The Metabolism of Ketone Bodies in the Baboon Leucocyte

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Declaration

This is to certify that the work presented in this thesis is my own, and has not been presented previously for examination.

Signed .....

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INDEX

LIST OF TABLES

Chapter I

INTRODUCTION

The major part of the work done on ketosis has centred very largely on the production of the so called ketone bodies, acetoacetate and 3-hydroxybutyrate by the liver and rumen of various mammaliam species (45, 46, 48, 99). More recently, interest has been shown in the metabolism of ketone bodies by extrahepatic tissues.

The investigations on ketone body metabolism in extrahepatic tissues have been varied; Krebs, Eggleston and D'Alesandro (55) measured the conversion of acetoacetate to 3-hydroxybutyrate with accompanying oxygen consumption in rat epididymal fat pads. They estimated that acetoacetate when added as the sole

substrate may account for more than 50% of cellular respiration. Hanson (41) demonstrated the oxidation of acetoacetate-3-C^{14} to $C^{14}O_2$ by mouse epididymal fat pads. Hanson (41) also investigated the utilisation of ketone bodies by mouse epididymal tissue in vitro. In this study an increased output of $C^{14}O_2$ from 3-hydroxybutyrate was noted in the presence of either glucose plus insulin, succinate, oxalacetate, L malate or L aspartate. Enhanced fatty acid synthesis from 3-hydroxybutyrate also occurred when either glucose plus insulin, L aspartate, L malate, oxalacetate or citrate were present. Nicotinamide stimulated the oxidation of 3-hydroxybutyrate, but did not effect the synthesis of fatty acid from either ketone body. The oxidation of acetoacetate-3-C_{14} by mouse epididymal fat pads and its effect on the pattern of glucose utilisation was studied by Hanson (42). He found that glucose and insulin did not change the rate of acetoacetate oxidation, but in the presence of insulin the ketone body altered the pattern of glucose utilisation. Carbon dioxide output and fatty acid synthesis were enhanced, while glyceride-glycerol

synthesis were decreased. This reduced glycerol synthesis was reflected in lowered triglyceride synthesis from palmitate.

Studying the metabolism of red and white muscle fibre groups in the rat, Beatty et al (8) reported that acetoacetate uptake by red muscle was higher than that of white muscle. Oxygen uptake and incorporation of C^{14} from acetoacetate-C^{14} into $C^{14}O_2$ were higher in red than in white muscle. Previously the same school (7) reported that adductor muscle from depancreatised rats took up less acetoacetate than muscle fibres from control animals. The fibres and diaphragm from alloxan diabetic rats also converted less C^{14} acetoacetate to $C^{14}O_2$ than the controls.

Krebs et al (56) investigated the metabolism of acetoacetate and lactate in the rat kidney cortex. When slices of kidney cortex were incubated with lactate and acetoacetate, lactate was almost quantitatively converted

to glucose whereas acetoacetate provided a major part of the fuel of respiration.

In the perfused rat heart, Williamson and Krebs (125) found that acetoacetate was rapidly removed from the perfusate, and at concentrations above 4mM about half was recovered as 3-hydroxybutyrate. At low concentrations (below 0.25mM) acetoacetate appeared to be completely oxidised. Added 3-hydroxybutyrate was as readily oxidised as acetoacetate. The results of Williamson and Krebs indicated that acetoacetate was oxidised in preference to glucose and endogenous substrate.

Neptune et al (76) studied the oxidation of labelled 3-hydroxybutyrate and acetoacetate to $C^{14}O_2$ by rat diaphragm homogenate. Added 3-hydroxybutyrate participated in the CO_2 production and the acetoacetate to an even greater extent.

As far as can be ascertained, very little work has been done on the utilisation of ketone bodies by leucocytes, although one paper (1) has appeared on the utilisation of 3-hydroxybutyrate by leucocytes from normal and tuberculotic humans, in which the authors claim that in the latter group the utilisation of this ketone body by leucocytes is increased 62% above that of controls.

On the other hand, the utilisation of acetate-C^{14} by leucocytes has been demonstrated by many workers (15, 43, 44, 69, 72, 83, 95). In normal blood most of the lipid synthesised from acetate is carried out by the leucocyte (15, 95). These cells were found to be 100 - 1000 times more active in the uptake of radioactivity from C^{14} acetate into their lipid fractions than were erythrocytes. Also the rate of lipid synthesis on a cell basis is approximately 70 fold greater in leucocytes than in platelets. In a few experiments where erythrocytes have been obtained

uncontaminated by leucocytes (less than 50/mm^3) the erythrocytes were found to be completely inactive (15). In two studies where the fatty acid synthesis in human leucocytes was studied (3, 63), acetate-C^{14} was found to be incorporated into fatty acid mainly by chain lengthening of pre-existing fatty acid.

The fact that leucocytes are capable of synthesising lipid from acetate-C^{14} raised the possibility that these cells would be especially suitable for the present study as they represent a readily available tissue for biopsy. In this respect they have an advantage over most other tissues. They are capable of suspension as single cells, free of blood vessels, connective tissue, and other encumbering elements that in the ordinary way make the biochemical characterisation of a tissue so difficult. This cell may be the ideal one for the study of cellular metabolic activities; whether the specialised functions of the leucocyte overshadow its general cellular metabolic functions remains to be established.

The aim of the present study was to determine the extent of the metabolism of the ketone bodies in the baboon leucocyte.

The initial problem encountered in the study of the metabolism of the leucocyte *in vitro* was to find a suitable medium. A combination of "HEPES" buffer and pork-skin gelatine was eventually found to be suitable for this purpose.

Respiration studies were then undertaken, firstly to ascertain the viability of the leucocytes during the incubation period, and, secondly to determine whether the ketone bodies could possibly influence respiration.

Radioactive tracer studies using labelled acetate, acetoacetate, and 3-hydroxybutyrate were then carried out to determine the extent of incorporation of

activity into (i) carbon dioxide produced during oxidation, and (ii) the various neutral lipid fractions. Acetate C^{14} was used in some experiments as a reference substrate; firstly, because of its position in intermediary metabolism, i.e. the possibility exists that both acetoacetate and 3-hydroxybutyrate could be metabolised by similar pathways; secondly, because the techniques used in the present study are quite different from those described by previous authors, it was considered advisable to repeat the work on the utilisation of C^{14} acetate for the purpose of comparison.

Chapter 2

THE ISOLATION OF THE LEUCOCYTES

(a) General

The two most common sources of leucocytes are from peritoneal exudates and from peripheral venous blood. The technique for obtaining leucocytes from peritoneal exudates has been used by Elsbach and co-workers (27, 28) and Sbarra and Karnowsky (100, 101) among others. Generally an irritant such as a caseinate is introduced into the peritoneal cavity and the exudates withdrawn by gravity drainage. This is a good method for obtaining almost pure suspensions of polymorphonuclear leucocytes from rabbits. In the baboon this technique was not practical, and so leucocytes were obtained from peripheral venous blood, as in the case of humans. Only the isolation of leucocytes from blood will be considered.

The most fundamental problem concerning the biochemistry of the leucocytes is its procurement, and the great obstacle to this study has been freeing the leucocytes from contamination with red cells, platelets, and other blood consituents. Numerous methods have been developed for the isolation of leucocytes. There have been attempts at mechanical methods (29,36), but they suffer from the disadvantage of isolating leucocytes in very low yield. The most popular procedures used in obtaining leucocytes involve differential sedimentation by the addition of one of several rouleaux promoting agents. These are substances of high molecular weight and include: Dextran, Fibrinogen, Phytahaemaglutinin, Polyvinylpyrollidone, Gammaglobulin, Dextrin, Glutamyl Peptide, Polyglucose and Gelatin. In fact, as shown by Oncley et al (80) the roleaux formation is dependent only on the presence of a long asymmetrical molecule. By addition of one of these compounds, it is possible within the course of about 35 - 45 minutes to produce a sedimentation approximately corresponding to the haematocrit. Skoog and Beck (107), who conducted

an exhaustive study on roleaux promoting agents found Dextran and Fibrinogen to have the widest application, and in leucocyte studies since 1960 these have become almost the only two in common use. Dextran was chosen for the present study as it is far easier to work with than Fibrinogen and according to Martin and Green (70) it is the only agent which maintains the necessary degree of the cells' chemical integrity. Many Dextrans tried by these workers contained an agent which was injurious to leucocytes. They found that Dextrans prepared by Pharmacia (Dextrans TDR 205 - 11 - B - 1) to be the most satisfactory. It is unfortunately not possible to isolate leucocytes of uniform age within the myeloid series or for the separation of granulocytes from lymphocytes. The leucocyte is extremely sensitive to many environmental factors such as trauma, alterations in pH, temperature and osmolarity (20, 30, 84).

A worker who has attempted to isolate leucocyted while paying careful attention to the environmental conditions

referred to above is Esmann (30), and with minor alterations the present author has followed his method of isolation. Esmann observed that as soon as the temperature dropped during any of the isolation operations, or if leucocytes were transferred to glassware at room temperature, a fine spontaneous agglutination took place. This observation has been confirmed by the present author. Tullis (116) described two types of agglutination or clumping, one reversible and the other irreversible. After the advent of irreversible clumping the leucocytes are dead, and no further use can be made of them. For these reasons all operations involved in the collection of blood and in the isolation of the leucocytes were carried out at 37°C, except for the short centrifugations. Furthermore, the Dextran Saline solution used in the present study for collection of blood and for its sedimentation was buffered with 0.05M phosphate buffer as used by Christlieb et al (19) containing glucose (100 mg%). Tullis and Baudanza (113) suggest that glucose should be added to the sedimentation system to support metabolism

whenever temperatures of 12°C and over are used. Various authors have stressed the importance of non-wettable surfaces in the handling of leucocytes (16, 32, 49, 107, 114) and without it severe damage occurs to the leucocytes. These changes not only cause a decrease in amoeboid motility and phagocytic index, but in respiration as well.

(b) Experimental

1 Animals

The animals used in this study consisted of 40 odd Chacma baboons (Papio ursinus) both male and female, housed in a colony in the Medical School. The animals were normally bled after an overnight fast (about 16 hours).

2 Siliconisation

All syringes, needles and glassware which came into contact with the leucocytes were siliconised. Two types of Silicone were used; the first, ICI 441 was used on the glassware only, as due to its acidid nature it cannot be used on metal surfaces, and the second, Siliclad (Clay Adams, New York) was water soluble and was used to siliconise the syringes and needles. The equipment was siliconised 6 times initially and subsequently after each experiment.

3 Reagents

A Dextran

(i) Phosphate buffer 0.05 M pH = 7.4

(a) 6.8g KH_2PO_4 dissolved in distilled water and made up to 1000 ml;

(b) 8.7g K_2HPO_4 dissolved in distilled water and made up to 1000 ml. (a) and (b) mixed in the ratio 19.81 parts by volume.

(ii) 5 g. of Dextran 250, 0.09 g. NaCl and 0.3 g. glucose were dissolved in 100 ml. of phosphate buffer, 0.05 M pH = 7.4 in a volumetric flask. This was then dispensed into vials in 12.5 ml. aliquots, sealed, and autoclaved at 15 lbs for 15 minutes and stored at 4°C.

B EDTA

4 g. EDTA (disodum salt, ethylenediamine tetracetic acid, British Drug Houses, Poole, England) and 0.9 g. NaCl dissolved in 100 ml. distilled water was similarly dispensed into vials in 1.6 ml. aliquots and autoclaved at 15 lbs for 15 minutes and stored at room temperature.

4 Collection

12.5 ml. of the 5% Dextran solution and 1.6 ml. of the 4% EDTA solution described above were transferred to a suitably graduated Erlenmeyer flask. The flask was

fitted with a ground glass stopper and placed in a Thermos jar containing water at 37°C. 50 ml. of blood was then added to this mixture, noting the precaution of Martin and Green (70) by making the venipuncture with a large needle (17 gauge), inserted in a rapid clean puncture, since tissue juices or blood clots markedly alter glycolysis and respiration as well as inducing early reversible agglutination of the cells. A syringe was not used in taking the blood as the pressure necessary for the removal of the contents caused haemolysis and was injurious to the leucocytes.

5 Isolation

As soon as possible after the collection of the blood, it was distributed in siliconised pyrex test tubes (14 x 125 mm.) previously placed in a constant temperature bath at 37°C. The red blood cells were allowed to sediment for exactly 45 minutes. The supernatant was then aspirated into a 50 ml. constricted neck centrifuge tube and centrifuged for 200 G for exactly 4

minutes. The supernatant was then decanted and the leucocyte pellet taken up in a little of the medium used and resuspended by aspirating slowly up and down with a Pasteur pipette. At this stage about 40 ml. of the medium was added and the contents of the tube carefully mixed with the pipette and centrifuged at 200 G for exactly 2 minutes. The supernatant was then decanted, and the leucocyte pellet made up to an appropriate volume with the medium. At this point aliquots were taken for leucocyte counting.

6 Leucocyte Counting

The leucocytes were counted in an improved Neubauer counting chamber after dilution (50:950) with 1% acetic acid tinged with gentian violet. 6 to 8 squares were normally counted with two pipettings.

Chapter 3

THE MEDIUM

A General

Studies on the metabolism of intact leucocytes *in vitro* have been hindered by the lack of a suitable medium. Leucocytes in whole blood have been used in tracer studies and then separated afterwards. Alternatively, the leucocytes have been pre-separated and suspended in homologous serum or plasma, or in serum or plasma diluted with buffer.

The latter media are not readily reproducible; the reasons why so many studies are carried out in these irreproducible media are twofold. Firstly, leucocytes agglutinate when suspended in buffers, and,

secondly, it is difficult to find a buffer system with sufficient buffer capacity to control the large quantities of lactic acid produced by the vigorous glycolysis of the leucocytes. Skoog and Beck (107) after observing a large number of leucocyte isolations concluded that clumping always occurs to a greater or lesser extent in leucocyte preparations. Tullis (115), Ruthberg and Terent'eva (96) and Leontovich and Abezgauz (62) found that 1 to 2% gelatine was capable of counteracting this agglutination, but these authors did not specify the kind of gelatine they had used.

Tullis (115), interested in the preservation of leucocytes over an extended period, tested a number of colloids, including albumen, each of the plasma fractions of Cohn and a number of plasma volume expanders. Gelatine was, however, the most beneficial in supporting normal functional activities. The manner in which gelatine acts so satisfactorily to

support viability is not known, but Tullis has suggested that the cells are kept discreet, thus ensuring even distribution of media and nourishment. 200 mg.% of sodium acetate was found by Tullis to have a similar restraining effect. Attempts by the present author to conduct experiments in the absence of sodium acetate always resulted in the formation of white cell clots before the white cells could be completely isolated.

Esmann (30) has described two buffers containing gelatine and sodium acetate for the study of leucocyte metabolism. (Table I)

KRPAGA is a Krebs - Ringer - Phosphate - Acetate - Gelatine - Ascorbic acid buffer. Esmann used the KRBAGA in all his studies as it maintained the pH throughout the incubations. However, this buffer is troublesome to use, and only allows the indirect

Table I

BUFFER COMPOSITIONS

	Esmann (30)		Present Author	
	KRBAGA (mM)	KRPAGA (mM)	KRPAGA (mM)	KRHAGA (mM)
NaCl	91.8	95.9	95.9	95.9
KCl	4.7	4.7	4.7	4.7
$CaCl_2$	2.5	2.5	2.5	2.5
KH_2PO_4	1.2	1.2	1.2	1.2
$MgSO_4$	1.2	1.2	1.2	1.2
Na-Acetate	24.4	24.4	24.4	24.4
$NaHCO_3$	25.0	-	-	-
NaH_2PO_4	-	1.9	1.9	-
Na_2HPO_4	-	12.5	12.5	-
Glucose	16.7	16.7	16.7	16.7
Gelatine	1%	1%	1% CSA	1% PSH
Vit.C.	0.06	0.06	0.06	0.06
pH	7.43	7.10	7.40	7.40

Warburg technique to be used. In the latter technique, the continuous uptake of oxygen cannot be followed, and in radioactive tracer studies the continuous collection of CO_2 is not possible. The KRPAGA on the other hand has insufficient buffer capacity, and the phosphate concentration cannot be increased, as it precipitates the calcium and the magnesium ions, which are present.

Delaunay and his co-workers (23) demonstrated that free Ca^{++} ions are necessary for normal surface activity and permeability. Mg^{++} ions are also important in intermediary metabolism, and Tullis showed that their absence depresses the function of the leucocyte.

The aim of the study was therefore to modify the KRPAGA buffer described by Esmann using a specially prepared gelatine, and a new buffer described by Good

et al (40). These authors have recently carried out studies on 12 new or little used hydrogen ion buffers covering the range pKa = 6.15 - 8.35. These they have prepared and tested in a variety of biological systems including the Hill reaction, and the phosphorylation coupled oxidation of succinate by bean mitochondria. Two buffers which they found outstanding were HEPES (N-2-hydroxyethanepiperazine-N-2-ethane sulphonic acid) and TES (N-Tris (hydroxymethyl)-methyl-amino-ethane sulphonic acid). TES and HEPES gave particularly active and stable mitochondrial preparations. These two also gave higher rates of protein synthesis in cell free bacterial preparations than do tris or phosphate. HEPES and TES emerged outstanding partly because they buffer so well (pKa values 7.55 - 7.50 respectively), and partly for reasons not yet understood. The combination of a de-ionised pork-skin gelatine and HEPES buffer has been investigated as regards its ability to keep the pH constant, and to prevent the agglutination of the leucocytes. In accordance with

the nomenclature above it has been called KRHAGA or Krebs - Ringer - Hepes - Acetate - Gelatine - Ascorbic acid buffer.

B Experimental

Studies were taken on the KRPAGA buffer, and the two components of this system were investigated in detail. These were firstly, the gelatine, and secondly, the buffer component.

The first brand of gelatine tested by the author was: -

(a) Calf-skin gelatine (Oxoid-Oxo, London)

This brand of gelatine, used at a concentration of 1%, caused a white precipitate of secondary phosphate in the presence of the phosphate buffer. This was due to the presence of $CaSO_4$ formed

during the production of this type of gelatine. After de-ionisation (for details see below) the gelatine was at the iso-electric point I.E.P. = 4.9. In the initial experiments the pH of this gelatine was raised to pH = 7.4 with 10% KOH. The medium was then made up to contain 1% of this gelatine, with the concentration of phosphate buffer 15.6 mM. A number of respiration experiments were done using this KRPAGA buffer and in all cases it was found to have no buffer capacity, and at the end of two hours the pH had dropped to the region of 6.0 - 6.4. To avoid this happening, tris-HCl, tris-maleate-NaOH, and imidazole were substituted for phosphate in the medium. This was not successful as these buffers in concentrations necessary to maintain sufficient buffer capacity, had a severely depressing effect on leucocyte respiration. Tris is a primary aliphatic amine of considerable reactivity, and is consequently often inhibitory. Estes (31) also found that the incubation of leucocytes in tris leads to a fall in oxygen uptake. Imidazole was found to be even less satisfactory in the present study, and at a concentration of 30mM inhibited

respiration completely.

(b) Pork-skin gelatine

The next gelatine investigated was an American pork-skin variety, which, when de-ionised, had an I.E.P.= 8.0. An attempt was made to blend this with the de-ionised calf-skin gelatine until the pH = 7.4, but this did not maintain the pH during leucocyte incubations. When de-ionised, pork-skin gelatine I.E.P. = 8.0 was mixed with the HEPES buffer adjusted to pH = 7.2 with 2N NaOH, the pH of the solution obtained was pH = 7.4. This gelatine, called PSH (pork-skin-HEPES) was used in all of the subsequent experiments and was the gelatine used in KRHAGA.

(c) Method of Preparation of PSH Gelatine

(i) 1 M HEPES (Calbiochem, Lucerne) 23.6 g. was dissolved in about 30 ml. of distilled water

and the pH adjusted to 7.4 with 2N NaOH. This was then transferred to a 100 ml. volumetric flask and made up to the mark.

(ii) 20 g. of American pork-skin gelatine with an iso-electric point I.E.P. = 8.0 was dissolved in 150 ml. of distilled water by heating to 40°C in a water bath. This gelatine was then transferred to the funnel of a completely jacketed column which was circulated with water at about 50°C. The gelatine was allowed to flow through the column packed with Amberlite MB3 Monobed resin (Rohm and Haas Co., Philadelphia) at the rate of 1/6 ml/ml resin/minute and collected in a 500 ml. volumetric flask. After all the gelatine w had passed through the column, the latter was washed through with warm distilled water. The gelatine solution was finally made up to the mark. The column should contain at least 100 ml. of resin, which is self indicating. Should the gelatine come off the column at a pH other than 8.0 it must be de-ionised again.

(iii) 25 ml. of the gelatine solution and 5 ml. of 1 M HEPES were transferred to 50 ml. McCartney bottles and autoclaved at 15 lbs for 15 minutes and finally stored at 4°C. This amount of the de-ionised gelatine and HEPES was sufficient to make up 100 ml. KRHAGA i.e., it is equivalent to 1% gelatine. The final pH of KRHAGA was always 7.4.

Chapter 4

RESPIRATION OF THE LEUCOCYTES

(a) General

Many studies have been published on the glycolytic and respiratory metabolism of the leucocyte. Much of this work is difficult to interpret, because of variations in experimental procedure, e.g., different incubation media or incubation periods, widely different leucocyte concentrations were used, or no leucocyte counts were recorded, and results expressed in terms of leucocyte dry weight. In the latter case, conversion factors have been published for the conversion of dry weight to numbers of leucocytes, although the values are not in agreement. Many of the studies have been done in plasma or serum which a fairly high oxygen consumption (Table II), or in various buffers containing serum or plasma.

Table II

COMPARISON OF RESULTS OBTAINED
FOR HUMAN LEUCOCYTES BY VARIOUS AUTHORS

Authors	n	Medium	Glucose	μ moles $O_2/10^{10}$ leucs/hr $\pm$ S.E.M.
Warburg et al (57)	5	Plasma + Saline	+	245 $\pm$ 25
Bird et al (58)	5	Plasma + Bicarb. Buffer	+	230 $\pm$ 38
Pastore (83)	3	Serum + Phosphate Buffer	+	178
Seelich et al (102)	9	Bicarb. Buffer-Krebs	+	402 $\pm$ 22
	9	Ringer with air as the Gas Phase	-	473 $\pm$ 27
Remmele (92)	3	Bicarb. Buffer-Krebs Ringer	-	213
Esmann (30)	77	Bicarb. Buffer (KRBAGA)	+	172
Bicz (11)	5	Bicarb. Buffer-Krebs Ringer + Tris	-	103
Seelich et al (102)	5	Phosphate Buffer-Krebs Ringer	+	219 $\pm$ 14
	7	Phosphate Buffer-Krebs Ringer	-	228 $\pm$ 17
McKinney et al	?	Phosphate Buffer-Hanks	+	92 $\pm$ 6
McKinney et al (75)	?	+ Resin Plasma	-	150 $\pm$ 7
Athens et al (3)	3	Phosphate Buffer-Hanks + Resin Plasma	-	173
Bisset & Alexander (13)	8	Phosphate Buffer (Krebs)	+	110 $\pm$ 6
Bisset & Alexander (14)	6	Containing 1.4 mM HCO_3^-	?	113 $\pm$ 11
Present Author	9	KRPAGA	+	73 $\pm$ 1.58
	11	KRHAGA	+	126 $\pm$ 4.05

This was the case in the studies of Warburg et al (121) and Bird et al (12) where citrated plasma was used. The results they obtained were 245 and 230 μ moles* respectively. Pastore (83) incubated in phosphate buffer plus 30% homologous serum and found an oxygen consumption of 178 μ moles.

Results obtained in bicarbonate buffer are also higher than in phosphate buffer, and Remmele (92) reported an oxygen consumption of 213 μ moles (although only three experiments were done and each value differed from the average by about 50%). Bicz (11) found the oxygen uptake of leucocytes to be 103 μ moles, but he did have tris present in the medium which probably inhibited the respiration. The values obtained by Seelich et al (102) of 402 and 473 μ moles are very high. These workers used bicarbonate buffer with air as the gas phase and presumably the pH of this system must also

* All results expressed as μ moles $O_2/10^{10}$ cells /hour.

have been very high. Esmann (30) in a very thorough study of leucocyte respiration in bicarbonate - CO_2 buffer (KRBAGA) found the oxygen consumption of leucocytes to be 172 μ moles.

The lowest figures for oxygen uptake where phosphate buffer was used were those of McKinney et al (74) and Bissett and Alexander (13 and 14) with buffer containing glucose, i.e., 92 μ moles and 111 μ moles respectively. In the absence of glucose the consumption appears to be somewhat higher, and McKinney et al (75) reported a value of 150 μ moles, and Athens et al (3) one of 170 μ moles. Even these results in phosphate buffer are not really comparable as McKinney et al and Athens et al used resin plasma in their media, and that of Bisset and Alexander contained bicarbonate.

Finally, for comparison, the result of measuring respiration in leucocyte homogenates can be mentioned. McKinney et al (74) found the oxygen consumption in the intact leucocyte to be approximately twice that of the cell free homogenate. Beck and Valentine (10) found that respiration in phosphate buffer was of the same order of magnitude as that of the intact cells, whilst the addition of ATP,NAD and cytochrome c increased the respiration considerably. Kurland et al (58) found respiration to be highly dependent on the presence of ATP and NAD.

In a review published in 1953, Beck and Valentine (9) summarised the knowledge of some aspects of leucocyte metabolism. Their section on leucocyte respiration included the work that had been done up until 1950. Esmann (30) in 1962 concluded that no further advance had been made in this field since 1950 and this is still the position today.

It was decided firstly to determine the respiration of the leucocytes in order to test the viability of the cells in different media. Although it has been postulated (71) that pyruvate and succinate are the only intermediates which stimulate the respiration of leucocytes. In this study the ketone bodies and a few other substrates were used in an attempt to determine their influence on the oxygen uptake.

(b) Experimental

The standard direct Warburg technique was used. 2 ml. of the leucocyte suspension (made up in one or other of the media) were pipetted into each flask pre-warmed in an incubator at 37°C. The centre well was fitted with 0.1 ml. 40% KOH. The paper projected about 3 - 4 mm. above the rim of the centre well. After a 10 minute equilibration

period the taps were closed and readings taken every 30 minutes during the 2 hour incubation period. 44 experiments were carried out on the respiration of the leucocytes, using various types of medium and various substrate additions.

I Media

The investigations on the medium showed that tris, either as tris-HCl, or as tris-maleate-NaOH was unsatisfactory and caused a 34% drop in oxygen uptake. Imidazole at a concentration of 30 mM caused a complete inhibition of respiration.

A comparison of the oxygen consumption of baboon leucocytes suspended in the KRPAGA medium containing both the CSA gelatine and phosphate buffer revealed results which were similar to those obtained by other authors using phosphate buffers and human leucocytes (Table II). Adjustment of the pH of the

CSA gelatine solution to pH = 7.4 with either KOH or Na_2CO_3 did not appear to influence the respiration.

The combination of the PSH gelatine and HEPES in the KRHAGA buffer made an appreciable difference to the uptake of oxygen. In nine experiments the oxygen consumption of baboon leucocytes was found to be 73 $\pm$ 1.58 μ moles in the case of KRPAGA containing CSA gelatine. In 11 experiments the oxygen consumption was found to be 126 $\pm$ 4.07 μ moles in the case of the KRHAGA containing the PSH gelatine. The standard error or the difference between means was 12.24. ($P < 0.0005$) (Fig.1.). Furthermore, the pH remained constant in the latter buffer system during the two hour incubation period.

Incubating leucocytes in the KRPAGA medium without glucose caused an increase in the oxygen uptake : - 102 as opposed to 73 μ moles.

Table III

The average uptake of oxygen with the addition of substrates and cofactors in KRPAGA medium without glucose.

(Number of experiments in parenthesis).

μl moles $O_2/10^{10}$ cells / hours

KRPAGA	3-hydroxy-butyrate	Cofactors	Fumarate	CPCA
102 (10)	102 (9)	124 (8)	(124)(2)	53 (4)

2 Substrates and Cofactors

Adding various cofactors, NAD, ATP, and coenzyme A to the incubation medium appeared to have some effect on the respiration, but the number of observations did not lend itself to statistical treatment of the results. The addition of 3-hydroxybutyrate was next attempted but had no noticeable effect on the respiration as shown on Table III. In the same series of experiments CPCA (Cyclopropanecarboxylic acid) was added to the Warburg flasks in some cases. If the ketone bodies had had an effect on respiration the purpose of the CPCA addition was to see if this compound could influence this respiration. CPCA has been claimed (124) to inhibit the oxidation of fatty acids and the utilisation of ketone bodies. CPCA in the few experiments attempted inhibited the respiration by approximately 50%.

Due to the negative results obtained with 3-hydroxybutyrate in an attempt to influence the oxygen uptake the respiration studies were abandoned in favour of radio-active tracer studies.

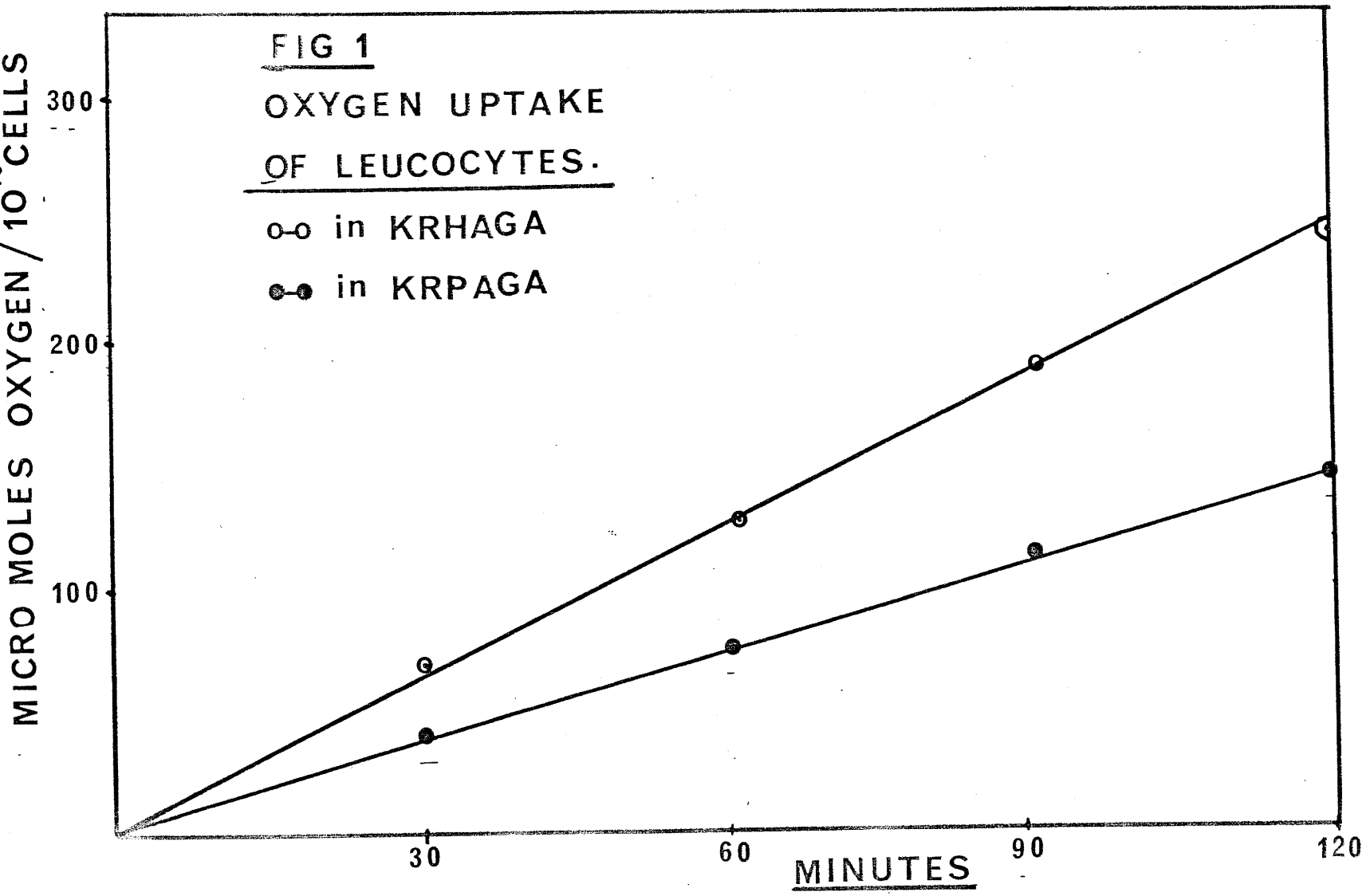


FIG 1
OXYGEN UPTAKE
OF LEUCOCYTES.
o-o in KRHAGA
●-● in KRPAGA
MICRO MOLES OXYGEN / 10^{10} CELLS
300
200
100
30
60
90
120
MINUTES

Chapter 5

RADIOACTIVE TRACER STUDIES

(a) Incubation of the Leucocytes

The leucocyte pellet obtained after the isolation of the leucocytes was normally made up to 10 ml. with KRHAGA medium, and transferred to a Saba-di Luzio flask (see below). The radioactive substrate was added to the suspension of leucocytes, and the flask sealed with either parafilm (Gallenkamp, London) or with a Suba seal bung, and fitted into an adaptor which could be used in the Braun Warburg bath. The temperature could be accurately maintained at $38^{\circ}C \pm 0.01$ and had a shaking rate of 84 strokes per minute. Before the commencement of the incubation the flask was pre-warmed for 15 minutes. After the addition of the leucocyte suspension the flask was equilibrated for a further 5 minutes before the collection of the $C^{14}O_2$ was started. As a rule, the incubations were of a 2 hour

duration, and the scintillation vials were changed every 30 minutes.

The following radioactive substrates were used in these studies (obtained from Amersham, Bucks., England) : -

(i) Na-acetate-1-C^{14} (0.1 mc)

Specific activity = 28.4 mc / mM

= 346 µc / mg

Radiochemical purity 98%

This was made up to 2 ml. in a volumetric flask with distilled water.

(ii) Na DL-3-Hydroxybutyrate 3-C^{14} (0.1 mc)

Specific activity = 5.74 mc / mM

= 45.6 µc / mg

Radiochemical purity 99%

Similarly made up to 2 ml with distilled water.

The radiopurity of this substrate was checked by chromatography on glass fibre paper using solvent system, butanol : acetic acid : water (50 : 11 : 25). The 3-hydroxybutyric acid spot was visualised with phenol red, and assayed by the technique of liquid scintillation counting. The radiopurity was found to be more than 99%.

(iii) Ethyl Acetoacetate-3-C^{14} (0.1 mc)

Specific activity = 5.7 mc / mM

= 44 µc / mg

Radiochemical purity 98%

The ethyl acetoacetate was converted to the sodium salt by using the method of Krebs et al (56). The vial was placed in a dry-ice acetone bath with the neck sticking out. The top of the vial was then placed in an incubator at 40°C for 1 hour. The contents were then transferred to a round bottomed flask with about 1 ml. of water and lyophilised. The dried Na acetoacetate was then transferred to a 2 ml. volumetric flask and made up to the mark with distilled water.

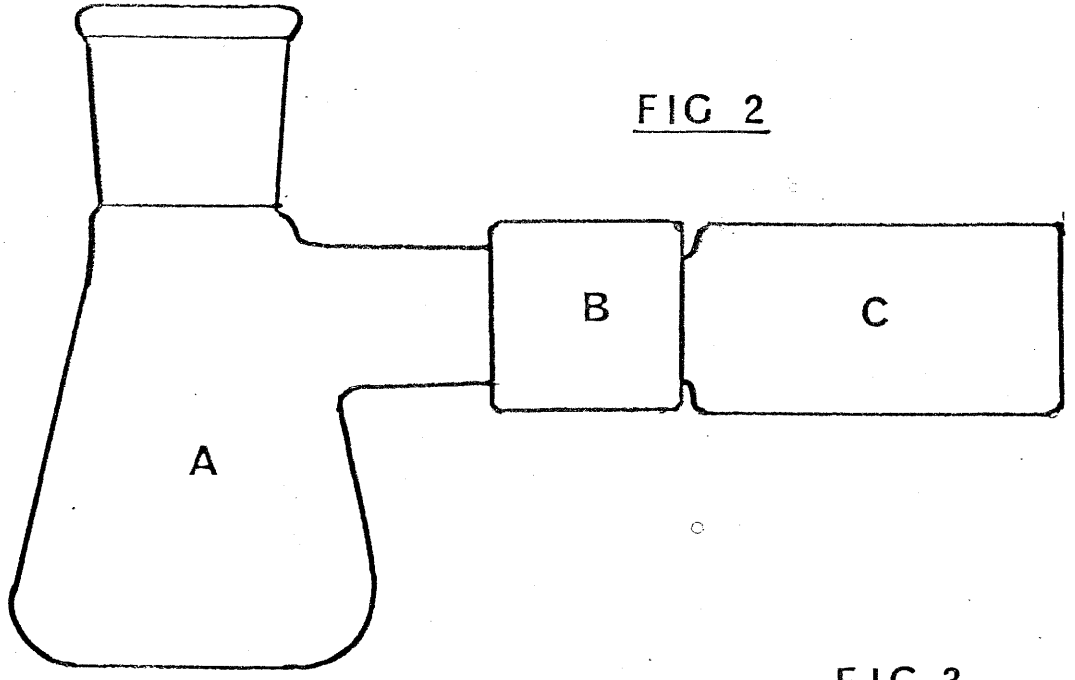
(b) Collection of CO_2

A novel method for the collection of CO_2 has recently been described by Saba and di Luzio (97). The metabolism flasks were to their design and tefion adaptors were made so that a scintillation vial could be attached directly to the flask (See Fig 2).

A strip of Whatman glass fibre filter paper GF/A (4.0 x 1.5 inches) was rolled up and inserted into a standard scintillation vial and soaked with 1 ml. of 10% KOH. After removal from the incubation flask, the scintillation vials were placed in a vacuum dessicator and dried over P_2O_5 for a day or more in an attempt to reduce the quenching found in aqueous systems. They were then ready for liquid scintillation counting.

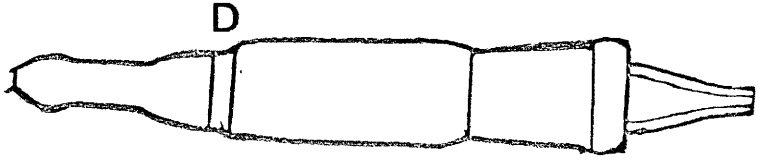
C

FIG 2



A. ERLENMEYER FLASK
B. TEFLON ADAPTOR
C. SCINTILLATION VIAL
D. SINTERED DISC

FIG 3



(c) Lipids

(i) Solvents

Iso-propyl ether was freed of peroxides by extraction with acidic ferrous sulphate, dried over $CaCl_2$ and redistilled. All of the other solvents were from freshly opened bottles of the A.R. grade.

(ii) Standard Lipids

Cholesterol (B.D.H.) was purified by the method of Fieser (33). Cholesterol stearate (B.D.H.) was recrystallised 3 times from acetone. Both products were then dried in a vacuum oven for 4 hours at 45°C, and then placed in a dessicator which was first evacuated and atmospheric pressure restored with nitrogen and stored at -20°C. Palmitic acid was obtained 99.9% pure from Applied Science Laboratories. Tripalmitin was re-crystallised from methanol.

(iii) Extraction of Lipids after Incubation with C^{14} Substrates

After the incubation of leucocytes with the C^{14} substrates, the cells were washed out of the incubation flask with KRHAGA medium and transferred to a 50 ml. constricted centrifuge tube and centrifuged at 2500 r.p.m. for 15 minutes. The supernatant was then decanted and the leucocyte pellet made up to a definite minimal volume with distilled water, and frozen and thawed three times. 20 volumes of ice cold chloroform : methanol (2 :1) were added. The mixture was then homogenised for 5 minutes in a Potter-Elverhejm homogeniser with a tefion pestle. The homogeniser was immersed in an ice bath during this procedure. This homogenisation appeared to increase the yield of lipid by a factor of at least 5. Except for this, the standard method of Folch et al (34) for the extraction of the lipid was followed.

After homogenisation, the mixture was allowed to

stand for 1 hour at 4°C and then filtered through fat free filter paper. The solid material was removed from the filter paper and extracted again with 5 volumes of chloroform methanol (2 : 1) at room temperature for a further 30 minutes and again filtered. This filtrate was then combined with the first in a 100 ml. beaker which was carefully filled with distilled water and cautiously submerged beneath a large volume of distilled water (10 L.) and left to wash overnight. The water was removed from the large container by use of a water pump and the beaker containing the lipid removed. The aqueous phase was removed very carefully using a fine Pasteur pipette and very low vacuum.

(iv) Fractionation of the Lipids

After the lipid fraction was freed from the aqueous phase, it was transferred to a tared sample tube, and the solvent evaporated off with mechanical agitation, in a water bath at 40°C, under a stream of nitrogen.

After complete removal of the solvent, the sample tube was again weighed. The lipid was finally taken up in a known volume of hexane or toluene containing 0.01% BHT (4 Methyl -2,6,- Di tert - Butyl Phenol), the final concentration being about 1%. The lipid solution was spotted on to thin layer plates (20 x 5 cm.) coated to a depth of 0.5 mm. with silica gel H (Merck & Co., Darmstadt). Skipsky's (106) two solvent system for the separation of all classes of neutral lipid was used. The first solvent system was isopropyl ether : acetic acid 96 : 4. Insofar as Skipsky used Camag DO silica gel, slight adjustments had to be made in the height each solvent system was allowed to ascend. The lipid solution was spotted 2.5 cm. from the bottom of the plate, and allowed to ascend 14 cm. in the first solvent system. On removal of the plate from the tank it was left to dry at room temperature for 45 minutes and then allowed to ascend to 18 cm. in the second solvent system : petroleum ether : ether : acetic acid (90 ; 10 : 1).

All the neutral lipid fractions were separated in these solvent systems. The components were detected by exposure to iodine vapour. As soon as the spots were visible the plate was removed and the spots marked out with a series of pinpricks. The iodine was then allowed to sublime at room temperature.

(v) Recovery of the Lipid Fractions

After the thin layer chromatography, and removal of the iodine, the area of each spot was carefully loosened with a sharp implement. A small column containing a sintered glass disc was used under vacuum to suck up the silica gel (94) (Fig. 3). The final scraping was done with the edge of a microscope cover slide. When all of the silica gel was transferred to the column it was eluted with three column volumes of chloroform methanol (1 : 1) into B 14 stoppered test tubes (1.8 x 10 cm.). The only exception was the free

fatty acid fraction, which was eluted into B 10 stoppered test tubes (8 x 1.5 cm.). The eluted samples were then dried under a stream of nitrogen at 40°C with mechanical agitation, and then suspended in hexane containing BHT (77, 126) at -20°C until ready for analysis.

The lipid components for radioactive assay were removed by scraping the spot onto a square of glossy paper and transferred to a liquid scintillation vial.

(vi) Analysis of the Neutral Lipid Fractions

(a) The Determination of Cholesterol and Cholesterol Esters

Badzio (6) has recently described a method for the determination of cholesterol and its esters in whole blood. The method has been adapted

for use by the present author after TLC of the lipid extract. The colour reaction was modified from that of Zlatkis (128).

Reagents

1 Anhydrous $FeCl_3$, 1.2% in ethyl acetate mixed with chloroform (1 : 1).

2 $H_2 SO_4$, 93%, mixed with ethyl acetate (1 : 1).

3 Cholesterol and cholesterol ester (purified as mentioned in the section on lipid standards).

The sulphuric acid was added to the ethyl acetate while stirring and cooling under tap water. After preparation, this solution should stand for about 24 hours before being used, and it will keep for about 2 weeks if stored in a glass stoppered flask in a refrigerator. The colour of the reagent does not interfere with the analysis.

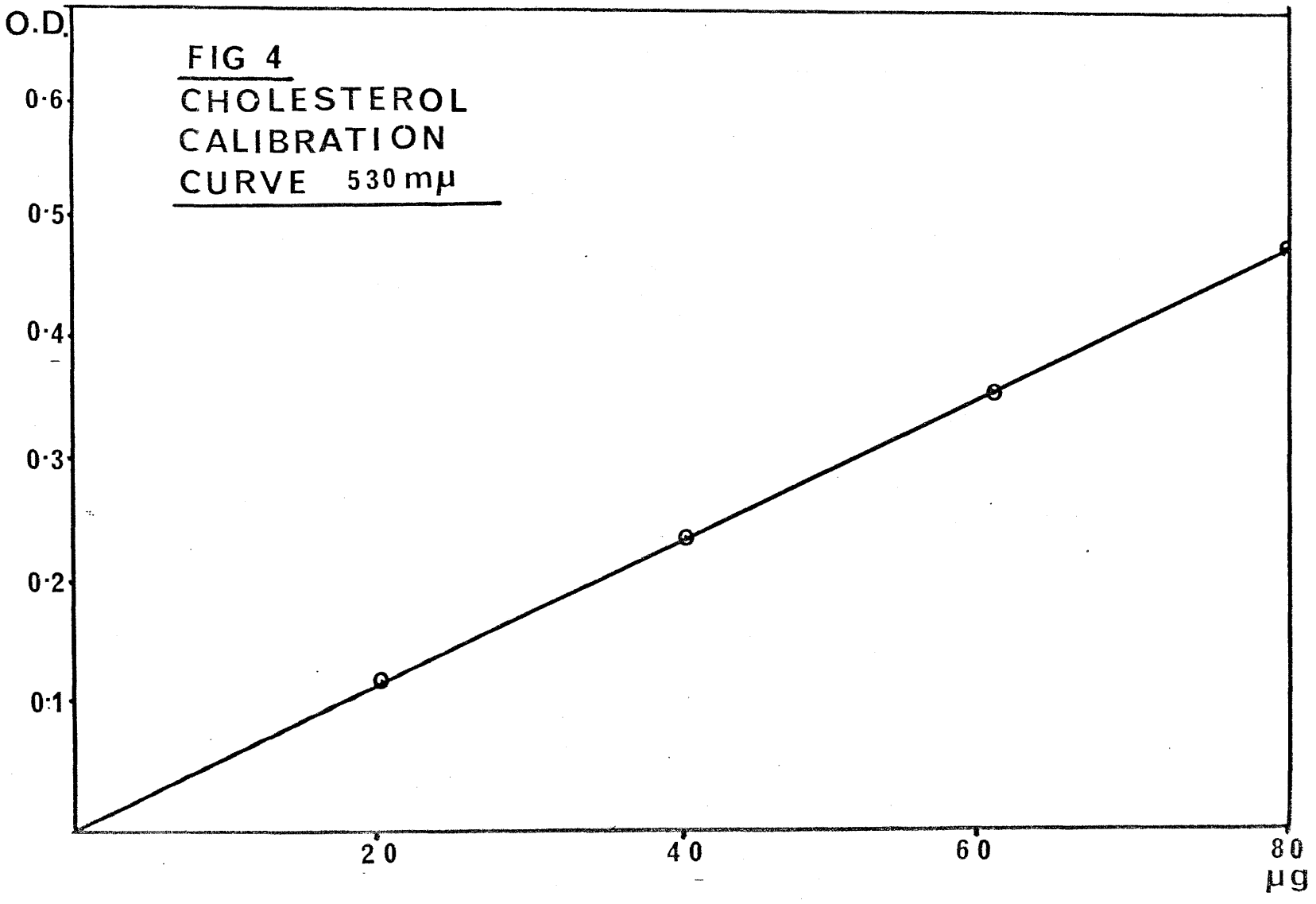


FIG 4
CHOLESTEROL
CALIBRATION
CURVE 530 mµ

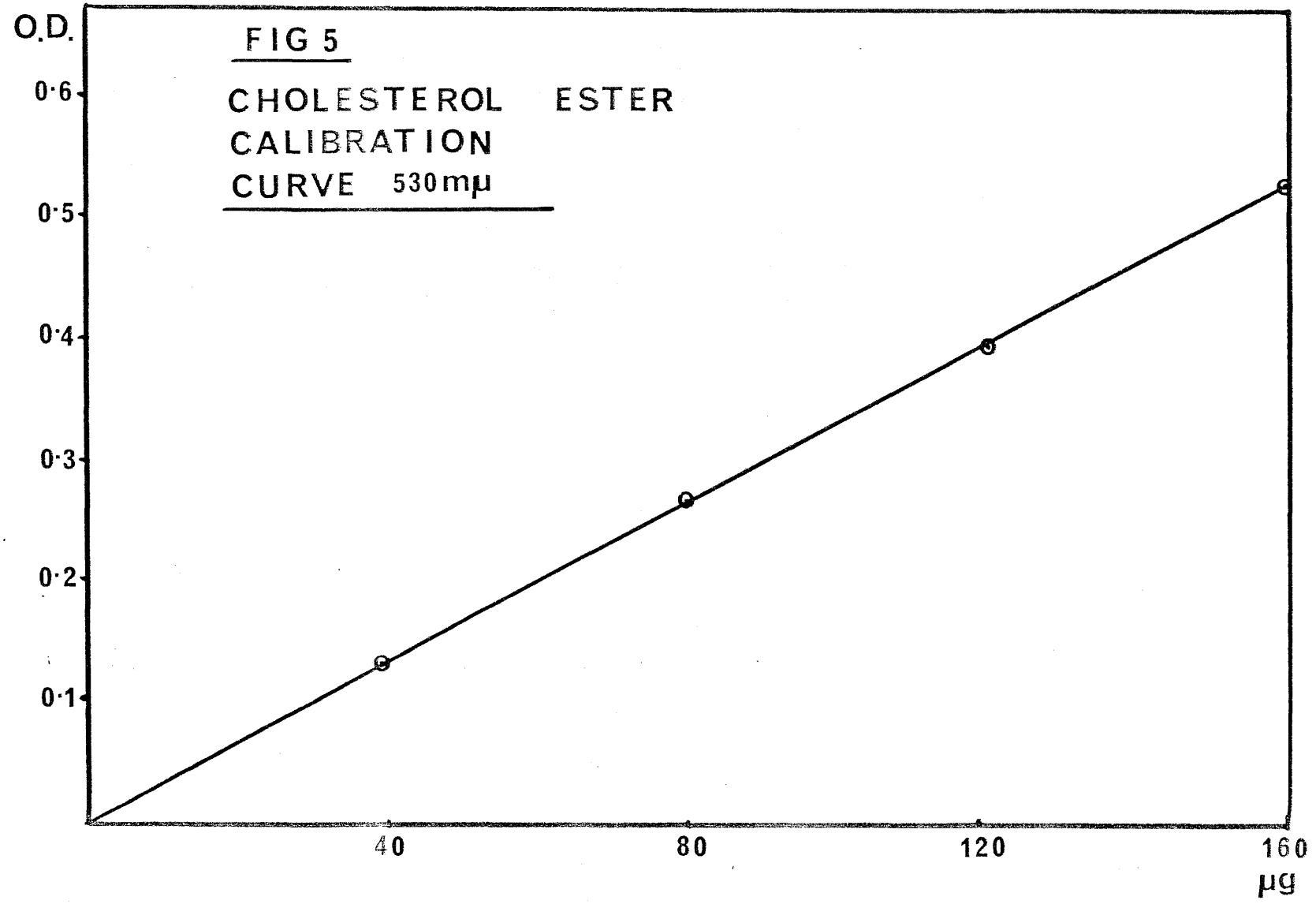


FIG 5
CHOLESTEROL ESTER
CALIBRATION
CURVE 530mμ
O.D.
0·6
0·5
0·4
0·3
0·2
0·1
40
80
120
160
μg

Method

0.5 ml. of the $FeCl_3$ reagent was added to the dried sample eluted from the silica gel, and allowed to stand for 5 minutes. 5 ml. of the H_2SO_4 : ethyl acetate was added, and the contents of the tube mixed well. The mixture was allowed to stand for 1 hour, protected from direct sunlight, and then read at 530 mu in a Beckman model B spectrophotometer. Standard curves were constructed for cholesterol with concentrations ranging from 20 - 100 ug and for cholesterol ester from 20 - 200 ug. (Figs. 4 and 5).

(b) The Determination of the Free Fatty Acids

The method used was adapted from the colorimetric method of Novak (78), in which cobalt soaps of the fatty acids are extracted with a solvent lighter than water and the complex

with ∝ nitroso β napthol measured at 530 mμ. Whereas Novak determined the free fatty acids in serum, the present author used the method after TLC of the lipid extract.

Reagents

I Solution A

(i) A saturated solution of K_2SO_4 saturated while boiling and stored in contact with crystals and filtered before use.

(ii) In a 100 ml. flask : -

80 ml. saturated K_2SO_4

6 g. $Co(NO_3)_2 6H_2O$

0.8 ml. glacial acetic acid

Made up to the mark with K_2SO_4 and kept at 37^oC.

2 Solution B

A saturated Na_2SO_4 solution prepared by adding Na_2SO_4 to boiling water and stored at 37°C.

3 Cobalt Reagent

Triethanolamine	1.35 volumes
Solution A	8.65 volumes
Solution B	7.00 volumes

This reagent is not stable and was prepared fresh for every series of analysis.

4 Indicator

Stock Solution : -

0.4% ∝ Nitroso β napthol in 96% ethanol.

Working Solution : -

Prepared Fresh daily, 3 ml. of the stock was diluted to 50 ml. with ethanol.

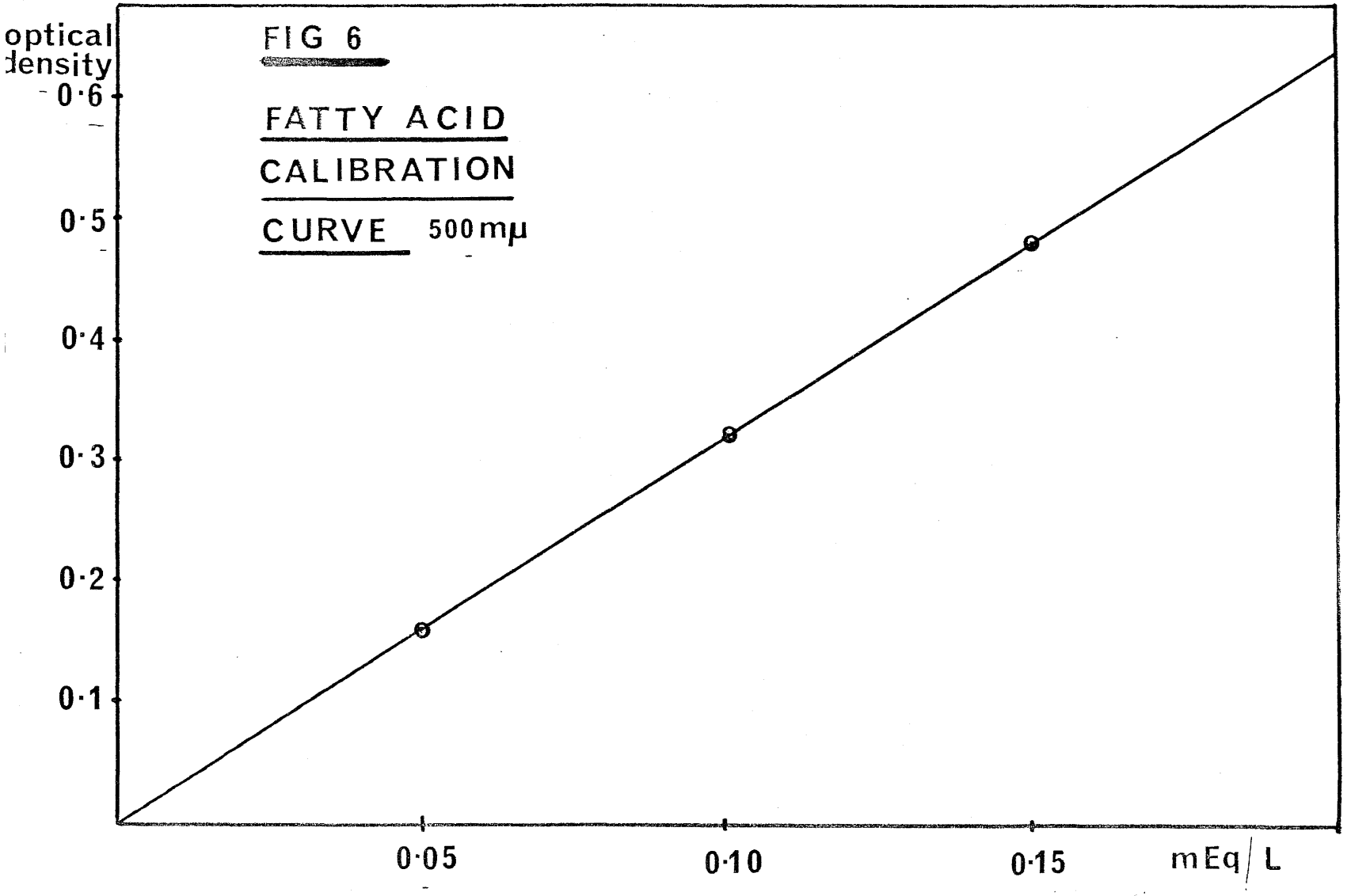
5 Chloroform : heptane 1 : 1

6 Palmitic acid

Method

The dried fatty acid sample eluted from the thin layer chromatogram was dissolved in 2 ml. of chloroform : heptane (1 : 1). Standards in the range 0.5 - 2.0 mEq/litre were included in each analysis. (Fig. 6). To the fatted acid dissolved in chloroform methanol was added 1 ml. of the cobalt reagent. Plastic stoppers were firmly inserted into the B 10 sockets of the tubes which were then very vigorously shaken for 5 minutes in a micro flask shaker so that the two phases were indistinguishable.

The tubes were then centrifuged at 3000 r.p.m. for 15 minutes. 1 ml. of the upper heptane phase was transferred to a clean test tube, and 2 ml. of the α nitroso β napthol were added. Values were read 30 minutes later at 500 mu. The colour was stable for several hours.


optical density
0·6
0·5
0·4
0·3
0·2
0·1
FIG 6
FATTY ACID
CALIBRATION
CURVE 500mμ
0·05
0·10
0·15
mEq/L

(c) The Determination of the Triglycerides

Triglycerides were determined by the modified hydroxaminolysis method (86, 111) in which esters react with alkaline hydroxy-; lamine to form a hydroxamic acid; the latter forms a purple iron chelate complex in the presence of ferric perchlorate.

Reagents

1 Stock Ferric Perchlorate

Normally this reagent was made up by dissolving 5 g. $Fe(ClO_4)_3$ in 10 ml. of 70% $HClO_4$ and 10 ml. of water, then diluting to 100 ml. with cold absolute ethanol. Due to the fact that $Fe(ClO_4)_3$ was unobtainable, the alternative method of Goddu et al (39) was used. 0.8 g of

iron wire was weighed into a 50 ml. beaker, and 10 ml. of 70% $HClO_4$ added. The beaker was then heated very carefully at low heat until the iron dissolved. When the beaker cooled, the contents were transferred to a 100 ml. volumetric flask with 10 ml. of water and diluted to volume with cold absolute ethanol.

2 Reagent Solution

4 ml. of the stock ferric perchlorate and 3 ml. 70% $HClO_4$ were diluted to 100 ml. with cold absolute ethanol.

3 Alkaline Hydroxylamine

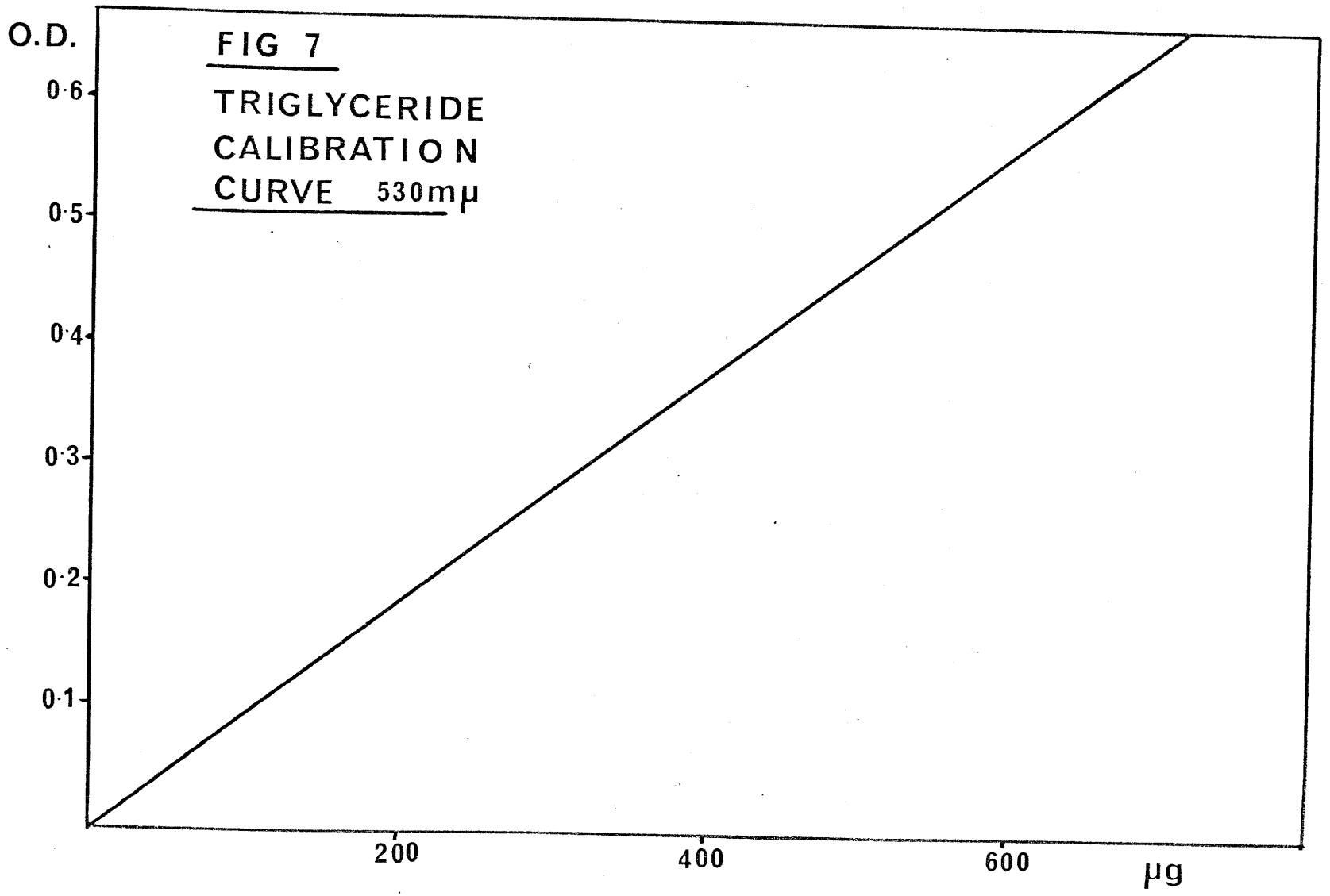
Equal volumes of ethanolic hydroxylamine solution (2.0 g. hydroxylamine hydrochloride dissolved in 2.5 ml. water, diluted to 50 ml. with absolute ethanol) and of an 8% ethanolic NaOH solution

(4.0 g NaOH dissolved in 2.5 ml. H_2O diluted to 50 ml. with absolute ethanol) were mixed in a stoppered cylinder. The NaCl was removed by centrifugation or by filtration through Whatman No 42 filter paper. This reagent was prepared fresh daily.

4 Tripalmitin

Method

1 ml. of alkaline hydroxylamine reagent was added to each of the dried triglyceride samples, which were then placed in a water bath at 65°C for 20 minutes. 2.5 ml. of the ferric perchlorate reagent (fresh daily) was added to the tubes, mixed, and after 30 minutes the purple colour was read at 530 mμ. The colour was stable for more than 1 hour. Standard solutions of tripalmitin were included in every analysis. (Fig. 7).


O.D.
FIG 7
TRIGLYCERIDE
CALIBRATION
CURVE 530mμ
0·6
0·5
0·4
0·3
0·2
0·1
200
400
600
μg

(d) The Measurement of Radioactivity

I Scintillation Solutions

(a) Phosphor for Counting CO_2

(i) 0.3 g. Dimethyl POPOP* (1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene. Scintillation grade.)

(ii) 5 g. PPO* (2,5-Diphenyloxazole. Scintillation grade.)

(iii) The dimethyl POPOP and PPO were made up to 1 litre in a volumetric flask with A.R. toluene.

(b) Phosphor for Counting the Lipid Fractions

Cab-O-sil* a thixotropic gel powder made up in the phosphor described in (a) above to 4% w/w.

* Packard Instrument Company, Illinois

2 The Preparation of Biological Samples

(a) Carbon Dioxide

The scintillation vials containing the glass fibre paper were removed from the desiccator, and 20 ml. of the solvent phosphor added.

(b) Lipid Fractions

The areas of silica gel corresponding to various neutral lipid fractions which had been transferred to scintillation vials, were suspended in 15 ml. of the Cab-O-sil mixture. (1 (b))

3 Radioactive Assay

(a) Method of Quench Correction

The instrument used in this study was the Packard Tri-carb Scintillation Spectrometer model 3000 with 4 π geometry

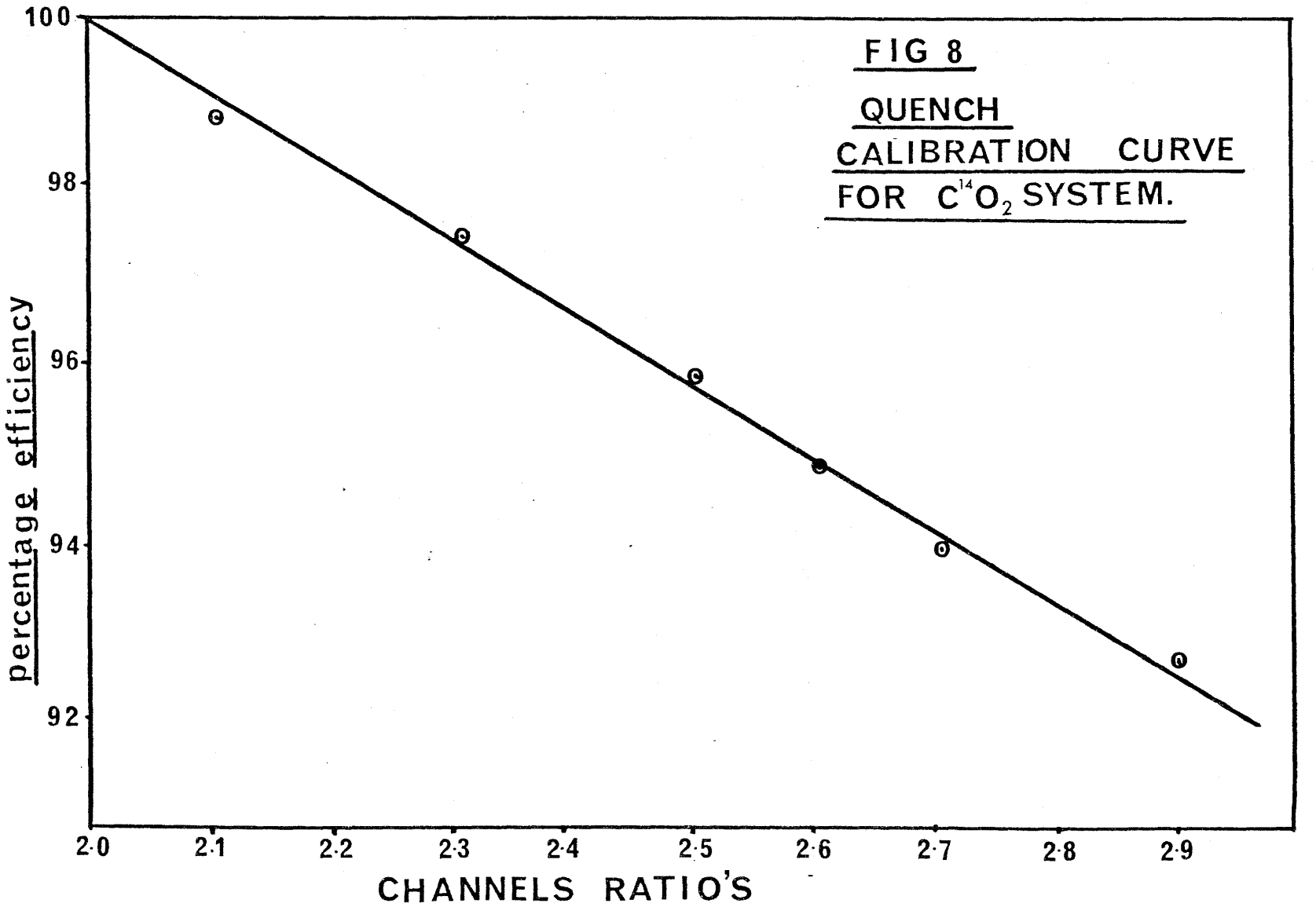
being observed throughout. Quenching was monitored by the channels ratio method, which has been studied by Rogers and Moran (93) and found to be very accurate for moderate degrees of quenching. Moderate quenching was found to be the case with the samples assayed in the present study.

(b) Construction of Quench Calibration Curves

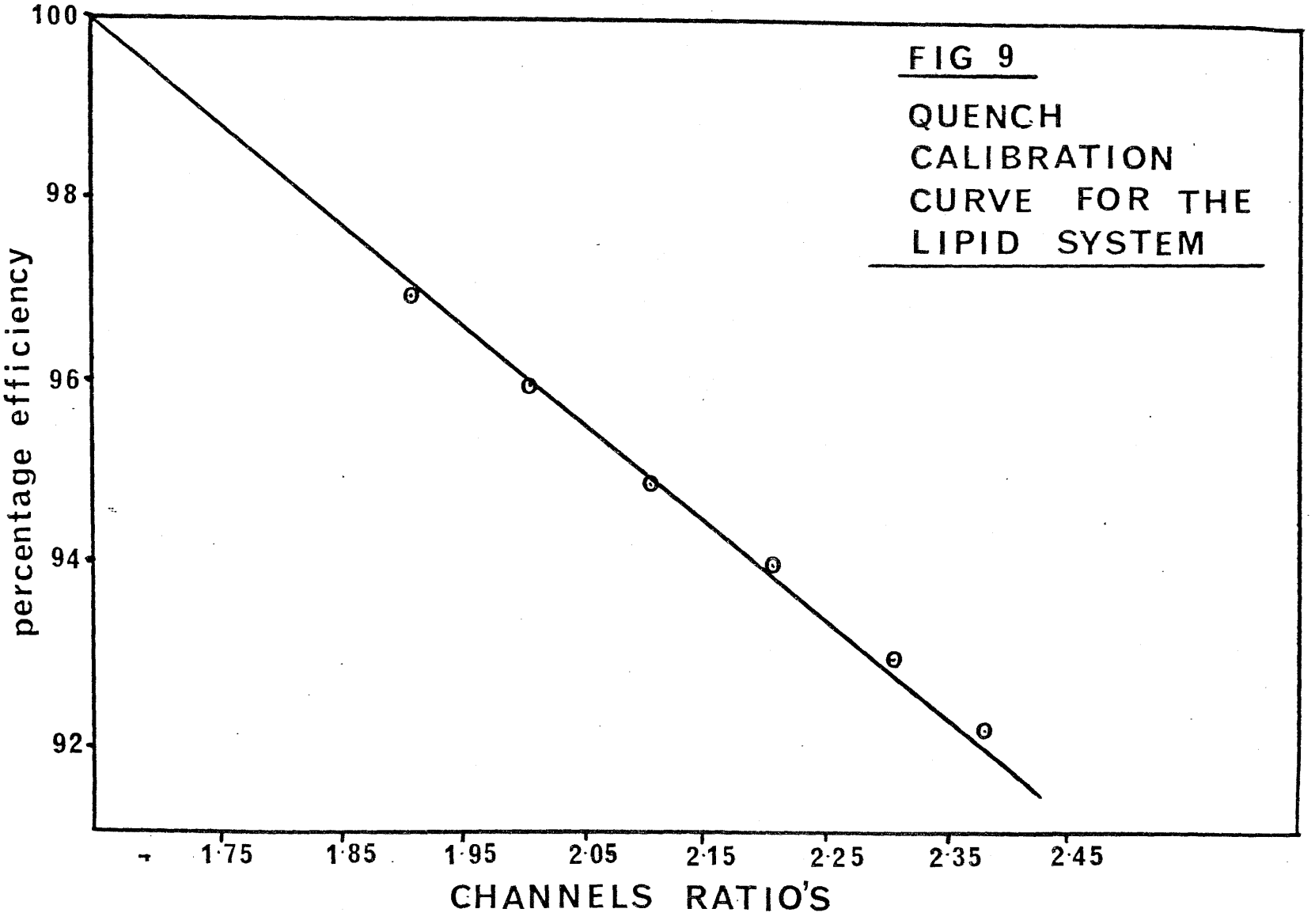
The scintillation vials to be used in the quench calibration curves were prepared exactly as in the case of the CO_2 and lipid samples. For the CO_2 curve a roll of Whatman GF/A paper (4.0X 1.5 inches) was placed in the scintillation vial and soaked with 1 ml. of 40% KOH. A series of scintillation vials prepared in this manner were then dried in a vacuum desiccator overnight. The curve for the lipid fractions

was prepared by adding silica gel from blank chromatograms prepared in the same manner as for the fractionation of the lipids. The relevant phosphor was then added to both series, and a measured amount of radioactive toluene-C^{14} (2.21×10^5 DPM/ml) added to each scintillation vial. In some cases benzoic acid -C^{14} (5,500 DPM/mg) was used. This was made up to a concentration of 1 mg/ml. and 1 ml. aliquots of this solution were dried in the scintillation vials in a vacuum desiccator, prior to the preparation of the vials as described in the case of the toluene -C^{14}. The samples were then counted and the CPM recorded for each sample. Thereafter, a range of concentrations of chloroform, which is a strong quenching agent, was added to each series of scintillation vials, and these were then counted again. Typical quenching curves are shown in Fig. 8 and 9 for the CO_2 and lipid samples.

100% on the y axis represents 74% overall efficiency.



100% on the y axis represents 73% overall efficiency.



(c) Counting Efficiency and Precision

15% gain was found to be optimal in both series of samples, and in the case of the CO_2 samples window settings of A-B 50-1000 and C-D 250-1000 were used. It was found necessary to raise the lower discriminator A-B 100-1000 in the case of the lipid samples due to low energy noise caused by a fluorescence effect in the Cab-O-sil. The CO_2 samples were counted at an efficiency of 74% and a background of 55 cpm, while the lipid samples were counted at an efficiency of 73% and a background of 30 cpm.

Chapter 6

DISCUSSION OF METHODS

(a) Separation of Lipids

Extraction of the lipid of the leucocytes from 50 ml. of blood yielded on an average 3 - 10 mg. of total lipid, and this had to provide lipid for both chemical and radioactive analysis. Several procedures have been described for the separation of different classes of neutral lipid, such as the early work of Mangold and his co-workers (65, 66, 68), Williams *et al* (123), Vogel *et al* (119) and others. Various investigators have used step wise or gradient systems for neutral lipids. Weicker (122) examined the serum lipids by thin layer chromatography and developed a chromato-plate with three solvent systems successively. Lipid mixtures are normally separated into classes by chromatography on layers

of silica gel G usually with solvent systems containing light petroleum and diethyl ether in various ratios, with added acetic acid (1 - 2%). Unfortunately, on a one dimensional chromatogram these systems do not separate all classes of neutral lipid normally present in human and animal tissue.

Skipski et al (105) has pointed out that it is very important to separate all of the fractions so that when it comes to analysis and radioactive counting there is no interference of one fraction with the next. It is possible to have one lipid spot on top of another without even realising its presence. The difficulty of separating all classes of neutral lipid on a one dimensional chromatogram with the solvent systems mentioned above has led to a description of a number of two step chromatograms, using two solvents of different polarity. Huhnstock and Welcker (47) analysed humans serum

lipids by two dimensional chromatography using a two phase solvent system; propyl alcohol - ammonia 2 : 1 in one direction and carbon tetrachloride in the other. This method was modified slightly by Sachs and Wolfman (98). The present author found these methods resolved the neutral lipids indistinctly.

Perhaps the only two methods which are satisfactory and which separate all of the lipid classes are those of Skipski et al (106) and Freeman and West (35). The method of Freeman and West has a two step development with two solvents. Solvent 1 is diethyleter - benzene - ethanol - acetic acid (40 : 50 : 2 : 0.2) and Solvent 2 diethylether - hexane (6 : 94), but it requires 34 cm plates for the resolution and can take 3 / 3½ hours for a single run. Also the tanks are fairly large and saturation with solvents is a far greater problem. Skipski's two step, two solvent develop-

ment was found to be by far the simplest and most reproducible. The method employs silica gel without binder, which was successfully used by the same school in the case of phospholipids. Using this method all classes of neutral lipid normally present in animals tissues are separated on a single one dimensional chromatogram. Good separations are obtained of not only cholesterol from diglycerides, but the two structural isomers 1,3 and 1,2 diglycerides as well.

(b) Quantitation

Complex mixtures of lipids can be separated rapidly by TLC, and in many cases the separations can not be reproduced by any other method. Therefore, many workers have devised quantitative methods for the estimation of the separated components.

Williams et al (123) obtained semi-quantitative estimations by eluting the components from the plates and weighing. It is, however, fairly inaccurate to weigh such small amounts and various alternative methods have been developed. There are two fundamentally different approaches to the quantitative analysis of neutral lipids by thin layer chromatography. The first is based on the assumption that the individual spots represent individual classes of lipid, free from non-specific chromogens. The quantitative analysis would therefore be a simple measurement of the total mass of organic material in these spots. Such measurement has been performed by the measurement of the area of the spot in relation to the concentration (90), or the measurement of the charred lipid spots directly on the chromatogram by photo-densitometery (87, 88, 89). Amenta (2) and Freeman and West (35) attempted the quantitative estimation of the lipid fractions by oxidation with dichromate solution, followed by the

measurement of the reduction of dichromate reagent in the solution by spectrophotometery. All of these procedures are non-specific.

The second approach to quantitative analysis of neutral lipids is based on the application of a specific chemical test for the particular lipid class after the lipids have been separated and in most cases eluted from the chromatograms. Vioque and Holman (118) separated esters of different types, the spots were removed and converted to iron - hydroxamate acid complexes, which were estimated colorometrically. Badzio and Boczon (6) adapted a thin layer chromatographic method for quantitative chemical determination of free and esterified cholesterol in serum. Gloster and Fletcher (38) applied thin layer chromatography in combination with the chemical quantitative determination of separated and eluted compounds for a more systematic characterisation of lipid extracts from human serum.

Very recently, Skipski et al (105) have published a quantitative analysis of neutral lipids by TLC. All the lipids were analysed by specific methods. The glycerides and the hydrocarbons were determined by infra-red spectrophotometery, free fatty acids by titration and cholesterol and its esters by a standard chemical procedure. This is very similar to the method used by the present author as described in the lipid methods.

(c) Cholesterol and Cholesterol Esters

No other lipids of the human body have been so thoroughly studied, and a massive volume of work has accumulated on methods for their determination. Several of the early methods are interesting primarily because of historic interest. They include gravimetric methods, in which cholesterol is precipitated as the digitonide, and after saponification and the digitonide weighed (67). Titrametric methods, in which the digitonide is

oxidised by a mixture of silver chromate and H_2SO_4 (Nicloux reagent), then KI is added and the excess of dichromate is titrated with thiosulphate (84), and manometric methods, in which the carbon of the digitonide is determined manometrically (54).

The methods of greatest interest are photometric, and the different colour reactions might be discussed. Cholesterol and other steroids intense colours when treated with acid reagents. In the Liebermann - Buchard reaction, cholesterol in chloroform is treated with acetic anhydride and concentrated sulphuric acid yielding a green colour. The Salkowski reaction eliminates the acetic anhydride and a red purple colour is formed. Other colour reactions, such as the Tschugaeff reaction (with acetyl chloride and zinc chloride in glacial acetic acid) and ferric chloride - sulphuric acid reactions probably have a similar

basis, but either employ weaker acids, and as a result are possibly more selective, or include an oxidising agent which can increase the length of the conjugated diene chain. The reaction with $FeCl_3$ - H_2SO_4 appears to involve oxidation because definite amounts of Fe^{+++} are reduced to Fe^{++}, the amount being proportional to the cholesterol present (127).

The Liebermann - Buchard reaction and its modifications rely on a complex colour chain, which is dependent and can be influenced by many variables, and is generally regarded as insensitive. The Salkowski reaction in which only H_2SO_4 is used appears to have been abandoned. Success appears to depend on low temperature and rigorous exclusion of moisture. The Tschugaeff reaction, despite the fact that it is 15 times more sensitive than the Liebermann - Buchard reaction, has also not become popular, possibly due to the stringent requirements for anhydrous $ZnCl_2$ (57).

By far the most used group of methods employ the $FeCl_3$ - H_2SO_4 reaction, introduced in 1953 by Zlatkis et al (98). Literally hundreds of papers have appeared using this reaction. Zlatkis et al first proposed using the method directly on serum, but later the cholesterol, free and esterified, was first extracted with various fat solvents, followed by washes of different types and the results were then found to agree with the reference methods of Shoenheimer and Sperry (103) and Sperry and Webb (112). The reagents from that used by Zlatkis et al to the present have been $FeCl_3$, H_2SO_4 and glacial acetic acid in different ratios and added in different orders. The big problem found in this laboratory is that the reagent is very viscous and inclined to trap air bubbles which give spurious results. The present author attempted to use the modification of Zlatkis et al as described by Vahouny et al (117). Whereas it worked well for cholesterol it could not be used for cholesterol esters due to the fact that

the esters were insoluble in acetic acid. Using this system Badzio (5) and Kurweg and Massman (59) showed that there was a difference in the molar extinction for free and esterified cholesterol. Badzio (6) has separated cholesterol and cholesterol esters on TLC and c claims that the colour reaction is far less influenced by temperature and the concentration of H_2SO_4. The present author modified the method and used it after the thin layer chromatography of the lipid extract of the leucocyte.

(d) The Free Fatty Acids

Nearly all the methods for free fatty acids are titrametric using thymol blue, phenol phthalein, or a pH meter for the end point. This technique is very difficult to perform when working with a very small quantity of biological tissue. The ultra-micro technique is extremely troublesome to use, and the end point with indicators is often very difficult to determine. Where the free

fatty acids are extracted from biological tissues there may be interference by lactic acid, phospholipids, exceptionally high levels of acetic acid, acetoacetate, and B-hydroxybutyric acid (24).

The development of a photometric method, which could simplify the estimation of free fatty acids enormoursly, has been the aim of many workers. The method of Coleman and Middlebrook (21) was one of the first photometric method described, and this was based on the interfacial enrichment of dye. Methylene blue is attracted by fatty acids to the interface between alkaline aqueous solutions and a water immiscible fatty acid solvent. The loss of dye from the aqueous phase was measured. Although this method is reported to be very sensitive, it develops colours of varying intensity, depending on the fatty acid composition. In 1962 Duncombe (25) suggested in a preliminary communication that the selective transfer of the

copper soaps of fatty acids into chloroform, the principle of which was first introduced by Ayers (4), may be applicable to colourimetric determination of fatty acids. The full report of the method (26) was published in 1963, and was very useful when combined to paper chromatography. When applied to biological material, however, billirubin and other chromogens soluble in lipid extracts may interfere with the determination. In addition, when an ultra-micro modification of these methods is used, it is difficult to take samples of the lower chloroform extract uncontaminated by the upper aqueous phase containing an excess of copper salts.

Novak (78) perfected this method by increasing the sensitivity, and extracting cobalt rather than copper soaps by means of a solvent lighter than water instead of chloroform.

Cobalt soaps give a colour reaction with α nitroso β napthol.

(e) The Determination of the Triglycerides

The most common colorimetric methods are those in which the triglyceride is saponified and glycerol is oxidised with periodic acid to formaldehyde. The excess periodic acid can be titrated, or the formaldehyde formed can be determined photometrically by the chromotropic reaction, or by the phenyl hydrazine-potassium ferricyanide reaction. The last two are probably the most used methods. The first, pioneered by Lambert and Neish (60), and the modification by Carlson and Wadström (18) is very widely used in clinical laboratories. It is, however, very time consuming, requiring 9 or 10 manipulations before the absorbance can be read. Laurell (61) has recently streamlined the method, using the same

principles but requiring less time, and has brought the operation time down to about 1½ hours. The phenyl hydrazine-potassium ferricyanide technique of Randrupp (91) has been modified by many authors, and they claim it to be far more rapid and sensitive than that using chromotropic acid. Jover (51) and Galetti (37) have recently modified the colour reaction.

As the triglycerides were separated from the other neutral lipid fractions by thin layer chromatography, the relatively non-specific technique of Snyder and Stevens (111) and of Pinter et al (86) could be used. Skidmore and Enteman (104) and Goddu et al (39) used this relatively non-specific method for cholesterol esters, phospholipids, and anhydrides. After TLC the method was found to be very satisfactory and Vioque and Martin (118) found recovery of esters from the plate better than 95%. Pinter et al determined the serum triglycerides after glass fibre chromatography and

found the method to be reproducible and very time saving. Snyder and Stevens used the method to estimate ester groups in lipids, and found the method timesaving, and a means of determining extremely small samples of lipid esters with excellent reproducibility.

(f) The Collection of CO_2

The collection of $C^{14}O_2$ for subsequent radioactive assay has long posed a problem in tracer studies. A number of techniques have been developed for the collection and subsequent counting of $C^{14}O_2$ liberated during metabolic studies.

Snyder and Godfrey (109) found great difficulty in transferring $C^{14}O_2$ from a Warburg flask to a scintillation vial. They eventually used a removable vessel which was positioned on top of the centre well in a Warburg flask. After the

completion of an incubation Hyamine hydroxide was injected into the removable vessel through a rubber cap fitted to the Warburg flask. The flask was then allowed to shake for three hours at 37°C. The vessel was then transferred *in toto* to a glass scintillation vial containing the solvent phosphor. As the methanolic Hyamine hydroxide commonly employed to trap the evolved $C^{14}O_2$ for liquid scintillation counting is toxic towards most biological systems, it is necessary to add it at the end of the incubation period. For the same reason Wakil (120) also introduced it at the end of the incubation period by suspending a paper strip moistened with Hyamine into the Warburg flask. Cuppy and Crevasse (22) described a modification of the Snyder and Godfrey technique in which Warburg flasks were replaced by 25 ml. Erlenmeyer flasks as the main vessel. The vessel for CO_2 collection was suspended on a stainless steel mount, and the Hyamine injected through a sleeve-type rubber stopper. Passman (82) avoided the difficulty of

using the toxic Hyamine by trapping respiratory CO_2 in NaOH and then after incubation, the contents of the flask were acidified and Hyamine was used for trapping the $C^{14}O_2$. All of these methods require transfer of the viscous Hyamine hydroxide to the scintillation vials, which is difficult without specially constructed flasks. Moreover, the collection of the $C^{14}O_2$ is possible only at the end of an incubation. Besides exhibiting a fairly substantial degree of quenching Hyamine does not permit the periodic collection of CO_2.

Mirsky (73) reported a technique for trapping $C^{14}O_2$ on papers moistened with NaOH injected through a vial seal into the conventional centre well. The strips were removed, dried and counted in a liquid scintillation counter. Buhler (17) trapped $C^{14}O_2$ on paper moistened with KOH in the Warburg centre well, and after the incubation transferred these directly to scintillation vials containing the solvent phosphor. Buhler

tested the trapping and counting technique using standard $Na_2C^{14}O_3$ and reported that the recoveries were quantitative and reproducible. As water is a fairly strong quenching agent, this system must have shown considerable quenching power, as was reflected in the counting efficiency of only about 40% in a special solvent phosphor for the assay of aqueous samples. In a toluene phosphor similar to the one used in the present study, only 5.6% of the radioactivity initially present in the Warburg flask was detected. However, if the strips were first dried in vacuo and then counted, Buhler found that the counting efficiency was increased to 45%. When compared with Hyamine hydroxide KOH is a particularly attractive trapping agent, when the CO_2 trapped contains large amounts of water vapour. The reason for this is that in the Hyamine hydroxide system it would be difficult to get rid of water, which would also contribute to quenching.

The method used in the present study (97) had a number of advantages over the methods mentioned above. The only modification made was to substitute Whatman GF/A glass fibre paper for Whatman 40 filter paper. As mentioned in the section on radioactive counting (Chapter 5d) this glass fibre paper did not give rise to a yellow colour, which was however produced on cellulose paper; this increased the efficiency of counting. The technique of Saba and di Luzio permits the repetitive determination of $C^{14}O_2$ evolved by the same sample. These authors found the time necessary for quantitative recovery of $C^{14}O_2$ with this method was significantly less than others which had previously been reported. In investigations to test the efficiency of the trapping system, Saba and di Luzio found that essentially all the radioactivity was trapped in 15 minutes. They also tested the removal and replacement of scintillation vials and found no detectible loss of activity when the trapping

chamber was replaced during the course of experimental procedure.

(g) The Measurement of Radioactivity

(i) Scintillation solutions

The solvent phosphor containing PPO and dimethyl POPOP is the most efficient yet described. Firstly, no primary solute with the solubility and emission characteristics of PPO has been found- dimethyl POPOP was used as it is rapidly replacing POPOP as a secondary solute for the following reasons: - its mean wave length of emission is somewhat longer, and also it is appreciably more soluble in the usual liquid scintillation solvents. Early studies of scintillation solvents (53) showed that the best performances resulted from alkyl benzene structures, and the simplest of these, toluene, has become the most widely used.

(ii) Carbon Dioxide

As mentioned in the section on the discussion on the method for the collection of CO_2, the cellulose fibre paper used by Saba and di Luzio was replaced by glass fibre paper. This was originally done by the present author to avoid the formation of a yellow tinge on the cellulose fibre paper. This was caused by KOH and gave rise to severe colour quenching. The use of glass fibre paper has been described in the literature. Pinter et al (85) used silica gel impregnated glass fibre paper to separate cholesterol 1-C-^{14} from a lipid mixture and afterwards the cholesterol area was cut out and added to a scintillation vial containing 15 ml. of scintillation fluid. Pinter et al found that glass fibre paper has a

60 - 70% higher efficiency than cellulose paper, and tested this by counting identical amounts of fucose -l-C^{14} in a non-polar scintillation fluid. Normally the glass fibre paper becomes transparent in toluene as they have a similar refractive index; borosilicate glass n=1.5097 and toluene n=1.497.

(iii) Lipids

The Cab-O-sil phosphor serves to keep the silica gel in suspension after the removal from the chromatoplate and saves the necessity of elution (110). This method gives quantitative recovery of radioactivity from the lipid fractions and the efficiency of the toluene system is not essentially altered by the addition of Cab-O-sil (108).

(iv) The Method of Quench Correction

The channels ratio method of quench correction, first proposed by Baillie (cited from (44)), is a very useful one, and has the following advantages:- the efficiency is measured while the sample is being counted and is immediately determinable upon completion of the count; for a two channel instrument only a single count is needed; the sample itself remains unchanged and can be recovered. If desired, it can be counted under different or improved conditions and mistakes or inaccuracies from addition of internal standard or dilution of the sample are eliminated; also, the technique is independent of sample volume over a considerable range of sample volume (unlike external standardisation).

Rogers and Moran (93) compared the external, internal, and channels ratio methods of quench correction and found the channels ratio to be the most efficient for moderate degrees of quenching. Herberg (44) has mentioned that all C^{14} colourless solutions, whatever the degree of quenching, and whatever the quenching agent, appear to fall on the same calibration curve. Herberg also found a parallel shift of calibration curves, determined over several weeks, amounting to a difference of 2 - 6 percentage efficiency units. A series of quenched standards should thus be counted with each series of samples. No colour quench calibration curves were constructed as this was not a problem in the present study.

Chapter 7

RESULTS

(a) Carbon Dioxide

The radioactive tracer studies were started using acetate-1-C^{14} as a substrate. Looking at the $C^{14}O_2$ productions (Table IV) in the first two experiments RAI and RAII, where 5 μ curies of the substrate were used, the graph of activity against time (Fig. 10) shows an initial fairly high production of $C^{14}O_2$, which then drops down to a steady level less than half the 30 minutes value. When the radioactivity added was increased to 25 μ curies this effect disappeared. The $C^{14}O_2$ productions were then similar to those found in the case of the ketone bodies. The total $C^{14}O_2$ production in the two experiments where a 5 μ curie quantity of acetate-C^{14} was used were

comparable to the two experiments in which a 25 μ curie quantity was used. Multiplying the total CO_2 production by a factor of 5, the results for the 5 μ curie acetate experiments were 4.1 and 2.7 cpmX$10^6/10^{10}$ cells/hour and for the 25 μ curie acetate experiments, 4.7 and 6.9 respectively.

In the experiments using Na-acetate-1-C^{14} there was a considerable isotope dilution effect, as Na-acetate was present in the medium at a concentration of 24.4 mM. 5 times as much of the labelled substrate (25 μ curies) had to be used before the incorporation of the label into $C^{14}O_2$ was comparable to the experiments where 3-hydroxybutyrate-3-C^{14} and acetoacetate -3-C^{14} were used (5 μ curies). The specific activity of the Na-acetate present in the medium was very low (5 μc in 20.144 mg. for 5 μc dose, and 25 μc in 20.720 mg. for 25 μc dose).

The 5 μ curie doses of the labelled ketone bodies resulted in total $C^{14}O_2$ productions that were very comparable (Table X), especially in the case of 3-hydroxybutyrate. The $C^{14}O_2$ productions from the ketone bodies as a function of time are shown in Fig. 11. After 30 minutes of incubation the label incorporated into $C^{14}O_2$ in the case of acetoacetate is progressively more than in the case of hydroxybutyrate.

The percentage of each substrate converted into $C^{14}O_2$ (Table XI b) was, acetate 11.86, 3-hydroxybutyrate 55.59 and acetoacetate 85.76, which represents 26.2, 0.488, and 0.745 μ moles/10^{10} cells/hour respectively. Alternatively, this can be represented as 1.78, 0.03, and 0.05 μ moles/100 mg. leucocyte dry weight, (using the average of 4 published conversion factors = 6.8×10^6 leucocytes = 1 mg. dry weight.

(b) Lipids

In the first two experiments RA I and RA II where Na-acetate-1-C^{14} was added to the cell suspension at a level of 5 μ curies there was no significant incorporation of the label into neutral lipid. In order to show incorporation of the label into lipid, it was necessary to increase the amount of acetate added to a level of 25 μ curies, and even then the incorporation was fairly low (Table V). Again the level of activity added to the leucocyte suspensions in RA III, IV and V was 5 times higher than in the case of the ketone body experiments. The percentage incorporation into the neutral lipid fractions was reasonably similar with roughly half the label being incorporated into the triglyceride fraction. Unfortunately, no analyses were done on the neutral lipid fractions so that no specific activities were available in the case of acetate -C^{14} (See Table V).

In the case of the ketone bodies, the incorporation of the label into the neutral lipid seemed to be higher on the average (more than three; times) and in only two of the experiments were the figures as low as for those using acetate (RBH V and RBH VI) (Tables VII and IX). The amount of radioactivity incorporated into the phospholipid fraction was always higher than for any other lipid fraction. The highest percentage incorporations were found in the case of 3-hydroxybutyrate, 76% as against 57% in the case of acetate and 53% in the case of acetoacetate (Table XII).

In Table XI a, where the μ moles of substrate incorporated into the lipid fractions are given, it can be seen that the bulk of the substrate is incorporated into the phospholipid fraction. In the same Table it is evident that acetate was incorporated into the total lipid at a level

about 10 times that found for the ketone bodies.

From the results of 15 separate analyses an average of 4.8 mg. of total lipid was obtained per 100 mg. of leucocytes (wet weight).

Table IV

UTILISATION OF C^{14}- ACETATE

INCORPORATION INTO $C^{14}O_2$

Exp.	Cells x 10^8	Time	CPM	Total CPM
RA I 5 µc	2.62	30 60 90 120	18,108 8,372 8,304 8,211	42,995
RA II 5 µc	2.04	30 60 90 120	9,987 4,452 3,813 4,361	22,613
RA III 25 µc	3.82	30 60 90 120	—	—
RA IV 25 µc	1.46	30 60 90 120	21,733 28,131 43,765 43,061	136,690
RA V 25 µc	1.32	30 60 90 120	10,640 37,000 61,280 73,800	182,270

Table V

UTILISATION OF C^{14}- ACETATE

INCORPORATION INTO NEUTRAL LIPIDS

Exp	Cells x 10^8	Fraction	CPM	% Activity
RA I 5 µc	2.62	Chol. F.A. Triglyc. Chol.Ester	No Sig. inc.	-
RA II 5 µc	2.04	Chol F.A. Triglyc. Chol.Ester	No Sig. inc.	-
RA III 25 µc	3.82	Chol F.A. Triglyc. Chol.Ester	28 41 181 63	9 13 58 20
RA IV 25 µc	1.46	Chol. F.A. Triglyc. Chol.Ester	76 55 204 44	20 14 54 12
RA V 25 µc	1.32	Chol. F.A. Triglyc. Chol.Ester	25 41 189 40	8 13 64 15

Table VI

UTILISATION OF 3-HYDROXYBUTYRATE-C^{14}

Incorporation into $C^{14}O_2$

Exp.	Cells x 10^8	Time (Mins)	CPM	Total
RBH I 5 µc	3.10	30 60 90 120	18,539 72,836 116,178 163,039	370,731
RBH II 5 µc	2.43	30 60 90 120	10,516 40,140 61,419 63,566	175,641
RBH III 5 µc	3.60	30 60 90 120	36,227 91,386 139,641 158,120	425,374
RBH IV 5 µc	7.54	30 60 90 120	31,219 110,697 181,815 197,159	520,890
RBH 5 µc	3.27	30 60 90 120	23,415 48,667 74,991 87,237	
RBH VI 5 µc	3.58	30 60 90 120	77,858 80,623 92,131	250,612

Table VII

UTILISATION OF 3-HYDROXYBUTYRATE-C^{14}

INCORPORATION INTO NEUTRAL LIPIDS

Exp.	Cells $\times 10^8$	Fraction	CPM	% Activity Inc	Specific Activity /100 mg.
RBH I 5µc	3.10	Chol.	193	16	
		F.A.	193	16	-
		Triglyc.	576	50	
		Chol.Ester	195	17	
RBH II 5 µc	2.43	Chol.	367	24	
		F.A.	287	19	-
		Triglyc.	716	47	
		Chol.Ester	141	9	
RBH III 5 µc	3.60	Chol.	234	16	780
		F.A.	226	16	1076
		Triglyc.	892	61	-
		Chol.Ester	98	7	3267
RBH IV 5 µc	7.54	Chol.	248	10	330
		F.A.	430	19	2388
		Triglyc.	1425	62	3166
		Chol.Ester	186	8	2657
RBH V 5 µc	3.27	Chol.	41	19	40
		F.A.	24	11	184
		Triglyc.	126	60	630
		Chol.Ester	18	9	450
RBH VI 5 µc	3.58	Chol.	69	12	431
		F.A.	114	19	633
		Triglyc.	356	60	791
		Chol.Ester	52	9	346

Table VIII

UTILISATION OF ACETOACETATE -C^{14}

INCORPORATION INTO $C^{14}O_2$

Exp.	Cells $\times 10^8$	Time (Mins)	CPM	Total
RAA I 5 µc	3.82	30 60 90 120	34,707 114,926 178,413 215,766	543,812
RAA II 5 µc	3.83	30 60 90 120	11,995 67,089 125,707 162,855	367,646
RAA III 5 µc	4.11	30 60 90 120	16,154 69,355 132,104 146,237	363,850
RAA IV 5 µc	3.05	30 60 90 120	51,749 167,556 268,431 249,623	737,359
RAA V 5 µc	4.56	30 60 90 120	20,603 100,103 153,674 186,628	461,008

Table IX

UTILISATION OF ACETOACETATE -C^{14}

INCORPORATION INTO NEUTRAL LIPIDS

Exp.	Cells $\times 10^8$	Fraction	CPM	% Activity	Specific Activity /100 mg
RAA I 5 µc	3.82	Chol. F.A. Triglyc Chol.Ester	252 370 1368 433	10 15 56 18	336 1193 2736 1082
RAA II 5 µc	3.83	Chol. F.A. Triglyc Chol.Ester	303 405 2127 674	9 11 61 19	439 2130 6065 8425
RAA III 5 µc	3.05	Chol. F.A. Triglyc. Chol.Ester	306 180 469 187	26 15 41 16	612 692 1042 1335
RAA IV 5 µc	4.56	Chol. F.A. Triglyc. Chol.Ester	275 244 842 335	16 14 50 20	1309 1876 2105 1861

Table X

TOTAL $C^{14}O_2$ PRODUCTION

CPM x 10^6 / 10^{10} CELLS / HOUR

Exp.	Acetate 25 µc	3-Hydroxybutyrate 5 µc	Acetoacetate 5 µc
1	4.1	5.9	7.1
2	2.7	3.6	4.8
3	-	5.9	4.4
4	4.7	3.4	12.0
5	6.9	3.6	5.0
6	-	3.5	-

Table XI (a)

μ MOLES SUBSTRATE INCORPORATED INTO THE LIPID FRACTIONS

(/100 mg. leucocyte dry weight)

	Acetate	3-Hydroxybutyrate	Acetoacetate
P-lipid	0.02	0.003	0.001
Chol.	0.002	0.000098	0.00014
FattyAcid	0.002	0.0001	0.00015
Triglyc.	0.009	0.00033	0.00058
Chol.Ester	0.002	0.00005	0.00019
Total	0.035	0.00358	0.00206

Table XI (b)

μ MOLES SUBSTRATE INCORPORATED INTO $C^{14}O_2$

(/100 mg. leucocyte dry weight)

Acetate	3-Hydroxybutyrate	Acetoacetate
1.78 (12%)	0.03 (55.6%)	0.05 (85.8%)

Table XII

INCORPORATION OF ACTIVITY INTO PHOSPHOLIPID

	CPM	% Inc		CPM	% Inc		% Inc	CPM
RA I	-		RBH I	9,671	90	RAA I	53	2,722
RA II	-		RBH II	2,886	67	RAA II	43	2,649
RA III	310	50	RBH III	12,394	90			
RA IV	716	65	RBH IV	-		RAA IV	64	2,045
RA V	416	56	RBH V	344	62	RAA V	53	1,930
			RBH VI	1,403				
	Mean=57			Mean= 76		Mean =	53	

Table XIII

% COMPOSITION OF LEUCOCYTE LIPID FRACTIONS

Cholesterol Esters	1%
Triglyceride + Fatty Acid	6.0%
Cholesterol	5.6%
Phospholipids	87.7%
Total	100.3%

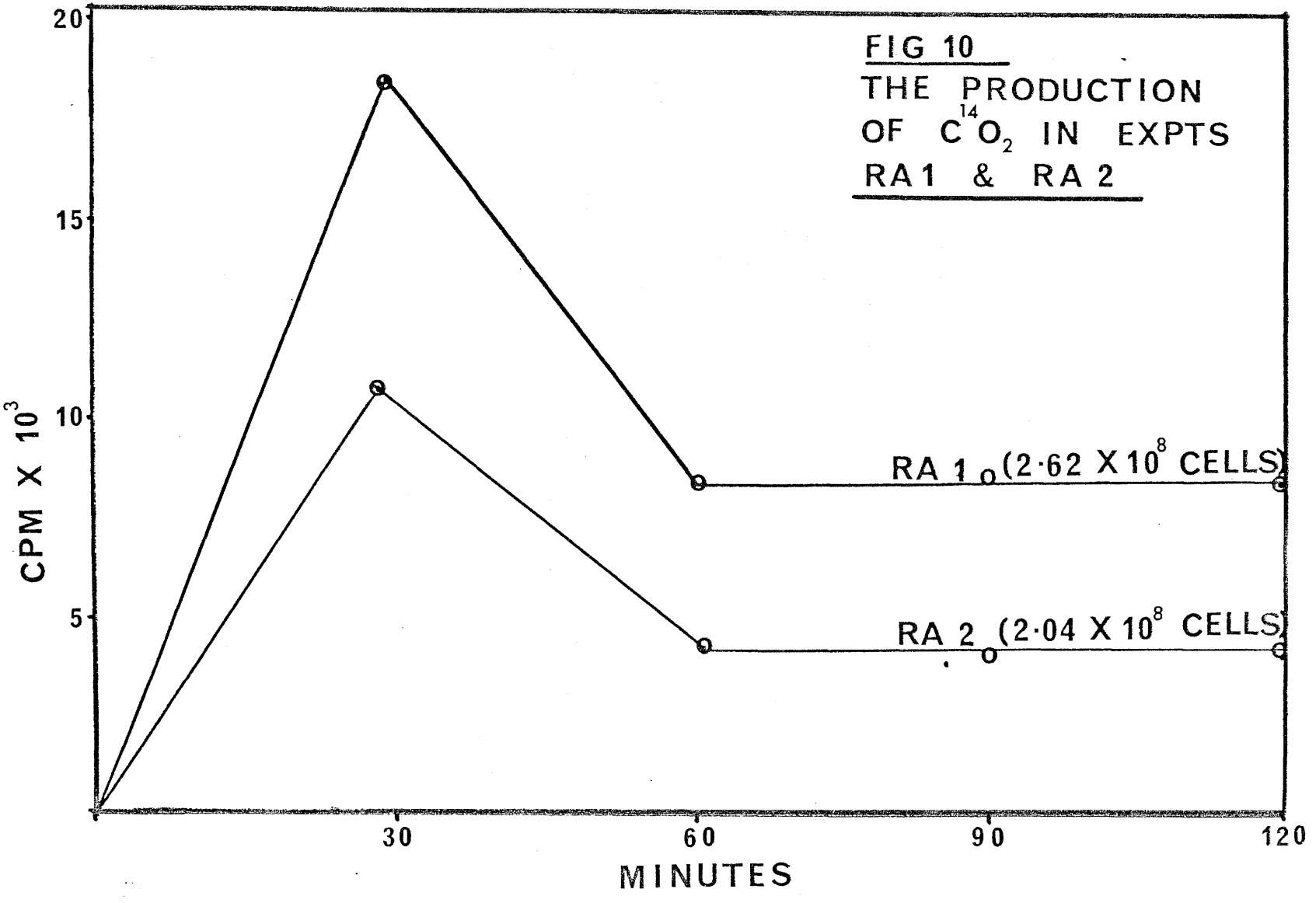


FIG 10
THE PRODUCTION OF $C^{14}O_2$ IN EXPTS RA1 & RA 2
RA 1 o (2·62 X 10^8 CELLS)
RA 2 o (2·04 X 10^8 CELLS)
CPM X 10^3
20
15
10
5
30
60
90
120
MINUTES

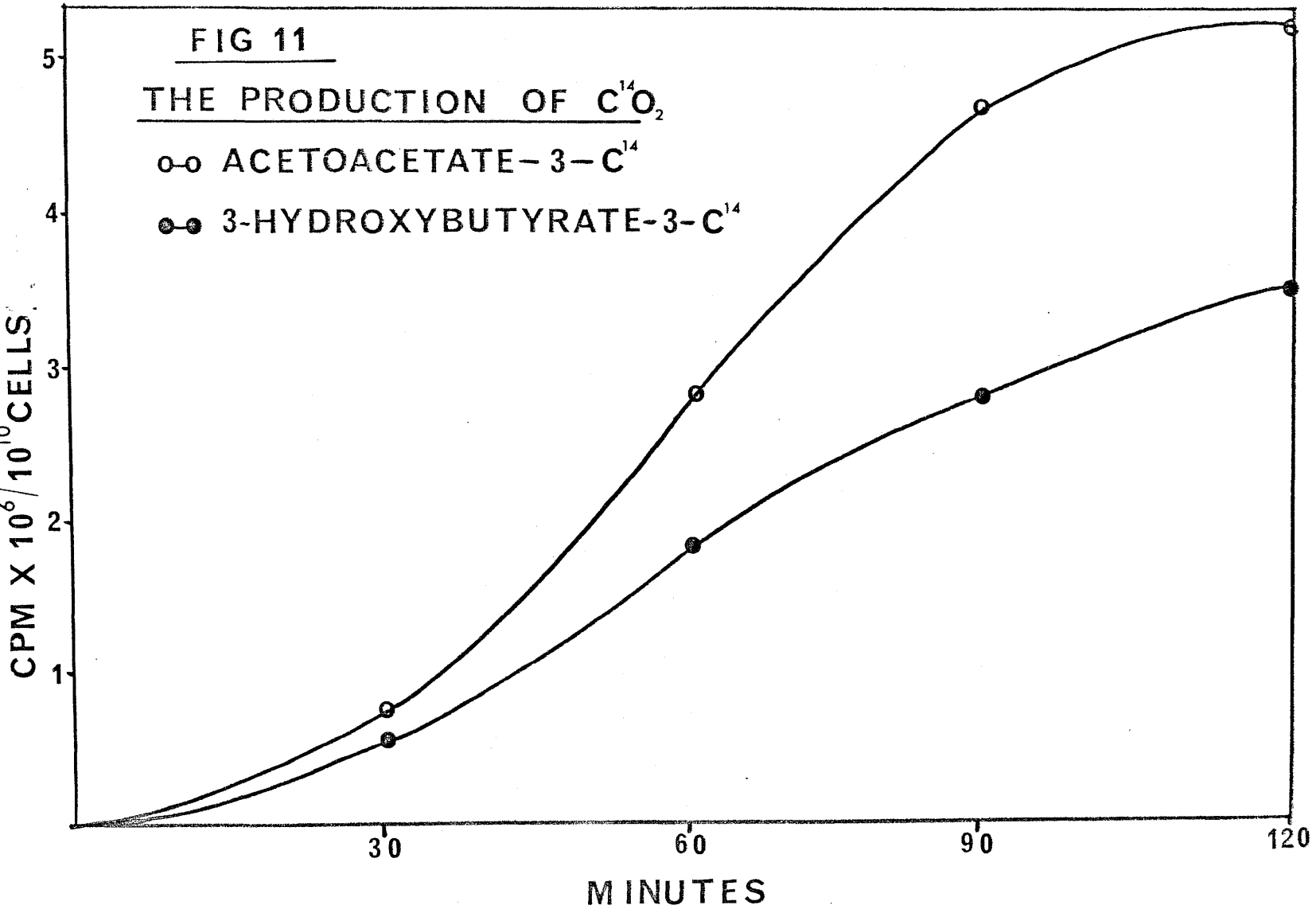


FIG 11
THE PRODUCTION OF $C^{14}O_2$
ACETOACETATE-3-C^{14}
3-HYDROXYBUTYRATE-3-C^{14}
CPM X $10^6/10^{10}$ CELLS
1
2
3
4
5
30
60
90
120
MINUTES

Chapter 8

DISCUSSION

As mentioned in the introduction, a great deal of work has been done on the incorporation of C^{14}-acetate into human leucocyte lipids (15,43,64, 83,95). The major drawback in these studies is that almost without exception the suspension medium has been either blood, the original serum, or plasma, or the original serum or plasma diluted with buffer. Some studies using homogenate (63) have also been used to evaluate the extent of lipid synthesis from acetate-C^{14}.

In the studies cited above, the leucocytes were incubated with C^{14}-acetate while suspended in whole blood (43,50,64,69,95) and the cells fractionated afterwards. Alternatively the

leucocytes were separated prior to incubation, washed, and then resuspended in plasma or serum (15,64,69,72) or serum or plasma diluted with buffer (83). Studies on whole blood have the drawback that both leucocytes and platelets incorporate acetate-C^{14} into triglyceride and phospholipid and transfer these into plasma lipids (64,69). No isotope incorporation into plasma lipids was detectable after incubation of cell free plasma with acetate-C^{14}. Thus of the total radioactivity incorporated into mixed lipids by the cells at the completion of a 2½ hour incubation 55% was recovered in the plasma lipids, 5% in the erythrocyte fraction, 15% in the platelet fraction and about 25% in the leucocyte fraction. These results were obtained without correction for contamination of one cell type by another. The same exchange of course occurs in pure plasma or serum (15) and serum diluted with buffer (83).

Although Marks et al (69) found that leucocytes separated from whole blood resuspended in plasma and incubated with acetate-C^{14} incorporated isotope into lipids, they found this pre-separation associated with an 80% decrease in the rate of lipid snythesis compared to that of leucocytes separated from whole blood subsequent to incubation. In contradiction to this finding Malamos et al (64) showed that little alteration was observed whether the incubations took place before or after the separation of leucocytes from erythrocytes. It should be emphasised that these workers still suspended the leucocytes in plasma. In the present study the leucocytes were always suspended in a buffer (KRHAGA) free of plasma.

Conflicting evidence was provided by O'Donnell (79) who reported that whereas rabbit reticulocytes possessed the ability to synthesise lipids,

normal human leucocytes did not incorporate acetate-C^{14} into lipids. The failure of these studies to observe lipid synthesis in leucocytes may be in part due to either the preparative methods employed to obtain leucocytes for subsequent incubation, or to the conditions used during the incubation. These points were also commented on by Marks et al (69). It should be noted that the possibility exists that a number of workers have performed experiments using clumped leucocytes. Both Pastore (83) and Buchanan (15) describe the presence of clumps of cells in their preparations.

In the work cited above, the methods whereby the incorporation of radioactive isotope into lipids were measured varied to some extent. In studies on the utilisation of acetate-C^{14} by leucocytes, Buchanan (15) merely measured the incorporation of acetate-C^{14} into total lipid. Rowe et al (95)

separated the lipids into saponifiable, non-saponifiable, and phospholipid. Marks et al (69) used column chromatography to separate the lipid into mixed lipid, neutral lipid, and phospholipid. Malamos et al (64) also used column chromatography to separate leucocyte lipid into all of the neutral lipid fractions. Pastore (83) isolated the total lipids according to the method of Folch et al (34) and the gross lipid fraction was plated and counted directly. It is evident that of these workers only Malamos et al attempted separation of all the lipid classes, and it is possible that the separation obtainable with thin layer chromatography was seldom achieved. These workers quoted the specific activity of the total lipid only, or otherwise quoted the percentage radioactivity incorporated into the different lipid fractions.

In the present study the total incorporation of acetate into baboon leucocyte lipids was found to be

0.035 μ moles*. This figure is in good agreement with results calculated from the reports of other authors using human leucocytes : - Pastore (83) 0.02 μ moles, Malamos et al (64) 0.032 μ moles and Marks et al (69) 0.0298 μ moles. Marks et al reported that increasing the concentration of acetate increased the incorporation of the label into mixed lipid. Above a concentration of acetate of 0.02M, however, the incorporation was constant. In the present study the level of acetate was 0.0244 M in all experiments. Although other authors have investigated the incorporation of acetate-C^{14} into leucocyte lipids, they have not supplied sufficient information in their papers for comparison of their results with those reported in the present study.

A study of the incorporation of radioactivity into the neutral lipids using acetate-C^{14} as a substrate

* All results expressed as μ moles/100 mg. leucocyte dry weight/hour.

showed that the triglyceride fraction incorporated an average of 58% of the isotope in the neutral lipids. Malamos et al, the only authors with whom a comparison can be made, found a rather lower figure of 34%.

The incorporation of activity into phospholipid as shown in Table XII reflects a 57% incorporation of the label into total mixed lipids when C^{14}-acetate was used as substrate. The incorporation was far higher than in any other lipid fraction. Malamos et al found that 58% of the label in C^{14}-acetate was incorporated into the phospholipid fraction in pre-separated leucocytes as opposed to 61% if whole blood was incubated with C^{14}-acetate and the cells separated after incubation. Thus these authors also found a greater incorporation into phospholipid than into the neutral lipids. Marks et al and Rowe et al in contradiction to this finding, report a greater incorporation into the neutral lipids than into the phospholipid fraction.

The extent of incorporation of C^{14}-acetate into $C^{14}O_2$ using human leucocytes was investigated by Pastore (83) and appears to be the only author to have done so. He obtained a value of 2.2 μ moles as compared to 1.78 μ moles obtained in the present study, although it appears that this figure was the result of 1 experiment. The pattern of incorporation using 5 μ curie of acetate in experiments RA I and RA II are difficult to interpret as the total amount of acetate present in the medium differs very little from that present when 25 μ curies were used. The total $C^{14}O_2$ productions using 5 μ curie are comparable to those obtained using 25 μ curies if the figures in the former case are multiplied by 5.

As the incorporation of the ketone bodies into the lipids and CO_2 of leucocytes from any species has not been attempted by other workers at the time of writing, these results were compared to those found in other extra-hepatic tissues.

Hanson (41) found that 1.05 μ moles of acetoacetate $-C^{14}$ were oxidised to $C^{14}O_2$ in mouse adipose tissue. In rat adductor muscle fibres, Beatty et al (8) reported an incorporation of 0.6 μ moles acetoacetate into CO_2. Comparing skeletal muscle and diaphragm tissue from rats Beatty et al (7) found 0.183 μ moles and 0.233 μ moles of aceto-acetate were incorporated into CO_2 from skeletal muscle and diaphragm respectively. Krebs et al (56) perfused rat kidney cortex with a perfusate containing C^{14}-acetoacetate and found 0.71 μ moles incorporated into CO_2. Lastly, Hanson and Ziporin (42) reported that 0.364 μ moles of acetoacetate were incorporated into CO_2 by mouse adipose tissue. These figures for the incorporation of acetoacetate isotope into CO_2 are very much higher than the figure of 0.05 μ moles obtained with leucocytes in the present study.

The only figure available for the incorporation of 3-hydroxybutyrate into CO_2, that of Hanson and

Ziporin, 0.038 μ moles, compared favourably with that obtained in the present study which was 0.03 μ moles. Hanson and Ziporin were also the only authors who provided sufficient information for their results to be calculated for the incorporation of the label from ketone bodies into fatty acids. They found that 0.0026 μ moles of 3-hydroxybutyrate and 0.005 μ moles of acetoacetate were incorporated. If most of the activity incorporated into lipid in the present study resides in the fatty acids of the lipids, then the figures of 0.0036 μ moles for 3-hydroxybutyrate and 0.0021 μ moles for acetoacetate are of the same order. No other studies where the incorporation of ketone bodies into lipid by extra-hepatic tissues were available.

The incorporation of isotope into phospholipid by leucocytes (Table XII) shows that the incorporation is higher than for any of the neutral lipid

fractions, 76% for 3-hydroxybutyrate and 53% for acetoacetate. The 3-hydroxybutyrate was used in this study as the racemic mixture, and from the point of view of adding ketone body (or the D (-) isomer), only half was present in this form. This leaves unanswered the question of how much of each or which isomer was being utilised.

The specific activities of the neutral lipids obtained in this study show that while the triglyceride has the highest specific activity and highest percentage incorporation of radioactivity, the free fatty acid and cholesterol ester have very high specific activities which are not reflected in their percentage incorporation of radioactivity. Cholesterol usually had the lowest specific activity.

Cline (20) and Malamos *et al* (64) have quoted figures for the lipid composition of leucocytes. The total lipid of a population of human leucocytes undifferentiated as to morphological type, compared to those obtained in the present study are : - Cline - cholesterol 20%, neutral fat 30%, and phospholipid 45%; Malamos *et al*- cholesterol ester 2.8%, triglyceride plus fatty acid 4.7%, cholesterol 13% and phospholipid 77%; present study (Table XIII) - cholesterol ester 1%, triglyceride plus fatty acid 6%, cholesterol 5.6%, and phospholipid 87.7%. Whereas Cline reported the total lipid to be 1 - 3% of the total mass of the white cell, a figure of 4.8 was found in the present study.

Whether the acetate present in the medium was competing with the ketone bodies for entry into the cell is difficult to say, and would require further investigation. Another factor which should be

investigated, would be the use of far higher concentrations of ketone bodies, as the concentrations used in this study were minimal. This ensured that the ketone body itself would have little effect on the metabolism of the leucocytes.

From the findings of other workers it is evident that the ketone bodies can be utilised by peripheral tissues. The leucocyte can be added to the list of extra-hepatic tissues which can utilise appreciable quantities of ketone bodies for the support of respiration and as a source of energy. As Nepture et al (76) found in the case of excised rat diaphragm, 3-hydroxybutyrate can participate to a considerable extent in the oxidative metabolism of the leucocyte, and acetoacetate to a greater extent.

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SUMMARY

The metabolism of the ketone bodies in baboon leucocytes was investigated. A new medium for the study of leucocyte metabolism *in vitro* was developed. The respiration of the leucocytes in various media, and in the presence of ketone bodies and other substrates and cofactors was studied. Radioactive tracer studies with acetate, and the ketone bodies were undertaken. The results of other authors who investigated the metabolism of acetate by leucocytes were confirmed. It was also shown that leucocytes could utilise the ketone bodies as a fuel of respiration, and could incorporate them into the various lipid fractions.

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CORRECTIONS

Page 1 Line 5 for mammaliam read mammalian.

Page 10 Line 13 for phytahaemaglutinin read phytahaemagglutinin.

Page 11 Line 18 for leucocyted read leucocytes.

Page 14 Line 4 for acidid read acidic.

Page 16 Line 19 at instead of for 200 G.

Page 27 Line 1 pH=7.4 should read pH=7.2.

Page 43 Insert after line 15 .....and 0.045 ml. NaOH, and 0.032 ml. water added to the ethyl acetoacetato, and then...

Page 44 Line 13 and Page 45 Line 13 for dessicator read desiccator.

Page 57 Line 6 for fatted read fatty.

Page 71 Line 13 for diethyleter read diethylether.

Page 74 Line 12 for coloromet rically read colorimetrically.

Page 76 At the end of line 9 insert the word give.

Page 81 Line 3 for colourimetric read colorimetric.

Page 81 Line 8 for billirubin read bilirubin.