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Localization By Autoradiography Of Phospholipid Synthesis In Rabbit Atherosclerotic Aorta[1]

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The phospholipid content of the atherosclerotic intima increases as the lesion progresses, and much of this phospholipid is located in the foam cells present (Day, 1962; Dunnigan, 1964). In view of this relationship, attempts have been made to determine whether the foam cells are responsible for the synthesis of phospholipid which occurs in the atherosclerotic lesion as a whole (Zilversmit, Shore and Ackerman, 1954; Zilversmit and McCandless, 1959). Initial studies (Day, Fidge, and Wilkinson, 1966) have shown that the incorporation of ^{32}P-labeled phosphate into phospholipid by macrophages is stimulated by the uptake of cholesterol by these cells. Foam cells isolated from rabbit atherosclerotic aortas take up various lipid precursors and incorporate these precursors into phospholipid and into other lipid fractions, notably, cholesterol esters (Day, Newman, and Zilversmit, 1966; Day and Wilkinson; 1967; Day and Tume, 1969). Autoradiographic techniques have also indicated that foam cells may be responsible for much of the lipid synthesis in the atherosclerotic lesion (Day and Wahlqvist, 1968; Wahlqvist, Day, and Tume, 1969). Incubation *in vitro* of atherosclerotic lesions with ^{14}C-labeled oleic acid is followed by the uptake of oleic acid and its subsequent incorporation into cholesterol ester and phospholipid by the foam cells present in the lesion. In these studies, however, the oleic acid is incorporated into both cholesterol ester and phospholipid.

In the present paper, by using ^{14}C-labeled choline as a precursor for the choline-containing phospholipids it has been possible to study, specifically, the localization of phospholipid synthesis in rabbit atherosclerotic intima. In addition, using oleic acid as precursor, and subjecting the tissues to acetone extraction, all the lipid moieties, with the exception of phospholipid, could be removed and specific localization of phospholipid synthesis in the intima observed.

MATERIALS AND METHODS

Choline chloride (methyl-^{14}C) (specific activity 32 mCi/m*M*) was obtained from the Radiochemical Centre, Amersham, U.K.. Purity was checked by paper chromatography on Whatman No. 1 paper, using *n*-butanol: water:acetic acid 120:50:30 as solvent system. The ^{14}C-labeled choline chromatographed as a single spot with an R_F of 0.4.

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Oleic acid-9-10-^{3}H, specific activity 3020 mCi/m*M*, was also obtained from the Radiochemical Centre, Amersham. Purity was checked by chromatography on silicic acid (as set out below) and after methylation by chromatography on silver nitrate-impregnated silicic acid (Morris, 1964). The ^{3}H-labeled oleic acid chromatographed as a single spot corresponding to unesterified fatty acid in the first case, and as a single spot corresponding to methyl oleate in the second.

Atherosclerotic aortas were obtained from male New Zealand white rabbits fed a diet of rabbit chow containing 1% cholesterol and 3% peanut oil, for periods varying from 1 to 4 months. All rabbits were 6 weeks old when cholesterol feeding was commenced.

In the first experiment, three rabbits fed cholesterol for 1 month, 2 months, and 3 months, respectively, were used. Rabbits were killed by Nembutal anesthesia, their thoracic aortas removed and divided longitudinally into halves; each half was then incubated in 5 ml of medium (50:50 Hanks':normal rabbit serum) to which was added a tracer dose of ^{14}C-labeled choline (3.04×10^{6} cpm). Incubation was carried out for 4 hours. After this period the aortic halves were washed thoroughly in 0.9% sodium chloride solution and both halves from each of the three rabbits were fixed in 1% calcium chloride in 4% formal saline for 4 days and finally washed in running water for a further period of 24 hours. One aortic half from each animal was reserved for autoradiography, the other half being reserved for radioassay. In the latter, the intima was stripped from the media and adventitia, and the two portions extracted separately with 2:1 chloroform:methanol according to Folch, Lees, and Sloane Stanley (1957). In order to determine the amount of ^{14}C-labeled choline present in nonlipid components, the upper phase of the Folch wash and the subsequent washings, together with the protein precipitate were collected and counted separately. An aliquot of the lipid extract was counted using the dioxane–water scintil-

TABLE I

UPTAKE AND CONVERSION OF ^{14}C-LABELED CHOLINE TO PHOSPHOLIPID BY RABBIT ATHEROSCLEROTIC AORTAS

Period rabbit cholesterol fed (months)	Incubation time (hours)	Intima			Media		
		% of Incubation medium ^{14}C-choline present as:		Phospholipid specific radioactivity (cpm/μg lipid P)	% of Incubation medium ^{14}C-choline present as:		Phospholipid specific radioactivity (cpm/μg lipid P)
		Lipid	Nonlipid		Lipid	Nonlipid	
1	4	10.8	0.19	—	—	—	—
2	4	11.3	0.31	—	—	—	—
3	4	10.8	0.16	—	—	—	—
3	1	1.21	—	2830	0.81	0.064	4560
3	3	1.31	0.083	6130	2.61	0.131	12200
3	4	2.69	0.089	8360	1.79	0.115	12500
4	0.25	0.13	0.006	—	0.022	0.002	—
4	0.5	0.39	0.011	—	0.088	0.004	—

TABLE II

PERCENTAGE DISTRIBUTION OF LIPID ^{14}C-LABELED CHOLINE IN RABBIT ATHEROSCLEROTIC AORTAS

Period rabbit cholesterol fed (months)	Incubation time (hours)	Intima phospholipids				Media phospholipids			
		Lyso-lecithin	Sphingo-myelin	Leci-thin	Other phospho-lipids	Lyso-lecithin	Sphingo-myelin	Leci-thin	Other phospho-lipids
1	4	6.5	5.0	87.8	0.7	—	—	—	—
2	4	6.4	3.8	89.2	0.7	—	—	—	—
3	4	6.8	4.2	88.3	0.8	—	—	—	—
3	1	13.4	2.6	81.9	2.3	9.0	4.1	83.7	3.2
3	3	12.2	1.2	84.7	2.0	7.2	3.0	87.2	2.7
3	4	7.5	3.2	86.6	2.8	11.7	2.4	83.9	2.1
4	0.25	7.2	2.5	87.5	2.6	6.0	4.2	81.9	7.7
4	0.5	6.5	2.0	89.1	2.4	5.9	2.8	88.9	2.4

lator described by Snyder (1964) and the remainder reserved for separation of phospholipids by thin-layer chromatography as described below. All counting was done in a Packard Tricarb spectrometer. The aortic half reserved for autoradiography was sectioned without prior embedding on an International Cryostat (Model CTI) and 6μ sections mounted and autoradiographs prepared using Kodak AR10 stripping film. After exposure and development, the autoradiographs were stained through the film with Sudan IV and hematoxylin.

In the second experiment, the thoracic aorta obtained from a rabbit fed cholesterol for 3 months was divided longitudinally into thirds, each third being incubated in a medium containing 50:50 Hanks':normal rabbit serum, to which had been added 7×10^6 cpm of ^{14}C-labeled choline. Incubation was carried out for 1, 3, or 4 hours, respectively, for each of the thirds. A segment of each third was reserved for autoradiography, and the remainder was processed for radioassay as set out above. In this experiment the specific activity of the total phospholipid at the three time intervals for both media and intima was also calculated. Lipid phosphorus was determined in aliquots of the lipid extract by the method of Bartlett (1959).

In the third experiment, short incubation periods of 15 and 30 minutes were used in duplicate halves of a thoracic aorta obtained from a rabbit fed cholesterol for 4 months. Incubation was carried out in 5 ml of Hanks':normal rabbit serum containing 25×10^6 cpm of ^{14}C-labeled choline. Most of the artery was used for radioassay, but portions were taken for autoradiography. Preparation procedures were as described for the first experiment.

In the fourth experiment, the thoracic aorta obtained from a rabbit fed cholesterol for 3 months was divided longitudinally into halves and both halves incubated in 5.5 ml of medium containing 50:50 Hanks':normal rabbit serum, together with a known amount (34.4×10^6 cpm of ^{3}H-labeled oleic acid). After incubation for 3 hours, the aortic halves were washed thoroughly in 0.9% sodium chloride solution and reincubated in nonlabeled 50:50

Hanks': normal rabbit serum for a further period of 1 hour. One half was reserved for autoradiography. In the other half the intima was stripped from the media/adventitia and the two portions extracted separately with 2:1 chloroform:methanol as set out above. In the half reserved for autoradiography, 6μ sections were cut as described above and mounted at each end of glass slides. One end of the slide was dipped into acetone at 4°C in order to remove from those sections the lipids other than phospholipid. The optimum time required to achieve such removal was found to be 25 minutes. Enough slides were set up to compare, by means of thin-layer chromatography, the effect of acetone extraction on the distribution of ^{3}H-labeled oleic acid among aortic lipids. Groups of sections on slides were extracted and aliquots of the lipid extract separated by thin-layer chromatography on silicic acid as set out below. The remaining slides were set up with Kodak AR10 stripping film and autoradiographs developed. Little sudanophilia remained in those sections which had been extracted with acetone in contrast to those untreated.

FIG. 1. Autoradiograph of an atherosclerotic lesion incubated for 30 minutes with ^{14}C-labeled choline. The aorta was obtained from a 23-week-old rabbit fed cholesterol for 17 weeks. The luminal aspect (L) is uppermost in the figure. Localization of silver grains representing essentially choline-containing phospholipids, to variously shaped foam cells (F) is shown. Hematoxylin and Sudan IV. Exposure time 8 days.

FIG. 2. Autoradiograph of an atherosclerotic lesion incubated for 4 hours with ^{14}C-labeled choline. The aorta was obtained from a 10-week-old rabbit fed cholesterol for 4 weeks. Cells at the luminal border, possibly endothelial (E), exhibiting some sudanophilia, have localized ^{14}C-labeled choline. Foam cells (F) in the intima have also localized label. The intimomedial junction is denoted IM. Some granulation is also evident in the media. Hematoxylin and Sudan IV. Exposure time 12 days.

Thin-layer chromatography

In the experiments in which ^{14}C-labeled choline was used as precursor, the lipid extracts, after initial counting, were separated into their individual phospholipid fractions by thin-layer chromatography on silicic acid by the method of Skipski, Peterson, and Barclay (1964). Identification of phospholipids was made by comparison with standards as previously described (Newman, Day, and Zilversmit, 1966). The individual phospholipids were scraped from the plates and counted directly using the method of Snyder (1964).

In the fourth experiment in which ^{3}H-labeled oleic acid was used as precursor, the lipid extracts from the portion of the artery reserved for radioassay, and the extracts of the acetone and control-treated sections, were separated into their individual lipid fractions by thin-layer chromatography on silicic

acid using the solvent system diethyl ether:acetic acid:hexane 38:3:100 v/v/v as developing solvent. The spots were eluted with chloroform:methanol 2:1 in the case of the cholesterol ester, triglyceride, fatty acid, and diglyceride spots, and with chloroform:methanol:acetic acid:water, according to the method of Arvidson (1967) for the phospholipid spots. The eluents were evaporated to dryness and the ^{3}H counted using 10 ml of scintillator containing 4 gm PPO and 100 mg POPOP per liter of toluene. Counting at an efficiency of 33% was carried out using a Packard Tricarb spectrometer.

RESULTS

The uptake of ^{14}C-labeled choline and its incorporation into phospholipid in intima and media of the atherosclerotic rabbit aortas is shown in Table I. ^{14}C-labeled choline is taken up by both intima and media and incorporated into phospholipid in each of the three experiments. In the second series of experiments, the specific activity of the phospholipid labeled was determined in relation to time. It is apparent, that in both the intima and media, increased

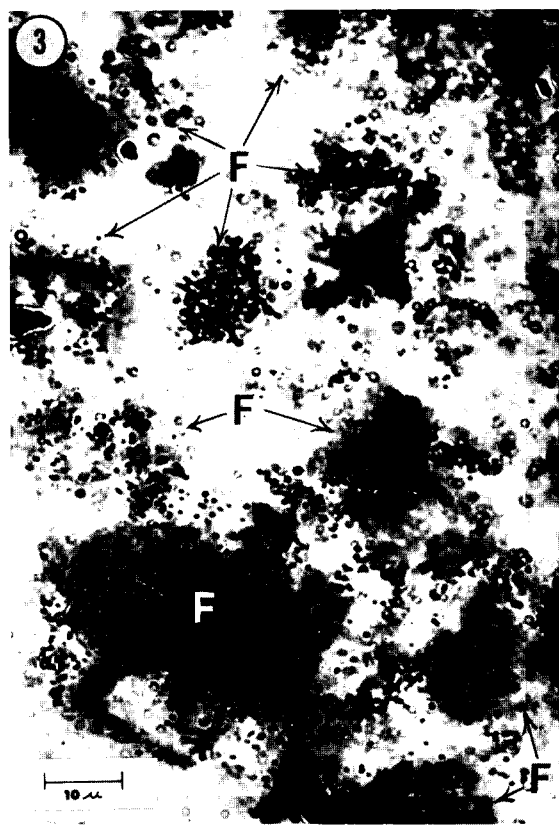

FIG. 3. Autoradiograph under oil immersion of an atherosclerotic lesion from the same artery as in Fig. 1, showing the concentration of silver grains in the region of intimal foam cells. Hematoxylin and Sudan IV. Exposure time 15 days.

TABLE III

GRAIN COUNTS (No. grains/100 μ^2)[a] IN AUTORADIOGRAPHS PREPARED FROM RABBIT AORTAS INCUBATED in Vitro WITH ^{14}C-LABELED CHOLINE

Period rabbit cholesterol fed (months)	Incubation time (hours)	Intima		Media
		Foam cells	Extracellular	
1	4	8.3	5.4	6.2
2	4	8.3	4.5	3.2
3	4	10.7	6.7	9.9
3	1	6.5	1.1	2.0
3	3	11.3	4.6	5.9
3	4	9.6	3.2	5.4
4	0.25	7.3	2.2	14.5
4	0.5	5.9	1.9	7.3

[a] At least 6000 μ^2 assessed for each feature.

TABLE IV

UPTAKE AND INCORPORATION OF ^{3}H-LABELED OLEIC ACID INTO LIPIDS OF ATHEROSCLEROTIC RABBIT AORTA INCUBATED in Vitro

	Percentage uptake from incubation medium	Percentage distribution				
		Phospholipid	Diglyceride	Fatty acid	Triglyceride	Cholesterol ester
Intima	2.69	28.3	3.2	10.7	5.7	52.2
Media	2.15	14.5	15.6	7.4	54.3	8.1

incorporation of choline into phospholipid occurs in relation to time. The incorporation in the intima was essentially linear but some leveling-off of specific activity in the media occurred after 3 hours.

In order to confirm that the ^{14}C-labeled choline present in the arteries used for autoradiography was present predominantly as phospholipid, the amount of ^{14}C-labeled choline present in non-lipid forms was determined in portions of the aortas subjected to the same washing procedures as those investigated autoradiographically, and these data are presented also in Table I. After the washing procedure used, very little nonphospholipid choline remained; autoradiographic localization of ^{14}C-labeled choline in the arteries can be taken to indicate therefore localization of ^{14}C-labeled phospholipid synthesized.

The distribution of ^{14}C-labeled choline between individual phospholipids in the intima and media for the three experiments is shown in Table II. Practically all of the ^{14}C-labeled choline was incorporated into choline-containing phospholipids, mainly into lecithin, but partly into sphingomyelin and lysolecithin. The pattern of incorporation is similar in the intima and the media, and in all of the arteries studied. It is apparent, therefore, that where localization is studied, primarily lecithin synthesis is being considered.

Localization of the ^{14}C-labeled phospholipid synthesized in the aorta is illustrated in Figs. 1, 2, and 3. Much of the ^{14}C is concentrated over the foam cell areas. These are quite extensive, however, and in many cases make up a considerable proportion of the lesion in the intima. There is some radioactivity, however, in areas between the cells and also in the media. This distribution of ^{14}C-labeled phospholipid in the foam cells as opposed to the extracellular material in the intima and in the media is shown quantitatively in Table III, where the grain counts are presented. It can be seen that in all cases, the concentration of grains in the foam cells exceeds that in other areas of the intima and in all but one case exceeds that in the media.

The uptake of ^{3}H-labeled oleic acid and its incorporation into different lipid fractions is shown in Table IV. In the intima, most of the oleic acid has been taken up and incorporated into cholesterol ester and into phospholipid with lesser amounts present in the triglyceride. In the media, most of the oleic acid has been taken up and incorporated into triglyceride with smaller amounts present as phospholipid and cholesterol ester. The localization of the ^{3}H-labeled oleic acid in the lesion is shown in Fig. 4. Most of the radioactivity is concentrated over the foam cell areas. This localization, however, represents ^{3}H-labeled oleic acid incorporated into cholesterol ester, phospholipid, and fatty acid, and gives no specific indication of the cells responsible for the phospholipid synthesis. In order to investigate this aspect, sections obtained from the same aorta were subjected to acetone extraction. Under these circum-

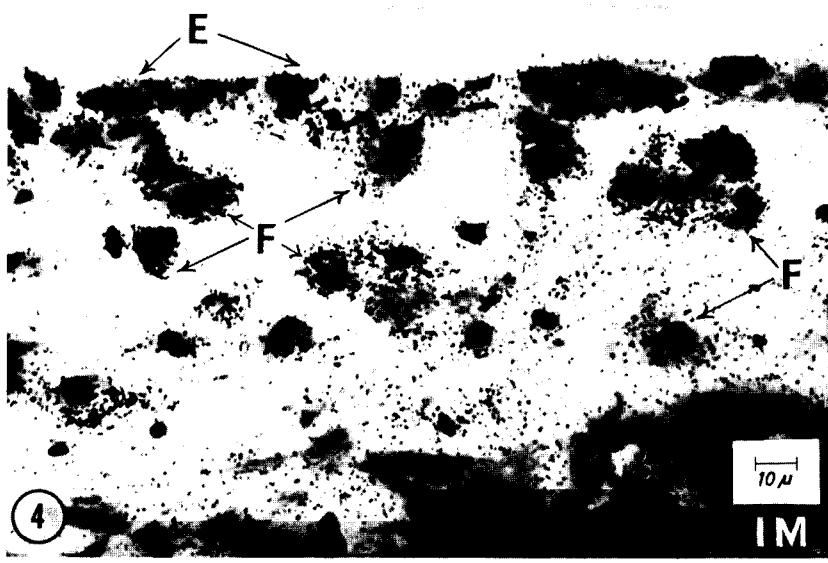

FIG. 4. Autoradiograph of atherosclerotic lesion incubated with ^{3}H-labeled oleic acid. The aorta was obtained from a rabbit aged 18 weeks and fed cholesterol for 12 weeks. Some endothelial cells (E) localize the label representing chiefly free fatty acid, phospholipid, and cholesterol ester. So also do superficial intimal foam cells (F). There is less label in the region of cells deeper in the intima. Intimo-medial junction is denoted IM. Hematoxylin and Sudan IV. Exposure time 9 days.

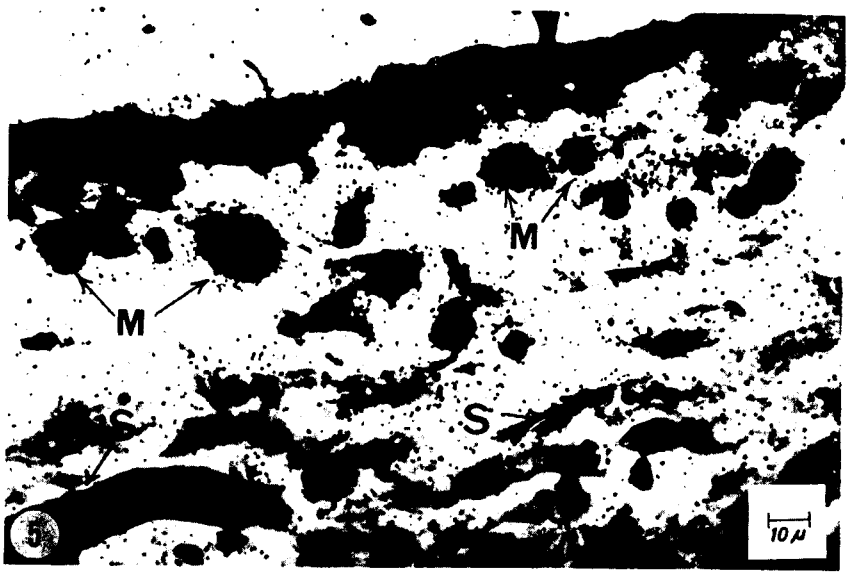

FIG. 5. An adjacent section of the same lesion as in Fig. 4, but extracted with acetone in the cold. It is apparent that phospholipid formed from oleic acid is localized to superficial intimal round mononuclears (M) with little label in the region of spindle-shaped cells (S). Hematoxylin and Sudan IV. Exposure time 9 days.

stances, most of the free fatty acid, triglyceride, and cholesterol ester was removed and in the sections studied 88.9% of the ^{3}H-labeled oleic acid was present in the phospholipid moiety, so that in the autoradiographs prepared from these sections, it is possible to obtain information regarding localization of the uptake of ^{3}H-labeled oleic acid and its incorporation specifically into phospholipid. Figure 5 is an autoradiograph prepared from sections so treated. The localization to foam cells is still apparent, although, of course, the total amount of radioactivity present is not so great as in those sections shown in Fig. 4. Quantitative information is given in Table V, where grain counts of the autoradiographs from both the control and acetone-extracted sections are presented. It can be seen that considerable concentration of label occurs in the foam cells present in the lesion, primarily in those nearer the luminal aspect. In the acetone-extracted sections, the grain counts in the foam cells are approximately half those in the controls, but still considerably above those in the other cells present in the intima, or in the media.

DISCUSSION

The uptake of ^{14}C-labeled choline by rabbit atherosclerotic aortas, and its incorporation into phospholipid confirms previously reported work (Newman, Day, and Zilversmit, 1966). The incorporation of choline into choline-containing phospholipids, lecithin with lesser amounts of sphingomyelin and lysolecithin, is the expected product of such incorporation. It will be noted, as observed previously (Newman, Day, and Zilversmit, 1966; Day and Wahlqvist, 1968) that the amount of sphingomyelin synthesized is considerably less than

that of lecithin. In the atherosclerotic intima the amount of sphingomyelin, of course, increases considerably, and in the rabbit lesion comprises approximately one third of the phospholipid present in the intima. It seems possible that the accumulation of sphingomyelin is not associated with its increased synthesis in the wall, but rather with reduced removal by hydrolysis, and there is some experimental evidence to support this conclusion (Rachmilewitz *et al.* 1967). In a recent paper by Morin (1968) it has also been shown that ^{14}C-labeled choline is taken up by normal and atherosclerotic rabbit aortas and incorporated into choline-containing phospholipids. The amount of choline taken up in these experiments, however, is extraordinarily low and a significant proportion of the choline appears in the non-choline-containing phospholipids. It is difficult to explain Morin's observations in terms of the known pathways for choline metabolism, and, of course, it is apparent from the findings of the present paper that this aspect has not been supported.

The main objective of the present paper was concerned with the localization of phospholipid synthesis to cells present in the intima. The phospholipid content of the atherosclerotic intima increases rapidly as the lesion progresses and it has been shown in a series of papers by Zilversmit and colleagues (1954, 1959) that most of this phospholipid arises by synthesis *in situ*. Previous work presented from this laboratory has suggested that the phospholipid synthesis which occurs in the atherosclerotic lesion takes place in part in the foam cells (Day, Newman, and Zilversmit, 1966; Day and Wilkinson, 1967; Day and Tume, 1969). Most of these studies, however, do not provide definitive evidence to support this conclusion. Although foam cells are able to incorporate ^{32}P-labeled phosphate and ^{14}C-labeled oleic acid into phospholipid when observed as isolated foam cells, such synthesis may not represent more than a small proportion of the phospholipid synthesis which takes place in the atherosclerotic arterial wall. The uptake of ^{14}C-labeled choline and its in-

TABLE V

GRAIN COUNTS (No. Grains/100 μ^2)[a] IN AUTORADIOGRAPHS PREPARED FROM RABBIT AORTAS INCUBATED WITH ^{3}H-LABELED OLEIC ACID

	Intima						Media
	Foam cells			Non-sudanophilic mononuclears	Spindle-shaped cells	Extracellular	
	Luminal	Intermediate	Deep/intimomedial				
Control[b]	23.3	4.2	1.0	0.9	—	2.4	0.6
Acetone extracted[c]	11.2	0.7	0.6	—	0.7	0.6	0.7

[a] At least 6000 μ^2 counted for each feature.

[b] "Foam cells" include lipid-containing cells of all kinds, including those of a more spindle shape. Endothelial cells are at times somewhat sudanophilic and localize label.

[c] In the case of the acetone-extracted section there is scarcely any sudanophilia and therefore "foam cells" are not recognizable by this feature. Round mononuclears are assessed instead and those spindle cells which were sudanophilic are now grouped under "spindle-shaped cells." Endothelial cells again apparently localize label in the acetone-extracted section.

corporation into phospholipid in the present work by the foam cells present in the intima do, however, provide some definite evidence in support of the conclusion that the foam cells are responsible for much of the phospholipid synthesis which occurs in the atherosclerotic intima. The localization studied is, as has been made clear in the Results, predominantly localization of lecithin synthesis. Almost all of the ^{14}C-labeled free choline present was removed by the washing procedures used, so that the label present, and studied autoradiographically, was predominantly ^{14}C-labeled phospholipid, and since most of the phospholipid was present as lecithin, was predominantly ^{14}C-labeled lecithin.

The localization of phospholipid synthesis from choline to foam cells was not so definitive as that when oleic acid was used as precursor, either in the present experiments or in those described previously (Day and Wahlqvist, 1968). It is apparent from Table III that there is some label present in other than foam cell areas in the intima and also in the media. Parker *et al.* (1966) have shown that the incorporation of fatty acid into phospholipid by rabbit atherosclerotic aortas correlates closely with the formation of membranous organelles and interprets this as evidence for active foam cell involvement in phospholipid synthesis. It is likely that some of the choline is incorporated into phospholipid in membranes of other cells both in the intima and the media, and that some spread of label occurs for this reason. One possible explanation of the more clear-cut localization of phospholipid synthesis in the intima when oleic acid is used as precursor may be related to the different pathways taken by the two precursors in their incorporation into phospholipid. Fatty acid may be incorporated into phospholipid in the normal rabbit artery by the Lands pathway, in which lysolecithin provides an acceptor for fatty acid, lecithin being formed predominantly (Eisenberg, Stein, and Stein, 1967). It is probable that such a pathway exists in the rabbit atherosclerotic aorta and has, in fact, been described in experimental atherosclerotic lesions in monkeys (Portman, 1967). Choline presumably is incorporated into phospholipid via the cytidine diphosphate choline pathway described by Kennedy and Weiss (1955) for liver.

SUMMARY

Autoradiographic techniques have been used to determine the portions of the arterial wall responsible for the phospholipid synthesis which occurs therein. Aortas obtained from cholesterol-fed rabbits have been incubated with ^{14}C-labeled choline or with ^{3}H-labeled oleic acid, and the uptake and incorporation into phospholipid of these two precursors studied biochemically and by autoradiography. ^{14}C-labeled choline was incorporated into choline-containing phospholipids, predominantly into lecithin, in the lesion and these changes were shown to occur predominantly in the foam cells present. ^{3}H-labeled oleic acid was taken up and incorporated into both phospholipid and cholesterol ester, but extraction by acetone of all the ^{3}H-labeled lipid present, except for the phospholipid, enabled localization of phospholipid synthesis to foam cells to be confirmed.

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