and manually saponified; fluorometric[c] aliquots taken to dryness and reconstituted in isopropanol.

Specimens 1–10 were pig serum; 11–20 were human serum.

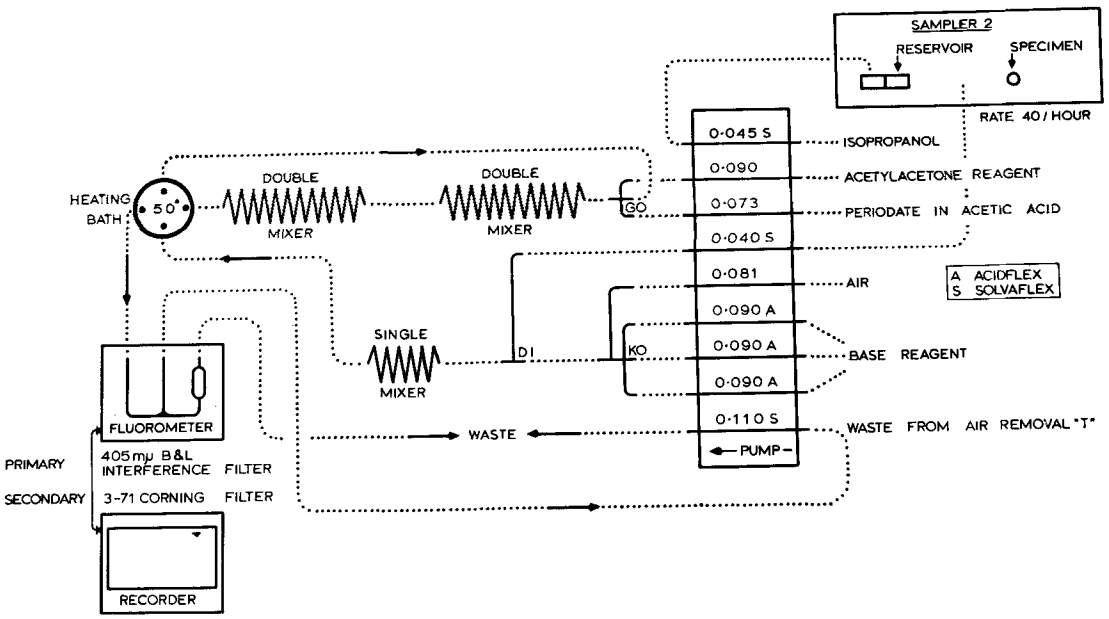


FIG. 3. Flow diagram for fluorometric glycerol determination (method of Kessler and Lederer [18]).

is activated at 405 mμ, and the fluorescence above 470 mμ measured and recorded. Blanks are determined by omitting the saponification stage. Both assay and blank runs are performed at 40 determinations per hour giving a net rate of 20 samples per hour. Full operating details and reagents required have been documented (18).

In my own laboratory I have used the above automated procedure on phospholipid-free serum lipid extracts obtained, as described in the previous section, with silicic acid and diisopropyl ether, and reconstituted in isopropanol. Blank determinations without saponification are unnecessary since the reconstituted extracts are free from interfering contaminants. The precision of the method has also been improved by the use of a 30, 1:1 cam in the sampler.

*Enzymatic Method.* Enzymatic methods for the determination of glycerol are based on its oxidation by ATP in the presence of glycerokinase and $Mg^{++}$, producing L(-)-glycerol-1-phosphate and ADP (25). Each of these products may then take part in subsequent enzymatic reactions involving the NADH/$NAD^+$ equilibrium with estimation of the amount of NADH formed or utilized by measurement of the extinction at 340 mμ or the fluorescence produced at 470 mμ after activation with U.V. light of 365 mμ.

An automated method for the enzymatic fluorometer assay of glycerol in hydrolysates has recently been developed in my laboratory. This method is based on the manual procedure of Kreutz (26) which utilizes the following series of enzyme-catalyzed reversible reactions:

$$\text{Glycerol} + \text{ATP} \xrightleftharpoons[Mg^{++}]{GK} \text{L(-)-Glycerol-1-phosphate} + \text{ADP}$$

$$\text{ADP} + \text{Phosphoenolpyruvate} \xrightleftharpoons{PK} \text{ATP} + \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightleftharpoons{LDH} \text{Lactate} + NAD^+$$

$$\text{Glycerol} + \text{Phosphoenolpyruvate} \rightleftharpoons \text{L(-)-Glycerol-1-phosphate} + \text{NADH} + H^+ + \text{Lactate} + NAD^+$$

By coupling the reactions catalyzed by glycerokinase (GK), pyruvate kinase (PK) and L-lactate: NAD oxidoreductase (LDH) a total reaction results in which the amount of $NADH_2$ oxidized is equivalent to the amount of glycerol displaced. At pH 7 in the presence of $Mg^{++}$ and excess $NADH_2$, the reaction proceeds rapidly to completion, and the change in fluorescence is directly proportional to the amount of glycerol present.

Glycerol hydrolyzates, obtained from specimens and standards as described under the colorimetric chemical method, and saponification blanks are taken up in 1.0 ml of 0.2 M potassium phosphate buffer pH7 instead of in 0.3 N $H_2SO_4$, and transferred directly to sample cups for automatic analysis according to the flow system shown in Fig. 4. The buffer-cofactor solution consists of the following reagents dissolved in a liter of 0.2 M phosphate buffer, and is freshly prepared before use: ATP, 0.6175 g; phosphoenolpyruvate, 0.1095 g; $NADH_2$ disodium salt, 0.0600 g; $MgSO_4 \cdot 7\ H_2O$, 0.6163 g; disodium ethylenediamine tetraacetate, 0.3500 g; LDH, 10 mg; PK, 8 mg and GK, 2 mg. Analytical grade reagents are used throughout, except for enzymes and cofactors which are of the highest purity commercially available (Boehringer Corporation, London, Ltd.).

In the flow system specimens, air and buffer containing enzymes and cofactors are pumped through a mixing coil into a time delay coil at 25C where the reaction proceeds over a period of approximately 6 min. Air is removed and the mixture passes through the flow cell of a fluorometer, where it is activated with U.V. light (Chance OX1 filter, 365 mμ) and the excited fluorescence measured (Wratten 45 filter, 470 mμ).

The procedure is started by pumping plain buffer solution (used also as a blank wash for the sampler 2 reservoir) through the system until a steady baseline is attained with the highest sensitivity of the fluorometer (Scale 1). Background fluorescence is reduced to zero by means of a potentiometer, and the fluorometer set at the desired sensitivity (Scale 2—approximately one-third of maximum sensitivity). Buffer containing enzyme, cofactors and $NADH_2$ is now pumped through the system for about 5 min until a new baseline is attained, and the sensitivity of the fluorometer adjusted to approximately 95%

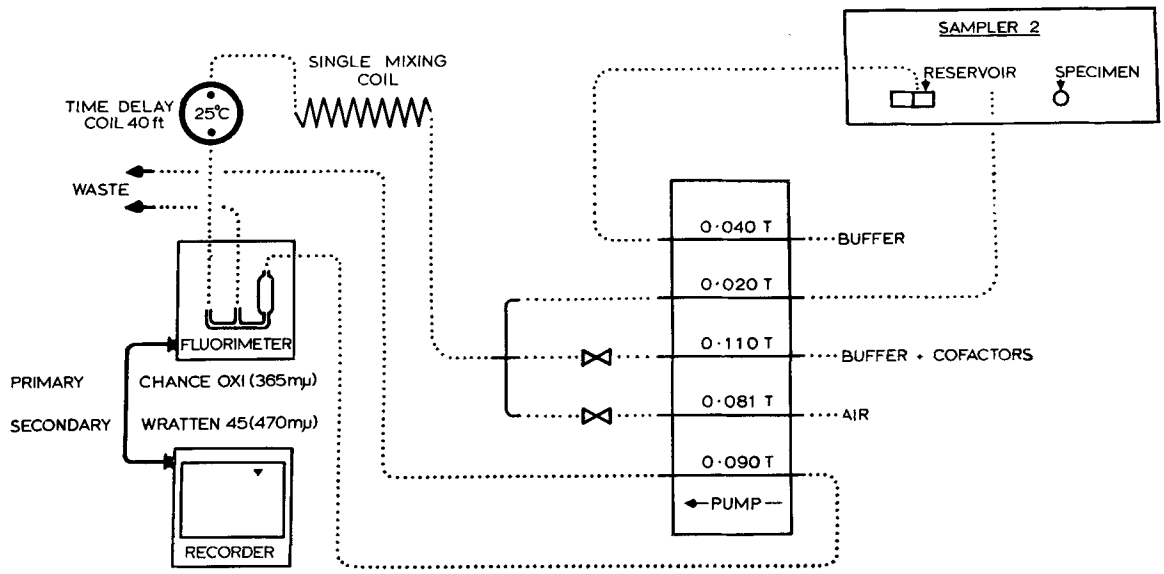


FIG. 4. Flow diagram for enzymatic fluorometric glycerol determination. Tygon tubing used for all pump and delivery lines; bore as indicated (inches).

transmission. Blanks, standards and specimens are then sampled at the rate of 60 per hour, using a 60, 1:1 cam, corresponding to 30 sec sampling of specimen (approximately 0.08 ml) followed by 30 sec of blank.

The presence of glycerol in the specimens produces a decrease in intensity of fluorescence directly proportional to concentration, and the range of glycerol concentration to be measured will depend on the excess of $NADH_2$ present. The concentration of $NADH_2$ given above covers a range of serum triglyceride concentration of up to 500 mg per 100 ml of serum when carried through the procedure described. Where higher serum triglyceride concentrations are indicated by complete reduction of fluorescence, specimens may be diluted and reestimated. The range may alternatively be increased by increasing the concentration of $NADH_2$ in the buffer, and reducing the sensitivity of the fluorometer.

The range of sensitivity, on the other hand, may be increased by using pump tubing of a larger internal diameter than 0.020 in. for sampling specimens. For example, using tubing with an internal diameter of 0.040 in., approximately 0.30 ml of specimen will be sampled over the 30-sec period, and complete reduction of fluorescence will correspond to a triglyceride concentration of approximately 100 mg per 100 ml serum. The choice of pump tubing of a particular internal diameter will therefore depend on the range of concentration expected in the samples of serum to be analyzed.

Full details of operating procedure, specificity and precision of the enzymatic method will be reported later in a separate communication.

#### Comparison of Results

Serum triglyceride levels estimated by the semiautomated procedures described above have been compared with the manual method of Van Handel and Zilversmit (19). Each serum specimen was extracted with silicic acid and diisopropyl ether, and duplicate aliquots were taken to dryness for each of the procedures investigated. For the colorimetric and enzymatic fluorometric procedures, dried specimens were manually saponified; for the chemical fluorometric method dried specimens were reconstituted in isopropanol. Results obtained by all the methods have shown excellent correlation as indicated in Table III. The three semiautomated procedures showed a greater degree of precision between duplicates than the manual method; the highest precision being obtained with reconstituted isopropanol extracts analyzed according to the method of Kessler and Lederer (18).

### Plasma Free Fatty Acid Concentration (FFA)

The titimetric technique of Dole (27), as modified by numerous investigators, has served as the most frequently used method for the determination of FFA concentration in plasma (or serum). While this method has provided excellent results, it requires skilled technical assistance, and is not very suitable for the rapid analysis of large numbers of specimens. In recent years, however, manual colorimetric methods for the determination of long chain fatty acids have been reported which lend themselves more easily to automation, and thus to rapid and precise determination. These methods are based on the solubility of copper or cobalt soaps in organic solvents with subsequent measurement of the extinction coefficients. Further modifications and improvements by complexing the metals have greatly increased the sensitivity of these methods. Barreto and Mano (28) and Duncombe (29) have complexed the copper with diethyldithiocarbamate, while Novak (30) has similarly used $\alpha$-nitrose $\beta$-napthol for cobalt. The method of Duncombe (29) has recently been adapted by Antonis (16) to give a semiautomatic determination of plasma (or serum) free fatty acids which is simple, rapid and reproducible. The method requires the preliminary preparation of a phospholipid-free plasma lipid extract which has been reconstituted in chloroform, and the chloroform solution of the free fatty acids is then analyzed automatically. The specimen in chloroform is pumped together with an aqueous copper nitrate-triethanolamine solution through a special mixing coil filled with glass beads before being separated into two phases. The aqueous copper phase together with a portion of the chloroform phase passes to waste, and the remainder of the chloroform

phase (containing the copper soaps) is redrawn through the pump and caused to react with a solution of sodium diethydithiocarbamate in butanol before passing through the flow-cell of a colorimeter with subsequent recording of the extinction at 440 mμ.

Acidflex tubing is used in the pump manifold for all lines in contact with chloroform. The following precautions must be observed, since the clean separation of the aqueous copper and chloroform phase is dependent on carefully defined rates of flow, and even slight contamination by traces of copper reagent will materially affect the results. In the published procedure (16) flow rates obtained with acidflex (Technicon, Ltd.) were similar to those specified for Tygon tubing of the same internal diameter. Recent batches of acidflex tubing of a stated diameter have given lower flow rates. Since it is essential to maintain a sufficient excess of chloroform entering the separator over that being redrawn through the pump (to prevent contamination by the copper reagent), flow rates of the acidflex tubing must be carefully checked before use.

The following flow rates of the acidflex pump tubes are necessary for efficient performance of the procedure: 1) line pumping specimen from sampler, approximately 3.2 ml/min; 2) line repumping chloroform from the sampler, approximately 1.9 ml/min; 3) line pumping reaction mixture from the colorimeter flow cell, approximately 1.5 ml/min. With recent batches of acidflex tubing these flow rates may be attained by using tubing of internal diameters of 0.110 in., 0.081 in. and 0.073 in., instead of internal diameters of 0.100 in., 0.073 in. and 0.065 in., respectively, as specified in the published method (16). Modifications of sampler 2, its coverplate, and the use of glass or polyethylene sample cups, as described for the fluorometric determination of cholesterol, are also essential. A special cam (30, 1:2) is required so that specimens are run at the rate of 30 per hour with 40-sec sampling of specimen (approximately 2.2 ml of extract) followed by 80 sec of chloroform (blank) drawn from the reservoir. The cam normally supplied (30, 2:1) would allow the sampling of approximately 4.3 ml of extract, and this would require the special construction of larger cups, resistant to chlorinated hydrocarbons.

Full details of the extraction and operating procedures, reagents and standards have been documented (16) together with a discussion of the precision of the method and its correlation with a titrimetric procedure. Over a range of FFA concentration of 220–1300 μeq/liter of plasma, the standard error of a single determination was 10.5 μeq. Comparison of results for FFA concentration of plasma determined by the above procedure, and by the manual titration procedure of Shafrir and Steinberg (31) showed no significant difference. Over the range of FFA concentration of 360–1400 μeq per liter of plasma, the average difference between the two methods was 12.1 μeq, with a standard error of differences between paired items of ± 11.6 μeq.

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