

# Lipid metabolism in pigeon aorta during atherogenesis

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**SUMMARY** The development of atherosclerosis has been studied in White Carneau and Show Racer pigeons. The results of analytical studies indicate that the disease process is characterized by the accumulation of various lipids, and especially of sterol esters and free sterols. By perfusion of arteries with blood serum or with tissue culture medium containing C<sup>14</sup>-labeled acetate, significant synthesis of labeled fatty acids has been shown to occur. As the aorta becomes relatively more diseased, the synthesis of fatty acids is enhanced, and the atherosclerotic plaque itself appears to be the site of most of the synthesis. Likewise, as the aorta becomes more diseased, relatively more of the newly synthesized fatty acid becomes esterified to cholesterol. The cholesterol to which the fatty acid is esterified appears to be that which is contained within the arterial wall, and the artery appears to have the necessary enzyme systems for carrying out the esterification.

**KEY WORDS** atherogenesis · fatty acid biosynthesis · aorta · pigeon · cholesterol feeding · cholesterol ester synthesis · esterifying enzymes

**T**HE AORTAS OF MAN and certain experimental animals are capable of synthesizing certain of the lipids which accumulate in atherosclerotic lesions (1, 2). Thus the fatty acids which appear in triglycerides and phospholipids of rabbit atheromata appear to be synthesized by the artery (3-5), whereas some evidence suggests that cholesterol is deposited from blood serum (6, 7).

The White Carneau pigeon is known to develop severe aortic atherosclerosis without the stimulation of dietary cholesterol (8), although the addition to the diet of small

amounts of cholesterol markedly exacerbates the disease (9). Likewise, the aortas of pigeons are capable of synthesizing fatty acids in vitro (10).

In previous studies on spontaneously diseased pigeons (10), we were able to show that the distal end of the aorta (where lesions most often develop) synthesizes more lipid in vitro than does the proximal end; we were not able, however, to show that the arteries from atherosclerosis-susceptible White Carneau pigeons synthesize more lipid than do those from the more resistant Show Racer pigeons.

As mentioned above, feeding cholesterol to White Carneau pigeons accelerates the development of aortic atherosclerosis. Morphologically, the disease resembles that which develops spontaneously. The studies described in this paper were undertaken (*a*) to compare the lipid composition of aortas of pigeons during cholesterol-aggravated atherogenesis with that of aortas from naturally diseased birds, and (*b*) to investigate certain aspects of lipid synthesis during this period of rapid lesion formation.

## MATERIALS AND METHODS

Pigeons of the White Carneau or Show Racer breed were obtained either from our stock colony, or from their original source, the Palmetto Pigeon plant, Sumter, S. C. The birds were maintained on a diet of pigeon pellets,<sup>1</sup> or on pellets plus cholesterol coated on pellets by dissolving in warm lard (final ration contained 10% lard, 1% cholesterol). In most experiments, young birds 6-12 weeks of age were maintained on the diets

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<sup>1</sup> Purina Pigeon Pellets, Ralston-Purina Co., St. Louis, Mo., consisting of crushed, pelleted grains.

containing cholesterol for periods of up to one year. Middle-aged birds, 6–8 yr of age, maintained on a grain diet without added cholesterol or lard were used for comparison. No attempt was made to separate the effects of feeding either cholesterol or lard alone in these experiments. Rather, we used a mixture which we knew to be atherogenic for pigeons.

#### *Analytical Studies*

In the first series of experiments, four groups of six week old pigeons (8 pigeons per group) were designated "Initial," "Two Month," "Four Month," and "Six Month" groups. The Initial group was sacrificed at the start of the experiment. The remaining groups were maintained on the diet containing cholesterol for the periods of time indicated. At necropsy, the aortas were removed, cleaned of adhering tissue, weighed, and opened longitudinally. The extent of grossly visible atherosclerosis was evaluated by calculating the percentage of the surface area of the aorta occupied by plaques. The aortas were assigned a grade ranging from 0 (no visible plaques) to 4 (>15% of the aorta involved).

Because of the small amount of lipid present in a single pigeon aorta, it was necessary to pool all eight aortas from each group. Lipids were extracted from the minced aortas with boiling ethanol, followed by three extractions with boiling ethanol-ether 2:1 (v/v). The lipid extract was taken up in chloroform, dried, weighed, and finally resuspended in Skellysolve B. Each lipid extract was fractionated into three classes on activated silicic acid-Celite (1:1) columns essentially as described by Van Handel (11). Two columns were run from each lipid extract, and all determinations were carried out in duplicate. The values reported in Table 1 are therefore the means of four determinations. Triglycerides were determined in Fraction II by the method of Van Handel (12), and lipid phosphorus was determined in Fraction III after incineration, by the method of Fiske and Subbarow (13). Cholesterol was determined in Fraction II, which contains both free sterols and triglycerides, by the Searcy procedure (14), and from the micromoles of cholesterol present in the sterol ester fraction (Fraction I), the amount of fatty acid bound to sterol was calculated.

#### *Perfusion Studies*

In our previous experiments on lipid synthesis in pigeons (10), minced aortas were incubated with acetate-1- $C^{14}$ . It was not possible to separate the thin pigeon aorta into its component layers. Hence the lipid synthesized represented that coming from adventitia, media, and intima. To avoid this complication, we devised a system whereby twelve aortas could be perfused simultaneously with blood serum to which sodium acetate-1-

$C^{14}$  is added (1  $\mu$ c and 0.6  $\mu$ mole/ml of medium). A photograph of the system is shown in Fig. 1, and a schematic diagram is shown in Fig. 2. Approximately 3 min are required to remove the aorta from the animal, attach it to the pump, and begin perfusion. In certain experiments, a tissue culture medium<sup>2</sup> was used as the perfusion medium instead of blood serum. The thoracic aorta of the pigeon has no branching arteries, hence only the intimal surface was exposed to the perfusion medium.

Following perfusion, the aortas were removed from the pump, cleaned, weighed rapidly, and scored for atherosclerosis as described above. Lipids were extracted from the aortas by homogenizing in all-glass hand homogenizers in chloroform-methanol 2:1 (v/v). After extraction and centrifugation to remove particulate matter, the lipids were obtained in the chloroform phase by the addition of a volume of water equal to that of the chloroform-methanol. The chloroform extracts were then washed with 5% sodium acetate, then twice more with water, and finally brought to a volume with chloroform. At this point, an aliquot was taken for the determination of the total lipid radioactivity, using liquid scintillation counting. The remaining lipid extract was then evaporated at 50° under nitrogen to a volume convenient for applying to thin-layer chromatographic plates coated with silicic acid. The developing solvent was a mixture of Skellysolve B (146 ml), diethyl ether (50 ml), and glacial acetic acid (4 ml). The location of the spots was determined by spraying the outer channels of the plate with Rhodamine 6G in ethanol, and viewing under ultraviolet light. In this system, sterol esters migrate to a position near the solvent front, followed by triglycerides and free sterols. Phospholipids remain at the origin. The spots containing the various lipid classes were scraped from the plates and eluted, and their radioactivity was measured. Results were expressed as cpm/g of aorta, or as percentage of the total lipid radioactivity found in all four lipid classes.

## RESULTS AND DISCUSSION

### ANALYTICAL STUDIES

Table 1 shows the changes in the lipid composition of pigeon aortas as the birds become progressively more diseased as a result of cholesterol feeding. In the last column the values obtained on 6–8 year old spontaneously diseased birds are presented. The arteries of the birds in the Initial group had no atherosclerotic lesions, whereas the aortas of the Two Month and Four Month groups were only slightly diseased. All aortas

<sup>2</sup> "Minimum Essential Medium (Eagle) Spinner," consisting of a balanced mixture of amino acids, vitamins, and salts. Hyland Laboratories, Los Angeles, Calif.

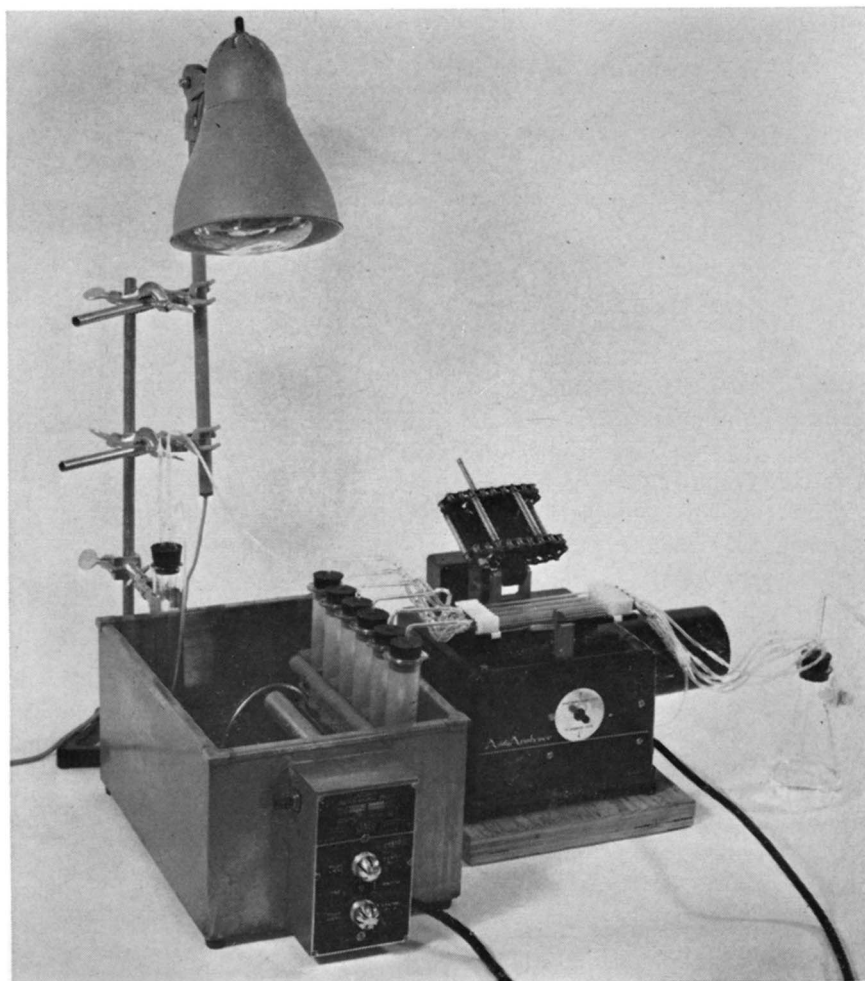


FIG. 1. Photograph of apparatus, assembled for perfusion of six aortas. The pump and chambers are duplicated on the opposite side when 12 aortas are perfused.

from both the Six Month and Spontaneously diseased groups had Grade 4 lesions. There was a striking similarity in lipid composition of arteries from birds in these last two groups. This observation suggests that the cholesterol-aggravated disease is basically similar to that which develops in pigeons naturally. This point may be of interest to other workers who might use this species for studies on atherosclerosis.

The data of Table 1 also show that as the aortas became more diseased there were absolute increases in the aortic content of all the lipid classes studied. Of special interest, however, was the increase in sterol ester, in which a 27-fold difference in aortic concentrations was found between birds of the Initial group and those of the Six Month group. In terms of percentage of the total lipid in the artery, sterol esters underwent a 13-fold increase, whereas phospholipids and triglycerides showed a relative decrease. These findings are consistent with the results of numerous studies on atherosclerotic lesions in human beings (15, 16).

## PERFUSION STUDIES

### *Total Lipid Synthesis*

In one of the first series of experiments using the perfusion technique, we attempted to establish the time course for the synthesis of lipid from acetate by isolated aortas. The results are shown in Fig. 3. The points on the curve for total radioactivity represent the mean values from five aortas for each time interval, and the vertical bars represent the standard errors of these means. Obviously there is a high degree of variability in lipid synthesis among the birds. We observed this type of curve and similar degrees of variability in each of several experiments. On the basis of the increasing radioactivity after 4 hr of perfusion, we chose this interval for subsequent experiments. This curve represents the sum of the synthesis of several lipid classes, as will be discussed later in this paper.

It has not been possible to calculate with certainty the total amount of acetate metabolized by the perfused

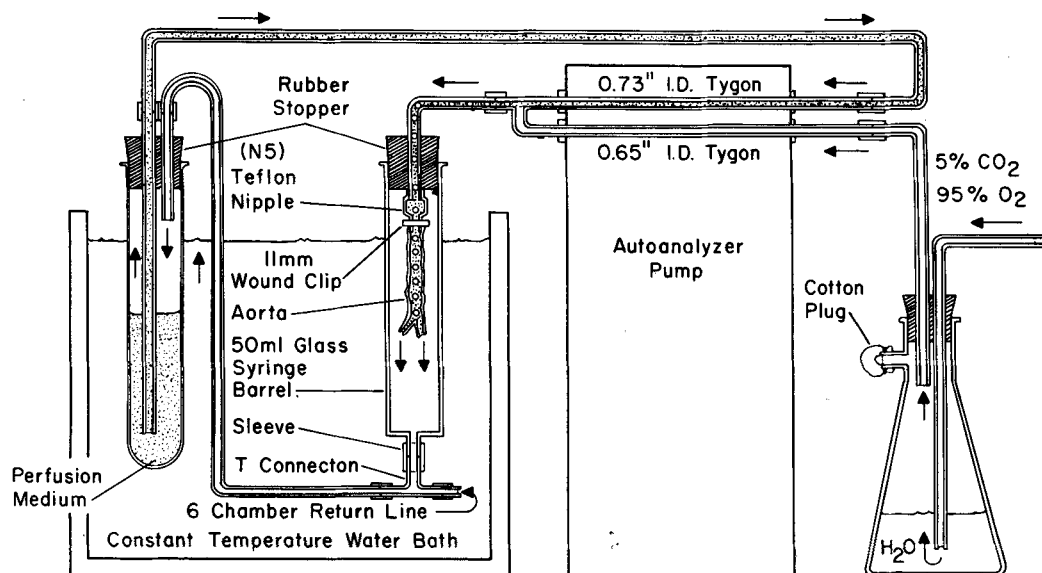


FIG. 2. Schematic diagram of perfusion system.

aortas. From the total radioactivity of the added acetate- $1\text{-C}^{14}$ , and from the radioactivity of the lipids recovered from the aortas, we have obtained values ranging from 0.2 to 0.7%. It seems apparent that these represent minimum values only, since the extent of dilution of the substrate is not known. Likewise we have not attempted to measure the amount of acetate which is converted to nonlipid substances, nor that synthesized into fatty acid and then metabolized further.

We next attempted to compare the lipid-synthesizing activity in the aortas of atherosclerosis-susceptible White Carneau pigeons with that of the more resistant Show Racers (Table 2). Differences among the breed and age groups were slight and not statistically significant. On the basis of these data we feel that we cannot demonstrate that the two breeds differ in this regard. It should be

pointed out, however, that lesions develop in the White Carneau breed over a 2 yr span (17), hence differences in the rate of lipid synthesis might be very slight and difficult to detect. However, as shown by the data of Table 1, the administration of cholesterol to White Carneau pigeons results in an accelerated rate of lipid accumulation in the aorta. Accordingly, young pigeons of both breeds were placed on an atherogenic diet for a 3 month period, then sacrificed, and their aortas perfused. The results shown in Table 3 demonstrate that the aortas of White Carneau fed cholesterol synthesize significantly more lipid than do their noncholesterol fed controls, or Show Racers on either dietary regimen. These findings suggest that cholesterol feeding results in the stimulation of synthesis of lipids from acetate in the aortas of the susceptible breed.

In Table 3 we have shown individual values, as well as means for the 6 birds in each group. Among the White Carneau receiving cholesterol, two aortas had radioactivity values which were considerably higher than any

TABLE 1 LIPID COMPOSITION OF PIGEON AORTAS\*

| Lipid Fraction    | Experimental Group |                 |                 |                |                 |
|-------------------|--------------------|-----------------|-----------------|----------------|-----------------|
|                   | Initial            | 2 Month         | 4 Month         | 6 Month        | Spontaneous     |
| Cholesterol ester | 0.44<br>(0.6)      | 5.9<br>(4.0)    | 7.3<br>(4.9)    | 11.9<br>(7.7)  | 10.8<br>(6.3)   |
| Free cholesterol  | 5.6<br>(7.8)       | 8.5<br>(5.8)    | 11.2<br>(7.6)   | 16.2<br>(10.5) | 19.1<br>(11.2)  |
| Triglyceride      | 46.7<br>(65.3)     | 109.6<br>(75.6) | 102.6<br>(70.2) | 88.8<br>(57.8) | 115.1<br>(67.9) |
| Phospholipid      | 18.7<br>(26.1)     | 20.9<br>(14.4)  | 25.0<br>(17.1)  | 36.7<br>(23.8) | 24.4<br>(14.4)  |

\* Top values are expressed as micromoles per gram of aorta; values in parentheses represent percentage of the total lipid.

TABLE 2 LIPID SYNTHESIS IN AORTAS OF PIGEONS OF TWO BREEDS AND AGES

| Breed of Pigeon | Number of Birds | Age        | Radioactivity of Newly Synthesized Lipid* |
|-----------------|-----------------|------------|---|
|                 |                 |            | <i>cpm/g of aorta</i>                     |
| White Carneau   | 12              | 12 weeks   | 52,600 ± 10,700                           |
| White Carneau   | 12              | 8-9 months | 41,800 ± 3,200                            |
| Show Racer      | 12              | 12 weeks   | 40,400 ± 5,800                            |
| Show Racer      | 12              | 8-9 months | 44,200 ± 6,100                            |

\* Mean values, followed by the standard errors of the means.

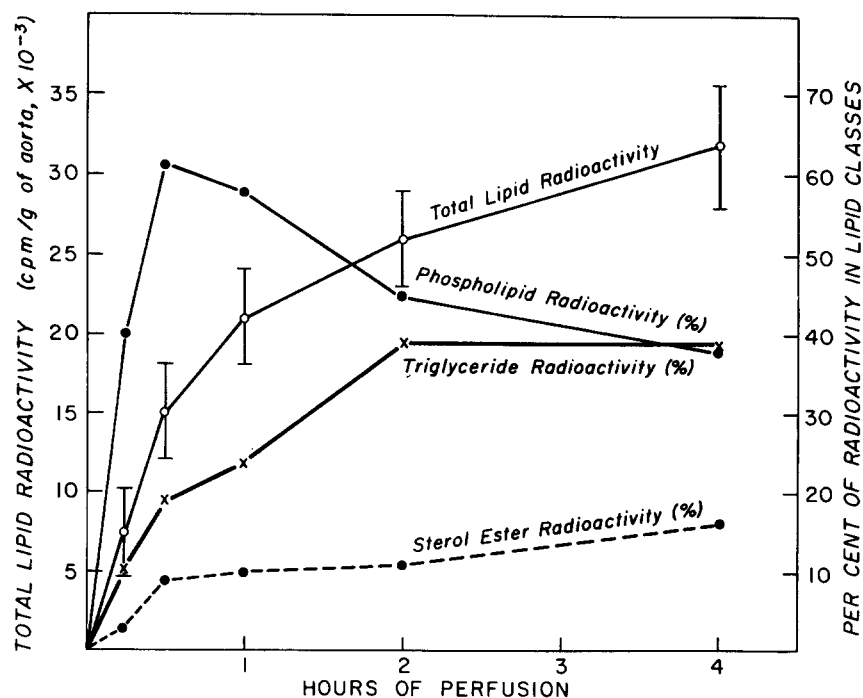


FIG. 3. Time-activity curve for the total lipid radioactivity and percentage curves for three lipid fractions.

other in the group. It was observed that these two birds were the most severely diseased of those studied, and had numerous grossly visible raised plaques in the intima. The other birds had a few small plaques, or fatty streaks only. These observations suggested to us that the rate of lipid synthesis in aortas is related to the severity of atherosclerosis.

In order to establish this point more firmly we examined by perfusion the aortas from a series of Show Racers whose natural resistance to aortic atherosclerosis had been overcome by the feeding of 1% cholesterol for 18 months, during which period the pigeons received almost 6000 roentgens of whole body irradiation. These birds were from a series of investigations of the effects of irradiation on atherogenesis in pigeons, the results of

which will be published elsewhere. However, among these birds were some that were extremely diseased, with virtually the entire aortic surface being occupied by plaques. Others were considerably less diseased. Following perfusion, the aortas were scored as being slightly, moderately, or severely diseased. Table 4 shows the radioactivity of newly synthesized lipids from these aortas. The differences among these three groups of birds are striking, and it seems clear that enhanced lipid synthesis parallels the increase in severity of atherosclerosis.

We have extended this observation by examining the radioactivity in different areas of the same perfused aorta. In many instances, atherosclerotic plaques in pigeons are discrete enough to allow excision of the

TABLE 3 LIPID SYNTHESIS IN AORTAS OF CHOLESTEROL-FED PIGEONS OF TWO BREEDS

| Breed of Pigeon | Dietary Treatment | Radioactivity of Newly Synthesized Lipid |         |                       |
|-----------------|-------------------|--|---------|-----------------------|
|                 |                   | Individual Values                        |         | Mean                  |
| White Carneau   | Cholesterol-fed*  | 345,600                                  | 280,000 | 133,900 $\pm$ 57,500† |
|                 |                   | 26,900                                   | 30,500  |                       |
|                 |                   | 57,400                                   | 63,100  |                       |
| Whit Carneau    | Control           | 21,000                                   | 13,200  | 24,600 $\pm$ 4,200    |
|                 |                   | 40,800                                   | 15,200  |                       |
|                 |                   | 28,900                                   | 28,300  |                       |
| Show Racer      | Cholesterol-fed   | 20,900                                   | 42,800  | 25,800 $\pm$ 4,300    |
|                 |                   | 20,200                                   | 13,900  |                       |
|                 |                   | 33,300                                   | 23,430  |                       |
| Show Racer      | Control           | 13,600                                   | 7,800   | 18,200 $\pm$ 3,600    |
|                 |                   | 23,800                                   | 17,000  |                       |
|                 |                   | 33,100                                   | 14,200  |                       |

\* Pigeon pellets, to which was added 1.0% cholesterol and 10% lard. Control, pigeon pellets only.

† Mean  $\pm$  SEM (n = 6 for each group).

plaque so that almost no normal tissue is included in the sample. The lipid radioactivity from the plaque was higher than that from the undiseased area of the same aorta (Table 5). The data suggest strongly that the plaque itself is the primary site of increased lipid synthesis.

#### Distribution of Lipid Among Various Classes

The lipid extracts from perfused arteries were separated by thin-layer chromatography into four classes, all of which were radioactive. We were especially interested in the consistent finding of 5–10% of the total radioactivity in the free sterol fraction, since plasma cholesterol is generally assumed to be the source of aortic cholesterol (6). When a pooled extract of lipids from perfused arteries was subjected to saponification, followed by isolation of sterols by digitonin precipitation, less than 0.1% of the total lipid radioactivity was recovered in the sterol fraction (from both the free and the esterified forms). We conclude that the synthesis of sterols from acetate in these systems is negligible, and that our observed values of 5–10% of the total activity in these fractions probably represent an artifact of the chromatographic system. Essentially all of the radioactive lipid synthesized is fatty acid.

As can be seen from Table 6, in undiseased arteries, most of the radioactive fatty acid is found in phospholipids and triglycerides. This type of distribution is also invariably seen in arteries in the early stages of atherosclerosis (fatty streaks only, or those with only minimal involvement with small raised plaques). On the other hand, in more severely diseased arteries, the sterol ester fraction contains a major portion of the newly synthesized fatty acid. In some experiments this fraction has accounted for as much as 60% of the total lipid radioactivity. When this observation is coupled with that of the remarkably enhanced synthesis of fatty acids in severely diseased aortas (as shown in Table 5), it seems obvious that accelerated atherogenesis in pigeons is characterized by increased fatty acid synthesis; the fate of much of the newly synthesized fatty acid appears to be esterification to cholesterol, or perhaps interesterification with preexisting sterol esters in the wall of the artery.

TABLE 4 RELATIONSHIP BETWEEN LIPID SYNTHESIS AND SEVERITY OF AORTIC ATHEROSCLEROSIS

| Severity of Disease | Radioactivity of Newly Synthesized Lipid<br><i>cpm/g of aorta</i> |
|---------------------|---|
| Slight              | 35,800 ± 3,400*   |
| Moderate            | 95,300 ± 6,700  |
| Severe              | 536,800 ± 250,000   |

\* Mean ± SEM (n = 4 in each group).

TABLE 5 LIPID SYNTHESIS IN DIFFERENT AREAS OF THE SAME AORTA

| Source of Material             | Radioactivity of Newly Synthesized Lipid<br><i>cpm/g of tissue</i> |
|--------------------------------|--|
| Undiseased area of aorta No. 1 | 24,100   |
| Plaque from aorta No. 1        | 160,000  |
| Undiseased area of aorta No. 2 | 46,900   |
| Plaque from aorta No. 2        | 212,700  |

The mechanism of esterification of cholesterol in the isolated perfused aorta is of interest. Recently, Glomset et al. (18) have described a fatty acid transferase system in the serum of rats and humans which transfers fatty acids from the  $\beta$ -position of phospholipids to free cholesterol. This observation was confirmed and extended by Shah, Lossow, and Chaikoff (19). Since most of our experiments have been carried out using fresh serum as the perfusion medium, it seems possible that the cholesterol esterifying system in arteries could come from such a serum transferase system. However, in Table 6, those values labeled with a dagger were obtained by perfusing the aortas with tissue culture medium only, and with no added serum. Thus it would seem that the aorta itself has the enzyme systems necessary to esterify the newly synthesized fatty acid with cholesterol, whether by the transferase system or by some other cholesterol esterase system. In this regard, the time course of synthesis of the various lipid classes is of some interest. In Fig. 3 the time course of synthesis of the various lipid classes is plotted, as well as the total lipid radioactivity. As can be seen, total phospholipid radioactivity increases steadily during the 4 hr of the experiment. However, the percentage of the total radioactivity found in phospholipid reaches a maximal value rather rapidly, then declines

TABLE 6 DISTRIBUTION OF RADIOACTIVITY AMONG CLASSES OF LIPID SYNTHESIZED DURING PERFUSION

| Source of Material       | Expt. No. | % of Total Radioactivity |                 |               |              |
|--------------------------|-----------|--------------------------|-----------------|---------------|--------------|
|                          |           | Phospholipid             | "Free Sterol" * | Tri-glyceride | Sterol Ester |
| Undiseased artery        | 1         | 35                       | 6               | 51            | 8            |
|                          | 2         | 38                       | 8               | 50            | 4            |
| Severely diseased artery | 3         | 37                       | 5               | 27            | 31           |
|                          | 4         | 24†                      | 4†              | 37†           | 35†          |
| Excised plaque           | 5         | 12                       | 2               | 42            | 44           |
|                          | 6         | 19                       | 6               | 39            | 36           |

\* Radioactivity found in this fraction may be artifactual. See text.

† Arteries perfused with tissue culture medium, plus acetate-1- $C^{14}$ , with no blood serum.

over the 4 hr period, whereas the activity of both triglyceride and sterol fatty acid continues to increase. It seems possible that phospholipid fatty acid could serve as precursor for that esterified to cholesterol. More evidence is needed, however, on this point.

From the data presented here, certain tentative conclusions can be drawn. It seems apparent that sterol ester is the fraction showing maximal percentage change as the aorta progresses from the undiseased to the diseased state. Free cholesterol also increases markedly. A characteristic of the diseased artery is the rapid synthesis of fatty acids, which become esterified (in large proportion) to cholesterol preexisting in the arterial wall. This activity appears to be localized in the plaque itself, which would then have a different metabolism from that of the surrounding normal tissue. These data do not rule out, however, the possibility that the fatty acids are synthesized in normal regions of the artery, then transferred to the plaque. It is tempting to think of this process as being a metabolic response to some tissue stimulus, which perhaps might be the deposition of free cholesterol in the artery from plasma. This must remain speculative, however, until further evidence is obtained.

In general, our data in regard to enhanced synthesis of phospholipids and triglycerides appear to be in good agreement with the work of Zilversmit and his co-workers (3-5, 7). Likewise, Whereat (20) has recently reported significant increases in the synthesis of fatty acids from acetate in atherosclerotic aortas of rabbits. His work, like ours, suggests that such synthesis is greatest where the lesions are the most advanced.

This work was supported by grants from the U.S. Public Health Service (H-4371) and from the American Heart Association (63-6-123).

A portion of this work was from a thesis submitted by Carl W. Hoffman in partial fulfillment of the requirements for the M.S. degree in Biochemistry, 1964, Bowman Gray School of Medicine of Wake Forest College.

Manuscript received July 23, 1964; accepted September 22, 1964.

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