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# THE INCORPORATION OF $P^{32}$-LABELED ORTHOPHOSPHATE AND GLYCEROL-1-3-$C^{14}$ INTO THE LIPIDS OF THE POLYVINYL SPONGE GRANULOMA *

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Subcutaneous implantation of polyvinyl alcohol sponge is one technique currently in use to secure weighable samples of inflammatory tissue for microscopic and biochemical analyses. Several reports dealing with preliminary characterization of the lipid constituents have been published (1–4). Although size of the sponge prosthesis and duration and site of implantation can affect the lipid content of the tissue (4–6), when these factors are carefully controlled, lipids constitute 15% of the tissue dry weight. In granulomas 21 to 42 days of age, the phospholipids are high, cholesterol low, and triglycerides intermediate in concentration. During the period of inflammatory tissue organization of the sponge implant, it has been demonstrated that increasing amounts of lipid accumulate (1, 4).

The present investigation was done to establish whether these lipids are derived predominantly from the blood, synthesized by the inflammatory cells *in situ,* or represent progressive accumulation of debris from cells destroyed during the early exudative phase of inflammation. A comparative study of incorporation of $P^{32}$ and glycerol-1-3-$C^{14}$ into lipids by the cells of the polyvinyl sponge granuloma and the liver, under the same experimental conditions, is also included in this report. After systemic administration of $P^{32}$ to intact animals, both tissues incorporate this isotope into tissue phospholipids. Use of these two isotopes *in vitro* has provided additional information on de novo synthesis of individual phospholipids, and glycerol-1-3-$C^{14}$ studies have supplied preliminary information on triglyceride synthesis by inflammatory cells.

## MATERIALS AND METHODS

*Tissues studied.* The technique involved in implantation and removal of polyvinyl sponge granulomas and the morphologic characteristics of this tissue have been previously described (5). After *in vivo* or *in vitro* exposure to $P^{32}$ or glycerol-$C^{14}$, 25- and 42-day sponge implants ($0.3 \times 1 \times 1$ cm) from the low dorsolumbar area of guinea pigs, samples of liver, interscapular brown fat pad, and brain were removed for study. Microscopic examination demonstrated complete penetration of the sponge implants by inflammatory tissue. The tissues were dried *in vacuo* over phosphorus pentoxide and fragmented into a coarse powder before lipid extraction.

*Preparation of lipid extracts.* Weighed samples of 50 to 100 mg of tissue powder were subjected to a preliminary extraction with chloroform: methanol (2:1, vol/vol) at room temperature as previously described (4). In order to reduce contamination of the total lipid extract by radioactive water-soluble substances, it was dried *in vacuo* or under a stream of nitrogen, resuspended in 10 ml chloroform, emulsified with 2.00 ml water, centrifuged, and the aqueous layer discarded. This procedure was repeated twice. In the $P^{32}$ experiments, the first wash consisted of .01 M sodium phosphate buffer, followed by a second wash with distilled water. The water-washed organic layer was again evaporated to dryness *in vacuo* and extracted three times with 6 ml diethyl ether or chloroform.[1] Previous studies have demonstrated that this procedure is required to free the tissue lipids from a fraction of the polyvinyl sponge that was soluble in chloroform: methanol after implantation in animals. The final supernatant fluid contained 90 to 95% of the tissue lipids and was used in the present investigation (4).

*Lipid chromatography.* The lipid extracts containing 3 to 10 μmoles of phosphorus were separated into neutral lipid and phospholipid fractions on 5-g silicic acid col-

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[1] When $NaHP^{32}O_4$ was added directly to dry sponge granulomas and this extraction procedure carried out, less than 0.01% of the radioactivity was recovered in the final lipid extract.

umns (Mallinckrodt, AR 100 mesh). In the $P^{32}$ experiments, the elution of neutral lipids by diethyl ether was followed by gradient elution of phospholipids with diethyl ether : ethanol : methanol as previously described (7). In the other experiments, the lipids were applied to the column and eluted stepwise with chloroform, and methanol : chloroform (7 : 1, 4 : 1, 7 : 3, and 1 : 1, vol/vol) and absolute methanol as described by Nye, Waterhouse, and Marinetti (8). In some experiments, fractions of 5 to 10 ml were collected, phosphorus was determined on each tube by the method of Bartlett (9), and other samples were assayed for radioactivity. Appropriate fractions were combined, reduced to dryness *in vacuo,* and further characterized as outlined below. In other cases, the eluates after each solvent change were collected in bulk.

The initial diethyl ether or chloroform eluates from these columns contained the neutral lipids. Samples were taken for total ester and sterol analyses (4) and determination of radioactivity. The remainder was applied to a 5-g silicic acid column, and neutral lipid was separated into cholesterol ester, triglycerides, free cholesterol, mono-, and diglycerides by elution with increasing amounts of diethyl ether in *n*-heptane as described by Marinetti, Griffith, and Smith (10).

Silicic acid paper chromatography was done on all fractions obtained by column chromatography and on samples of the original total lipid extracts. The technique of qualitative and quantitative silicic acid paper chromatography of the phospholipids was performed as recently outlined in detail by Marinetti (11). On each chromatogram, migration of purified phospholipids was compared with the unknown constituents.[2]

Paper chromatography of neutral lipids was carried out with *n*-heptane : 2,6-dimethyl-4-heptanone (96 : 6, 96 : 3), and *n*-heptane : benzene : glacial acetic acid (91 : 9 : 1) as described by Marinetti and Stotz (14). The lipid components were identified by their mobility and staining reaction with rhodamine 6G. Phospholipids were further identified by staining with Ninhydrin and by the choline spot test (11). Radioautograms of the chromatograms were made to assist in precise localization of individual components on the unstained chromatograms and to determine which were radioactive.

Phospholipids from the column fractions and samples of the total lipid extracts were subjected to Dawson's micromethod of alkaline, mild acid, and strong acid hydrolysis (15). The resulting water-soluble phosphorus-containing compounds were chromatographed on paper with phenol : water (4 : 1), phenol : water : acetic acid : ethanol (90 : 10 : 10 : 12), and methanol : formic acid : water (80 : 13 : 7). Two-dimensional chromatography with the last two solvents was also employed. The phosphorus-containing compounds were localized by radioautography and use of Dawson's modification of the acid molybdate spray reagent (15). The spots were cut from the paper, and assayed for radioactivity and phosphorus content.

*In vitro incubations.* Twenty-five-day and 42-day sponge granulomas and liver were sliced by hand and incubated at 37° C in 3 ml of Krebs-Ringer bicarbonate buffer at pH 7.4, containing glucose (200 mg per 100 ml). Five or occasionally 10 $\mu$c $Na_2HP^{32}O_4$ or glycerol-1-3-$C^{14}$ (3 $\mu$c per $\mu$mole) was added to the flask. The flasks were shaken with a gas phase of 95% oxygen and 5% $CO_2$ in a Dubnoff metabolic incubator for an appropriate time. At the end of the incubation period, the tissue was removed from the flasks and blotted on filter paper, and lipid extracts of the tissue were made as outlined above.

*Radioactivity determinations.* In the $P^{32}$ experiments, radioactivity of samples from the whole lipid extract, column fractions, and incubation media was determined after drying on metal planchets. Radioactivity of individual components on a chromatogram was determined by cutting out the spot and affixing it to a planchet for counting. A thin-window gas flow counter was used for all determinations of $P^{32}$ radioactivity. Counting efficiency was such that 1 $\mu$c was equal to $7.5 \times 10^5$ cpm. In the glycerol-$C^{14}$ experiments, counting was done in a Packard Tri-Carb liquid scintillation counter, and 1 $\mu$c was equivalent to $7.2 \times 10^5$ cpm. The scintillation system contained 0.1 g 1,4-*bis*-2(5-phenyloxazolyl)-benzene, and 4.0 g 2,5-diphenyloxazole per L toluene.

## RESULTS

*In vivo incorporation of $P^{32}$-orthophosphate.* Three groups of four animals each were used in this study; each animal had six low dorsolumbar sponge implants made 25 days before administration of isotope. The first group received 50 $\mu$c of $P^{32}$ by intraperitoneal injection, while the second group each received 100 $\mu$c of $P^{32}$ by the same route of administration. In the third group, 5 $\mu$c of $P^{32}$-orthophosphate was injected directly into one of the six implants in each animal. Two other implants were injected with equivalent amounts of physiologic saline. The sponge granulomas,

[2] Chromatographically pure preparations of egg lecithin, soybean monophosphoinositide, and synthetic and enzymatically produced phosphatidic acid were kindly supplied by Dr. Bernard W. Agranoff, The Mental Health Research Institute, The University of Michigan. Commercially available phosphatides—inositol phosphatide (Nutritional Biochemicals Corporation, Cleveland, Ohio), phosphatidyl L-serine, synthetic L-$\alpha$-lecithin dipalmitoyl, and L-$\alpha$-cephalin dipalmitoyl (Mann Research Laboratories, Inc., New York, N.Y.)—with the exception of the last compound, were found to be heterogeneous on initial chromatographic and chemical analysis. The synthetic cephalin was chromatographically pure phosphatidyl ethanolamine. Sphingomyelin was prepared from brain by the method of Carter, Haines, Ledyard, and Norris (12) and the procedure of Rapport and Lerner (13). The latter compounds were further purified by solvent fractionation and column chromatography and were used as additional standards.

specimens from liver, interscapular brown fat pad, and brain were removed, and lipid extracts were prepared of the tissues 5, 22, 48, and 96 hours after administration of the isotope.

Figure 1 shows the time course of incorporation of $P^{32}$ into the phospholipids for each of the tissues studied. Comparable results were obtained after intraperitoneal injection of 50 $\mu$c and 100 $\mu$c, although the larger dose increased the specific activity of the phospholipids in each tissue approximately 25%. The polyvinyl sponge granuloma and liver appeared to have comparable capacity for incorporation of $P^{32}$ orthophosphate, whereas modest levels of radioactivity were found in the phospholipids of brown fat and only traces in the brain.

In Figure 2, the result of direct injection of 5 $\mu$c of $P^{32}$ into one sponge granuloma is compared with that for five adjacent granulomas, liver, and brown fat pad. For the implants that had not been injected directly with isotope, the time course of incorporation was similar to that seen in the previous two experiments, although the specific activity of the phospholipids for these granulomas was one-sixth of that found after intraperitoneal injection. No difference in capacity for $P^{32}$ incorporation was observed for the implants injected locally with saline and those that were uninjected. The specific activity of the phospholipids of the injected implant from each animal reached an apparent plateau at 48 and 96 hours. Only trace activity was detected in the liver and moderate activity in the phospholipids of the interscapular brown fat pad. The low level of radioactivity found in the liver indicates that systemic distribution of $P^{32}$ was minimal, and that alterations in blood supply known to occur as a result of subcutaneous implantation of the sponge may have allowed more direct local circulation of isotope to the uninjected implants and the adjacent interscapular brown fat pad.

The phospholipids from the polyvinyl granu-

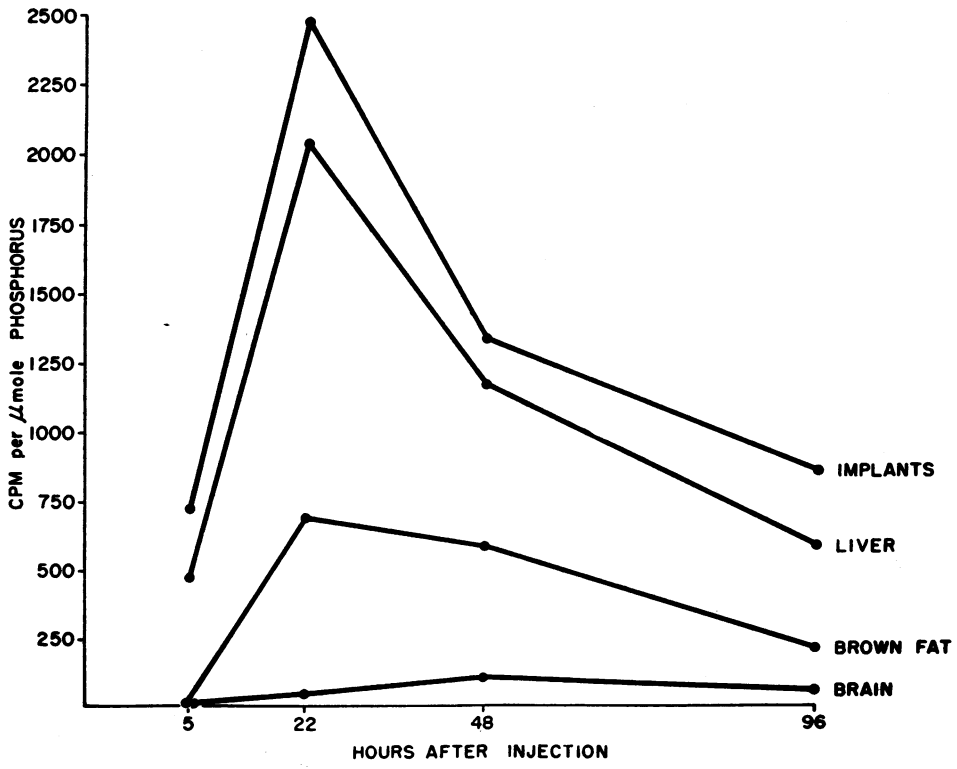


FIG. 1. IN VIVO $P^{32}$ INCORPORATION. Specific activity of 25-day sponge granuloma phospholipids (implants) compared to liver, interscapular brown fat, and whole brain phospholipids after ip injection of guinea pigs with 50 $\mu$c $NaHP^{32}O_4$. Tissue samples and sponge implants were removed at the times indicated on the abscissa.

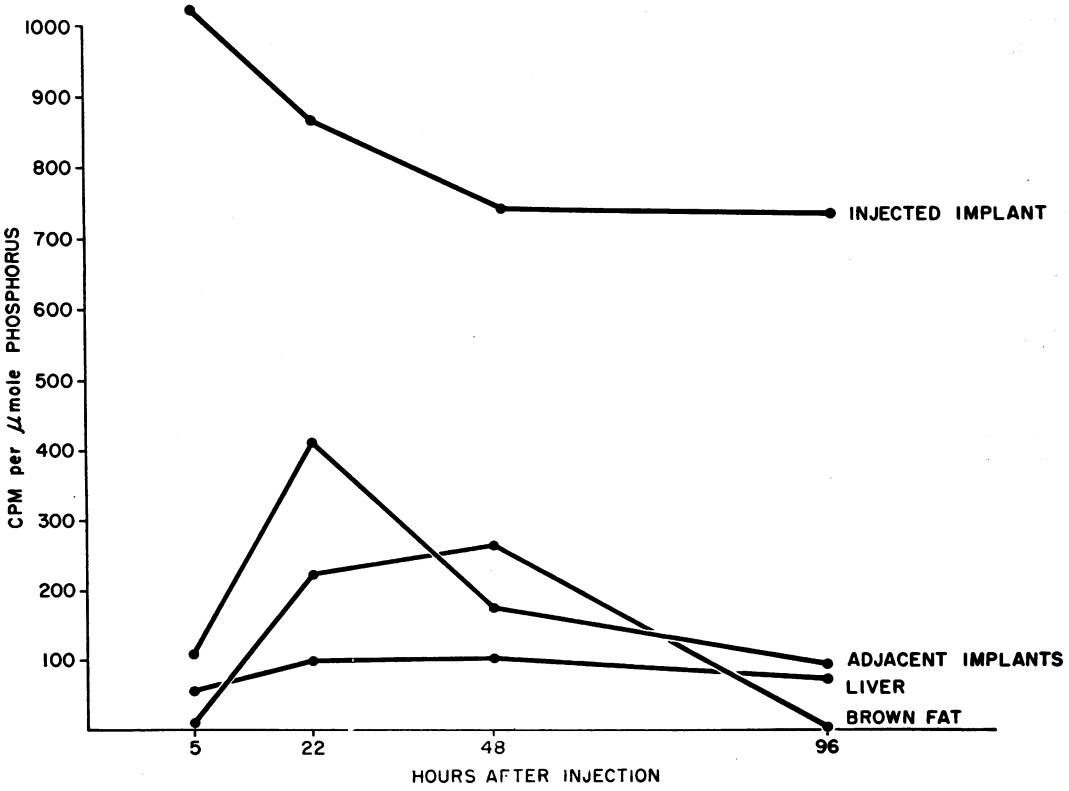


FIG. 2. IN VIVO $P^{32}$ INCORPORATION. Comparison of specific activity of 25-day sponge granuloma phospholipids after direct injection of 5 μc $NaHP^{32}O_4$ into one of six sponge implants. The sponge granulomas and specimens from liver and brown fat pad were removed from each animal at the times indicated for determination of phospholipid specific activity.

lomas were subjected to silicic acid column chromatography using a diethyl ether, ether : ethanol : methanol gradient. Ninety per cent or more of the total radioactivity in the whole lipid extract was recovered from the column in the phospholipid fractions. There was no evidence that the phospholipid column fractions were contaminated by inorganic $P^{32}$.

*In vitro incorporation of $P^{32}$-orthophosphate.* In these experiments, 25-day granuloma slices were incubated *in vitro* for 0 and 40 minutes, and 1½, 2, 3½, and 5 hours. Radioautograms of the silicic acid chromatograms of the whole ether-soluble lipid fraction (Figure 3A) showed that phosphatidyl ethanolamine, phosphatidyl choline, sphingomyelin, and monophosphoinositide had been labeled. These radioactive components were identified by direct comparison of their $R_f$ values with the standard compounds applied in spots on each chromatogram. All components stained with rhodamine 6G and gave typical color reactions when the wet chromatograms were viewed under ultraviolet light (11). Component g (Figure 3A) was Ninhydrin-positive. Weak reactivity occurred at the origin on some chromatograms. Components f and e gave positive choline spot tests. The low-mobility component near the origin may represent diphosphoinositide (11), but it has not been possible to characterize this component adequately. Equivocal or faint spots with the $R_f$ of authentic phosphatidic acid were observed on a few of the radioautograms. Inorganic $P^{32}$ remained at the origin when it was chromatographed in 2,6-dimethyl-4-heptanone : acetic acid : water (40 : 20 : 3).

Three samples of the whole lipid extract from triplicate incubation at each period were chromatographed on silicic acid-impregnated paper. After chromatography and radioautography, the spots were cut out and the radioactivity deter-

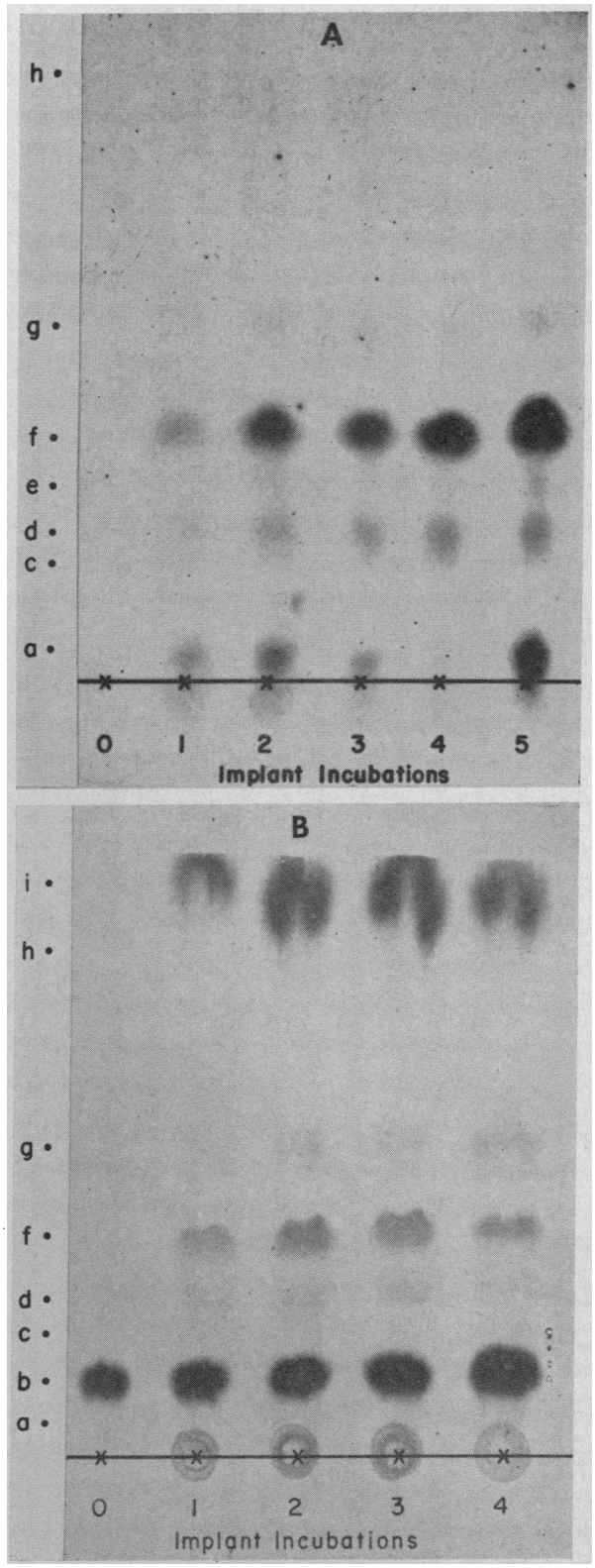


FIG. 3.

mined, followed by elution and quantitative estimation of the phosphorus in each component. In Figure 4 are presented the specific activities of each of the major phospholipids after 40 minutes to 5 hours of incubation *in vitro*. At each period studied, phosphatidyl choline had the highest specific activity. Increase in specific activity for monophosphoinositide, phosphatidyl ethanolamine, and sphingomyelin also occurred with time, but was less marked in each instance.

*In vitro incorporation of glycerol-1-3-$C^{14}$.* Granuloma slices 25 and 42 days of age were incubated *in vitro* from 0 time to 8 hours. Radioautogram of one of the silicic acid paper chromatograms is shown in Figure 3B. The $R_f$ of standard compounds chromatographed simultaneously is indicated by letters at the left of the figure. Chromatography of the phospholipids from whole lipid extracts demonstrated labeling of phosphatidyl ethanolamine, phosphatidyl choline, and monophosphoinositide. The radioactivity near the solvent front indicated isotope incorporation into neutral lipids. The prominent radioactive component with low mobility noted at 0 time and in the other lipid extracts was glycerol-1-3-$C^{14}$. In these studies, contamination of the whole lipid extracts by free glycerol constituted 5 to 15% of the total detected radioactivity. This was established for each lipid extract by combined use of silicic acid column and paper chromatography followed by radioautography and determination of radioactivity in each component on a chromato-

FIG. 3. RADIOAUTOGRAMS OF PAPER CHROMATOGRAMS OF WHOLE LIPID EXTRACTS OF 25-DAY SPONGE GRANULOMA SLICES EXPOSED TO $P^{32}$ (A) AND GLYCEROL-1-3-$C^{14}$ (B) IN VITRO. For each extract, 0.25 μmole of P was applied to the chromatograms, which were developed in 2,6-dimethyl-4-heptanone : acetic acid : water (40 : 20 : 3) at room temperature before radioautography. Identity of components: a = diphosphoinositide (tentatively), b = free glycerol-1-3-$C^{14}$, c = lysophosphatidyl choline, d = monophosphoinositide, e = sphingomyelin, f = phosphatidylcholine, g = phosphatidyl ethanolamine, h = phosphatidic acid, and i = neutral lipids. A. Results obtained after *in vitro* incubation with $NaHP^{32}O_4$. 0 = zero time, 1 = 40 minutes, 2 = 1½ hours, 3 = 2 hours, 4 = 3½ hours, and 5 = 5 hours. Letters at the left of the figure identify labeled components and indicate $R_f$ values of phospholipid standards run simultaneously on the same chromatogram. B. Results obtained following *in vitro* incubation with glycerol-1-3-$C^{14}$. 0 = zero time, 1 = 1½ hours, 2 = 3 hours, 3 = 5 hours, and 4 = 8 hours.

gram. Radioactivity in neutral lipids present in the initial chloroform eluate from the columns was identified in mono-, di-, and triglycerides by paper chromatography using three solvent systems. The glycerol-$C^{14}$ present in the total lipid extracts was eluted from the columns by chloroform: methanol, 7:3 (vol/vol), during fractionation of the phospholipids. This was demonstrated by paper chromatography of samples from the phospholipid column fractionations, and was quantitated by measurement of radioactivity in each component. In this study, radioactivity contributed by presence of free glycerol-$C^{14}$ has been determined individually for each lipid extract, and substracted from that reported for glycerol-$C^{14}$ incorporation into total lipids and total phospholipids. Since the specific activity of the several phospholipids has been calculated from triplicate analysis of radioactivity and phosphorus content for each individual component on a chromatogram, it was unaffected by this contamination.

In Figure 5, the specific activity for monophosphoinositide, phosphatidyl choline, and phosphatidyl ethanolamine at $1\frac{1}{2}$, 3, and 5 hours are given for 25-day granuloma slices. In these experiments, monophosphoinositide had the highest specific activity at each period studied. This is in contrast to the *in vitro* $P^{32}$ studies where phosphatidyl choline was the most highly labeled phospholipid. When the difference in observed specific activity for inorganic $P^{32}$ ($8.6 \times 10^5$ cpm per $\mu$mole) and glycerol-1-3-$C^{14}$ ($1.6 \times 10^6$ cpm per $\mu$mole) of the initial incubation media was taken into consideration, incorporation of $P^{32}$ into phosphatidyl choline was 100 times, phosphatidyl ethanolamine 33 times, and monophosphoinositide 10 times that achieved with glycerol-$C^{14}$.

The glycerol-$C^{14}$-labeled phospholipids were subjected to mild alkaline hydrolysis (15); over 90% of the total radioactivity in the whole phospholipid fraction was recovered in the aqueous phase of the hydrolysate. Chromatography of the organic phase on silicic acid-impregnated paper, before further acid hydrolysis, demonstrated that residual radioactivity was due to trace amounts of phosphatidyl choline and lysophosphatidyl choline remaining in the organic layer. Sphingomyelin present in this fraction was found to be unlabeled. In Figure 6, the results of radioautography and acid molybdate spray reaction for phosphorus on a two-dimensional chromatogram from a 3-hour incubation are schemati-

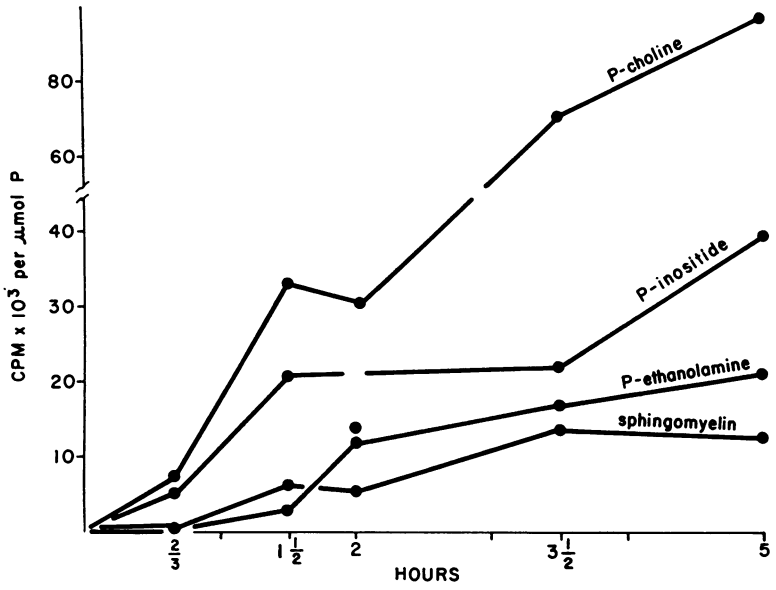


FIG. 4. SPECIFIC ACTIVITY DETERMINATIONS FOR INDIVIDUAL PHOSPHOLIPID COMPONENTS FROM 25-DAY SPONGE GRANULOMAS AFTER VARIOUS EXPOSURES TO $NaHP^{32}O_4$ IN VITRO. P-choline = phosphatidyl choline, P-inositide = monophosphoinositide, and P-ethanolamine = phosphatidyl ethanolamine.

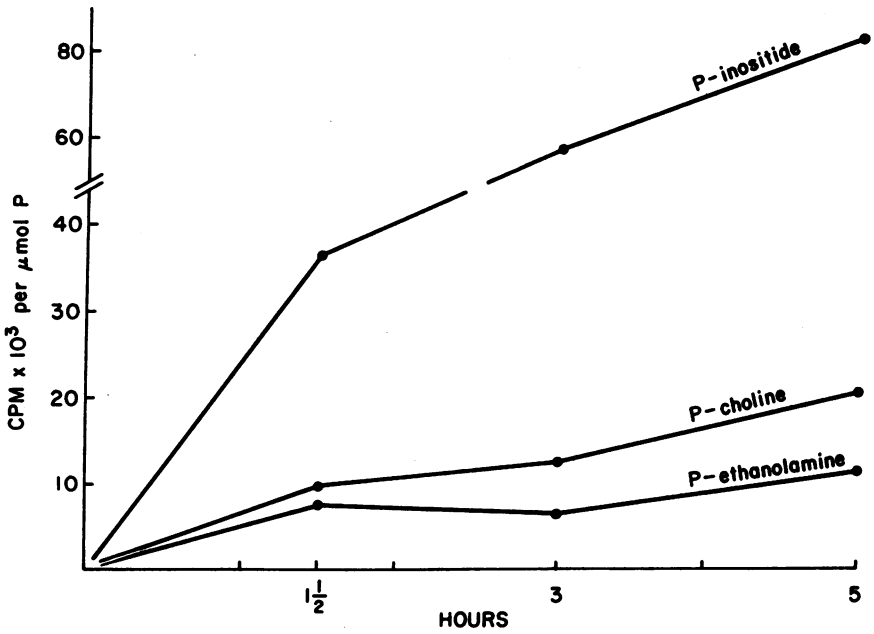


FIG. 5. SPECIFIC ACTIVITY DETERMINATIONS FOR PHOSPHOLIPID COMPONENTS FROM 25-DAY GRANULOMAS AFTER VARIOUS EXPOSURES TO GLYCEROL-1-3-$C^{14}$ IN VITRO. P-inositide = monophosphoinositide, P-choline = phosphatidyl choline, and P-ethanolamine = phosphatidyl ethanolamine.

cally represented. In the two-dimensional systems employed, free glycerol-$C^{14}$ was separable from glycerylphosphoryl ethanolamine and the water-soluble hydrolysis products of the other alkali-labile phospholipids. The analogues of phosphatidyl serine and phosphatidic acid and an unidentified component were also detected. These three constituted a small fraction of the total phosphorus applied to the chromatograms, and allowed qualitative detection, but not quantitative determinations of specific activity. In Table I, the specific activities for phosphatidyl choline determined by each of the methods used in this study demonstrated good agreement. The values for phosphatidyl ethanolamine determined by silicic acid paper, silicic acid column, and paper chromatography of intact phospholipids, and two-dimensional chromatography of the alkaline hydrolysates show somewhat greater variability. Those given for unidimensional chromatography were high due to inadequate resolution of glycerylphosphoryl ethanolamine from free glycerol-$C^{14}$ present in the total phospholipid fraction.

A comparative study of the *in vitro* capacity to incorporate glycerol-$C^{14}$ by 25-day and 42-day granulomas was carried out. Maximal *in vitro* incorporation of glycerol-1-3-$C^{14}$ into lipids of 25-day granuloma slices reached 5% at 5 hours of incubation, and was essentially equal for neutral lipids and phospholipids; their values at 1½ hours were 1.58 and 1.46%, at 3 hours, 2.12 and

TABLE I

*Variation in phospholipid specific activities as influenced by the method of analysis (glycerol-1-3-$C^{14}$, 3-hour incubations)*

| Component | Silicic acid paper chromatography | Silicic acid column and paper chromatography | Alkaline hydrolysis [1]* | Alkaline hydrolysis [2]* |
|---|---|---|---|---|
| | *cpm/ μmole P* | *cpm/ μmole P* | *cpm/ μmole P* | *cpm/ μmole P* |
| Phosphatidyl choline | 9,900 | 14,100 | 10.200 | 7,400† |
| | 9,600 | 17,100 | 10,300 | 7,800 |
| | 10,900 | | 10,700 | 9,600 |
| | 11,400 | | | |
| | 12,300 | | | |
| Phosphatidyl ethanolamine | 4,600 | 10,300 | 58,800 | 5,200† |
| | 5,000 | 21,700 | 57,300 | 11,200 |
| | 5,300 | | 93,300 | |

* [1] and [2] indicate results obtained after uni- and bidirectional chromatography of the water-soluble phosphorus-containing components.

† Replicate analyses on the same lipid extract.

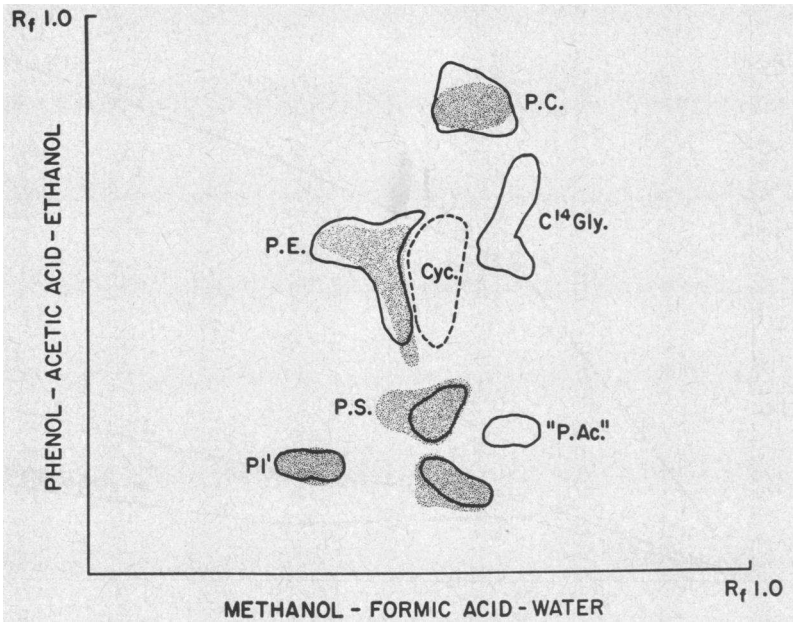


FIG. 6. TRACING OF A TWO-DIMENSIONAL PAPER CHROMATOGRAM OF THE PHOSPHORUS-CONTAINING PRODUCTS OF MILD ALKALINE HYDROLYSIS OF 2.3 μMOLES OF 42-DAY SPONGE GRANULOMA PHOSPHOLIPIDS. Three-hour *in vitro* exposure to glycerol-1-3-$C^{14}$ was carried out before lipid extraction and hydrolysis. Encircled areas represent radioactive components demonstrated by radioautography, and stippled areas identify color development after the acid molybdate spray for phosphorus (15). The abbreviations indicate the hydrolysis product (the parent phospholipid is given in parentheses): P.C. = glycerylphosphoryl choline (phosphatidyl choline); P.E. = glycerylphosphoryl ethanolamine (phosphatidyl ethanolamine); P.S. = glycerylphosphoryl serine (phosphatidyl serine); PI' = glycerylphosphoryl inositol + phosphoryl inositol (monophosphoinositide); unlabeled = unidentified, radioactive, phosphorus-containing component; "P.Ac." = glycerophosphoric acid (phosphatidic acid?); Cyc. = cyclic products of alkaline hydrolysis, including glycerophosphate obscured by alkali-metal present in the hydrolysate; "P.Ac." and Cyc. reacted faintly with acid molybdate on some chromatograms; $C^{14}$ Gly. = free glycerol-1-3-$C^{14}$.

2.33%, and at 5 hours, 2.80 and 2.32%, respectively. Total incorporation by 42-day granulomas was half as great at 1½ and 3 hours and was also equally distributed between neutral lipids and phospholipids. When, however, the decrease in total tissue solids that occurred in these granulomas between 25 and 42 days (5) was considered and the data were expressed as millimicromoles lipid formed per hour per milligram tissue, this difference was no longer apparent. The values for neutral lipids and phospholipids of 25-day granulomas were: at 1½ hours, 0.43 and 0.41; at 3 hours, 0.25 and 0.28; and at 5 hours, 0.24 and 0.20 mμmole per hour per mg. For 42-day granulomas they were at 1½ hours, 0.27 and 0.41, and at 3 hours, 0.32 and 0.30 mμmole per hour per mg, respectively. In Table II, the mean specific activities of phospholipids from these granulomas are given for 1½ and 3 hours of incubation. The values for monophosphoinositide, phosphatidyl choline, and phosphatidyl ethanolamine were derived from phosphorus and radioactivity measurements on these components separated by silicic acid paper and column chromatography, and by chromatography after alkaline hydrolysis. Tentative identification of phosphatidic acid or polyglycerol phosphatide in these lipid extracts has been indirect and is subject to the qualifications expressed by other investigators (11, 15,

TABLE II

*Comparison of phospholipid specific activities by tissue age after two periods of in vitro exposure to glycerol-1-3-$C^{14}$**

| | 1½-hour incubations | | | | 3-hour incubations | | | |
|---|---|---|---|---|---|---|---|---|
| | "P.Ac." | PI′ | P.C. | P.E. | "P.Ac." | PI′ | P.C. | P.E. |
| | *cpm/μmole P* | | | | *cpm/μmole P* | | | |
| 25-Day granulomas | 33,400 [2] | 36,750 [4] | 9,850 [4] | 7,450 [4] | 138,100 [5] | 58,100 [5] | 12,500 [5] | 6,350 [4] |
| 42-Day granulomas | † | 11,300 [3] | 9,800 [3] | 7,700 [3] | 59,000 [3] | 9,200 [7] | 11,400 [7] | 5,200 [6] |

* "P.Ac." = phosphatidic acid, PI′ = monophosphoinositide, P.C. = phosphatidyl choline, and P.E. = phosphatidyl ethanolamine. Mean values are given. Number of observations are given in brackets.
† Not determined.

16). It was impossible by chromatography of the whole lipid extract on silicic acid paper to separate clearly this component from glycerol-$C^{14}$-labeled neutral lipids migrating near the solvent front. After column chromatography and rechromatography of samples eluted by chloroform: methanol, 7: 1, a discrete component was identified on radioautograms with the $R_f$ of authentic phosphatidic acid. Addition of known amounts of carrier phosphatidic acid, as described by Hokin and Hokin (17), resulted on rechromatography in a decrease in radioactivity of this component. By two-dimensional chromatography, a radioactive component with an $R_f$ value similar to glycerolphosphate was also observed (Figure 6). Owing to the low phosphorus values and indirect identification, the specific activities included in the table should be considered only semiquantitative estimates. The specific activity of monophosphoinositide in 25-day implants was much higher at 1½ and 3 hours than those observed at 42 days. This was a consistent finding, irrespective of the method of separation and identification of this component. Between 25 and 42 days, the spe-

TABLE III

*In vitro studies on incorporation of $P^{32}$ and glycerol-1-3-$C^{14}$ by liver and granuloma slices*

| Mean values | $P^{32}$-orthophosphate, 2-hour incubations | | Glycerol-1-3-$C^{14}$, 3-hour incubations | |
|---|---|---|---|---|
| | Liver | Granuloma | Liver | Granuloma |
| Isotope Incorporation | | | | |
| Total cpm: | | | | |
| Zero time | 2,070 | 1,500 | 2,760 | 3,840 |
| Total lipid | 89,770 | 61,540 | 96,800 | 121,100 |
| Phospholipids | 89,770 | 61,540 | 65,400 | 63,500 |
| Neutral lipids | | | 31,400 | 52,600 |
| Per cent: | | | | |
| Total lipid | | | 3.25 | 4.45 |
| Phospholipid | 1.16 | 0.82 | 2.27 | 2.33 |
| Neutral lipids | | | 1.08 | 2.12 |
| Incorporation, mμmole per hour per mg tissue | | | | |
| Total lipid | | | 0.12 | 0.53 |
| Phospholipids | 1.1 | 4.2 | 0.08 | 0.28 |
| Neutral lipids | | | 0.04 | 0.25 |
| Specific activity, cpm per μmole P | | | | |
| "Phosphatidic acid" | 106,000 | 0 | 41,600 | 138,100 |
| Phosphatidyl ethanolamine | 9,300 | 10,700 | 1,190 | 6,350 |
| Phosphatidyl choline | 7,000 | 48,600 | 1,240 | 12,540 |
| Sphingomyelin | 1,750 | 8,800 | 0 | 0 |
| Monophosphoinositide | 19,100 | 19,000 | 5,800 | 58,100 |
| Isotope specific activity, observed cpm per μmole | $8.6 \times 10^5$ | $8.6 \times 10^5$ | $1.7 \times 10^6$ | $1.6 \times 10^6$ |
| No. of observations | 2 | 4 | 4 | 3 |

cific activity of phosphatidyl choline and phosphatidyl ethanolamine remained stable within the experimental error of these methods of analysis.

*Comparison of $P^{32}$ and $C^{14}$-glycerol incorporation by granuloma and liver slices.* Simultaneous *in vitro* incubations of 25-day granuloma and liver slices from the same animal were performed. In Table III, the data are given for incorporation of these two isotopes into lipids under the *in vitro* conditions employed throughout this investigation.

The value for $P^{32}$-orthophosphate incorporation, expressed as total radioactivity and as the percentage of isotope incorporated after 2 hours of incubation, was similar for each tissue. Glycerol-$C^{14}$ incorporation into neutral lipids and phospholipids was also similar, although labeling of neutral lipids was somewhat greater in the granuloma slices. Upon these determinations, a direct comparison between the two isotopes should not be made, since duration of incubation and observed specific activity for the two isotopes were different, as indicated in Table III. Although the tissue mass in each incubation flask was judged to be equivalent, presence of the polyvinyl sponge prosthesis in the granulomas resulted in net tissue dry weights per flask that were $\frac{1}{3}$ to $\frac{1}{6}$ those for liver (8.4 to 48 mg granuloma, 46 to 150 mg liver slices). When each of these variables was taken into consideration by expressing the data as millimicromoles of lipid formed per hour per milligram of tissue, both tissues showed greater incorporation of $P^{32}$ than glycerol-$C^{14}$ into phospholipids, and the granuloma slices demonstrated at least four times the capacity of liver slices to incorporate each isotope. A slightly greater difference was observed for glycerol-$C^{14}$ incorporation into neutral lipids.

Two major differences were found on comparison of the specific activities for $P^{32}$-labeled phospholipids. In the liver slices, "phosphatidic acid" had the highest activity, whereas in the granuloma, labeling was undetected, and in it phosphatidyl choline was found to have the highest specific activity. This difference demonstrable with $P^{32}$ was not found with glycerol-$C^{14}$. In each tissue, the glycerol-$C^{14}$ relative specific activities were similar to those found for liver after exposure to $P^{32}$, i.e., phosphatidic acid > monophosphoinositide > phosphatidyl choline or phosphatidyl ethanolamine > sphingomyelin.

## DISCUSSION

These studies demonstrate that cells present during the proliferative phase of inflammation are capable of incorporating radioactive inorganic phosphorus and glycerol into lipids. Systemic and local administration of $P^{32}$ resulted in labeling of phospholipids, providing indirect evidence for *in situ* incorporation by inflammatory cells. Confirmation of these preliminary findings has been provided by the *in vitro* studies. Under identical conditions in a simple *in vitro* system, granuloma and liver slices have shown similar capacity for $P^{32}$ incorporation into phospholipids. Since the purpose of the present investigation was to gain evidence for local de novo synthesis of phospholipids and triglycerides, the studies with radiophosphate have been compared with the *in vitro* incorporation of glycerol-$C^{14}$. It has been emphasized by several investigators (16, 18, 19) that incorporation of $P^{32}$-orthophosphate into phospholipids *in vivo* or *in vitro* can occur by exchange of the phosphate moiety of the molecule without true synthesis of new phospholipid. For glycerol-$C^{14}$ to be incorporated into a lipid, formation of new ester bonds between glycerol and fatty acids or phosphate must occur. Identification of radioactivity in phospholipids or triglycerides from this source, based upon currently established biosynthetic pathways (18, 20), offers more direct evidence that net lipid synthesis has occurred. Although isotope tracer studies have led in many instances to better understanding of enzymatic differences existing between tissues (10, 18, 20), an analysis of the precise reactions involved in incorporation of this isotope into lipids of the polyvinyl sponge granuloma is beyond the scope of the present investigation. Direct phosphorylation of glycerol catalyzed by glycerokinase has been specifically demonstrated only in liver, kidney, and heart (20), although incorporation of this isotope has been reported for several other tissues (7, 21, 22). In the $P^{32}$ studies, labeling of phospholipids of the sponge granuloma may have occurred through a series of kinase-catalyzed reactions resulting in the introduction of radiophosphate into a phospholipid (phosphatidyl choline) without de novo synthesis of the entire molecule (18).

Boucek and Noble (23, 24) have previously

reported that polyvinyl sponge granulomas incorporate acetate-$C^{14}$ into cholesterol. In their studies, incorporation *in vitro* was only a fraction of that demonstrated for liver. They emphasized that the capacity was significant, considering the relatively cell-poor nature of this inflammatory tissue compared with that of the liver. These investigators were concerned with the possible relationship between their observations and the problem of atherosclerosis. More recently, Newman, McCandless, and Zilversmit (25) demonstrated that atheromatous lesions in the aorta of rabbits apparently synthesize phospholipids and triglycerides *in situ,* although they were unable to demonstrate cholesterol synthesis. A preliminary report by Jackson and Levin (26) indicates that cells of the carrageenin granuloma will incorporate acetate-$C^{14}$ into lipids. Several investigations (7, 10, 20-22) have shown that individual tissues incorporate different isotopes at variable rates into phospholipids and neutral lipids. Hokin, Hokin, and Benfey (21, 22, 27) have demonstrated in several tissues that the pattern of labeling of phospholipids by $P^{32}$ and glycerol-$C^{14}$ *in vitro* differs for resting cells and after stimulation of the tissue by a variety of agents. Direct comparison of such isotope data is difficult, since the presently established biosynthetic schemes for the synthesis of individual lipids in many instances offer several alternative routes for incorporation, depending on the isotope tracer employed.

Differences in labeling of phospholipids after *in vitro* exposure to $P^{32}$ or glycerol-$C^{14}$ found in the present study have been observed in other investigations (21, 22). Explanation of this difference by the *in vitro* conditions employed or the methods of isolation and identification of the phospholipids cannot be excluded. In an attempt to establish a basis for comparison between this study and other investigations, the *in vitro* incubations of liver slices were done simultaneously under the same experimental conditions, with each of the isotopes. The inability to detect $P^{32}$-labeled phosphatidic acid in the granuloma has been explained in other tissues by the demonstrated presence of phosphatidic acid phosphatase, by the influence of *in vitro* conditions, or by its rapid utilization and low concentration *in vivo* (18). "Phosphatidic acid" has been recognized as an important intermediate in phospholipid synthesis, and in the glycerol-$C^{14}$ studies, this compound was tentatively identified. A decrease in the specific activity of monophosphoinositide has been observed between tissue 25 and 42 days of age. The possible physiologic significance of this finding remains unknown.

These data, and those of Boucek and Noble (23, 24) and Jackson and Levin (26) indicate that inflammatory tissue cells will incorporate isotopically labeled precursors into lipids. Chemical studies and histochemical observations (1, 4-6, 28) on the lipid constituents of experimentally induced inflammatory tissue need not be ascribed in whole or in part to cell degeneration or passive deposition from the blood. Leukocytes from peripheral blood have been shown to sequester lipids in extracellular location (29, 30). Studies on cell membrane integrity (31, 32), transport (33), phagocytosis (34), and coagulation (35) suggest that lipid synthesis is involved in each of these phenomena. Collateral observations of this type emphasize that further investigations of lipid biosynthesis by inflammatory cells should contribute to a better understanding of the complex intracellular and extracellular events that occur during the inflammatory response.

## SUMMARY

Inflammatory tissue induced by subcutaneous implantation of polyvinyl sponge will incorporate $P^{32}$-orthophosphate and glycerol-1-3-$C^{14}$ into phospholipids and neutral lipids *in vivo* and *in vitro.* Under the same experimental conditions, this capacity was found to be similar to that of liver.

There was a definite difference observed *in vitro* between the labeling pattern of inflammatory tissue phospholipids after exposure to $P^{32}$ and glycerol-$C^{14}$. With $P^{32}$, major incorporation occurred in phosphatidyl choline, > monophosphoinositide > phosphatidyl ethanolamine > sphingomyelin, whereas with glycerol-$C^{14}$, monophosphoinositide and a component tentatively identified as phosphatidic acid were most highly labeled, > phosphatidyl choline and phosphatidyl ethanolamine. Approximately half of the glycerol-$C^{14}$ incorporated into lipids was found in the neutral lipid fraction, i.e., mono-, di-, and tri-

glycerides. The pattern of incorporation of both isotopes into liver phospholipids was found to be similar to that for glycerol-$C^{14}$ incorporation by inflammatory tissue.

*In vitro* incorporation of glycerol-$C^{14}$ indicates that local de novo synthesis can contribute to the lipid content of the polyvinyl sponge granuloma; cell degeneration or deposition from the blood does not alone account for this constituent of inflammatory tissue.

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## REFERENCES

1. Boucek, R. J., and N. L. Noble. Connective tissue: a technique for its isolation and study. Arch. Path. 1955, **59**, 553.
2. Noble, N. L., and R. J. Boucek. Lipids of the serum and connective tissue of the rat and rabbit. Circulat. Res. 1955, 3, 344.
3. Boucek, R. J., N. L. Noble, and K-Y. T. Kao. The conjugated lipids of connective tissue of the rat and rabbit. Circulat. Res. 1955, 3, 519.
4. Bole, G. G., Jr., S. Roseman, and W. E. M. Lands. Studies on the lipid content of polyvinyl sponge biopsy connective tissue. J. Lab. clin. Med. 1962, **59**, 730.
5. Bole, G. G., Jr., and W. D. Robinson. Histochemical and biochemical variations in the connective tissue in polyvinyl alcohol sponge implants. J. Lab. clin. Med. 1962, **59**, 713.
6. Dasler, W., R. V. Hilliser, and R. E. Stoner. Influence of site of implantation on lipid content of subcutaneous sponge biopsy connective tissue. J. Lab. clin. Med. 1960, **56**, 760.
7. Lands, W. E. M. Metabolism of glycerolipides: a comparison of lecithin and triglyceride synthesis. J. biol. Chem. 1958, **231**, 883.
8. Nye, W. H. R., C. Waterhouse, and G. V. Marinetti. The phosphatides of human plasma. I. Normal values determined by paper and column chromatography. J. clin. Invest. 1961, **40**, 1194.
9. Bartlett, G. R. Phosphorus assay in column chromatography. J. biol. Chem. 1959, **234**, 466.
10. Marinetti, G. V., M. Griffith, and T. Smith. The incorporation of [1-$^{14}$C] glycerol, [1-$^{14}$C] acetate and DL[3-$^{14}$C] serine into the lipids of rat-heart and liver homogenates. Biochim. biophys. Acta (Amst.) 1962, **57**, 543.
11. Marinetti, G. V. Chromatographic separation, identification, and analysis of phosphatides. J. Lipid Res. 1962, 3, 1.
12. Carter, H. E., W. J. Haines, W. E. Ledyard, and W. P. Norris. Biochemistry of the sphingolipides. I. Preparation of sphingolipides from beef brain and spinal cord. J. biol. Chem. 1947, **169**, 77.
13. Rapport, M. M., and B. Lerner. A simplified preparation of sphingomyelin. J. biol. Chem. 1958, **232**, 63.
14. Marinetti, G. V., and E. Stotz. Direct chromatography of serum lipids without solvent extraction. Biochim. biophys. Acta (Amst.) 1960, **37**, 571.
15. Dawson, R. M. C. A hydrolytic procedure for the identification and estimation of individual phospholipids in biological samples. Biochem. J. 1960, **75**, 45.
16. Firkin, B. G., and W. J. Williams. The incorporation of radioactive phosphorus into the phospholipids of human leukemic leukocytes and platelets. J. clin. Invest. 1961, **40**, 423.
17. Hokin, L. E., and M. R. Hokin. The presence of phosphatidic acid in animal tissues. J. biol. Chem. 1958, **233**, 800.
18. Kennedy, E. P. Biosynthesis of phospholipides. Fed. Proc. 1957, **16**, 847.
19. Agranoff, B. W., R. M. Bradley, and R. O. Brady. The enzymatic synthesis of inositol phosphatide. J. biol. Chem. 1958, **233**, 1077.
20. Kennedy, E. P. Biosynthesis of complex lipids. Fed. Proc. 1961, **20**, 934.
21. Hokin, L. E., and M. R. Hokin. Phosphoinositides and protein secretion in pancreas slices. J. biol. Chem. 1958, **233**, 805.
22. Hokin, L. E., and M. R. Hokin. Acetylcholine and the exchange of inositol and phosphate in brain phosphoinositide. J. biol. Chem. 1958, **233**, 818.
23. Boucek, R. J. and N. L. Noble. Biochemical studies on cholesterol in *in vivo* cultivated connective tissue. Circulat. Res. 1957, **5**, 27.
24. Noble, N. L., and R. J. Boucek. Hormonal effects upon *in vitro* cholesterol synthesis. Circulat. Res. 1957, **5**, 573.
25. Newman, H. A. I., E. L. McCandless, and D. B. Zilversmit. The synthesis of $C^{14}$-lipids in rabbit atheromatous lesions. J. biol. Chem. 1961, **236**, 1264.
26. Jackson, D. S., and E. Levin. Lipid synthesis in the carrageenin granuloma *in* Summary reports, 4th Ann. Meeting, Research Fellows and Investigators, Helen Hay Whitney Foundation. Princeton, 1961, p. 15.
27. Hokin, M. R., B. G. Benfey, and L. E. Hokin. Phospholipides and adrenaline secretion in guinea pig adrenal medulla. J. biol. Chem. 1958, **233**, 814.
28. Edwards, L. C., L. N. Pernokas, and J. E. Dunphy. The use of a plastic sponge to sample regenerating

tissue in healing wounds. Surg. Gynec. Obstet. 1957, **105**, 303.

29. Marks, P. A., A. Gellhorn, and C. Kidson. Lipid synthesis in human leukocytes, platelets, and erythrocytes. J. biol. Chem. 1960, **235**, 2579.
30. Buchanan, A. A. Lipid synthesis by human leucocytes *in vitro*. Biochem. J. 1960, **75**, 315.
31. Allison, A. C., M. Kates, and A. T. James. An abnormality of blood lipids in hereditary spherocytosis. Brit. med. J. 1960, **2**, 1766.
32. Phillips, G. B. Quantitative chromatographic analysis of plasma and red blood cell lipids in patients with acanthocytosis. J. Lab. clin. Med. 1962, **59**, 357.
33. Hawthorne, J. N. The inositol phospholipids. J. Lipid. Res,. 1960, 1, 255.
34. Karnovsky, M. L., and D. F. H. Wallach. III. The metabolic basis of phagocytosis. Incorporation of inorganic phosphate into various classes of phosphatides during phagocytosis. J. biol. Chem. 1961, **236**, 1895.
35. Marcus, A. J., and T. H. Spaet. Platelet phosphatides: their separation, identification, and clotting activity. J. clin. Invest. 1958, **37**, 1836.