# STUDIES IN BIOCHEMICAL MICRO-ANALYSIS

Part I. The Determination of Lactic Acid in Muscle.

Part II. A Method for the Estimation of Glycerol in Blood.

by

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Contents.

# THE DETERMINATION OF LACTIC ACID IN MUSCLE.

## INTRODUCTION.

Lactic acid is a substance of prime importance in life and has been studied extensively. This is especially true with regard to its relationship to carbohydrate metabolism. It is not surprising therefore that a great variety of methods have been proposed for its quantitative estimation in biological fluids and tissues. These methods may be divided into five classes:

(a) Oxidative methods,
(b) Colorimetric methods,
(c) Measurement as zinc lactate,
(d) Physical methods, i.e. by the use of polarised light,
(e) Gasometric methods.

The number and variety of these methods show that lactic acid, although a fairly simple organic substance presents a number of difficulties to accurate and rapid analysis when present in small quantities. The difficulties which arise are not entirely due to the low concentration of the acid, but also to the fact that lactic acid is usually associated in living material with other chemically similar substances. These substances under treatment give products which behave/

behave similarly to those obtained from lactic acid itself. Methods of separation, e.g. as zinc salt, rarely satisfy the required conditions of completeness, specificity and delicacy.

The use of oxidising agents to obtain from lactic acid substances which are more easily estimated than the acid itself has been made the basis for the greater number of methods. The oxidising agents used are the usual ones, namely potassium permanganate, sulphuric acid and chromic acid. The methods themselves may be divided into two classes: (a) where the product is acetaldehyde, (b) where some other product of oxidation is estimated or where the amount of oxidising agent used is estimated by difference. In the former case, the estimation of acetaldehyde has been made in a number of ways. Boas (1) introduced dilute permanganate as the oxidising agent and trapped the acetaldehyde so formed in alkaline iodine solution. The iodoform formed was estimated either directly or by difference. This method was shown to be liable to grave errors by von Fürth and Charnas (2). Previously Ripper (3) had used sodium bisulphite solution to trap the acetaldehyde, determining the excess bisulphite by titration with standard iodine solution/

solution. Von Fürth and Charnas (2) incorporated this method and made the improvement of leading the acetaldehyde into the bisulphite by aeration. Bellet (4) trapped the acetaldehyde in ammoniacal silver nitrate solution and estimated the unreduced silver. What proved to be a step in the right direction was made by Clausen (5) who, as others had done, caught the acetaldehyde in bisulphite and titrated the excess with iodine. He then liberated the bisulphite from the acetaldehyde-bisulphite compound by addition of a solution of sodium bicarbonate. The liberated bisulphite was estimated by titration with standard iodine. The alkaline iodine method used by Boas (1) was reintroduced by Scott and Flynn (6) with various additional precautions but the method was still not so satisfactory as the one devised by Clausen(5). Meissner used concentrated sulphuric acid to oxidise the lactic acid to acetaldehyde and carbon monoxide according to the equation:

$$CH_3.CHOH.COOH - H_2O \longrightarrow CH_3.CHO + CO.$$

He trapped the CO and measured it. This method was also used by Scheyner (8) but was criticised by Mayer (9) on the grounds that biological fluids and/

and tissues contain many other substances which will give carbon monoxide under such treatment, even if acetaldehyde is not formed. Paessler (10) estimated the lactic acid by measuring, by difference, the amount of oxidising agent used up (in this case, potassium dichromate).

The colorimetric methods do not differ vastly in principle from the oxidative methods, since oxidising agents are used to break down the lactic acid to substances which are more easily estimated. Of these colorimetric methods perhaps the best known is that of Ryffel (11) who used concentrated sulphuric acid as an oxidising agent and Schiff's reagent for the production of a colour. Codeine was suggested as a substitute in the case of urine by Polonowski (12) and guaiacol by Harrop (13). Both workers used sulphuric acid as an oxidising agent.

The earliest type of estimation of lactic acid was one involving the separation of the zinc salt and its gravimetric determination. This must necessarily mean the employment of large volumes of blood and urine, in which fluids the concentration of lactic acid is very low. In addition there must be a complicated system of separation of the other substances/

substances present in the biological fluid and the final purification of the zinc lactate. Fletcher and Hopkins (14), during the course of their work on lactic acid in muscle, adapted the method to give more reliable results. The method was used by Wolf (15) to estimate lactic acid in blood. The results obtained were considered reliable, but it is evident that the method presents too many opportunities for a loss of lactic acid on the one hand and on the other, an increase in the apparent amount of acid due to the fact that there are substances present which are very similar to lactic acid. These will not be removed quantitatively and will give zinc salts almost identical with that given by lactic acid.

The chief indirect methods for the estimation of lactic acid are (a) use of polarised light, (b) measurement of the changes produced in the carbon dioxide capacity or the oxygen dissociation curve of blood when lactic acid is added to it. The former method was used by Hoppe Seyler and Araki(16) and by Yoshikara (17). However as the optical activity of zinc lactate is only $[\alpha]_D^{20} \pm 8.6$, the method cannot be considered reliable in estimations on very dilute solutions. The second method/

method was used by Verzàr (18) who estimated the lactic acid, formed in the blood of animals, after stimulation of certain muscles, by changes in the oxygen dissociation curve. Barr, Himwich and Green (19) attempted the estimation by studying the fall in the carbon dioxide capacity of blood but variations were large. This may have been due, as is suggested by Long (20), to the presence of formed elements (corpuscles) with constant membrane equilibria.

A gasometric method which has been used depends on the liberation of carbon dioxide from sodium bicarbonate by lactic acid.

On analysis the more dependable methods are those which involve preliminary oxidation of the acid to more easily estimated substances. It is evident that any errors introduced in the oxidation will affect the estimation. With potassium permanganate lactic acid is said to oxidise to pyruvic acid (Avistoff (21), Schoorl (22) ), but in dilute solutions acetaldehyde is the resulting product. This does not preclude the possibility of some pyruvic acid being present. The formation of such a substance in aeration methods would cause an apparent loss of lactic acid. Another factor

which may cause a loss of lactic acid is the fact that acetaldehyde is a substance which is easily oxidised and some may undergo decomposition by the action of the permanganate. The latter is a strong oxidising agent and may split off volatile bi-sulphite-binding substances from compounds, other than lactic acid, present in biological fluids and tissues. This possibility was discussed by Clausen (5). It was indicated by Jerusalem (23) that the yield of acetaldehyde and the specificity of the reaction depend on several factors - namely, the concentration of the potassium permanganate, the rate at which it is run in, and the rapidity of the air current used for aeration purposes. Oxidation by sulphuric acid while free from the above difficulties presents several others. Sulphuric acid is the oxidising agent used in all the colori-metric methods mentioned. Temperature is an important factor and although Clausen (5) found heating at 140°C. with 50% sulphuric acid to be satisfactory, Long (20) favours more drastic treat-ment, namely a higher concentration of acid at a temperature of 160°C.

In the oxidative methods loss of lactic acid may/

may occur prior to oxidation in the removal of the proteins and sugars. Heat-coagulation of proteins may carry down as much as one third of the total lactic acid adsorbed on the coagulum (Mondschein (24) ). When the Van Slyke method (25) is used for the removal of sugar there is no loss of lactic acid (Long (2) ).

The oxidative method now in general use takes these difficulties into account and is a modification of the Clausen method by Friedmann, Cotonio and Shaffer (26). The proteins are removed by the Folin-Wu (27) technique, which involves precipitation with tungstic acid. An aliquot of the filtrate is then treated with copper sulphate and lime according to Van Slyke (25), the filtrate then being oxidised with potassium permanganate ( N/200) in the presence of manganese sulphate and sulphuric acid in a Kjeldahl flask. The acid-sulphate reagent used to catalyse the oxidation is made up by dissolving 10 g. $MnSO_4$ in 90 c.c. of a 10 N. solution of sulphuric acid. 2.5 c.c. of this solution are used for each estimation. The procedure of oxidation is as follows. The flask carries a thistle funnel at the top for the permanganate, with a screw-clip to regulate the flow/

flow. The flask is connected to a condenser, the end of which rests under the surface of the bisulphite. The fluid for estimation is placed in the Kjeldahl flask with 2.5 c.c. of the acid-sulphate mixture and a glass bead to prevent bumping, and is gently heated. When the liquid begins to boil the N/200 permanganate is allowed to drop in. The rate is regulated so that the liquid remains pale pink. After about 5 minutes the permanganate is no longer decolourised, and the mixture goes black. The screw-clip is then closed and distillation continued at the same rate for 5 minutes, to complete removal of the acetaldehyde. At the end of this time the bisulphite flask is removed, the end of the condenser being washed down with distilled water, and bisulphite and washings are neutralised with N/10 iodine, using starch as an indicator. The bisulphite is one-twentieth normal and 20-25 c.c. are used. The iodine is run in until the colour is definitely blue. By means of a pipette a drop or two of bisulphite is added until the colour disappears, then N/200 iodine is run in until the colour just reappears. 10 c.c. of saturated

sodium/

sodium bicarbonate are then added to liberate the bound bisulphite, which is then titrated directly with N/200 iodine. It was found that the blue colour of the end-point disappeared readily, necessitating the addition of more iodine, and it was found necessary to choose some standard of time for the duration of the colour. Long has suggested two minutes as being suitable and this is the standard chosen. The iodine is standardised in the usual way and also against a solution of lithium lactate ( 1 c.c. = 0.5 mgm. lactic acid). The lithium salt was chosen because it can be prepared readily in the anhydrous state and is not hygroscopic. On solutions of lithium lactate this method was found to be accurate within the limits of experimental error. Aeration and the introduction of a reflux condenser was found to be unnecessary.

It is evident that, in common with similar methods, this one has the disadvantage that it will estimate as lactic acid all volatile bisulphite-binding substances and, also, all substances which give volatile bisulphite-binding compounds on oxidation. Such substances are almost certainly present in biological fluids, but consideration of their possible identity, in the light of what we know of the constituents of such fluids, suggests that/

that under ordinary conditions at least they introduce no very great error into the estimation of lactic acid. Moreover, again under ordinary conditions, it is probable that the concentration of the interfering substances does not alter greatly, so that apparent changes in the lactic acid are, at least, approximately real. The method of estimation of lactic acid thus resembles the older methods for estimation of sugar in blood - it indicates at least the approximate amount present, and gives a fairly reliable indication of alterations in the concentration.

Such a method, however, is obviously inadequate without further examination for use under abnormal conditions - conditions, for example, which, by alteration of the relative activities of the components of the lactic acid producing enzyme system may well lead to marked alteration in the concentration of the interfering substances. This is the more important since among the possible interfering substances are known or hypothetical intermediates in the formation of lactic acid from glycogen.

In order to obtain an accurate method of estimation it was considered necessary to determine the "interfering" substances and evolve a method for/

for their removal prior to oxidation.

## EXPERIMENTAL.

### Method.

The most obvious of interfering substances is methylglyoxal, which is volatile in steam, combines with bisulphite and has been found by many workers to be present in muscle and other tissues.

A simple experiment was carried out (Table I) which showed that methylglyoxal alone among the probable precursors of lactic acid, interferes seriously with the determination. Each of the several possible interfering substances was added to a solution of lithium lactate and the estimation made in the ordinary way. It may be noted at this point that the Van Slyke method for the removal of sugar has been shown by many workers to remove quantitatively such possible interfering substances as citric, succinic, malic, maleic and tartaric acids.

Table I /

Table I.

All solutions contain 0.5 mgm. lactic acid.

| Substance added | Mgm. lactic acid found. |
|---|---|
| 1 mgm. glucose | 0.49 |
| 1 mgm. pyruvic acid | 0.55 |
| 1 mgm. methylglyoxal | 0.92 |
| 1 mgm. dihydroxyacetone | 0.59 |
| 1 mgm. glyceric aldehyde | 0.50 |
| 1 mgm. glycerophosphoric acid | 0.53 |
| 1 mgm. phosphoglyceric acid | 0.59 |
| nil | 0.50 |

The interfering substances are present in concentrations amounting to twice that of the lactic acid, i.e. amounts which in practice could only arise under very abnormal conditions.

The results show that methylglyoxal was the only substance which increased the apparent amount of lactic acid. It was also evident that this increase was greater than would be anticipated if it were distilling unchanged or were converted to acetaldehyde. The problem therefore was to devise a means for the removal of methylglyoxal prior to oxidation, and, as a subsidiary point, to determine the changes undergone by the methylglyoxal in the estimation of lactic acid.

The method which first suggested itself for the removal of methylglyoxal was a preliminary distillation after the removal of sugar but before the addition of permanganate. Such a process would be very simple in use, the minimum of additional manipulation would be achieved and there would be no increase in the number of measurements.

This was carried out but it appears that, while methylglyoxal undoubtedly distils and binds an equimolecular amount of bisulphite, it undergoes some change in the presence of copper sulphate and lime which destroys its volatility but does not prevent its oxidation to a volatile reducing substance by permanganate. Indeed the bisulphite binding/

binding power of methylglyoxal is increased by about 40% by treatment with copper sulphate and lime followed by permanganate oxidation. The substance bringing about this change is the lime, which can be replaced by other alkalies. (Table II).

The preliminary distillation had then to be discarded since it was obviously desirable to retain the copper-lime treatment for removal of sugar, etc. and another method tried. Methylglyoxal, and incidentally other reducing substances, have the power to form hydrazones with 2:4 dinitrophenylhydrazine. It was therefore considered possible to remove these reducing substances by standing the solution with the hydrazine either subsequent to the removal of glucose or in place of the copper-lime treatment, depending on whether or not the substance formed from methylglyoxal by lime retained a suitable reactive group. After treatment with lime it was found actually that methylglyoxal still has the ability to react with 2:4 dinitrophenylhydrazine, forming a substance almost insoluble in dilute acid.

Table II /

Table II

| Vol. of methyl-glyoxal (0.1%) c.c. | Treated with lime | $MnSO_4$ + $H_2SO_4$ | $KMnO_4$ | Titration - c.c. | |
|---|---|---|---|---|---|
| | | | | a. | b. |
| 1 (added direct to $NaHSO_3$ | - | - | - | 1.35 | 1.31 |
| 1 (distilled 30 min.) | - | - | - | 1.30 | 1.28 |
| 1 | - | + | - | 1.10 | - |
| 1 | - | - | + | 0.25 | 0.23 |
| 1 | + | - | - | 0.00 | 0.00 |
| 1 | + | + | - | 0.00 | 0.00 |
| 1 | + | + | + | 1.79 | 1.80 |

The methylglyoxal solution was distilled and the distillate trapped in $NaHSO_3$ where the bound bisulphite was determined by the usual $I_2$ titration method. Before distillation (or during it) the methylglyoxal underwent the treatment indicated by a plus sign, that indicated by a minus sign being omitted.

Synthetic mixtures were then taken, subjected to the copper-lime treatment and aliquots allowed to stand with a hydrazine solution made by dissolving 0.5 g. 2:4 dinitrophenylhydrazine in 30 c.c. hydrochloric acid and making to 100 c.c. with water. To ensure maximum removal of the methylglyoxal the solutions were allowed to stand overnight. After filtration the estimation was carried out in the usual way. Titration of the bisulphite was impossible as the solution turned green on the addition of sodium bicarbonate. This was undoubtedly due to part of the excess of 2:4 dinitrophenylhydrazine, still in solution, distilling over into the sodium bisulphite. An attempt was made to remove the excess of the 2:4 dinitrophenylhydrazine by shaking the solutions several times with ethyl acetate. The acetate layer was then removed roughly by suction and the last traces by a preliminary distillation prior to oxidation. Under these circumstances the results were invariably low. It was found that there was a volume increase in the solution after repeated extraction with ethyl acetate. This increase was found to be about one-tenth the volume of the solution and a suitable correction

was consequently introduced. The corrected results were still low and it was found that there was a variable loss during the extraction with ethyl acetate due to the solubility of lactic acid in the latter solvent. As would be expected this loss increased with the number of extractions. The solution at this stage was acid from the addition of the hydrochloric acid solution of the hydrazine, but even from alkaline solutions the wet ethyl acetate still removed some lactic acid. There is also another possibility which could account for this loss. A little lactic acid may have been oxidised by the 2:4 dinitrophenylhydrazine to pyruvic acid. This may be suggested as a possible explanation of the isolation from tissues of small amounts of pyruvic acid 2:4 dinitrophenylhydrazone.

There remains the possibility of removing methylglyoxal by distillation prior to the removal of sugar with copper sulphate and lime. This procedure was tried and it was found that the distillation had to be continued for one hour for the complete removal of the methylglyoxal. During this time it was necessary to add water to the contents of the distillation flask to replace that lost by evaporation. An extra volume adjustment was also necessary.

necessary. In the mixtures whose analysis is shown in Table III an aliquot from the filtrate from the tungstic acid precipitation was distilled for one hour.

Table III.

| Solutions contained (mgm.) per cent. | | | Lactic acid found |
|---|---|---|---|
| Lactic acid | Glucose | Methyl glyoxal | |
| 8.0 | - | - | 8.0 |
| 8.0 | 5.0 | 5.0 | 8.0 |
| 8.0 | 5.0 | 10.0 | 7.8 |
| 8.0 | 10.0 | 5.0 | 8.2 |
| 16.0 | 5.0 | 5.0 | 15.5 |

The residual liquid was quantitatively transferred to a 50 c.c. flask, the requisite amounts of copper sulphate and lime were added and the volume made up to 50 c.c. with distilled water. The solutions were then allowed to stand for half an hour and the subsequent oxidation was then carried out in the usual way.

Up/

Up to this point all analyses had been carried out on synthetic mixtures, for which the method was accurate. With tungstic acid filtrates from muscle obtained from frogs however, it was found that the results were invariably higher than those obtained by the standard method. The variations were not in agreement but in every case the results were higher. The reason for this is not clear but it was found that removal of the tungstic acid precipitate by filtration through Kieselguhr also removed the interfering substance or substances.

The nature of the interfering substance in this case is a subject for speculation. There is no interference in synthetic mixtures, only in the muscle extracts used. As these are made acid by addition of tungstic acid (in the removal of protein) and boiled for one hour to remove methylglyoxal, it may conceivably be glycerol and other products of the hydrolysis and oxidation of lipoid material. It cannot be derived from glucose as the latter substance is present in the synthetic mixtures.

Using/

Using the Kieselguhr method of filtration the modified method gave results invariably lower than the standard method (Table IV).

Table IV.

| | Wt. of muscle in g. | Apparent lactic acid per g. of muscle | | |
|---|---|---|---|---|
| | | Old method mgm. | New method mgm. | Difference mgm. |
| 1. | 1.00 | 3.00 | 2.50 | 0.5 |
| 2 | 1.52 | 2.90 | 2.55 | 0.45 |
| 3 | 1.42 | 3.20 | 2.78 | 0.42 |
| 4 | 1.41 | 3.35 | 2.93 | 0.42 |
| 5 | 1.20 | 1.96 | 1.56 | 0.40 |
| 6 | 1.70 | 1.19 | 0.74 | 0.45 |

Muscles 1-4 were completely fatigued, 5 was partially fatigued and 6 was fresh. In the case of 6 no special precautions were taken in isolating.

This decrease is not due to any loss of lactic acid in the filtration as "synthetic" mixtures are estimated accurately when subjected to the Kieselguhr filtration. In the case of one muscle/

muscle the distillate obtained by boiling the tungstic acid extract for one hour was trapped in the hydrochloric acid solution of 2:4 dinitrophenyl-hydrazine. This was then allowed to stand, after evaporation to small volume and approximately 2 mgm. of a red hydrazone was obtained. This was identified as that of methylglyoxal by the formation of a blue colour with an alcoholic solution of potassium hydroxide. The total amount of hydrazone to be expected, on the basis of the lactic acid estimation was about 3 mgm. The mother liquor still contained a little of the hydrazone in solution, sufficient in fact to account for the difference between the theoretical yield and the amount actually obtained. Thus it is evident that the difference between the results yielded by the two methods is entirely due to methylglyoxal.

An attempt was made to solve the problem of the changes undergone by methylglyoxal during treatment with copper sulphate and lime, with subsequent permanganate oxidation. A certain amount of success was achieved, but one must consider the problem as being not completely solved. A solution of methylglyoxal was mixed with aqueous sodium hydroxide and/

and allowed to stand for 30 minutes. Part of the solution was then distilled into bisulphite and the titration carried out in the normal manner. There was no bisulphite liberated on the addition of sodium bicarbonate, showing that on treatment with alkali, methylglyoxal loses its volatility, i.e. is converted to some substance which is either not volatile or is unable to bind bisulphite. This is confirmed to a certain extent by the fact that there was no 2:4 dinitrophenylhydrazone formed on trapping the distillate in a hydrochloric acid of the hydrazine. The logical assumption is therefore conversion to a substance which is either non-volatile or is not a reducing substance capable of forming a bisulphite compound or a 2:4 dinitrophenylhydrazone. After standing with alkali for 30 min. methylglyoxal solution gives an amorphous yellowish brown hydrazone with a very low melting point. This is completely different from the red crystalline compound from methylglyoxal itself. This compound is easily prepared and crystallises rapidly from solution. The yellowish substance from the alkaline solution has not been obtained in/

in crystalline form suitable for analysis or melting point determination. Hence its structure is still a matter for conjecture but one may suggest that it is the hydrazone of some polymeride of methyl glyoxal. Standing over alkali for 30 min. at room temperature may cause some intermolecular rearrangement in the methylglyoxal. If the alkaline solution of methylglyoxal containing this hypothetical polymer is boiled with acid permanganate and the distillate is passed into a hydrochloric acid solution of 2:4 dinitrophenylhydrazine, a golden yellow crystalline hydrazone is formed. This was recrystallised several times from methyl alcohol, and in another case from methyl alcohol and water. From the former solvent the crystals are orange-yellow in colour, from the latter they are pure yellow. In both cases they are long needles, identical in shape. In either case the crystals melt at 161°C. A specimen of the 2:4 dinitrophenylhydrazone of acetaldehyde was prepared and the melting point taken after purification. This was recorded as 161°C. A mixture of the hydrazone prepared from the "polymeride" of methylglyoxal, after oxidation and the 2:4 dinitrophenylhydrazone of acetaldehyde was subjected to a mixed melting point/

point determination. There was no lowering of the melting point of the latter substance. All this evidence is in favour of the assumption that the two hydrazones are identical. The melting point of the acetaldehyde-hydrazone is given in the literature as 148°C. by Case and Cook (28). All the melting points done in this work gave concordant results of 161°C. at which point the hydrazone melted unchanged. Case and Cook apparently re-crystallised their compound from nitrobenzene or pyridine. A sample was prepared and recrystallised from pyridine, washed several times with benzene and light petroleum and dried in air. This melted at 161°C. The crude hydrazone obtained from the oxidation of the alkaline solution of methyl-glyoxal was subjected to microscopic examination in an endeavour to discover any other hydrazone which might be present. It was impossible to detect any such substance with the exception of a minute amount of the red hydrazone of methylglyoxal itself. It is highly probable therefore that acetaldehyde is the sole product formed which is capable of forming a 2:4 dinitrophenylhydrazone.

The simultaneous production of formic acid, which does not form a compound with 2:4 dinitro-phenylhydrazine/

phenylhydrazine would account for the increased bisulphite binding power already referred to. The conversion of methylglyoxal into acetaldehyde and formic acid involves no oxidation and, as is shown by Table I, the participation of permanganate is essential for the changes observed. The problem of the change must therefore be regarded as being only partly solved.

As this research was considered to be in the first instance an investigation of methods of biochemical micro-analysis no further work was done along this line. The method for the estimation of lactic acid was adopted by Stewart and Gaddie in a series of investigations. Their results are at the moment in the course of publication, but as they consider the method suitable for their purpose, it may prove interesting to set down a summary of their work. In their investigation they made use of muscle extracts which had been dialysed to remove co-enzymes. They found that such extracts produced lactic acid from glycogen, glucose, hexose diphosphoric acid, or an equimolecular mixture of pyruvic and glycerophosphoric acids, in greater amounts/

amounts in the presence of magnesium ions, adenylic acid pyrophosphate and reduced glutathione, than in the presence of the first two of these co-enzymes only. In the absence of glutathione, methylglyoxal tends to accumulate while addition of glutathione tends to diminish and may prevent this accumulation. In the absence of adenylic acid pyrophosphate, magnesium ions and glutathione allow the production of lactic acid from glycogen by dialysed muscle extracts, but omission of the glutathione in this case does not result in a corresponding accumulation of methylglyoxal. These workers also find that during the formation of lactic acid, methylglyoxal accumulates at first and then disappears in part. This amount which disappears is not fully accounted for by conversion to lactic acid.

From these results it is evident that the method employed for the estimation of lactic acid must be accurate and be uninfluenced by such substances as methylglyoxal. It is also evident that the removal of methylglyoxal must be complete. It may be suggested that by comparing results obtained by the standard method it is also possible to obtain an indirect method for the estimation of methylglyoxal.

## SUMMARY.

1. The ordinary method for the estimation of lactic acid in muscle etc. appears to give results which are too high owing to interference by methylglyoxal. A method is described for its removal by distillation prior to treatment with copper sulphate and lime.

2. Copper sulphate and lime do not remove methylglyoxal but convert it into some substance which may be a polymeride and which is oxidised by acid potassium permanganate to acetaldehyde. No other substance has been detected among the oxidation products of the hypothetical polymeride but the amount of bisulphite bound corresponds to about one and a half molecules of acetaldehyde per molecule of methylglyoxal.

3. The modified method gives results in muscle about 10% lower than the standard method.

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# PART II.

## A METHOD FOR THE ESTIMATION OF GLYCEROL IN BLOOD.

## PART II.

## A METHOD FOR THE ESTIMATION OF GLYCEROL IN BLOOD.

### Introduction

A considerable amount is known about the metabolism of fats but there is also a great deal which remains obscure. We know that all living cells contain "lipins" (to use a single term which includes not only true fats or triglycerides, but also phospholipins, galactolipins, sterols and possibly other more or less fat-like substances). We know, in the case of some tissues what particular group or even what particular lipins are present, although not always their absolute amount. In other cases, however, our knowledge is scanty. In order to know and understand the functions and metabolism of the various lipins, it is obviously necessary to know where they occur, in what amounts, and under what conditions they alter in absolute and relative concentration.

There is no need to stress the difficulties of identifying lipins. These difficulties are obvious, and are due to their instability and their similarity/

similarity, chemical and physical. The difficulties are such that methods of isolation are often useless, since, even for the proper separation of groups and sub-groups, sufficient material cannot be obtained. Attention has thus been directed to the possibility of obtaining adequate information by indirect means. Such means consist of the determination of some key-atom or reactive group and can, provided the conditions of analysis are properly controlled, give information as to the amount of the corresponding class of lipins present. For example the determination of "lipoid phosphorus" is held to indicate the amount of phospholipins present. The key atom in this case is the phosphorus atom. The method has been shown by Stewart and Hendry (1) to give a true estimation of the phospholipin content but sheds no light on their constitution. It is known that lecithin and kephalin occur in blood and some workers claim to have found sphingomyelin. There is no exact information as to the relative concentration of these substances. Stewart and Hendry, by separating the phospholipins and comparing the "lipoid phosphorus" content with that of the fatty acids, have shown that they do not consist/

consist entirely of lecithin and kephalin. This was also suggested by Channon and Collinson (2) who determined the N:P ratio of the lipins. Others (e.g. Maclean (3) ) have also studied the N:P ratio of the precipitated phospholipins, but the results obtained by these workers were very variable, and were usually much higher than could be accounted for by any mixture of known phospholipins. It is probable therefore that they are quite unreliable and simply indicate contamination of the material with some nitrogenous substance. As Stewart and Hendry consider the method for the separation of the phospholipins, namely precipitation by acetone and alcoholic magnesium chloride, would exclude any galactosides which might be present, they have suggested that the phospholipins may consist, in part, of sphingomyelin. This is based on the fact that the ratio of fatty acid molecules to phosphorus atoms in the precipitated phospholipins is approximately 1.5:1. For lecithin and kephalin the ratio is 2:1 and for sphingomyelin 1:1. Lecithin and kephalin contain one molecule of glycerol per phosphorus atom, while sphingomyelin contains none. On these considerations it is obvious that by estimating/

estimating the glycerol content of the phospholipins confirmation of the suggestion of Stewart and Hendry could be obtained, or the suggestion could be refuted, according to the result of such an analysis

There is no really satisfactory method at present for the estimation of free fatty acids in blood. The concentration of these substances has been obtained indirectly by the difference between the total fatty acids and those calculated from the various other lipins present. Stewart and White (4) estimated inorganic base in blood extracts, and by a process of exclusion, considered that this procedure indicated the presence of and estimated small amounts of free fatty acid (present in the extracts as soaps). The amounts they found were small and of course the proof of identity is far from rigid.

There are conflicting views as to the presence of triglycerides. Channon and Collinson, working on samples of fasting ox blood by isolation methods produced results which indicate that glycerides are virtually absent. Herbert (5) investigated two cases of diabetic lipaemia and noted/

noted that at the height of lipaemia there is an abnormally high concentration of total fatty acids. This increase could in no way be accounted for by increase in cholesteryl esters and phospholipins. It was shown that, after deducting the phospholipins, cholesterol, cholesteryl esters and non-sterol unsaponifiable matter contained in 100 gm. total ether-soluble material from blood, the residual fat amounted to 69.6 gm. The amount of fatty acid in 100 gm. fat was estimated as 66.6% showing that 69.6 gm. "residual" fat yielded 66.6 gm. fatty acid, i.e. the residual fat is 95.7% of its weight fatty acid. Assuming this "residual" fat to be triglyceride the percentage fatty acid in the material would be 96.7%. Herbert therefore suggests that this residual fat is triglyceride and that it reaches a maximum concentration during the height of diabetic lipaemia. It is improbable that the metabolic disturbances leading to diabetic lipaemia would result in the appearance of an entirely new substance in the blood, and it is more reasonable to suppose that if Herbert is right, normal blood contains some simple fat, though possibly only a small amount/

amount.

Nitrogen estimations having failed to give useful information, it becomes necessary to search for other possibilities, and the estimation of glycerol at once suggests itself as a procedure likely to be of value. Applied to a purified phospholipin fraction, it could give information supplementary to determination of the fatty acid/P ratio used by Stewart and Hendry. Once the true state of affairs in this fraction has been established estimation of glycerol, in conjunction with total fatty acid, lipoid phosphorus, and cholesterol, provides information as to the presence of tri-glycerides. The evolution of a method capable of estimating glycerol accurately on a micro scale is evidently well worth while for these two purposes alone.

Experimental/

## EXPERIMENTAL.

### Method for the Estimation of Glycerol.

At the commencement of this investigation there was no reliable published method for the estimation of glycerol in blood. A micro-method for free and bound glycerol had been evolved by Kataoka (6) but had not been applied to blood and was not as we found very satisfactory. It depended on the conversion of glycerol to acrolein by means of potassium bisulphite, the trapping of the acrolein in Bertrand's copper colution and the estimation of the excess of copper sulphate by titration with permanganate. As a first approach to this problem, the writer, before finding Kataoka's paper, used the same basic reaction. A solution of sodium bisulphite was used instead of the copper solution, as in the determination of lactic acid and the bound bisulphite determined iodometrically. It was found, however, that the conversion of glycerol to acrolein was by no means quantitative and was not really sufficiently regular/

regular for quantitative work. Indeed Kataoka does not claim quantitative conversion. Such a method which depends on an incomplete reaction cannot be more than a makeshift. The possible errors which could be introduced are many and great and it was therefore considered undesirable to proceed further along these lines.

It was then decided to attempt the use of alkaline permanganate oxidation, with determination of precipitation of calcium or cerium oxalate, and permanganate titration of the oxalic acid formed by dissolving the precipitate in sulphuric acid. Izumi (7) points out that an error is introduced in the estimation of small amounts of oxalic acid as the calcium salt due to the fact that calcium oxalate has a solubility, in acid solutions, which is too great to be neglected. Precipitation in alkaline solutions obtained from biological fluids gives rise to additional products which interfere with the estimation. Dobbin and Mebane (8) and Dodds and Gallimore (9) found that the addition of ammonium phosphate tended to have a favourable influence on the yield of calcium oxalate. The same/

same result was reported by Merz and Maugeri (10) who used magnesium sulphate in place of ammonium phosphate. Excess of these substances however tends to increase the solubility of the calcium oxalate and at the same time to delay the precipitation (Fiske and Logan (11) ). Izumi replaced calcium as a precipitating agent by cerium on account of the more limited solubility of cerium oxalate, especially in dilute acid solutions. Solubility of cerium oxalate given by Izumi to be 0.0004 gm. per litre at 25°C., that of calcium oxalate being 0.0068 gm. per litre at the same temperature. The solution containing oxalic acid is brought to pH 2.5-3.0 (Congo red paper changes to bluish-violet) and the acid precipitated as the cerium salt by the addition of 0.25% aqueous cerium chloride. The precipitate is then washed, dissolved in sulphuric acid and titrated directly with centi-normal potassium permanganate. An alternative was suggested by dissolving the precipitate in normal sulphuric acid, adding 1 c.c. of 30% aqueous potassium carbonate and hydrogen peroxide, giving a brown solution for colorimetric determination. Suzuki (12) claimed that the titrometric method was liable/

liable to error in warming with permanganate and introduced a modification. He added excess permanganate to the cerium oxalate dissolved in sulphuric acid along with a little manganese sulphate as a catalyst. A little solid potassium iodide was then added and the liberated iodine titrated with thiocyanate. As this method seemed to be as liable to error as Izumi's original method, the latter was investigated. A standard solution of oxalic acid was used and the results are shown in Table I. The oxalic acid solution was placed in small centrifuge tubes and the cerium chloride added. The precipitation was aided by "scratching" the tubes allowed to stand for half an hour and centrifuged. The precipitate was washed twice with water, care being taken to ensure thorough washing. For each titration 5 c.c. of N. sulphuric acid was heated to about 60-70°C. and added to each tube immediately before the addition of the N/100 permanganate.

From these results it is evident that the precipitation method gives results which agree closely with those obtained by direct titration. The addition of the warm sulphuric acid to the precipitate/

precipitate instead of heating them together seems to rule out Suzuki's objection, as there is no significant loss of oxalic acid when determined by the former procedure. In order to find the minimum time for complete precipitation a series of tubes were set up, the analyses being given in Table II, the procedure for titration being identical with that of the previous experiment. These results show that precipitation is complete in fifteen minutes. It was found that a large excess of $CeCl_3$ interfered with the estimation by inhibiting the precipitation of cerium oxalate. This may probably be due to increase in volume of solution resulting in a relatively larger amount of the cerium oxalate remaining in solution.

This method of precipitation was then tried out on a solution (M/800) of A.R. glycerol which had previously been dried by standing in a vacuum desiccator over sulphuric acid for several days. Aliquots of the glycerol solution were placed in centrifuge tubes, make alkaline with 1 c.c. 10% aqueous KOH and 5% potassium permanganate run in drop by drop until there was no green colour even after/

after standing for one hour. The solutions were then nearly black. They were then decolorised with a few drops of concentrated hydrogen peroxide made up to 10 c.c. and the precipitated manganese oxide centrifuged off. Excess of hydrogen peroxide was avoided. An aliquot of the clear solution was then mixed with 2 c.c. of glacial acetic acid (to bring to pH 3.0) in a centrifuge tube and 2 c.c. of 0.25% cerium chloride solution run in. The solution was "scratched" with a glass rod to facilitate precipitation and allowed to stand for 30 minutes. After centrifuging the supernatant liquid was removed by suction, the precipitate was washed several times with distilled water, dissolved in 5 c.c. N. sulphuric acid and titrated with N/100 permanganate. From the results, given in Table III, it is obvious that the conversion to oxalic acid and the subsequent precipitation and estimation is quantitative, giving a degree of accuracy well within the limits of experimental error usually associated with micro-chemical analysis.

The problem of "bound" glycerol was then investigated. From an approximately M/800 alcoholic solution of purified tristearin aliquots were taken and/

and saponified by the method used by Stewart, Gaddie and Dunlop (13). This consists in evaporating, on a hot plate, with 5 c.c. N/10 alkali, the last traces of liquid being removed in the steam-oven. The saponified fats are then allowed to stand overnight with sufficient hydrochloric acid to liberate the fatty acids. The solutions are now filtered, the fatty acids being washed 3-4 times with 5% sodium chloride, in which they are insoluble. The filtrate, containing the glycerol, is made alkaline with 1 c.c. 10% KOH and the glycerol is then estimated by the procedure already described. The filter paper is placed in the hydrolysis flask and the fatty acids dissolved in alcohol. The volume is made up to 10 c.c. and aliquots are titrated with N/10 sodium hydroxide in a Rehberg burette. In this way the fatty acid and the glycerol derived from it are estimated on the same sample. Table IV. shows that the method gives a true measure of the bound glycerol, the estimated agreeing closely with the calculated values. They also agree closely with the figures calculated for an M/800 solution. The method chosen, however, was considered preferable to depending entirely on the weighing of the tristearin.

The/

The experiment was repeated with triolein to determine the accuracy of the method for unsaturated fats but the glycerol estimated was invariably higher than that calculated from the fatty acid found. This may have been due to two causes. There was the possibility that the unsaturated fat was giving a product which by interaction with the cerium chloride was estimated as apparent glycerol. On the other hand the oleic acid formed in the hydrolysis may not have been completely retained by the filter paper, thus giving a loss in fatty acid. Actually both errors were involved, since the figures for oleic acid were lower than those expected from a M/800 solution, while the glycerol figures were higher.

It has been shown that the introduction of a small amount of cholesterol to the triolein solution inhibits the tendency of oleic acid to pass through the filter. The analysis was repeated, with the addition of a few mgm. of cholesterol to the original alcohol-ether solution of triolein and the glycerol found was then found to be in agreement with the calculated value. (Table V).

The possibility of interference by sugars was the next consideration. As the method involves boiling/

boiling with alkali it is possible that sugars will yield substances capable of being oxidised to oxalic acid or to acids which may give insoluble cerium salts. A series of M/800 suspensions of triolein were then set up containing varying amounts of glucose, alcohol-ether extracts were made and the saponification etc. carried out as before. It was found that although concentrations of glucose comparable to that of normal blood have little or no effect, glucose in amounts which could easily be found in abnormal blood, such as in diabetes, gave an apparent amount of glycerol much higher than could be expected or than agreed with the fatty acid found. The"apparent"glycerol increased with the amount of glucose present, the amount of triolein being kept constant. Note that the criterion of accuracy is agreement between found fatty acid and glycerol, since it was necessary to use a <u>suspension</u> of fat in the aqueous sugar solution and "duplicate" aliquots did not necessarily contain equal amounts of fat. It was obviously desirable to remove the sugar before saponification with alkali. For this a modified procedure was adopted. Aliquots of the alcohol-ether extract of a solution of triolein and glucose/

glucose were evaporated to dryness on the hot plate, the last traces of solvent being removed by a current of $CO_2$ to prevent oxidation. The residue was extracted five or six times with ether, previously dried over sodium. It was found that the combined extracts contained a small quantity of insoluble material, probably glucose. This was removed by centrifuging and the ether solution then evaporated down to about 5 c.c. 15 c.c. alcohol and 5 c.c. N/10 sodium hydroxide were then added and the saponification carried out in the usual way. The completeness of the extraction was tested by estimating the glycerol in a solution of triolein which contained no glucose. Duplicate estimations were carried out at the same time without the additional extraction and the results are given in Table VI. These show that there is no loss of fat in the extraction or in the removal of insoluble matter by centrifuging. To test the efficacy of this method for glucose a standard solution of triolein was made, aliquots taken and varying amounts of glucose added to each. Analyses were carried out both with and without the extraction with anhydrous ether. The results, shown in Table VII, indicate/

indicate that glucose does not inferfere in the modified method even in a concentration of 1000 mgm. per cent.

The possibility of interference of other substances present in blood is not, however, excluded and to determine the presence of any such substance a solution was made up containing those substances which occur in appreciable amounts in blood. This solution contained glucose 0.1%, urea 0.04%, uric acid 0.01%, gelatine 0.1%, cholesterol 0.2%, triolein 0.3% with phosphate Ringer to 100 c.c. This is obviously not a true solution but was made as homogeneous as possible by prolonged shaking prior to the removal of aliquots. 10 c.c. of this solution were then extracted with alcohol-ether in the usual way and made to 250 c.c. For analysis aliquots were taken, evaporated to dryness with $CO_2$ in the final stages, extracted with dry ether and the fatty acids and glycerol estimated by the method already described (Table VIII).

The difference between the figures for extract A and those for extract B may be explained by the fact that although they are made from the same solution, the latter is not homogeneous and even after prolonged shaking/

shaking can hardly be expected to give duplicate extracts. The important point is that the figures for the estimation of glycerol agree closely with those calculated from the amount of fatty acid found. While this work was in progress Freeman and Friedmann (14) published their method for the estimation of glycerol in lymph. They use oxidation of the glycerol to oxalic acid by excess alkaline permanganate followed by iodometric determination of the excess permanganate. Apart from the crudity of the procedure, this method involves, as the results just given show, errors due to the presence of unsaturated fatty acids, and of sugar in the extracts. (Freeman and Friedmann do not separate the fatty acids at all). The interference of fatty acids is probably obviated in part at least by their "blank" estimation on the extract without saponification, but this does not eliminate the glucose error, since the alkaline hydrolysis alters the effect of the permanganate on glucose. It is evident from the writer's experiments that separation of sugar and fatty acids is a sine qua non.

Applications /

## Applications of the Method.

The method was now considered sufficiently accurate for the investigation of the problems already mentioned, namely (1) the constitution of the phospholipins and (2) the presence of triglyceride in normal blood.

The method adopted for the precipitation and purification of the phospholipins is essentially that devised by Bloor (15) in 1929 and later modified by Boyd (16). 4 c.c. of non-oxalated blood were extracted with boiling alcohol-ether in the ratio 3:1 and made to 100 c.c. Suzuki has found that there is free oxalic acid in whole blood to the extent of 4 mgm. per cent. Oxalic acid is soluble in dry ether to the extent of 2.5 gm. per 100 c.c. but during the method of analysis this will be converted to sodium oxalate. The possibility of this latter substance being present in sufficient quantity to necessitate a correction of the glycerol figures was considered. Excess of sodium oxalate was shaken with 100 c.c. anhydrous ether, at room temperature, filtered and the ethereal filtrate shaken with excess of a solution of cerium chloride. The ether was removed and the cerium oxalate precipitated/

precipitated by the method described and the solution centrifuged. Only a trace of precipitate could be detected which was certainly less than 0.5 mgm. This amount will not interfere to any appreciable extent with the method used for the determination of glycerol, and consequently no correction was introduced in this respect. 75 c.c. of the alcohol-ether extract are evaporated to dryness on the hot plate, the last traces of solvent being removed by a current of purified hydrogen. The dried residue is extracted 5-6 times with dry ether, the extracts being combined in a large centrifuge tube and insoluble material removed by centrifuging. It cannot be over-emphasised that the ether used in this precipitation and subsequent purification of the phospholipins must be free from peroxide. Even the slightest traces of peroxide rapidly bring about decomposition of the precipitate. The ethereal extract free from insoluble material, and contained in a large centrifuge tube, is evaporated down to 1 c.c. in a water bath. 10 c.c. pure acetone are added followed immediately by 2-3 drops of a saturated alcoholic solution of magnesium chloride. Stewart and Hendry found this latter substance to be/

be essential to the precipitation. Attempts to dispense with it or replace it with alcoholic solutions of lithium chloride or calcium chloride invariably resulted in a loss of material. The precipitate is allowed to stand in the dark for 30 minutes and centrifuged. Standing in excess of this time causes the precipitate to decompose, the almost pure white colour changing to brown. After centrifuging, washing several times with acetone, the precipitate is extracted several times with moist ether. The combined extracts having a volume of about 40 c.c. are placed in a standard 50 c.c. flask and concentrated to about 15 c.c. Alcohol is then added to make the volume up to 50 c.c. The concentrated ethereal solution is milky, probably due to magnesium chloride, but addition of the alcohol renders it clear. Aliquots of this solution are then analysed for fatty acid, glycerol and lipoid phosphorus. The method for the estimation of the first two substances involves the boiling with sodium hydroxide. During this saponification there is a small but appreciable precipitate thrown down. This has been suggested by Stewart and Hendry to consist/

consist of magnesium soaps formed during the saponification as a result of the presence of a magnesium complex in the ethereal extract. These two workers found that in the early stages of the saponification the precipitate consists of magnesium hydroxide which can be removed most effectively by centrifuging. This method of removal involves no loss of fatty acid or phosphorus. After removal of the magnesium hydroxide the saponification and estimation are carried out in the usual manner. The lipoid phosphorus was estimated by the method evolved by Stewart and Hendry. Aliquots are placed in Pyrex test tubes, a glass bead is introduced and the extract evaporated to dryness. The organic matter is charred with concentrated sulphuric acid, the carbon being oxidised away with a few drops of "perhydrol". The solution is diluted to about 8 c.c. 0.5 c.c. of 2.5% ammonium molybdate followed by 0.4 c.c. of 1.amino-2-naphthol-4-sulphonic acid. The tubes are placed in a boiling water bath for 10-15 minutes, after which time the production of the blue colour, on which the estimation depends, is complete. The colour developed is compared colorimetrically against/

against that developed in a standard solution of potassium dihydrogen phosphate. Table IX gives the results obtained from the analysis of five samples of normal blood. On investigation the average ratio of mgm. fatty acid to mgm. of phosphorus is approximately 14:1. This is slightly higher than the average found by Stewart and Hendry, their figure being 13.2:1. The results have a greater significance when expressed in millimols. The average ratio of fatty acid molecules to phosphorus atoms is approximately 1.5:1, a ratio also found by Stewart and Hendry. This, as they have suggested, indicates that the phospholipoid material is not entirely lecithin and kephalin, which, in their fatty acid, phosphorus and glycerol content are identical. A substance such as sphingomyelin may be present. Lecithin and kephalin contain two molecules of fatty acid and one molecule of glycerol for every phosphorus atom, and sphingomyelin has 1 molecule fatty acid and no glycerol per phosphorus atom. First, using the phosphorus and fatty acid figures, the amounts of sphingomyelin and mixed lecithin and kephalin were calculated and were found to be of the order suggested by the results of Stewart and Hendry.

From/

From this result the amount of glycerol to be expected was calculated. The results of the final calculation given in Table X agree fairly well with the actual amounts of glycerol found. It is hardly to be expected that there should be very close agreement as any errors in the fatty acid and phosphorus estimations are reflected in the calculated amount of glycerol. It is obvious that a substance similar to sphingomyelin if not sphingomyelin itself must be present in the phospholipin fraction as the amount of glycerol from lecithin and/or kephalin alone would be very much larger than that actually found.

The question of the presence of triglycerides was studied by an estimation of the various fat constituents in whole blood. The blood was extracted with Bloor's alcohol-ether and aliquots taken for the analysis of total fatty acid, glycerol, lipoid phosphorus, free and total cholesterol. The first three estimations were carried out by the methods already described. The cholesterol was determined by the digitonin method, the amount present as ester being calculated as the difference between the total and the free cholesterol. The

results/

results expressed in millimols are shown in Table XI. The excess fatty acid is found by subtracting from the total fatty acid present the fatty acid obtainable from cholesterol and phospholipoid (as measured by the lipoid P). The excess glycerol is calculated by difference between glycerol found and that obtainable from phospholipoid. An average factor was used for the calculation of the fatty acid and glycerol content of the phospholipoid material and of course will be expected to give variation when applied to individual results. Considering the excess fatty acid to arise from triglycerides, it is found, on calculating the glycerol obtainable from it, that this calculated value is of the same order as the excess glycerol found. In the first two analyses the values are very close together. It is obviously not to be expected that the two values, the actual and the calculated, will be in absolute agreement. The factors which will tend to prevent this are (1) the use of an average factor for the calculation of fatty acid and glycerol from the lipoid phosphorus - a procedure which entirely neglects the possibility of variations in individual samples and/

and (2) the method of calculation tends to accumulate all the errors into the figures for "excess" glycerol and fatty acid. It is sufficient to obtain the calculated glycerol of the same order as that found. The results expressed in Table XI consequently indicate that triglycerides in small quantities are present in human blood.

It must further be noted that the "excess" fatty acid and glycerol are both small. Taking into account the expected analytical errors in the various estimations, they are however real. It is significant in this connection that in every analysis made there was excess fatty acid and excess glycerol. Had none actually existed, negative values would, on the laws of probability, have been obtained approximately as often as positive ones.

Summary/

## SUMMARY.

A method is described for the estimation of glycerol in blood. The estimation may be carried out on the fraction being analysed for fatty acid, thus involving the minimum of additional manipulation.

The constitution of the phospholipins are discussed and results given by the method indicate that they are definitely not entirely lecithin and/or kephalin and may be composed of lecithin and/or kephalin and sphingomyelin or some similar substance in the ratio 1:1 (approximately).

The method gives results which indicate that triglycerides are present in small quantities in normal blood.

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Table I.

| Mgm. Oxalic acid present | Mgm. Oxalic acid found | |
|---|---|---|
| | (a) by direct titration | (b) by titration after precipitation by $CeCl_3$ |
| 0.45 | 0.44 | 0.442 |
| 0.90 | 0.87 | 0.86 |
| 1.35 | 1.34 | 1.34 |
| 1.80 | 1.75 | 1.73 |

N/100 oxalic acid was used 1 c.c. = 0.45 mgm. oxalic acid

Table II.

Each tube contained 0.45 mgm. oxalic acid.

| Tube | Time | Mgm. oxalic acid found |
|---|---|---|
| 1 | 15 min. | 0.43 |
| 2 | " | 0.44 |
| 3 | 30 min. | 0.44 |
| 4 | " | 0.445 |
| 5 | 45 min. | 0.43 |
| 6 | " | 0.43 |
| 7 | 1 hour | 0.43 |
| 8 | " | 0.44 |
| 9 | 2 hours | 0.44 |
| 10 | " | 0.45 |
| 11 | overnight | 0.44 |
| 12 | " | 0.45 |

The second column gives length of time solutions were in contact with 1 c.c. 0.25% $CeCl_3$.

Table III.

| c.c. M/800 Glycerol | Mgm. Oxalic Acid found | Mgm. Oxalic Acid from Glycerol (calculated) |
|---|---|---|
| 4 | 0.44 | 0.45 |
| 8 | 0.87 | 0.90 |
| 12 | 1.28 | 1.35 |
| 16 | 1.74 | 1.80 |
| 20 | 2.20 | 2.25 |
| 24 | 2.63 | 2.70 |

Table IV.

| c.c. M/800 Tristearin | Mgm. Fatty Acid found | Mgm. Glycerol found | Mgm. Glycerol calc. from Fatty Acid. |
|---|---|---|---|
| 2 | 2.05 | 0.217 | 0.221 |
| 4 | 4.00 | 0.415 | 0.432 |
| 6 | 6.03 | 0.640 | 0.651 |
| 8 | 8.01 | 0.850 | 0.865 |
| 12 | 12.14 | 1.150 | 1.311 |
| 20 | 20.17 | 2.120 | 2.178 |

Table V.

| c.c. M/800 Triolein | Mgm. Fatty Acid found | Mgm. Glycerol found | Mgm. Glycerol calculated from Fatty Acid |
|---|---|---|---|
| 2 | 2.110 | 0.2190 | 0.2296 |
| 4 | 4.15 | 0.443 | 0.4515 |
| 6 | 6.10 | 0.645 | 0.6637 |
| 8 | 8.27 | 0.878 | 0.8998 |
| 12 | 12.50 | 1.340 | 1.3900 |
| 20 | 20.85 | 2.20 | 2.2685 |

Table VI.

| c.c. M/800 Triolein | Mgm. Glycerol Found | |
|---|---|---|
| | Unmodified | Modified |
| 2 | 0.225 | 0.220 |
| 4 | 0.438 | 0.440 |
| 6 | 0.665 | 0.658 |
| 8 | 0.876 | 0.870 |
| 10 | 1.103 | 1.092 |
| 15 | 1.620 | 1.583 |
| 20 | 2.173 | 2.152 |

Table VII.

20 c.c. of alcohol-ether extract from triolein solution used for each estimation.

| Sample | Mgm. % Glucose present | Mgm. Glycerol | |
|---|---|---|---|
| | | Unmodified | Modified |
| 1 | nil | 0.213 | 0.210 |
| 2 | 0.05 | 0.224 | 0.220 |
| 3 | 0.10 | 0.240 | 0.213 |
| 4 | 0.15 | 0.263 | 0.200 |
| 5 | 0.50 | 0.381 | 0.210 |
| 6 | 1.00 | 0.726 | 0.204 |

Table VIII.

| c.c. Extract | | Mgm. Fatty Acid found | Mgm. Glycerol found | Mgm. Glycerol calculated from Fatty Acid |
|---|---|---|---|---|
| A | 10 | 2.852 | 0.299 | 0.310 |
| | 20 | 5.216 | 0.544 | 0.567 |
| | 30 | 8.289 | 0.841 | 0.901 |
| | 40 | 10.598 | 1.074 | 1.152 |
| B | 10 | 1.490 | 0.154 | 0.162 |
| | 20 | 3.140 | 0.340 | 0.342 |
| | 30 | 4.885 | 0.525 | 0.531 |
| | 40 | 6.955 | 0.731 | 0.756 |

A and B are extracts from the same solution.

Table IX.

| Fatty Acid | | Lipoid Phosphorus | | Glycerol | |
|---|---|---|---|---|---|
| Mgm. | Milli-mols | Mgm. | Milli-atoms | Mgm. | Milli-mols |
| 1. 151 | 0.535 | 10.3 | 0.33 | 14.7 | 0.16 |
| 2. 156 | 0.553 | 12.2 | 0.40 | 17.5 | 0.19 |
| 3. 123 | 0.436 | 8.5 | 0.28 | 12.0 | 0.13 |
| 4. 160 | 0.567 | 11.4 | 0.37 | 15.6 | 0.17 |
| 5. 148 | 0.524 | 10.0 | 0.32 | 13.5 | 0.146 |

Table X.

| | Glycerol found millimols. | Glycerol calculated. millimols |
|---|---|---|
| 1 | .16 | .20 |
| 2 | .19 | .153 |
| 3 | .13 | .156 |
| 4 | .17 | .197 |
| 5 | .146 | .20 |

Table XI.

All the substances are expressed in millimols.
(Phosphorus in milliatoms).

| Total fatty acid found | Total glycerol found | Total cholesterol found | Free cholesterol found | Cholesterol as ester (calc.) | Fatty acid from cholesterol (calc.) | Lipoid phosphorus found | Fatty acid from lipoid P. (calc.) | Glycerol from lipoid P. (calc.) | Excess fatty acid (calc.) | Excess glycerol (calc.) | Glycerol from excess fatty acid (calc.) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1.124 | 0.404 | 0.495 | 0.305 | 0.19 | 0.19 | 0.46 | 0.80 | 0.35 | 0.144 | 0.054 | 0.048 |
| 0.936 | 0.380 | 0.469 | 0.409 | 0.06 | 0.06 | 0.476 | 0.833 | 0.357 | 0.056 | 0.023 | 0.018 |
| 1.00 | 0.446 | 0.452 | 0.4068 | 0.0452 | 0.0452 | 0.458 | 0.801 | 0.354 | 0.1638 | 0.092 | 0.054 |