Incorporation of C^{14}-labeled acetate into lipids by macrophages in vitro

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SUMMARY The incorporation of C^{14}-labeled acetate into lipids by macrophages was investigated using rabbit peritoneal macrophages incubated in vitro. Incorporation of acetate into lipid was shown to occur, the specific activity of the cholesterol labeled being 10–15 times that of the fatty acid. Fractionation of the labeled lipid on silicic acid columns demonstrated the incorporation of the C^{14}-labeled acetate into cholesterol, cholesterol ester, triglyceride, mono- and diglyceride, and phospholipid. The importance of lipid synthesis by macrophages was indicated by the conversion to lipid of 27% of the acetate taken up by the macrophages. Of this 70–80% was incorporated into the nonsaponifiable fraction, 20–30% into fatty acid and about 1% into lipid glycerol.

In atherosclerosis much of the lipid in the arterial wall is present intracellularly in macrophages and the suggestion has been made that some of this arterial lipid accumulates as a result of metabolic changes taking place within these particular cells (1). In support of this suggestion it has been shown that macrophages in vitro can bring about metabolic changes to lipid preparations following the ingestion of such preparations (2–4). Furthermore, fatty acids that are taken up by macrophages in vitro are incorporated into triglyceride, phospholipid, and cholesterol ester and the possibility that macrophages might synthesize lipid in arterial lesions has been postulated (5). This possibility was strengthened by the demonstration that intimal macrophages were responsible for the synthesis of the phospholipid which accumulates in experimental atherosclerotic lesions (6).

In the present paper investigations concerning the synthesis of lipid by macrophages have been extended. The incorporation of C^{14}-labeled acetate into lipid by macrophages in vitro has been examined in order to establish that macrophages can svnthesize lipid from acetate and to obtain information about this synthesis and its possible significance in the pathogenesis of atherosclerosis.

MATERIALS AND METHODS

C^{14}-Labeled sodium acetate (specific activity 278 μc/mg) was obtained from the Radiochemical Centre, Amersham, U.K. Periodical checks for radioactive contaminants were made by paper chromatography (7), and when necessary the stock sodium acetate-C^{14} was purified by chromatography and distillation (8).

Liquid scintillators used were (*a*) 0.3% (w/v) 2,5-diphenyloxazole in toluene for lipid extracts and for column eluates, at a counting efficiency of 75%, and (*b*) 0.4% (w/v) 2,5-diphenyloxazole in dioxane as described by Bray (9) for the aqueous preparations, at a counting efficiency of 67%. Radioassay was carried out using an Ecko N662 Liquid Scintillation Counter.

Rabbit macrophages were obtained from the peritoneal cavity of adult rabbits as described previously (5).

Experimental Procedure

Siliconized Warburg flasks each containing a known number of macrophages (30–50 $\times$ 10^6), 1.0 ml of rabbit serum, 10 μc of sodium acetate-C^{14}, and made up to a final volume of 5 ml with Hanks' solution, were incubated at 37°. Duplicate flasks were removed at $^1/_2$, 1, 2, 4, and 6 hr, the oxygen uptake being recorded manometrically throughout the experiment. Control runs were included to which medium and sodium acetate-C^{14} were added, but no cells. After the desired incubation time, the cells were separated by centrifuging (1500 rpm for 5 min) and washed twice with 0.9%

sodium chloride solution, and the lipids were extracted from the cells using chloroform–methanol 2:1 (v/v), as described by Folch, Lees, and Sloane Stanley (10). Portions of this extract were counted and the remainder reserved for saponification and separation of labeled cholesterol and fatty acid as described below.

Total Lipid Specific Activity Determination

About 100 × 10^6 macrophages from each experimental batch were reserved for determination of total esterified fatty acid by the method of Stern and Shapiro (11). The specific activity of the total lipid at the various time intervals was expressed as counts per second per milligram of total esterified fatty acid.

Saponification

The total lipid extract was evaporated to dryness and saponified by refluxing with 0.5 ml of 30% potassium hydroxide solution and 0.5 ml of ethanol for 2 hr at 80–100°. The nonsaponifiable material was removed by extracting three times with 5 ml of 15% ethanol in petroleum ether (bp 60–80°). The combined petroleum ether extracts were washed twice with small portions of 2 N KOH solution, followed by a further four washings with water. The KOH washings and the first two water washings were returned to the aqueous phase. The latter was acidified with 6 N hydrochloric acid and the fatty acids were extracted with three to five washings of 15% ethanol in petroleum ether. Distribution of counts between the nonsaponifiable and the fatty acid fractions was obtained by scintillation counting.

Cholesterol Specific Activity

The nonsaponifiable fraction was dried over sodium sulfate–sodium bicarbonate 1:1 (w/w), filtered, and evaporated to dryness. The fraction was then chromatographed by the method of Marinetti and Stotz (12) (see below), and the cholesterol spots, located by radioautography, were cut out and eluted with alcohol–ether 3:1 (v/v). This purified cholesterol extract was filtered to remove silicic acid powder, a portion was counted by scintillation counting and the cholesterol in the remainder determined chemically by the method of Zlatkis, Zak, and Boyle (13). Blanks were used in each assay, in which a piece of silicic acid-impregnated paper corresponding in size to the spots eluted was also extracted with alcohol–ether and filtered as above. The specific activity was expressed as counts per second per milligram of cholesterol.

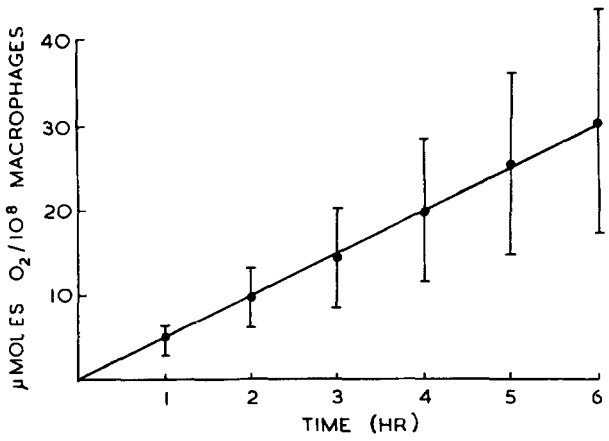

FIG. 1. Oxygen uptake of macrophages incubated in vitro. The mean O_2 uptake together with the standard deviation of the mean is plotted against time for the six experiments for which data on lipid synthesis are presented.

Fatty Acid Specific Activity

The methyl esters of the fatty acids obtained following saponification were prepared as described by Stoffel, Chu, and Ahrens (14). The specific activity was then determined by gas–liquid chromatography using a Pye Argon Chromatograph with polyethylene glycol adipate (10% on Embacel 100–120 mesh) at 180° as stationary phase. The fatty acid mass was calculated from the combined peak areas measured by planimetry while the labeled methyl esters were collected as they emerged from the detector (15) and counted, the columns having been calibrated with a C^{14}-labeled methyl palmitate standard of known specific activity. To facilitate collection and prevent condensation prior to collection, a heated outlet tube was fitted to the detector.

Glycerol Purification

The extent of glycerol labeling from C^{14}-labeled acetate was determined in a separate series of experiments in which the specific activities of individual fatty acids were estimated.[1] Higher activities in the lipid extracts were aimed at in these experiments. After methylation and removal of the fatty acids, 2 mmoles of unlabeled glycerol were added as carrier, the solution was deionized by passing through Dowex 1 Cl^- and AG 50W (H^+) columns, and then evaporated to dryness in vacuo. Glycerol was precipitated as glycerol tribenzoate as described by Rose et al. (16), amounts being scaled down for our purposes. The purified glycerol obtained after hydrolysis of the glycerol tribenzoate and subsequent deionization was dissolved in absolute alcohol and counted. To check recovery the glycerol was determined quantitatively (17) before and after purification. In all cases about 85% of the glycerol was recovered, and correction was made for the loss in determination of the C^{14} incorporated into the glycerol.

[1] A. J. Day, N. H. Fidge, and G. K. Wilkinson. *Biochim. Biophys. Acta*, in press.

Total Intracellular Incorporation of Sodium Acetate-C^{14}

A separate group of experiments was carried out in order to determine the amount of incorporation into lipids relative to that incorporated into other substances. Preparation and incubation of macrophages were carried out in 20 oz McCartney bottles as described for previous experiments (5). A known amount (approx 1 μc) of sodium acetate was added to the medium and incubation was carried out for 4 hr at 37°. The medium was then removed and centrifuged (2000 rpm for 5 min) to deposit the macrophages that had become detached from the glass during incubation. These macrophages were washed with 0.9% sodium chloride solution and recentrifuged. The macrophages still adherent to the glass incubation vessel were detached using 3 ml of 1% detergent solution (Teepol, Shell Oil Co. Ltd.) and combined with those that had been deposited after centrifuging the medium. The cell preparations were then lyophilized. In each experiment incubations were carried out in pairs, the cells of one bottle being reconstituted with 5 ml of water for determination of total uptake of C^{14}, and those from the other being extracted with 20 ml alcohol–ether 3:1 at 70°. This alcohol–ether extract was filtered and the filtrate evaporated to near dryness. One milliliter of 1 M unlabeled sodium acetate was added and the lipid extracted by means of three 10-ml washings of 15% ethanol in petroleum ether (bp 60–80°). Portions of both the reconstituted cells and the lipid extract were counted by liquid scintillation counting using scintillator (*b*).

Chromatography

Paper chromatography of lipid extracts was carried out on silicic acid-impregnated paper using the method of Marinetti and Stotz (12) for neutral lipid separation but using the solvent systems as described before (5). Radioautographs were prepared by the usual techniques.

Fractionation of the lipid extracts by column chromatography using the method of Hirsch and Ahrens (18) was carried out in several experiments. Stepwise elution was used; 20-ml fractions were evaporated to dryness and counted.

RESULTS

Figure 1 shows the O_2 uptake plotted against time for the experiments in which detailed data on lipid synthesis are presented below. The macrophages took up oxygen steadily during the 6 hr incubation period, the mean O_2 uptake being 5.0 μmoles/10^8 cells per hr.

The incorporation of C^{14}-labeled acetate into total lipid is shown in Fig. 2. The specific activity of the total lipid increased progressively with time to a mean of 2600 cps/mg after 6 hr. This represents about 0.4% of the

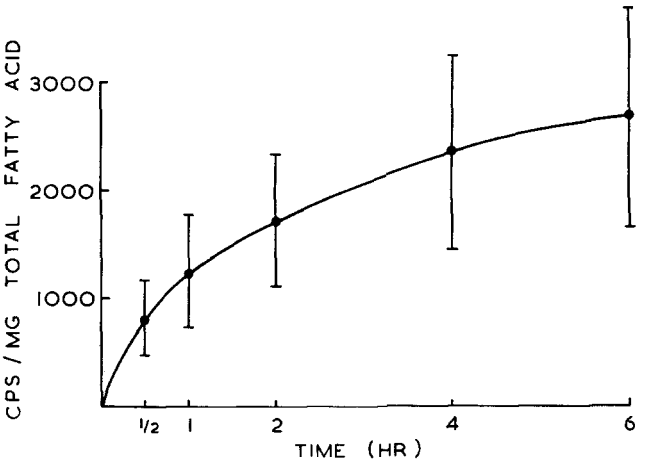

FIG. 2. Specific activity of total lipid (expressed as counts per second per milligram of total esterified fatty acid) following incubation of macrophages with sodium acetate-C^{14} plotted against time. The mean of six experiments together with the standard deviation of the mean is shown.

C^{14}-labeled acetate added to the media. The pattern of incorporation of C^{14}-labeled acetate in relation to time was similar in all experiments although the amount incorporated varied somewhat with different batches of cells.

The amount of acetate incorporated into lipid is compared in Fig. 3 with the total amount incorporated into all substances by the macrophages. The results of five paired experiments are shown. A mean of 1.6% of the acetate added to the media was taken up by the cells, and about one-quarter of this was incorporated into lipid.

The distributions of the C^{14} between the nonsaponifiable and fatty acid fractions of the total lipid are shown in Fig. 4 for the five time intervals investigated. Of the

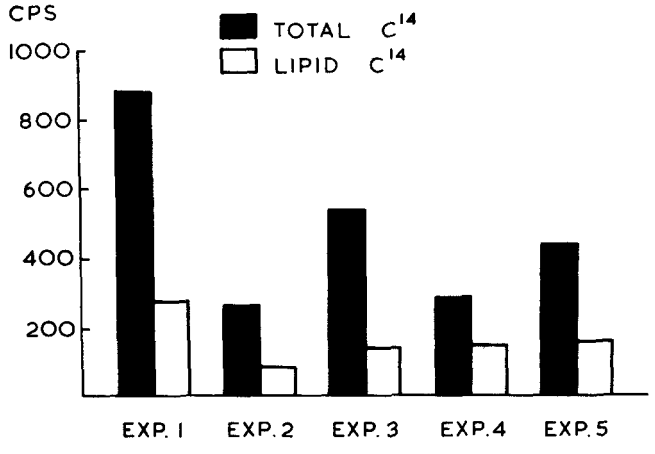

FIG. 3. The total C^{14} present in all forms in macrophages after 4-hr incubation with sodium acetate-C^{14} compared with the amount present as lipid. Data from five paired experiments.

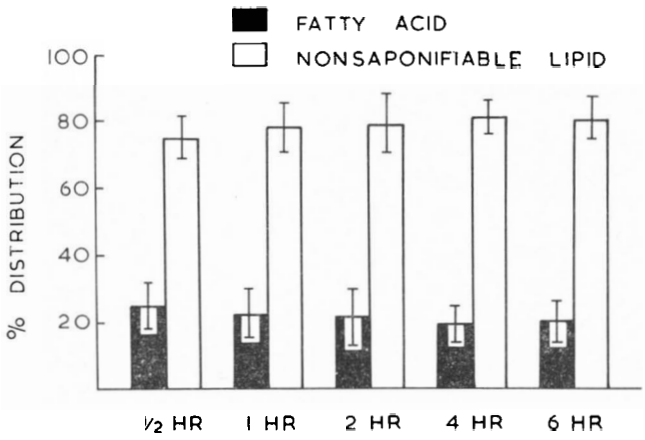

FIG. 4. Percentage distribution of the total C^{14}-labeled lipid between the nonsaponifiable and fatty acid fractions at various time intervals following incubation of macrophages with sodium acetate-C^{14}. The mean of six experiments together with the standard deviation of the mean is shown at each time interval.

C^{14}-labeled acetate incorporated into lipid, 70–80% was present in the nonsaponifiable fraction at all time intervals studied. The incorporation of C^{14}-labeled acetate into lipid glycerol by macrophages is shown in Table 1. These experiments were carried out using 20 μc of C^{14}-labeled acetate incubated with macrophages for 6 hr and then combining five to six batches of cells to obtain a total lipid extract with a relatively high radioactivity. In other respects these experiments were similar to those in which the distribution of label between the other lipid fractions was studied. Some incorporation of acetate into the lipid glycerol occurred, but the amount accounted for only 1–1.3% of the acetate incorporated into total lipid.

TABLE 1 INCORPORATION OF C^{14}-LABELED ACETATE INTO LIPID GLYCEROL BY MACROPHAGES IN VITRO

Total Lipid	Glycerol	% of Counts Incorporated into Glycerol
cps	*cps*	
1,050	12.6	1.2
11,000	127	1.2
8,800	89	1.0
13,860	175	1.3

The incorporation of labeled acetate into cholesterol and into fatty acid is shown in Fig. 5. Both of these moieties are labeled, the specific activity rising to a mean of 8890 cps/mg at 6 hr for cholesterol and of 680 cps/mg at 6 hr for fatty acid. The pattern of incorporation of acetate into both cholesterol and fatty acid was similar in all experiments, but there was rather a wide variation in the amount of labeling between the individual experiments, as can be seen from the standard deviation plotted at each time interval. The mean values given in

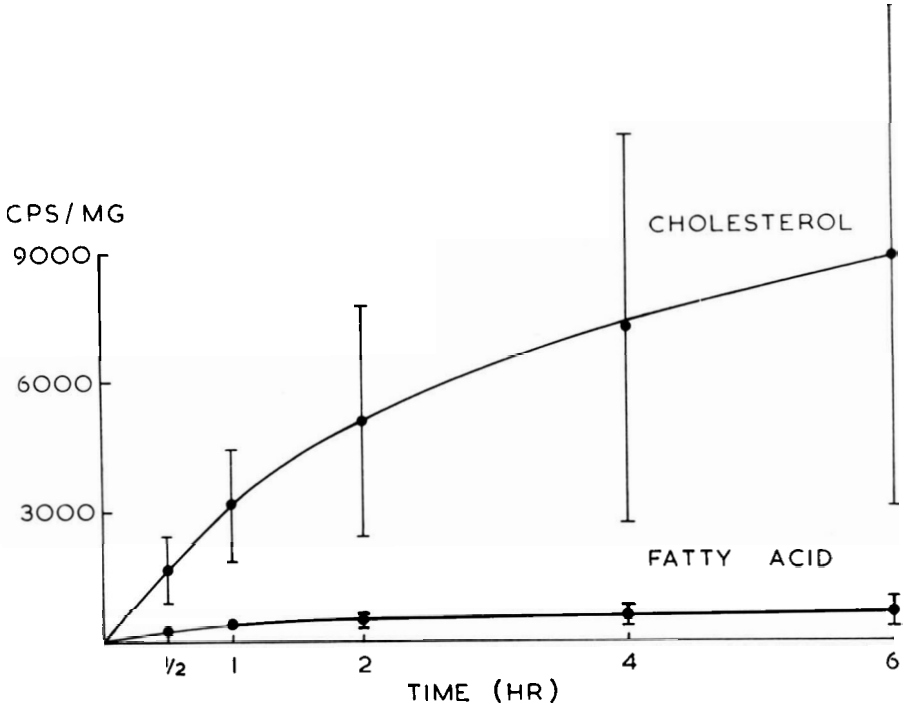

FIG. 5. Specific activity of cholesterol (counts per second per milligram of cholesterol) and of fatty acid (counts per second per milligram of fatty acid) following incubation of macrophages with sodium acetate-C^{14} plotted against time and to the same scale. The mean of six experiments is given for the cholesterol data, and of three for the fatty acid data. The standard deviation in each case is shown.

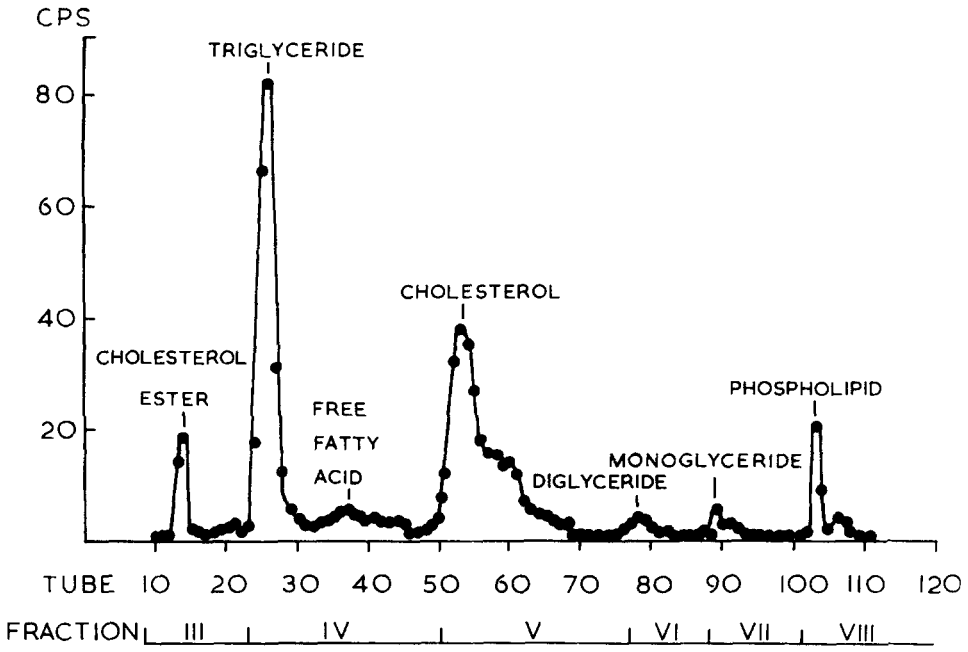

FIG. 6. Fractionation on a silicic acid column of the C^{14}-labeled lipid components of macrophages after 4-hr incubation with sodium acetate-C^{14}. Column load 590 cps of C^{14}-labeled lipid together with approximately 10 mg each of unlabeled cholesterol ester, tripalmitin, palmitic acid, cholesterol, and lecithin.

Fig. 5 for the specific activity of fatty acid in relation to time are those from the three experiments in which the total lipid synthesis was maximal. For this reason the contrast in specific activity between cholesterol and fatty acid should be even more marked than is indicated by Fig. 5.

Fractionation of the lipid synthesized following incubation of C^{14}-labeled acetate with macrophages is shown in Fig. 6. Most of the C^{14}-labeled lipid is present in the triglyceride and cholesterol fractions with smaller amounts in the cholesterol ester, free fatty acid, and di- and monoglyceride fractions. Appreciable labeling of the phospholipid was also demonstrated in this fractionation.

DISCUSSION

The oxygen uptake of the macrophages was constant over the 6 hr period, indicating that the cells were in a steady metabolic state during this time. The uptake compared closely with that reported for macrophages by other workers (19–21).

The uptake and incorporation of C^{14}-labeled acetate into lipid by macrophages in vitro indicates that these cells are able to synthesize lipid, and the question naturally arises whether this lipid synthesis represents a special function of the macrophage or is merely an indication of metabolism in general. Under the conditions studied about 0.1% of the acetate added to the media was converted to lipid after 30 min and about 0.4% after 6 hr incubation. However, what is perhaps more valid is the proportion of acetate (about a quarter) actually taken up by the macrophage that is diverted to lipid metabolism. In most tissues known to synthesize lipid from acetate the proportion of acetate converted to lipid is much smaller. For example Holdsworth and Neville (22), using sheep heart preparations incubated with labeled acetate in vitro, have shown that less than 1% of the acetate that is incorporated into the preparation is present as long-chain fatty acids, the bulk of the acetate being incorporated into nonlipid products. It seems reasonable, therefore, to consider that the much greater lipid synthesis shown by macrophages represents a more specific function of these cells and is not just a general manifestation of metabolism.

Most (70–80%) of the acetate was incorporated into the nonsaponifiable fraction. In contrast, polymorphonuclear leucocytes, which have also been shown to synthesize lipid from acetate (23–25), channel most of the acetate to fatty acid (23). The synthesis of cholesterol from acetate by macrophages is further highlighted when the specific activity of the C^{14}-labeled cholesterol relative to that of fatty acid is considered. If the pool sizes can be assumed to be constant, it can be concluded that the smaller cholesterol pool is turning over at a much greater rate than is the fatty acid pool. Some labeling of the lipid

glycerol occurs, but this does not represent a significant amount. Since glycerol is readily formed from glucose, it would be freely available from the relatively large amount of glucose present in the medium.

The chromatography studies indicate that most of the labeled lipid is present in the cholesterol and the triglyceride fractions but that some labeling of cholesterol ester and phospholipid occurs. The small amount of labeled free fatty acid present in the lipid extract is consistent with the ready incorporation of fatty acid into triglyceride and phospholipid that has been shown previously (5). The synthesis of phospholipid by macrophages shown to occur in these present experiments using C^{14}-labeled acetate has also been confirmed using P^{32}-labeled phosphate,[2] so that it is possible to conclude that significant amounts of cholesterol, fatty acid, and phospholipid are synthesized by the macrophages.

In vitro synthesis of lipid by macrophages suggests that synthesis by macrophages in vivo in the arterial wall may be one cause of the accumulation of lipid in atherosclerotic lesions. However, whether these cells are in fact concerned with the deposition of lipid by synthesis in the arterial wall awaits further evaluation by studies aimed at investigating the metabolism of lipid by macrophages in atherosclerotic lesions themselves.

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[2] A. J. Day and N. H. Fidge, data to be published.

References

1. Day, A. J. *J. Atherosclerosis Res.* in the press.
2. Day, A. J. *Quart. J. Exptl. Physiol.* **45:** 220, 1960.
3. Day, A. J., and P. R. S. Gould-Hurst. *Quart. J. Exptl. Physiol.* **46:** 376, 1961.
4. Day, A. J. *Quart. J. Exptl. Physiol.* **46:** 383, 1961.
5. Day, A. J., and N. H. Fidge. *J. Lipid Res.* **3:** 333, 1962.
6. Day, A. J. *J. Atherosclerosis Res.* **2:** 350, 1962.
7. Kennedy, E. P., and H. A. Barker. *Anal. Chem.* **23:** 1033, 1951.
8. Bartley, W. *Biochem. J.* **53:** 305, 1953.
9. Bray, G. A. *Anal. Biochem.* **1:** 279, 1960.
10. Folch, J., M. Lees, and G. H. Sloane Stanley. *J. Biol. Chem.* **226:** 497, 1957.
11. Stern, I., and B. Shapiro. *J. Clin. Pathol.* **6:** 158, 1953.
12. Marinetti, G. V., and E. Stotz. *Biochim. Biophys. Acta* **37:** 571, 1960.
13. Zlatkis, A., B. Zak, and A. J. Boyle. *J. Lab. Clin. Med.* **41:** 486, 1953.
14. Stoffel, W., F. Chu, and E. H. Ahrens. *Anal. Chem.* **31:** 307, 1959.
15. James, A. T. *Methods Biochem. Analy.*, **8:** 1, 1960.
16. Rose, I. A., R. Kellermeyer, R. Stjernholm, and H. G. Wood. *J. Biol. Chem.* **237:** 3325, 1962.
17. Lambert, M., and A. C. Neish. *Canad. J. Res.* **28:** Section B, 83, 1950.
18. Hirsch, J., and E. H. Ahrens, Jr. *J. Biol. Chem.* **233:** 311, 1958.
19. Harris, H., and W. R. Barclay. *Brit. J. Exptl. Pathol.* **36:** 592, 1955.
20. Stähelin, H., M. L. Karnovsky, and E. Suter. *J. Exptl. Med.* **104:** 137, 1956.
21. Pavillard, E. R. J., and D. Rowley. *Aust. J. Exptl. Biol. Med. Sci.* **40:** 207, 1962.
22. Holdsworth, E. S., and E. Neville. *Biochim. Biophys. Acta* **70:** 338, 1963.
23. Pastore, E. J., and F. Lionetti. *Federation Proc.* **18:** 299, 1959.
24. Elsbach, P. *J. Exptl. Med.* **110:** 969, 1959.
25. Sbarra, A. J., and M. L. Karnovsky. *J. Biol. Chem.* **235:** 2224, 1960.