

Effect of cholesterol in suspension on the incorporation of phosphate into phospholipid by macrophages in vitro

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ABSTRACT. Macrophages obtained from the peritoneal cavity of rabbits were incubated with phosphate-³²P in order to investigate the synthesis of phospholipid by these cells. After 6 hr of incubation 0.25% of the phosphate added to the medium had been incorporated into phospholipid by the macrophages, mainly into lecithin and sphingomyelin, but partly also into phosphatidyl ethanolamine and inositol phosphatide.

The addition of cholesterol to the macrophage suspensions increased the rate of incorporation by 20% with 1 mg of cholesterol added, and 44% with 2.5 mg added. The increase was similar for all the phospholipid fractions, and was not accompanied by any increase in oxygen uptake by the cells. The addition of carbon particles (as a specific check for phagocytic effects) had only a small effect on the rate of incorporation.

The data provide support for the concept that cholesterol stimulates phospholipid synthesis by similar cells in the arterial wall during atherogenesis.

KEY WORDS phosphate-³²P · incorporation · phospholipid · biosynthesis · macrophages · rabbit · cholesterol · carbon · suspension · oxygen uptake · phagocytosis · randomized block · atheroma

IT IS WELL KNOWN that in both experimental and human atheroma the phospholipid content of the arterial wall increases with that of cholesterol. Zilversmit and coworkers (1, 2) have demonstrated that this phospholipid accumulation occurs as a result of synthesis in the arterial wall rather than by deposition from the blood. The phospholipid that accumulates in the experimental atheromatous lesion, however, does so intracellularly in the foam cell (3); this suggests that during development of experimental atheroma, the phospholipid synthesis that is occurring in the arterial wall takes place in the foam cell. Recently Dunnigan (4) has

demonstrated that in human atheroma also, phospholipid accumulates intracellularly in foam cells. In view of these facts, some further investigation of lipid synthesis by these cells or their precursors is indicated.

Investigation of the *in situ* synthesis of lipid by foam cells is impracticable by other than histochemical means, and a more thorough investigation of their lipid metabolism requires a preparation free from other cells. Although it was previously thought that foam cells arise from tissue macrophages, there is evidence that some of these cells may arise from smooth muscle cells (5). However, a large proportion of them is derived from blood monocytes which infiltrate the lesion, become macrophages, and engulf lipid (6). Following the injection of liquid paraffin, monocytes are mobilized into the peritoneal cavity; these cells can be collected and their lipid metabolism studied. While it cannot be asserted with certainty that the metabolism of such cells is the same as that of arterial wall macrophages or of foam cells, it is likely to be similar.

In previous papers (7, 8) it has been shown, with monocytes from the peritoneal cavity of rabbits, that ¹⁴C-labeled fatty acid and ¹⁴C-labeled acetate both can be incorporated into phospholipid by macrophages *in vitro*. We have now studied the incorporation of ³²P-labeled phosphate into macrophages *in vitro*, in order, firstly, to establish that synthesis of phospholipid by macrophages occurs, secondly, to identify the phospholipid synthesized, and thirdly, to determine the effect of cholesterol uptake on this process.

MATERIALS AND METHODS

³²P-labeled carrier-free orthophosphate in dilute HCl was obtained from the Radiochemical Centre, Amer-

sham, U.K. This was diluted with 0.9% sodium chloride solution to give a stock solution for use.

Phospholipid standards for chromatography were obtained as follows. Synthetic dipalmitoyl lecithin, phosphatidyl ethanolamine, phosphatidyl serine, and inositol phosphatide were obtained from the Nutritional Biochemicals Corporation (Cleveland, Ohio). Sphingomyelin was prepared from beef lung as described by Thannhauser, Benotti, and Boncoddio (9). This preparation gave positive tests for choline (described later) and sphingosine (10) and the R_F value was identical with that of a sample of sphingomyelin supplied by the Commonwealth Serum Laboratories (Melbourne, Australia). Lysolecithin was prepared from synthetic dipalmitoyl lecithin by the method of Renkonen (11) and had the same R_F value as a sample of lysolecithin supplied by the Commonwealth Serum Laboratories (Melbourne, Australia). It also gave a positive test for choline.

Carbon suspension [carbon C11/1413a (Gunther Wagner, Hanover, Germany)] 32 mg/ml was diluted with water before use. The mean particle diameter was 0.05 μ .

A cholesterol suspension was prepared by slowly adding a warm solution of 500 mg of cholesterol in acetone to 200 ml of water. The acetone was removed by evaporation, and the suspension concentrated to 10 mg of cholesterol per milliliter by boiling. This suspension was stable, did not separate on standing even over a period of many months, and contained particles with a mean diameter of 1 μ .

Rabbit macrophages were obtained from the peritoneal cavity of rabbits as previously described (7).

Experimental Procedure

Two series of replicate experiments were carried out, the first to demonstrate the incorporation by macrophages in vitro of ^{32}P -labeled phospholipid and to identify the labeled phospholipid by fractionation, and the second to study the effect, on the rate of incorporation of labeled phosphate into phospholipid, of either added cholesterol or added carbon particles, the latter as a control for phagocytic effects.

In each experiment siliconized Warburg flasks containing an aliquot ($5-6 \times 10^7$ cells, approx 35 mg wet wt) of macrophages from a single freshly prepared stock, 60 μc of carrier-free ^{32}P -labeled orthophosphate, and, in the second series of experiments, additions of either cholesterol or carbon suspension or an equivalent volume of water, were made up to a final volume of 4 ml with Hanks' solution-serum 2:1 (v/v) and incubated for various periods of time at 37°.

The first series comprised seven replicate experiments in which flasks were incubated in duplicate for 1, 2, 4, 6, and (in five out of seven experiments) 12 hr. The second

series comprised two sets, (a) and (b), of four and three replicate experiments respectively, in which 12 flasks (in three groups of four with either added cholesterol, carbon, or distilled water as described above) were incubated for 1, 2, 4, and 6 hr respectively. The amounts of added cholesterol were either (a) 2.5 mg or (b) 1.0 mg respectively; and of carbon either (a) 0.5 mg or (b) 1.0 mg. [In the set (a) of experiments the amounts of added cholesterol or carbon suspension contained comparable numbers of particles; in (b) they were equal by weight.] Additional flasks containing dead cells (killed by heating at 70° for 10 min) were investigated in some experiments.

Oxygen uptakes were recorded throughout incubation in all experiments, with manometer readings at 20-min intervals. After the appropriate incubation time, the cells were separated by centrifugation (1500 rpm for 5 min), the medium being discarded. The cells were washed with 0.9% sodium chloride and centrifuged again, and the lipid was extracted with chloroform-methanol 2:1 (v/v) by the method of Folch, Lees, and Sloane Stanley (12). A portion of the lipid extract was taken for determination of lipid phosphorus as described by Brown (13), and a second portion for liquid scintillation counting, appropriate correction for ^{32}P decay being made.

Duplicate samples from the remainder of the extract were chromatographed on silicic acid-impregnated paper, using the solvent system diisobutylketone-acetic acid-water 40:25:5 (v/v/v) of Marinetti (14). After development, the papers were cut into strips 1.5 inches wide and scanned with a Nuclear-Chicago 4 π Actigraph paper chromatograph scanner. The area of the peaks obtained by triangulation was then used to measure the ^{32}P -labeled phosphate incorporated into individual phospholipids at the various time intervals. Radioautographs were prepared for further inspection of the separation achieved, and the papers were then stained with Rhodamine 6G or other spot test reagents as set out below. In some experiments the spots were cut out and eluted with 1.7 N HCl in methanol and the lipid phosphorus was determined in the eluates. However, since there was too little lipid phosphorus in any spot but that containing lecithin, it was possible to determine only the distribution of radioactivity and not the specific activity of the individual phospholipids.

Identification of Phospholipids

The phospholipid components separated on silicic acid-impregnated paper were identified by comparing their mobilities with those of reference phospholipids, by the use of specific spot tests, and by co-chromatography (15). The R_F values of the unknown spots were compared with those of the reference compounds and the papers were then stained with either 0.25% ninhydrin in ace-

tone-lutidine 9:1 (v/v) for detection of the aminophospholipids, with phosphomolybdate-stannous chloride reagent for detection of choline-containing phospholipids, or with Rhodamine 6G, which fluoresced with all phospholipids (14). For co-chromatography, the spot located by radioautography was cut out, combined with the corresponding spot from other chromatograms, and eluted for three successive 24 hr periods with chloroform-methanol-water 75:25:5 (15). The combined eluates were centrifuged to remove particles derived from the paper, the supernatant solutions were evaporated to dryness under N₂, and the residues co-chromatographed with the appropriate reference compound. The solvent system diisobutylketone-acetic acid-water was used, either in the proportions 40:25:5 or 40:30:7 (see Results). Radioautographs were then prepared, the radioactive spots being marked and the papers stained according to the type of reference phospholipid. If the radioactive spot coincided accurately with the stained spot, the unknown labeled material was considered to be identical with the reference compound.

Statistical Methods

Specific activities (cps/μg lipid P) and assays of total phospholipid (P, μg) were transformed to logarithms (base 10) for statistical analysis to obtain additivity of systematic effects and homogeneity of experimental variability. Transformation was, however, unnecessary for the percentage counts in the distribution of counts between phospholipid fractions, these values being already expressed on a relative basis.

Both series of experiments have the standard "randomized blocks" structure with replicate experiments constituting the blocks, and in the second series the 12 treatments have the 2-way factorial structure of three initial treatments (addition of cholesterol, carbon, or water) × four periods of incubation. This structure and the assumption of additivity of treatment and replicate effects determines the primary analysis of variance, in which error variation is measured by the treatment × replicate interaction mean square. However, there was a considerable degree of nonadditivity in the experimental data; a different preparation of macrophages was used in each experiment, and there was in consequence appreciable variation between experiments in the observed kinetic effects. It was necessary, therefore, to partition the analysis of variance further and eliminate the variation attributable to nonadditive effects from the estimate of error variation.

In the tabulation of results means of logarithms have been reconverted to natural units, and comparisons of logarithmic values expressed as relative differences, with appropriate standard errors. In Table 2 (ii) the entries have a constant *relative error* (coefficient of variation)

so that, for comparisons of entries, percentage differences should be calculated. (The coefficients of variation given with the table are the appropriate standard errors for testing percentage differences.)

In the graphical presentation of results (Figs. 1-3) the logarithmic scales used in the analysis have been retained so that the plotted points are all of the same accuracy visually. However, the scales have been calibrated in natural units for ease of reference. The time scale is also logarithmic, so that a hypothetically constant rate of uptake should be represented by a straight line with a slope of 1.

RESULTS

Viability of Macrophages during Incubation

During the first 4 hr of incubation, oxygen uptake proceeded at a constant rate in all experiments of approximately 5 μmoles of oxygen per 10⁸ cells per hr, a value similar to that reported previously (8). In some experiments a slight drop in rate of uptake occurred in the 4-6 hr interval, but in most experiments there was no drop in rate until after 6 hr. The mean values for total phospholipid extracted from the macrophages (Table 1) also remain constant during the first 4 hr but show some reduction at 6 and 12 hr. These results indicate that there was no appreciable loss in viability during the first 4-6 hr of incubation.

Incorporation by Macrophages of ³²P-Labeled Phosphate into Phospholipid

No significant incorporation of ³²P-labeled phosphate into phospholipid was detected in test flasks containing dead cells only; oxygen uptake was also negligible.

The results obtained for viable cells are summarized in Table 2. The data presented in Table 2 are for the controls in both series of experiments; the effects of added cholesterol in series II on the rate of incorporation are shown separately in Table 3. The results are illustrated graphically in Fig. 1. Synthesis of phospholipid by the macrophages is demonstrated by the increasing

TABLE 1 AMOUNT OF LIPID PHOSPHORUS EXTRACTED FROM MACROPHAGES AFTER VARIOUS PERIODS OF INCUBATION

Incubation Time	Total Lipid P*
hr	μg
1	41.6
2	39.1
4	39.6
6	35.5
12	32.3
	(SEM 1.20)

* Means at each time interval for the two series of experiments together with the combined standard error of the mean.

TABLE 2 INCORPORATION BY MACROPHAGES IN VITRO OF ³²P-LABELED PHOSPHATE INTO PHOSPHOLIPID, IN TWO SERIES OF EXPERIMENTS
(i) Mean Rate of Incorporation

	Over-all mean rate <i>cps/μg lipid P/hr</i>	Ratio mean rate/over-all mean rate in expt.						
		1	2	3	4	5	6	7
Series I	15.8	1.42	1.18	0.83	0.91	0.64	1.46	0.87
Series II	19.1	0.36	1.64	0.48	1.51	1.39	1.86	0.91

(ii) Specific Activity of ³²P-Incorporated Relative to Specific Activity After 6 Hr

Incubation Time <i>hr</i>	Means		Individual Experiments (Series II only)						
	Series I	Series II	1	2	3	4	5	6	7
			<i>% of specific activity at 6 hr</i>						
1	11	15	23	12	9	21	11	12	20
2	33	36	42	20	30	45	36	27	61
4	66	83	86	70	81	81	83	85	93
6	100	100	100	100	100	100	100	100	100
12	129								
Coefficients of variation for ratios of entries (%):									
In same column:	6.1	2.7	7.8				5.3		
In different columns:	6.7		11.7			9.4		7.9	

specific activity with time of the ³²P-labeled phospholipid.

The over-all mean rate of incorporation for all control experiments [Table 2 (i)] (averaged on a logarithmic scale over all times of incubation) was approximately 17.5 cps (³²P)/μg lipid P per hr, which represents approximately 0.04%/hr of the ³²P-labeled phosphate added to the incubation flasks. The mean rates of incorporation were appreciably different from experiment to experiment, that is to say, between different batches of macrophages, the lowest and highest rates differing by a factor of 5.

The rate of incorporation varied during incubation [Table 2 (ii)]. After 4 hr of incubation there was a progressive reduction in rate of incorporation, indicating transition towards an equilibrium state, though the rates during this period may also have been affected somewhat by the reduced viability of the macrophages. The kinetic curves of incorporation [see Table 2 (ii)] also varied appreciably between different batches of macrophages. However, there does not appear to be any close correlation between these variations in trend and the corresponding variations in mean rate of incorporation [Table 2 (i)].

TABLE 3 INCREASE IN INCORPORATION OF ³²P-LABELED PHOSPHATE INTO PHOSPHOLIPID DUE TO ADDED CHOLESTEROL

Incubation Time <i>hr</i>	Series IIa (cholesterol 2.5 mg, carbon 0.5 mg)					Series IIb (cholesterol 1.0 mg, carbon 1.0 mg)			
	Expt.					Expt.			
	1	2	3	4	Mean	5	6	7	Mean
	<i>% over control*</i>					<i>% over control*</i>			
1	13	44	79	12	34	-1	10	13	7
2	23	80	60	17	42	13	30	14	19
4	63	80	56	37	58	17	31	19	22
6	51	60	26	39	43	20	46	29	31
	(SEM 9.6)			(SEM 4.8)		(SEM 6.5)			(SEM 3.7)
Mean	36	65	54	26	44	12	29	19	20
	(SEM 4.8)			(SEM 2.4)†		(SEM 3.2)			(SEM 1.9)†

* I.e., a mean of two control values (for added carbon and for distilled water). Mean increases in incorporation due to added carbon suspension were (IIa), 7.7 ± 2.8 (SEM) % and (IIb) 2.7 ± 2.2 (SEM) %. Individual means of controls were adjusted to eliminate these differences.

† Significance level 0.1%.

SPECIFIC ACTIVITY
cts/sec / μg LIPID P

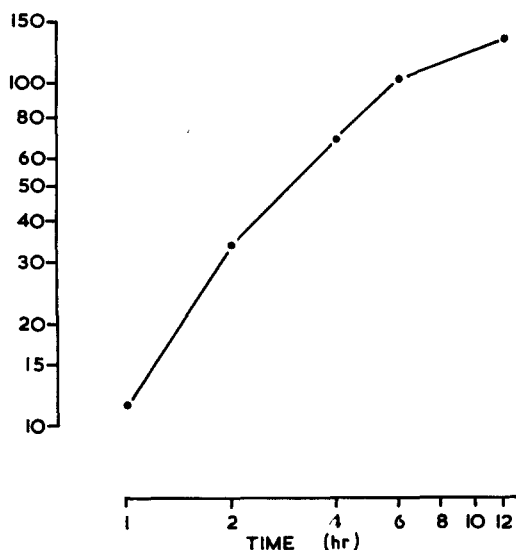


FIG. 1. Specific activity of phospholipid (cps/ μg lipid phosphorus) following incubation of macrophages with ^{32}P -labeled phosphate (logarithmic scales: see text and Appendix). Mean of seven experiments.

Effect of Added Carbon Particles

A very small increase (average 5.2%) in rate of incorporation was detected with added carbon particles. This effect was slight in comparison with that induced by added cholesterol (see below).

Effect of Added Cholesterol

The increases attributable to added cholesterol in the rate of incorporation of phosphate- ^{32}P into phospholipid are given in Table 3, expressed as percentages of the corresponding control values, and are illustrated in Figs. 2 and 3. The over-all mean increases in rate for the series of experiments IIa and IIb were 44 and 20% respectively, the increases in both series being highly significant ($P < 0.01$). These values are closely commensurate with the amounts of added cholesterol in each case, 2.5 and 1.0 mg respectively. The addition of cholesterol, however, produced no detectable increase in oxygen uptake.

There were appreciable differences in kinetic behavior according to the amount of cholesterol added. With the lower level of added cholesterol the difference in the amount of incorporated ^{32}P increased progressively from 7% at 1 hr to 31% of control at 6 hr, whereas with the higher level of added cholesterol the difference reached a peak of 58% at 4 hr and decreased thereafter. These results are kinetically consistent in that it would be expected that, with a higher rate of incorporation initially, transition towards equilibrium would occur some-

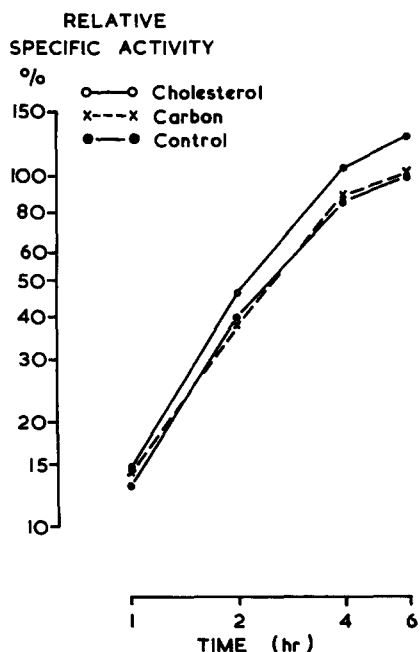


FIG. 2. The effect of 1 mg each of cholesterol and of carbon suspension on the incorporation of phosphate- ^{32}P into phospholipid by macrophages in vitro. Logarithmic scales (see text). Mean relative specific activity for three experiments (percentage of that present in the control at 6 hr) against time.

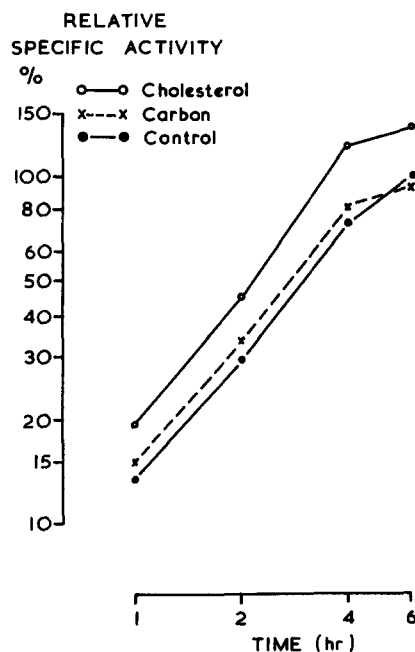


FIG. 3. The effect of 2.5 mg of cholesterol and 0.5 mg of carbon suspension on the incorporation of phosphate- ^{32}P into phospholipid by macrophages in vitro. Logarithmic scales. Mean relative specific activity for four experiments (percentage of that present in the control at 6 hr) against time.

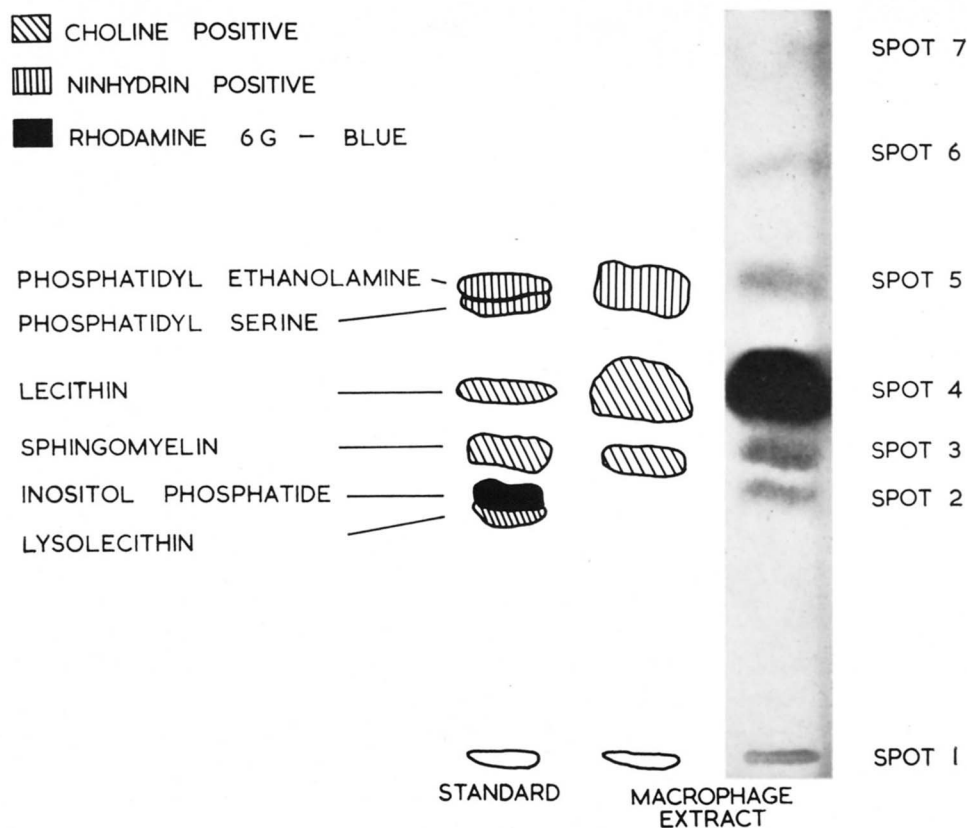


FIG. 4. Identification of ^{32}P -labeled phospholipids synthesized by macrophages in vitro. Separation of the labeled macrophage extract on silicic acid-impregnated paper followed by radioautography or specific spot tests is shown and compared with separation of standard phospholipids identified by spot tests and R_F values. Solvent system: diisobutyl ketone-acetic acid-water 40:25:5 (v/v/v).

what sooner and more rapidly. However, it could also be inferred, particularly from the data for series IIb, that the equilibrium level of incorporation of phosphate- ^{32}P has also been affected by the uptake of cholesterol, though the data are not conclusive on this point.

The kinetic effects of added cholesterol varied appreciably between experiments, mainly in series IIa, but in a consistent way. In experiment 3 (Table 3) the initial increase in uptake due to cholesterol was very large (79%) and the difference then decreased progressively, whereas in the other experiments with lower initial increases, the differences increased progressively during the first 4 hr of incubation. These differences in kinetic behavior due to cholesterol do not seem to be closely correlated with the kinetic behavior of the corresponding controls [see Table 2 (ii)].

Identification of ^{32}P -Labeled Phospholipid Fractions

The chromatographic separation of the individual labeled phospholipids is shown in Fig. 4. Seven radioactive components were detected in the lipid extract (spots 1-7 respectively). Spots 1 and 6 contained small and somewhat variable counts and were not identified.

Spot 7 moved with the solvent front and, although not positively identified, probably consisted of phosphatidic acid. Spot 3 was identified as sphingomyelin and spot 4 as lecithin. Spot 2 did not contain enough material to produce a color reaction. Its R_F value was similar to that for inositol phosphatide and for lysolecithin (which overlapped each other when developed with diisobutylketone-acetic acid-water 40:25:5). Spot 5, according to its staining reaction and R_F value, could have been either phosphatidyl serine or phosphatidyl ethanolamine. When the proportions of the solvent system were altered to 40:30:7, a better resolution of the standards were obtained. Spot 2 was then shown to be inositol phosphatide and spot 5 to be phosphatidyl ethanolamine. In some chromatograms a further unidentified radioactive spot occurred above phosphatidyl ethanolamine (see Fig. 5). It was provisionally identified as ethanolamine plasmalogen.

Distribution of ^{32}P Counts between Phospholipid Fractions

The percentage distributions of ^{32}P counts between the labeled phospholipid fractions for each period of in-

cubation are shown in Table 4. There was very little change in the percentage distribution with incubation time (except with respect to spot 7), which indicates that the incorporation of phosphate- ^{32}P into phospholipid proceeded at essentially the same rate for all phospholipids. In the case of spot 7, which was probably phosphatidic acid, the percentage counts decreased from 8.6 at 1 hr to 0.7 at 6 hr. Most of the radioactivity was associated with lecithin (68–76%) and sphingomyelin (10–15%).

There was no detectable effect of added cholesterol on the percentage distribution of ^{32}P counts between phospholipid fractions at any time of incubation (see Table 5). This indicates that the increase in rate of incorporation induced by added cholesterol is essentially the same for all measured phospholipid fractions.

DISCUSSION

The incorporation of ^{32}P -labeled phosphate into phospholipid by macrophages has been demonstrated and this, together with the previous evidence for incorporation of both ^{14}C -labeled fatty acid and ^{14}C -labeled acetate into phospholipid, is consistent with the conclusion that phospholipid is synthesized by these cells. The amount of ^{32}P -labeled phosphate incorporated into phospholipid

in these experiments is relatively high when compared with that reported for polymorphonuclear leukocytes (16, 17). In the latter experiments insufficient data were given to calculate the amount of phosphate- ^{32}P incorporated into phospholipid, but if certain reasonable assumptions are made regarding the lipid content of the leukocytes it would seem that only between 0.001 and 0.005% of the phosphate added to the medium was converted to phospholipid by the leukocytes after incubation for 30 min. Maximal phagocytosis of particles increased this to a variable extent, which is perhaps not surprising. In the work reported in the present paper, a mean of 0.25% of the ^{32}P -labeled phosphate was converted by macrophages to phospholipid in 6 hr.

The addition of cholesterol to the incubation medium caused a highly significant increase in the incorporation of phosphate into phospholipid by the macrophages. Cholesterol suspensions similar to the one used in the present experiment have been previously shown to be taken up by macrophages *in vitro* (18). Since such uptake is temperature-dependent, it seems probable that some phagocytosis of the suspension is occurring. Further, in experiments using the electron microscope, phagocytic uptake of 1 μ cholesterol particles by macrophages has been demonstrated (19). It appears therefore that in the present work some of the cholesterol has been taken up by phagocytosis.

TABLE 4 DISTRIBUTION OF ^{32}P COUNTS BETWEEN PHOSPHOLIPID FRACTIONS

Chromatograph Spot No.	Fraction	Incubation Time (Hr)					SEM
		1	2	4	6	12	
		% of total counts					
1	Unidentified	2.4	1.0	2.5	2.2	2.7	0.33
2	Inositol phosphatide	5.2	5.6	5.7	5.5	7.2	0.83
3	Sphingomyelin	10.0	9.0	9.0	10.4	14.6	0.77
4	Lecithin	68.2	76.6	74.6	76.3	70.5	2.10
5	"Cephalin"	4.0	3.0	3.1	3.3	3.9	0.70
6	Unidentified	1.7	1.1	0.5	0.6	0.4	0.35
7	?Phosphatidic acid	8.6	3.8	1.6	1.7	0.7	0.64

TABLE 5 LACK OF EFFECT OF CHOLESTEROL UPTAKE ON THE DISTRIBUTION OF COUNTS BETWEEN INDIVIDUAL PHOSPHOLIPIDS DURING ^{32}P INCORPORATION*

Incubation Time		Spot 1	Spot 2	Spot 3	Spot 4	Spot 5	Spot 6	Spot 7
		Unidentified	Inositol Phosphatide	Sphingo- myelin	Lecithin	"Cephalin"	Unidentified	?Phosphatidic Acid
<i>hr</i>		% of total counts						
1	Control	2.1	8.5	9.3	66.8	2.2	1.6	9.5
	Cholesterol	1.8	7.1	8.6	67.6	1.3	1.5	12.1
2	Control	1.1	5.0	6.4	79.5	2.1	0.7	5.1
	Cholesterol	1.8	6.3	8.1	76.2	1.4	0.8	5.4
4	Control	—	4.9	8.0	81.6	2.3	0.6	2.5
	Cholesterol	1.3	7.2	8.2	75.9	2.1	0.9	4.3
6	Control	0.7	5.2	8.9	80.0	2.3	0.3	2.5
	Cholesterol	1.0	7.6	7.5	79.5	1.6	0.5	2.3

* Data from one experiment.

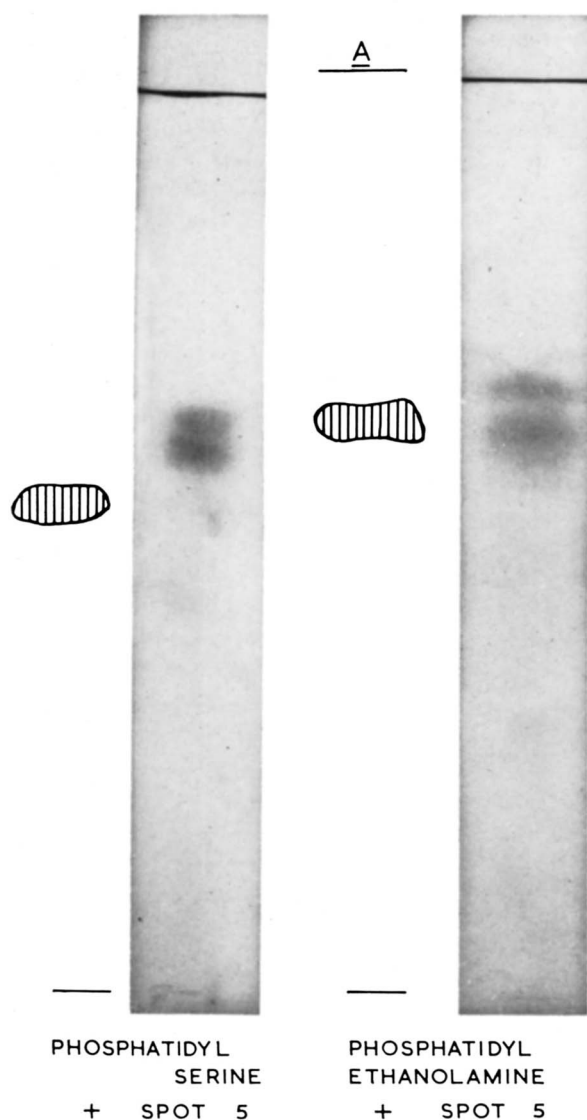


FIG. 5. Identification of spot 5 by co-chromatography on silicic acid-impregnated paper using the solvent system diisobutyl ketone-acetic acid-water 40:30:7 (v/v/v).

Phagocytosis of particulate matter by both leukocytes and monocytes is associated with increased glycolysis, and Karnovsky and coworkers have shown (16, 17, 20) an increase in oxygen uptake and an increase in phospholipid turnover in leukocytes as a result of phagocytosis. The possibility exists, therefore, that the stimulation of phospholipid synthesis associated with cholesterol uptake may be due to its nonspecific phagocytosis and not to a specific response to the presence of the cholesterol. In the experiments described by Karnovsky (20) for leukocytes the increased phospholipid turnover accompanied an increased oxygen uptake, very large concentrations (2–4 mg/ml) of 1μ polystyrene spherules or starch particles having been added “in an attempt to force maximal uptake” (17). In the present experiments

much lower concentrations of 1μ cholesterol particles were present (0.25 or 0.625 mg/ml) so that it is perhaps not surprising that no increase in oxygen uptake could be detected. Nevertheless, significantly increased incorporation of phosphate into phospholipid did occur and it seems reasonable to suggest that the effect of cholesterol on phospholipid synthesis by macrophages is not simply a nonspecific effect resulting from its phagocytosis. In any case, the pattern of the phospholipid labeled following cholesterol uptake is similar to that of the cells in the absence of cholesterol. The labeling of both lecithin and sphingomyelin is increased whereas in the case of stimulation of phospholipid synthesis by polymorphonuclear leukocytes during phagocytosis, these phospholipids do not share the increase (17).

The relationship of phospholipid synthesis by peritoneal macrophages to that of lipid-containing cells in the arterial wall might be disputed, and it is readily acknowledged that one cannot necessarily extrapolate the observations described in this present work to macrophages in another situation. With this proviso, however, the present work lends support to the concept that macrophages present in the arterial wall in atherosclerosis synthesize more phospholipid following infiltration of cholesterol from the blood. Whether such increased synthesis of phospholipid by macrophages then results in removal of the cholesterol from the wall into the blood stream awaits further evaluation.

APPENDIX ON STATISTICAL ANALYSIS

It may not be widely appreciated that, with experiments of the kind described in this paper, it is almost always necessary for the experimental data to be transformed to logarithms before statistical analysis, in order to obtain additivity of systematic effects (so far as possible) and homogeneity of experimental variation. In the present series of experiments the measured counts increased by a factor of 10 from 1 hr to 6 hr incubation time, and differed in average levels by as much as a factor of 5 between different batches of macrophages. It is these multiplicative factors, or relative differences, that remain consistent under variation of other factors rather than actual differences, which would vary widely under the same conditions; and the multiplicative effects become additive on the logarithmic scale. The same is true for error variations: homogeneity of relative variation observed on the natural scale corresponds to homogeneity of absolute variation on the logarithmic scale. For these reasons also, geometric means (which result from calculating the antilogarithms of means of logarithms) are both statistically more accurate and more relevant in interpretation of the results.

Since even with the application of a logarithmic transformation, various other nonadditive effects may still be present, great care must be exercised in the analysis to ensure that the estimates of error variation are not grossly inflated by the presence of such effects, as otherwise the analysis may fail to detect important effects as significant; and in more complex kinetic studies nonlinear curve fitting may be necessary. In the present

series of experiments all comparisons of initial treatments and of incubation times were effected with aliquots taken from a common stock of macrophages in each experiment, so that the only relevant error variation is that arising from the experimental handling of the incubation flasks and subsequent assay errors. In the second series of experiments there were no duplicates for estimating error variation directly, and if standard randomized block analyses had been applied, the error mean squares obtained would have been approximately six times larger than those finally obtained after the elimination of non-additive effects. Most of the nonadditive effects arose from the very appreciable differences in kinetic behavior of different batches of macrophages.

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