# COMPOSITION AND SYNTHESIS OF LIPIDS IN RESTING AND PHAGOCYTIZING LEUKOCYTES

By PETER ELSBACH,* M.D.

*(From The Rockefeller Institute)*

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White blood cells from various sources synthesize fatty acids *in vitro* (1, 2). During phagocytosis leukocytes show general metabolic stimulation as evident from an increase in glucose utilization, lactate production, $O_2$ uptake, and glycogen synthesis (3); lipid synthesis is also enhanced and it has been suggested that phospholipids are incorporated at an increased rate into cell membrane and related structures (4).

This communication presents results of studies on the composition of lipids of rabbit polymorphonuclear leukocytes in relation to the fatty acid pattern of dietary and serum lipids. The composition and turnover of cell lipids have also been compared in resting and actively phagocytic white cells.

## *Experimental Procedures and Methods*

The rabbits used in these experiments were fed an *ad libitum* diet of constant composition (Rockland, Decatur, Illinois). The fat in this diet consisted largely of soybean oil and contributed roughly 4 to 5 per cent of the calories in the food.

Polymorphonuclear white blood cells were obtained from peritoneal exudates produced in these rabbits as previously described (5). Following collection, the pooled exudates of several animals were strained through a fine wire mesh to remove clumped white blood cells and fibrin strands. A total and differential cell count were done for each experiment.

When lipid synthesis during phagocytosis was studied a suitable quantity of $C^{14}$-1-labelled Na-acetate dissolved in water was added to the unmodified exudate which was then divided into two equal portions. To one of these, heat-killed streptoccci of the strain S43G were added in a ratio of roughly 10 bacteria to one white blood cell. Strain S43G is a Group A streptococcus which is non-encapsulated and devoid of demonstrable M protein; it is readily phagocytized *in vitro*. Each of the two portions was then equally divided into 3 Erlenmeyer flasks and incubated under constant agitation at 37°C.

The occurrence of phagocytosis was established as follows:—

A drop of exudate was stained with Gram stain and examined microscopically for the presence of bacteria inside the cells. In the usual experiment from 70 to 90 per cent of the leukocytes appeared to contain one or more cocci after 20 minutes of incubation. Secondly, concentrated suspensions of white blood cells in ascitic fluid were placed in Warburg vessels with a suspension of streptococci in saline in the side arm. $O_2$ uptake was measured after equilibration for at least 1 hour before and after tipping of the bacteria into the main chamber. A brisk rise in $O_2$ uptake was taken as evidence that phagocytosis occurred (3, 6). Cultures were

* Present address: New York University, College of Medicine, New York.

taken of the cell suspension at the end of the experiment to exclude the presence of viable bacteria.

At intervals of 20 minutes over a 1 hour period a control flask and a flask containing heat-killed streptococci were removed and immediately placed in an ice bath. Following cooling the exudate was transferred to tared centrifuge tubes and spun at 400 *G* for 20 minutes. The supernate was poured off and the tube was reweighed for determination of wet weight of the cell button. In parallel experiments smaller quantities of exudate were incubated in centrifuge tubes for additional determination of dry weight in order to assess changes in water content and dry weight. After the wet weight was determined, the cell button was washed with saline to remove lipid-containing medium, centrifuged again, and homogenized in 20 volumes of a mixture of chloroform-methanol (2:1). Lipid extraction was completed by refluxing with this mixture for 2 hours at 70°C. The white blood cell residue was then filtered off, the filtrate washed 3 times with water and brought to constant volume. Aliquots were taken for determination of total lipid content and radioactivity. The remainder was prepared for gas chromatographic analysis of the total fatty acids. The total lipid content was determined microgravimetrically as described by Craig, Hausmann, Ahrens, and Harfenist (7).

Radioactivity in the total lipid extract was measured in a 5 ml. aliquot, pipetted into a counting flask. The solvent was evaporated in a nitrogen stream and the remaining lipid was redissolved in toluene containing a suitable phosphor (2,5-diphenyl oxazole and 1,4-bis-2-(5-phenyloxazole)benzene in a ratio of 40:1). Counting was carried out in a Packard tricarb liquid scintillation spectrometer model 314-DC (Packard Instrument Company, La Grange, Illinois). The effectiveness of washing the lipid extract free of radioactivity associated with water-soluble material was determined by repeated washings and subsequent removal of aliquots for counting. After the second wash no further loss of radioactivity occurred.

The fatty acids in the lipid mixture were methylated by refluxing for 2 hours with HCl—"superdry" methanol at 110°C. as described by Stoffel, Chu, and Ahrens (8). Following methylation the fatty acids were sublimated at a pressure of 0.1 mm. Hg (8), collected, and stored in petroleum ether (b.p. 30–60°C.) under a nitrogen atmosphere. Lipid extracts of food pellets, serum, ascitic fluid, and red blood cells were treated in a similar manner.

For gas chromatographic separation two types of stationary phase were used: (*a*) reoplex 400 (9) and a polymer of ethylene glycol and adipic acid (both polar polyesters), and (*b*) apiezon L vacuum stop-cock grease (a non-polar hydrocarbon). By utilizing two stationary phases with different physical and chemical properties reliable identification of most fatty acids was possible (10). For quantitative comparison of different samples however, apiezon was used because the polyester stationary phases yielded less reproducible results. As a detection device the ionization chamber of Lovelock and James was used (11). The areas of individual peaks on the chromatogram were calculated by triangulation and expressed as per cent of the total area.

Label ($C^{14}$) incorporated in the various fatty acids was recovered following chromatographic separation, by collecting the vapors in a glass tube which fitted on an outlet in the detection chamber, made for this purpose (12). The tubes contained a loosely packed cotton plug impregnated with toluene, at the opposite end. The fatty acids in the vapor condensed on the walls of the collecting tube or were trapped in the cotton plug. Radioactive material adherent to the walls of the tube or present in the cotton plug was rinsed into a counting flask by washing with toluene. Phosphor dissolved in toluene was added prior to counting. About 70 per cent of the radioactivity in the chromatographed sample was recovered in this manner. Collections were guided by following the direct recording of the sequence in which the various fatty acids reached the detector.

The total lipid extract was analyzed for the relative content of phospholipids, triglycerides,

and cholesterol esters by separation and quantitation on the silicic acid column as described by Hirsch and Ahrens (13).[1]

The NEFA[2] content of the rabbit leukocytes was determined by extracting spun cells directly with the extraction mixture used for determination of NEFA in serum (14). The upper (heptane) phase of the extraction mixture was reextracted with blank lower phase to remove non-NEFA"-titrable acidity.

*Presentation of the data.*—The choice of grouping of the fatty acids was guided by consideration of the proportions in the total mixture. For example, the small amounts of lauric, myristic, and pentadecanoic acid, usually present in the mixtures dealt with here, were not separately represented. Acids occurring in amounts of less than 0.5 per cent have been arbitrarily ignored in the calculations. The C18 acids with 2 and 3 double bonds and the C20 acids with 4 and 5 double bonds are shown combined, because the stationary phase used for chromatographic separation does not distinguish between these pairs. Two unidentified

TABLE I

*Content and Composition of Lipids of Rabbit Polymorphonuclear Leukocytes*

| | |
|---|---|
| Total lipid content (per cent of dry weight of cells)* | 8.7 ± 2.9 per cent |
| Lipid composition (per cent of total lipid) | |
| Phospholipids | 62 per cent |
| Triglycerides | 22 per cent |
| Cholesterol esters | 9 per cent |
| Free cholesterol | 5 per cent |
| Non-esterified fatty acids | 2 per cent |

* 27 determinations.

components appeared on the chromatogram between the C18 and C20 series. Since one of these was only present in trace amounts, the question mark which designates the two components refers almost exclusively to the one with a retention volume of 1.28, relative to stearic acid (stationary phase: apiezon).

The radioactivity recovered in the lipid extracts of leukocytes incubated with and without added streptococci, was expressed as per cent of the activity in the control sample after 60 minutes of incubation at 37°C.

## RESULTS

*1. Composition of lipids of polymorphonuclear leukocytes.*—Table I shows that lipids constituted 8.7 ± 2.9 per cent (27 determinations) of the dry weight of leukocytes. More than 60 per cent of all lipid consisted of phospholipids, about 20 per cent was triglyceride, and the remainder was composed of cholesterol esters, free cholesterol, and a small amount of non-esterified fatty acids (2 to 4 per cent).

The results of gas chromatographic separation of the fatty acids of whole

[1] These determinations were carried out by Dr. Jules Hirsch.

[2] NEFA, non-esterified fatty acid.

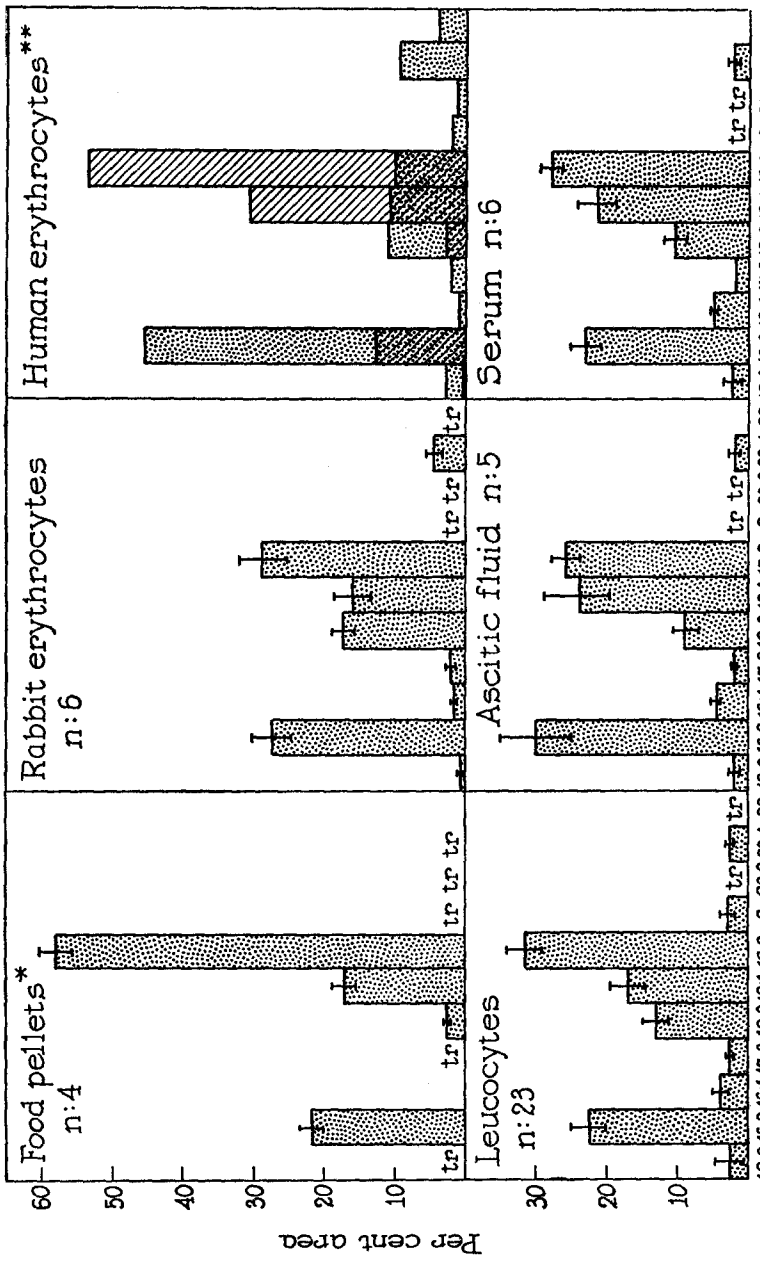


FIG. 1. Fatty acid composition of whole lipid extracts of rabbit leukocytes, ascitic fluid, serum, food pellets, and red blood cells, determined by gas-liquid chromatography.

* The bars represent the areas on the chromatogram, of the fatty acids indicated along the base of the figure, as per cent of the total area. The standard deviation from the mean of the indicated (n) number of determinations is given by the vertical line through the top of the bars.

** The cross-hatched superimposed pattern represents the fatty acid composition of corn oil.

lipid extracts of leukocytes, serum, ascitic fluid, erythrocytes and food pellets appear in Fig. 1. The composition of leukocytes was found to be quite constant,

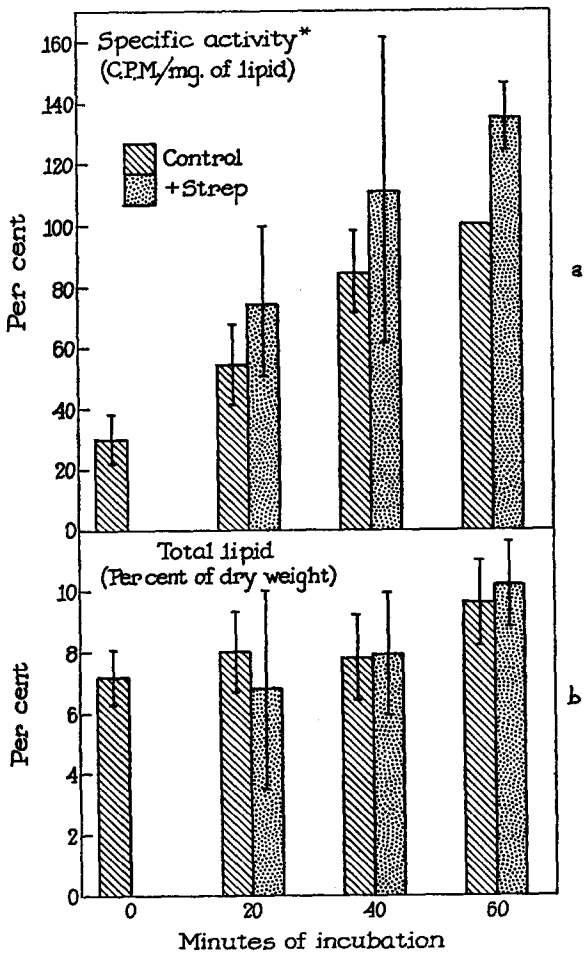


FIG. 2. Lipid content (Fig. 2 *b*) and incorporation of $C^{14}$-1-acetate into lipids (Fig. 2 *a*) of leukocytes incubated with ("+ Strep") and without ("Control") heat-killed streptococci.

* The specific activities (C.P.M./mg. of lipid) have been expressed as per cent of the control value after 60 minutes of incubation. The standard deviation from the mean of 3 determinations is given by the vertical line through the top of the bars.

as is apparent from the small standard deviation from the mean of 23 determinations.

Erythrocytes, serum, and ascitic fluid differed little but consistently from leukocytes in their fatty acid pattern. Perhaps the most striking finding was

the presence in leukocytes of 2.8 ± 1.0 per cent of an unidentified component (retention volume 1.28, relative to stearic acid) of which only traces occurred in rabbit food, serum, and red cells.

It is evident that the composition of the fatty acids in the food differed from the composition of serum or of white and red blood cells. C 18 acids with 2 and 3 double bonds predominated in the food, while the shorter chain acids and C 16:1 and C 20:4 and 5 occurred only in trace amounts.

The fatty acid composition of red blood cells of a patient on a long term

TABLE II

*Incorporation of $C^{14}$-Acetate in Relation to Composition of Lipids of Rabbit Leukocytes, before and after Incubation with Heat-Killed Streptococci, as Determined by Fractionation on a Silicic Acid Column into Phospholipids and Triglycerides plus Cholesterolesters*

| | | Content (per cent of total lipid) | Total C.P.M. | C.P.M./mg. lipid |
|---|---|---|---|---|
| $T_0$ | Triglycerides, Cholesterolesters | 27.5 37 | | |
| | Phospholipids | 72.5 63 | | |
| $T_{60}$ | Triglycerides, Cholesterolesters | 34 | 7,370 | 2,860 |
| | Phospholipids | 66 | 5,620 | 1,125 |
| Total C.P.M. recovered* ..... | | | 12,990 | |

$T_0$, before incubation. (The 2 values shown represent fractionation of 2 leukocyte extracts)

$T_{60}$, after addition of heat-killed streptococci and incubating for 60 minutes.

* Radioactivity of whole lipid extract prior to silicic acid column separation: 13500 C.P.M. (background: 18 C.P.M.).

formula diet containing corn oil as the sole fat is shown for comparison. The fatty acid composition of the corn oil resembled rather closely that of the soybean oil of rabbit food pellets. The fatty acid profile of the human erythrocytes, however, was strikingly different. Red cell samples from other human beings on the same or different diets show much the same composition (15).

*2. Lipid synthesis during the resting and phagocytic states.*—$C^{14}$-labelled acetate was rapidly taken up into the lipids of the leukocytes during incubation at 37°C. (Fig. 2 *a*). At the end of the 10 minute period required to prepare the first sample for centrifugation, an appreciable amount of label had already been incorporated. It is clear that the specific activity (C.P.M./mg. of lipid) after 20, 40, and 60 minutes of incubation was higher in the phagocytizing than in the resting cells ($P < 0.01$). No net synthesis, however, had taken

place, because the lipid content of the leukocytes, expressed as mg. of fat/$10^9$ cells, remained within the same range in the phagocytizing and control cells.

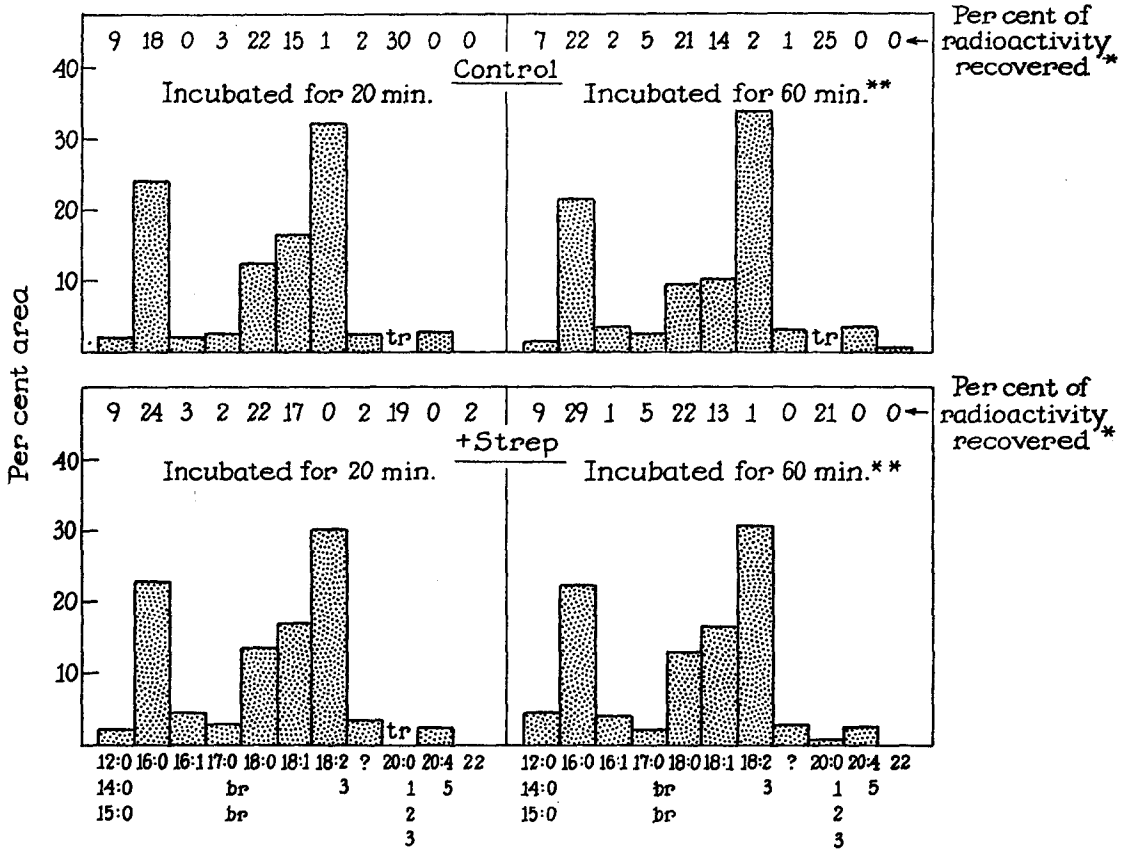


FIG. 3. Gas chromatographic analysis of the fatty acids of whole lipid extracts of leukocytes incubated for 20 and 60 minutes with ("+ Strep") and without ("Control") heat-killed streptococci.

* Radioactivity collected from the effluent gas, expressed as per cent of the total radioactivity recovered.

** The results obtained after incubation for 60 minutes are presented as the averages of three independent experiments.

Since it was noted that the dry weight of packed cell buttons of phagocytizing cells was sometimes less than that of the resting cells, the possibility was considered that the process of phagocytosis might cause a more rapid disintegration of leukocytes. Unless a correction were made for loss of dry weight,

as an index of cell mass, a greater net synthesis of lipid in a smaller surviving cell population might be missed. Therefore dry weight of the samples was estimated by employing the ratio between wet and dry weight, obtained in parallel experiments, of smaller aliquots of exudate, incubated with and with-

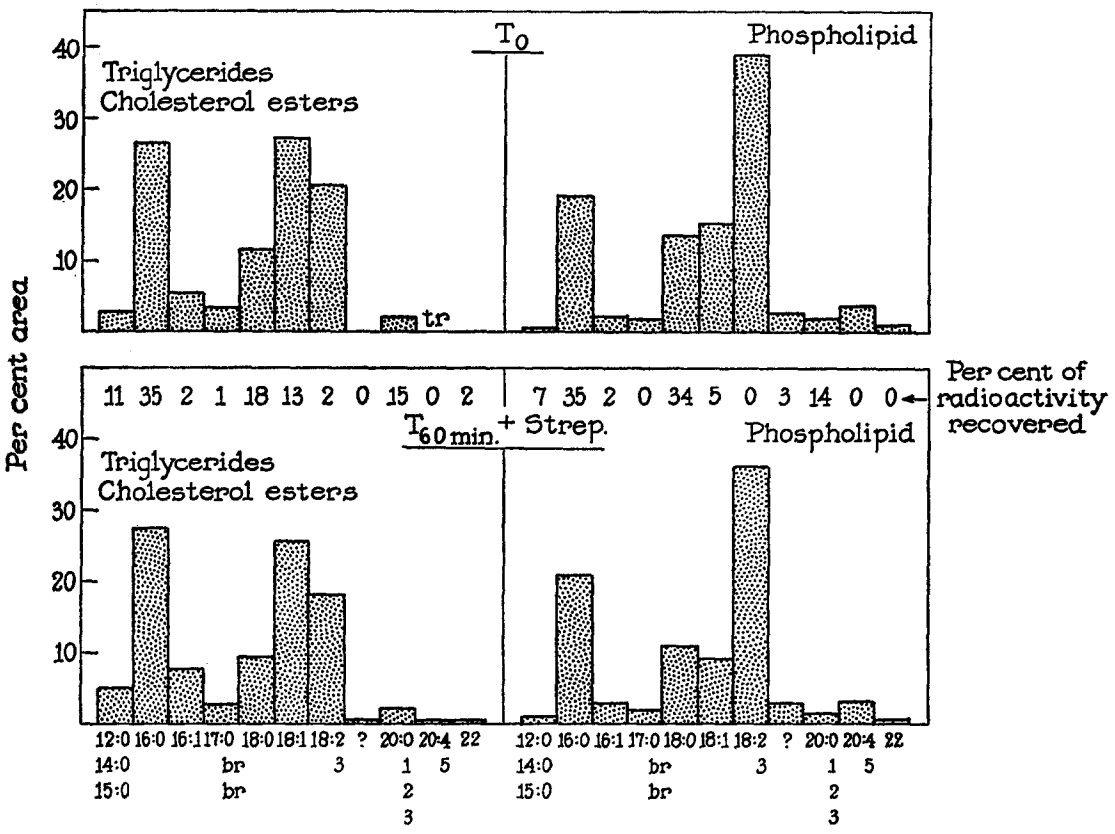


FIG. 4. Gas chromatographic analysis of the fatty acids of the phospholipid and triglyceride plus cholesterol ester fractions of whole lipid extracts of rabbit leukocytes, before ($T_0$) and after incubation with heat-killed streptococci for 60 minutes ($T_{60}$).

Radioactivity collected from the effluent gas, expressed as per cent of total radioactivity recovered.

out added streptococci. The lipid content, expressed as percentage of dry weight (Fig. 2 *b*), again showed closely similar figures for both series ($P > 0.5$).

Washed lipid extracts of the supernatant fluid of 60 minute samples contained an insignificant amount of radioactivity, indicating that little or no newly synthesized lipid was released into the medium. The possibility was

investigated that the phospholipid fraction of the total lipids would be synthesized in preference or at the cost of the other fractions. That this was not the case is apparent from Table II which shows that the phospholipid content of phagocytizing leukocytes did not increase in relation to the total lipid content. Moreover, the radioactivity per milligram of triglycerides and cholesterol esters together was 2½ times greater than that of the phospholipids.

The effect of lipid synthesis during incubation with and without heat-killed streptococci on the fatty acid composition of the leukocyte was studied by gas-liquid chromatography and collection of the labelled fatty acids in the effluent gas. Figs. 3 and 4 contain the results of such collection runs. The composition of the fatty acids of the whole lipids and of the phospholipid and cholesterol ester plus triglyceride fractions was closely similar in the resting and phagocytic cells. The distribution of radioactivity among the fatty acids in these fractions was also unaffected by the phagocytic process, while the specific activities of the different acids remained constant over a period of 60 minutes in both phagocytizing and control cells.

The label was predominantly located in the saturated C 16:0 and 18:0 acids. A considerable amount of activity also appeared in C 18:1 and in C 20:0, 1, 2, and 3. It is interesting that no significant activity is associated with the C 18:2 and 18:3 nor with 20:4 and 20:5 acids, supporting the evidence that these acids cannot be synthesized by mammalian tissues.

The rate of incorporation of radiocarbon into the fatty acids was independent of the relative amounts of individual fatty acids in the total mixture. This was particularly striking for the C 20 acids (excepting C 20 with 4 and 5 double bonds), which occurred only in traces, but which yielded from 15 to 30 per cent of the total radioactivity, indicating a rapid turnover.

## DISCUSSION

In order to study the fatty acid composition of a cell type under different metabolic conditions, variations due to changes in the ingested fat have to be excluded. The rabbits used in this investigation were fed a diet of constant composition. The imprint of the fatty acid pattern of the lipids in this diet on the fatty acid composition of serum and white and red blood cells was incomplete. This is not surprising, since even the well established dietary effect on the total fatty acids of serum lipids represents a trend, rather than an exact replication of the fatty acids of the fed fat (16, 17). Moreover, lipids of tissues seem to undergo much less of a dietary effect as demonstrated by Hirsch *et al.* (18), who found that human subcutaneous fat only very slowly assumes the fatty acid composition of the lipids in the diet. Similarly red blood cells change their fatty acid pattern relatively little in the face of radical changes in the composition of dietary fat (15).

The leukocyte can depend on two mechanisms for maintenance of its lipid

composition: selective transport across the cell membrane and intracellular production or modification of fatty acids and complex lipids. The relative importance of each is not known, but an effective regulatory mechanism must exist, since the leukocyte was found to keep its composition remarkably constant under the different metabolic conditions examined in this study.

The specific activities of the different fatty acids remained constant over a period of 1 hour, both at rest and during phagocytosis. This suggests that the incorporation of label took place by addition of acetate molecules to pre-existing carbon chains of various lengths rather than by a complete *de novo* synthesis of new acids.

Since the lipid content of phagocytizing leukocytes was not greater than that of resting cells and since no newly synthesized lipid was released into the medium, it may be concluded that no net synthesis took place. However, a higher specific activity was found in the lipids of leukocytes during phagocytosis, indicating a more rapid turnover. This may merely reflect the generally stimulated metabolic activity of phagocytizing leukocytes, rather than representing synthesis of phospholipids for new membrane as postulated by Karnovsky and Sbarra (4). Their hypothesis was based in part on studies with the electron microscope which appeared to show that engulfed particles were surrounded by a membrane-like structure. In support of a general stimulus of lipid metabolism, rather than a specific one directed at the production of membrane, is our observation that the turnover rate of the phospholipid fraction was considerably smaller than that of the triglycerides plus cholesterol esters.

While it is still possible that during phagocytosis changes in distribution of lipid and particularly phospholipid may reflect a redistribution of membrane for sequestration of ingested particles, no evidence has been found in these experiments to suggest an actual increase in membrane material.

Only energy derived from anaerobic glycolysis appears to be essential for phagocytosis (3, 4), since blocking of oxidative metabolism does not affect the ability of the leukocyte to engulf particles (4). An acceleration of certain metabolic processes, such as a moderate increase in turnover of lipid or a marked rise in oxygen consumption, may simply accompany a stimulated general metabolic state, rather than serving the phagocytic process directly.

## SUMMARY

The lipid content of rabbit polymorphonuclear leukocytes, obtained from peritoneal exudates, constituted 8.7 ± 2.9 per cent of the dry weight of these cells; 60 per cent of all lipids were phospholipids, 20 per cent triglycerides, and the remainder cholesterol and cholesterol esters and a small amount of non-esterified fatty acids (2 to 4 per cent).

The composition of the fatty acids in leukocytes, as determined by gas-

liquid chromatography, was slightly different from rabbit serum and red blood cells, but markedly different from the dietary fat.

The synthesis, turnover, and composition of lipids in rabbit leukocytes at rest and during phagocytosis *in vitro* were compared. Lipid content and composition were not affected by the phagocytic process. However, active phagocytosis resulted in an increase in the rate of turnover of lipids. This stimulation of lipid metabolism was more marked in triglycerides and cholesterol esters than in phospholipids. It is suggested that the increased turnover of lipid during phagocytosis may reflect a general metabolic stimulation accompanying this process, rather than a specific synthesis of phospholipid for the production of new cell membrane.

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