Composition and synthesis of fatty acids in atherosclerotic aortas of the pigeon

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ABSTRACT The composition, synthesis, and esterification of fatty acids were studied in aortas of White Carneau and Show Racer pigeons after perfusion of the aortas with a medium containing acetate-1-^{14}C.

For both breeds of pigeons the principal change in aortic fatty acids, in response to an atherogenic diet, was a marked increase in the percentage of oleic acid in the cholesteryl ester fraction.

In atherosclerotic aortas incorporation of acetate-1-^{14}C into the phospholipid and glyceride fractions increased 2-fold, while a much greater increase (up to 10-fold) was seen in incorporation into cholesteryl esters. In those birds receiving the atherogenic diet, palmitic acid accounted for approximately 50% of the fatty acid radioactivity, compared with approximately 25% from control aortas. Calculation of fatty acid synthesis showed the major newly synthesized fatty acids to be stearic acid in the phospholipid fraction; stearic, palmitic, and oleic acids in the glycerides; and oleic acid in the cholesteryl esters. The pattern of fatty acid synthesis was closely similar to the actual fatty acid composition of the aorta.

In atherosclerotic aortas an increased synthesis of all fatty acids was seen, but the greatest increase was seen in the synthesis of oleic acid and its esterification to cholesterol.

SUPPLEMENTARY KEY WORDS perfused aorta · chain elongation · desaturation

THE ACCUMULATION of lipids within the arterial wall is characteristic of atherosclerosis. Both in vivo and in vitro studies in rabbits (1, 2) have shown that the phospholipids arise largely from local synthesis within the artery. When acetate labeled with ^{14}C is used as a lipid precursor, the arterial wall is able to incorporate radioactivity not only into phospholipids, but also into triglycerides and cholesteryl esters. Most of this radioactivity is found in esterified fatty acids, very little if any being incorporated into the steroid nucleus of cholesterol (3–5). Studies with pigeons (5, 6) and rabbits (3) have indicated that the presence of atherosclerosis stimulates synthesis of fatty acids by the artery. The percentage of these newly synthesized fatty acids that are esterified to cholesterol increases as atherosclerosis becomes more extensive (5).

Using thin-layer chromatographic techniques, Day and Wilkinson (7) have shown that of the fatty acids synthesized by the atherosclerotic rabbit aorta, chiefly saturated fatty acids are esterified to phospholipids while predominantly monounsaturated fatty acids are found in the cholesteryl ester fraction. Howard (8) has demonstrated de novo synthesis as well as elongation of fatty acids in subcellular fractions from the aortic tissue of squirrel monkeys. In somewhat similar preparations of subcellular fractions from rabbit aorta, however, the only significant fatty acid synthesis that Whereat (9) was able to show was that resulting from mitochondrial chain elongation. Rabbits fed a cholesterol-containing diet had enhanced synthesis of fatty acids by this mitochondrial system.

In this report we describe quantitative and radiochemical studies of fatty acid synthesis in the aortas of two breeds of pigeons (the aortic atherosclerosis-susceptible White Carneau pigeon and the aortic atherosclerosis-resistant Show Racer pigeon). The influence of a cholesterol-containing diet on fatty acid synthesis in the two breeds of pigeons was assessed after perfusion of the intact aortas with a medium containing acetate-1-^{14}C.

Abbreviations: WC, White Carneau; SR, Show Racer; AI, atherosclerosis index; GLC, gas–liquid chromatography. Fatty acids are designated by chain length:number of double bonds.

MATERIALS AND METHODS

102 White Carneau (WC) and 48 Show Racer (SR) pigeons from our stock colony were maintained on a diet of pigeon pellets (Ralston Purina Co., St. Louis, Mo.), or on pigeon pellets coated with lard and cholesterol to a final concentration of 5% lard and 0.25% cholesterol. The pigeon pellets are made entirely from cereal grains containing no added cholesterol and 2.5% total fat. There were 42 WC and 12 SR pigeons in the cholesterol-fed group, 60 WC and 36 SR birds in the control group. After being on one of these diets for a period of 6 months to 1 yr (at this time the birds were 12–15 months old), the birds were killed by decapitation and their aortas were perfused by means of an apparatus described elsewhere (6). Each aorta was perfused continuously for 4 hr with 10 ml of a buffered tissue-culture medium (Minimum Essential Medium [Eagle] Spinner, consisting of a balanced mixture of amino acids, vitamins, and salts; Hyland Laboratories, Los Angeles, Calif.) containing 4100 units of penicillin G, 2.0 mg of streptomycin sulfate, and 8.34 μc of sodium acetate-1-^{14}C (2.0 mc/mmole). The acetate-1-^{14}C was purchased from New England Nuclear Corp., Boston, Mass., and used without further purification. The perfusion technique was utilized so that only the intimal surface of the aorta would be exposed to the perfusing medium (there are no branching vessels in the segments of pigeon aortas used for perfusion). The medium is, however, free to diffuse into the artery so that the results obtained probably reflect the contribution of the several different cell types found in the arterial wall. Perfusions were carried out at 40°C, while the medium was continuously gassed with a mixture of 95% oxygen and 5% carbon dioxide.

After the perfusion, the aortas were cleaned of adhering tissue, weighed, and opened longitudinally, so that the extent of atherosclerosis could be determined (10). They were then rinsed several times in distilled water and finally homogenized in chloroform–methanol 2:1 (v/v) with all-glass homogenizers. After the homogenate had been allowed to stand overnight, the residue was separated by centrifugation. The supernatant fraction was decanted and the residue was washed twice with small amounts of the chloroform–methanol mixture. The combined supernatant fractions were then washed twice with water and once with a 5% solution of sodium acetate. This procedure removed virtually all of the sodium acetate-1-^{14}C. In order to be assured of adequate amounts of radioactive fatty acid, we pooled the extracts of several aortas. Usually, the lipid extract of 12 aortas from control birds and 6 aortas from cholesterol-fed birds gave sufficient radioactivity with which to work.

After being dried at 40°C under a stream of nitrogen, the remaining residue was dissolved in petroleum ether for chromatography on columns of silicic acid and Celite (columns 1 cm in diameter filled with 1 g of silicic acid–Celite 1:1). All solvents used in chromatography were redistilled before use. Cholesteryl esters were eluted with 15 ml of petroleum ether–chloroform 9:1, glycerides and free cholesterol with 20 ml of chloroform, and phospholipids with 20 ml of methanol. Completeness of separation was checked by thin-layer chromatography in Skellysolve B–diethyl ether–glacial acetic acid 146:50:4.

After the classes of lipids had been separated by chromatography, methyl esters of the component fatty acids were prepared (11). Samples were then stored under a nitrogen atmosphere at −20°C until they could be analyzed. GLC was carried out in $^1/_4$ inch stainless steel columns filled with 15% diethylene glycol succinate polyester on 60–80 Gas-Chrom P (packed columns from Applied Science Laboratories Inc., State College, Pa.) in a Micro Tek GC-2000R chromatograph (Micro Tek Instruments, Baton Rouge, La.). Columns were temperature programmed at 2°C/min from 140 to 190°C and held at 190°C until all radioactive methyl esters had been eluted. Nitrogen was the carrier gas at a flow rate of 80 ml/min. The effluent stream was split before entering the hydrogen flame detector in such a way that 50% of the gas was diverted to a Packard fraction collector via a line heated to 250°C. Individual fatty acids were collected for analysis of radioactivity on cartridges of *p*-terphenyl coated with silicone oil (12). Using this technique, and injecting known amounts of a mixture of radioactive fatty acids similar in composition to those to be analyzed in this study, we recovered more than 98% of the injected radioactivity. All radioactivity appeared in the upper $^1/_3$ of the collection cartridge.

Radioactivity was determined in a Beckman DPM-100 scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif.). Samples were dissolved in 10 ml of a solution of toluene containing 6 g of 2,5-diphenyloxazole per liter, and counted until the 2-sigma counting error was less than 3%. Quenching was corrected for by external standardization.

Relative concentrations of fatty acids were calculated by multiplication of peak height by the width at half the height. Quantitative results with fatty acid standards (quantitative standards KA, KB, KC, and KD from Applied Science Laboratories) agreed with the stated composition data with a relative error of <3% for major components (those comprising more than 10% of the total mixture), <11% for components comprising 5–10% of the total mixture, and <60% for minor components (those comprising less than 5% of the total mixture). There was some overlap in the separation of stearic and oleic acids, amounting to about 5%. Somewhat less than 5% overlap in the separation of palmitic and palmitoleic acids was seen.

After the radioactivity of individual fatty acid methyl esters separated by GLC had been determined, fatty acids were recovered from the scintillation fluid by the method of Howard and Kittinger (13). Individual fatty acids were then decarboxylated by means of the Schmidt reaction, as below. For this purpose, fatty acids were placed in 50-ml Erlenmeyer flasks and dried under a stream of nitrogen, and the flasks were placed on ice. After the addition of 1 ml of sulfuric acid–fuming sulfuric acid 3:1 and 50–60 mg of sodium azide, an empty shell vial was added and the flasks were stoppered with serum caps. About 30 ml of air was evacuated from each flask with a 50 ml syringe, and the flasks were placed in a water bath at 70°C with constant shaking. After 1 hr, they were removed and placed on ice. The serum cap was punctured with a 26-gauge needle so that 0.5 ml of Hyamine (*p*-(diisobutylcresoxy-ethoxyethyl) dimethyl benzyl ammonium hydroxide, hydroxide of Hyamine 10-X, Packard) could be added to the shell vial by means of a syringe. The flasks were shaken for an additional hour at room temperature and the shell vials were removed and thoroughly washed on the outside. The vials and their contents were then placed in a counting vial, and scintillation fluid was added. Photoluminescence often made it necessary to dark-adapt the samples for several hours after addition of the scintillation fluid.

RESULTS

Table 1 shows the extent to which acetate-1-^{14}C is incorporated into lipids by the aortas of WC and SR pigeons. As is indicated by their atherosclerosis index (AI), control birds of both breeds had no grossly visible atherosclerosis. In cholesterol-fed birds, the AI averaged 28% for the White Carneaux and 2% for the Show Racers. The amounts of radioactivity incorporated into lipids were larger in the aortas of cholesterol-fed pigeons than in controls, and much larger in the more extensively diseased aortas from cholesterol-fed WC pigeons. This correlation between aortic lipid synthesis and the extent of atherosclerosis has been observed before (6). In the control birds, there was no difference between the two breeds in the ability of the aorta to synthesize lipids from acetate-1-^{14}C.

The fatty acid composition of the lipid classes shown in Table 1 was determined by GLC, and the results are presented in Tables 2 and 3. In both breeds of pigeons, the major fatty acids of the phospholipid fraction were palmitic acid, stearic acid, and oleic acid. Each comprised 16–27% of the total fatty acids of this fraction. Glycerides contained similar amounts of palmitic acid (approximately 20%) but had significantly more oleic acid and less stearic acid. The major polyunsaturated fatty acid of the phospholipid fraction was arachidonic acid, and for the glyceride fraction, linoleic acid. Ingestion of the cholesterol-containing diet had little influence on the fatty acid composition of aortic glycerides, other than a slight elevation of oleic acid in the White Carneau. The only changes noted in the phospholipid fraction were slight increases in palmitic acid and linoleic acid.

In the cholesteryl ester fraction from the aortas of control WC pigeons, the oleic acid content was approximately 45%. Linoleic acid accounted for 20% of the fatty acids in this fraction; palmitic acid, for 15%; and stearic acid, for 10%. The composition of the cholesteryl ester, unlike that of the phospholipid and glyceride fractions, changed markedly in response to cholesterol feeding. In cholesterol-fed birds, the proportion

TABLE 1 INCORPORATION OF ACETATE-1-^{14}C INTO LIPIDS OF PIGEON AORTAS

	Control				Cholesterol-Fed				Relative Increase over Controls¶
	No.*	Mean AI†	Radioactivity‡	Acetate-1-^{14}C Incorporated§	No.	Mean AI	Radioactivity	Acetate-1^{14}-C Incorporated	
		%	*dpm/g/4 hr*	*mμmoles/g/4 hr*		%	*dpm/g/4 hr*	*mμmoles/g/4 hr*	
White Carneau	7	0			7	28			
Phospholipids			52,000 ± 6,200‖	11.80			124,000 ± 24,000	28.20	2.4
Glycerides			35,000 ± 3,200	7.95			73,000 ± 11,100	16.60	2.1
Cholesteryl esters			3,000 ± 300	0.80			37,000 ± 11,200	8.41	10.6
Show Racer	5	0			2	2			
Phospholipids			64,000 ± 16,000	14.55			89,000	20.20	1.4
Glycerides			39,000 ± 7,600	8.87			54,000	12.30	1.4
Cholesteryl esters			3,200 ± 500	0.73			9,000	2.04	2.8

* We pooled extracts of several aortas in order to have adequate amounts of radioactive lipids with which to work. For the White Carneau control group, 60 aortas were used (seven pools), with 42 in the cholesterol-fed group (seven pools). For the Show Racer control group, 36 aortas were used (five pools), with 12 in the cholesterol-fed group (two pools).

† Atherosclerosis index (the percentage of intimal surface showing gross atherosclerosis).

‡ Results represent the mean of the total dpm/g of tissue (wet wt) incorporated during the 4 hr period of perfusion.

§ mμmoles/g of tissue (wet wt).

¶ The relative increase was obtained by dividing acetate incorporation in the cholesterol-fed group by that in the control group.

‖ Mean values ± SEM. Standard errors were not calculated for the cholesterol-fed Show Racer group because of the small sample size.

TABLE 2 INFLUENCE OF CHOLESTEROL-AGGRAVATED ATHEROSCLEROSIS ON FATTY ACID COMPOSITION OF AORTIC LIPIDS IN WHITE CARNEAU PIGEONS

	Number of Samples†	Fatty Acids*						
		16:0	16:1	18:0	18:1	18:2	18:3	20:4
		% of total fatty acids						
Phospholipids								
Cholesterol-fed	6	21.5 ± 0.7‡	2.0 ± 0.4	24.0 ± 0.9	21.0 ± 0.5	9.1 ± 0.3	0.6 ± 0.1	13.9 ± 0.8
Control	6	15.9 ± 0.6	1.4 ± 0.2	25.8 ± 1.4	20.2 ± 0.5	6.7 ± 0.2	1.6 ± 0.9	14.6 ± 0.4
Glycerides								
Cholesterol-fed	7	21.0 ± 1.1	6.3 ± 0.7	8.0 ± 0.7	46.2 ± 1.1	14.3 ± 0.6	0.7 ± 0.1	1.5 ± 0.3
Control	7	19.9 ± 0.6	7.8 ± 0.8	9.6 ± 0.7	41.6 ± 1.0	15.2 ± 1.2	1.1 ± 0.2	1.8 ± 0.3
Cholesteryl esters								
Cholesterol-fed	5	7.7 ± 0.3	7.3 ± 1.2	1.9 ± 0.4	61.9 ± 1.3	15.0 ± 0.4	0.6 ± 0.1	2.3 ± 0.4
Control	2§	13.1	5.8	9.3	45.8	20.5	0	4.1
		15.2	8.0	10.7	43.5	19.5	0	1.4

* Fatty acids are designated by chain length: number of double bonds.

† In order to have enough radioactivity for significant counting, we pooled 6 aortas from cholesterol-fed pigeons and 12 aortas from grain-fed pigeons. Thus, six samples actually represent the extract from the aortas of 36 cholesterol-fed pigeons or 72 controls.

‡ Mean values ± SEM.

§ Because of the small amount of lipid in this fraction, only two chromatograms were suitable for quantification; results from both of these are presented.

TABLE 3 INFLUENCE OF CHOLESTEROL-AGGRAVATED ATHEROSCLEROSIS ON FATTY ACID COMPOSITION OF AORTIC LIPIDS IN SHOW RACER PIGEONS

	Number of Samples†	Fatty Acids*						
		16:0	16:1	18:0	18:1	18:2	18:3	20:4
		% of total fatty acids						
Phospholipids								
Cholesterol-fed	2	18.1	1.5	26.2	20.4	7.7	0.8	15.5
		16.8	1.1	27.1	23.7	8.7	0.5	14.3
Control	5	13.3 ± 0.8‡	1.3 ± 0.2	28.3 ± 1.3	20.7 ± 1.3	6.3 ± 0.2	0.6 ± 0.2	17.3 ± 2.0
Glycerides								
Cholesterol-fed	2	18.3	5.7	9.1	51.5	11.9	1.2	0.7
		18.1	5.6	9.4	50.6	11.8	1.0	2.0
Control	5	19.1 ± 0.8	8.4 ± 0.6	10.3 ± 0.9	44.0 ± 0.8	14.3 ± 1.5	1.0 ± 0.4	0.9 ± 0.4
Cholesteryl esters								
Cholesterol-fed	2	7.6	6.3	4.6	70.2	11.7	—	—
		6.3	6.4	0.9	61.5	22.6	1.4	0.9
Control§	—	17.6	2.9	8.6	27.9	34.6	—	3.1

* Fatty acids are designated by chain length: number of double bonds.

† In order to have enough radioactivity for significant counting, we pooled 6 aortas from cholesterol-fed pigeons and 12 aortas from control pigeons. Thus, six samples actually represent the extract from the aortas of 36 cholesterol-fed pigeons or 72 controls.

‡ Mean values ± SEM.

§ The amounts of lipid present in this fraction were so small that no chromatograms were suitable for quantification. Values given are from a previously published study (15).

of cholesteryl oleate increased to the extent that it eventually accounted for 60–70% of the fatty acids in this fraction.

Even though the cholesterol-containing diet produced little *relative* change in the composition of fatty acids of the phospholipid and glyceride fractions, there was an *absolute* increase in all fatty acids proportionally (Table 4). The absolute concentration of each fatty acid in μmoles/g (wet wt) was calculated from previously published data on the lipid composition of aortas from WC pigeons (5) and the fatty acid composition data shown in Table 2. Although it would have been desirable to know the lipid composition of the aortas actually used in this study, this was not done because of the necessity of preserving as much radioactive fatty acid as possible. We feel, however, that the use of previously published lipid composition data from WC pigeons of similar genetic backgrounds, that had received the same diet for similar lengths of time, and had comparable aortic atherosclerosis, was sufficiently similar to allow the calculation of a reasonably accurate estimate of aortic fatty acid concentration.

TABLE 4 CONCENTRATIONS OF PRINCIPAL FATTY ACIDS IN THE AORTAS OF WHITE CARNEAU PIGEONS*

	Fatty Acids†			
	16:0	18:0	18:1	18:2
	μmoles/g wet wt			
Phospholipids				
Control	5.94	9.64	7.56	2.50
Cholesterol-fed	15.8	17.6	15.4	6.68
Absolute increase over controls‡	9.84	7.98	7.86	4.18
Relative increase over controls§	2.6	1.8	2.0	2.7
Triglycerides				
Control	27.9	13.4	58.1	21.2
Cholesterol-fed	56.0	21.3	123	38.1
Absolute increase over controls‡	28.1	7.86	65.0	17.0
Relative increase over controls§	2.0	1.6	2.1	1.8
Cholesteryl esters				
Control	0.06	0.04	0.19	0.09
Cholesterol-fed	0.92	0.23	7.35	1.78
Absolute increase over controls‡	0.86	0.19	7.16	1.69
Relative increase over controls§	15.3	5.7	38.7	19.8

* The concentration of individual fatty acids shown in this table was calculated from the percentage fatty acid composition as shown in Table 2 and the lipid composition of aortas from previously published data from White Carneau pigeons (5). The following equations were used for this calculation.

Phospholipid (PL) fatty acids = μmoles PL × percentage concentration of individual fatty acids × 2/100.

Triglyceride (TG) fatty acids = μmoles TG × percentage concentration of individual fatty acids × 3/100.

Cholesteryl ester (CE) fatty acids = μmoles CE × percentage concentration of individual fatty acids × 1/100.

† Fatty acids designated by chain length:number of double bonds.

‡ This value was obtained by subtracting control values from cholesterol-fed values.

§ The relative increase was obtained by dividing cholesterol-fed values by control values. A value of two means that twice the concentration of that fatty acid was found in aortas of cholesterol-fed birds than found in controls.

It is obvious that cholesterol ingestion does increase the total content of esterified fatty acids of the pigeon aorta, as would be expected from the observed increases in the total lipid content of atherosclerotic arteries (5). The fatty acids of the phospholipid and glyceride fractions were approximately doubled, with a much greater increase seen in the cholesteryl ester fraction. Although the *relative* increase is greatest in the cholesteryl ester fraction, triglycerides accounted for the largest *absolute* amount of lipid, followed by phospholipids, then cholesteryl esters.

The pattern of radioactivity of fatty acids differed considerably from their analytic composition. In Tables 5 and 6 the amount of acetate-1-^{14}C incorporated into each fatty acid is shown as a percentage of the total fatty acid radioactivity recovered. In the phospholipid and glyceride fractions, approximately 50% of the radioactivity of the control group was recovered in palmitic acid and stearic acid. In the cholesteryl ester fatty acids, substantial amounts of radioactivity were seen in oleic acid as well as in palmitic acid.

In those birds fed the cholesterol-containing diet a significantly greater percentage of the radioactivity was found in palmitic acid in all lipid classes studied. Incorporation of the radioactive label into monoenoic fatty acids of the cholesteryl ester fraction, however, remained quite active in the aortas of cholesterol-fed birds. This was particularly true in the Show Racers.

The results given in Tables 5 and 6 suggest that arterial tissue has the capacity to synthesize fatty acids de novo from precursor acetate-1-^{14}C, as well as to elongate and desaturate newly synthesized and preexisting fatty acids. In order to determine which method of biosynthesis was responsible for the production of specific acids, we subjected fatty acids, previously separated by GLC, to decarboxylation. Results are given in Table 7. For purely de novo synthesis, the ratio of total fatty acid radioactivity to carboxyl radioactivity would be 8:1, whereas elongation of preexisting, nonradioactive fatty acids would result in a ratio of 1:1. The results in Table 7 indicate that 16-carbon fatty acids arise primarily from de novo synthesis, while 18-carbon fatty acids are the result of chain elongation.

Knowing the amount of acetate-1-^{14}C incorporated into lipids (Table 1) along with the percentage incorporation into each fatty acid (Tables 5 and 6) and the molecular ratio of acetate utilization (Table 7), we were able to calculate the amount (in mμmoles) of newly synthesized and esterified fatty acids. Results from such a calculation are shown in Table 8. In the phospholipid fraction stearic acid was synthesized to the greatest extent while the fatty acids mostly synthesized in the glyceride fraction were palmitic, stearic, and oleic acids. In the cholesteryl ester fraction oleic acid was the major fatty acid synthesized. The synthesis of all fatty acids was stimulated by cholesterol feeding but the greatest influence appeared to be an increased synthesis of monoenoic fatty acids esterified to cholesterol.

DISCUSSION

Fatty Acid Composition of Pigeon Aortas

In WC and SR pigeons, cholesterol-aggravated atherosclerosis did not significantly alter the fatty acid composition of the phospholipid and glyceride fractions from the aortas except for a slight increase in percentages of palmitic acid and linoleic acid (Tables 2 and 3). This finding is in general agreement with those of similar studies on arteries from man and animals (14). Among birds fed cholesterol there was a marked alteration in the fatty acid composition of the cholesteryl ester fraction.

TABLE 5 DISTRIBUTION OF RADIOACTIVITY AMONG FATTY ACIDS FROM THE AORTAS OF CHOLESTEROL-FED AND CONTROL WHITE CARNEAU PIGEONS AFTER PERFUSION WITH ACETATE-1-^{14}C*

Fatty Acids†	Phospholipids		Glycerides		Cholesteryl Esters	
	Control	Chol-Fed	Control	Chol-Fed	Control	Chol-Fed
			% of total radioactivity			
<16:0‡	1 ± 0.4	2 ± 0.3	4 ± 0.6	6 ± 0.5	2 ± 1.4	4 ± 1.1
16:0	29 ± 1.5	46 ± 2.0	38 ± 2.4	54 ± 2.5	27 ± 6.3	43 ± 2.3
16:1	2 ± 0.3	5 ± 0.9	3 ± 0.6	7 ± 1.0	4 ± 2.5	18 ± 3.9
>16:1 < 18:0	0	1 ± 0.5	1 ± 0.3	2 ± 0.6	1 ± 0.7	1 ± 0.4
18:0	27 ± 2.5	20 ± 1.3	13 ± 0.9	8 ± 0.9	11 ± 0.5	4 ± 0.9
18:1	9 ± 0.4	9 ± 0.8	10 ± 1.0	8 ± 0.6	24 ± 4.9	17 ± 1.0
18.2§	1 ± 0.3	2 ± 0.3	2 ± 0.2	1 + 0.2	3 ± 2.3	2 ± 0.8
18:3 + 20:0	4 ± 0.6	3 ± 0.5	9 ± 1.1	5 ± 0.8	8 ± 6.5	3 ± 0.6
>18:3 < 20:4¶	11 ± 2.2	5 ± 0.5	12 ± 1.1	5 ± 0.7	13 ± 4.5	4 ± 1.1
20:4 + 22:1	5 ± 0.6	4 ± 0.6	4 ± 0.5	2 ± 0.5	6 ± 3.3	2 ± 1.1
>20:4‖	10 ± 3.4	1	5 ± 1.1	1 ± 0.3	1 ± 0.5	1 ± 0.5
Number of experiments	7	7	6	7	4	6
Number of birds	60	42	54	42	48	36

Mean values ± SEM (n given at bottom of table). Chol, cholesterol.

* All counting, including background, was carried out for a sufficient length of time to exceed a 2-sigma counting error of 3%. Actual activity ranged from a low of 41 dpm to a high of 10,787 dpm.

† Fatty acids are designated by chain length: number of double bonds.

‡ Collected from the time of injection up to the appearance of the 16:0 peak.

§ Radioactivity in the 18:2 area is probably due to incomplete separation from 18:1.

¶ This fraction includes 20:1, 20:2, 20:3, and 22:0.

‖ This fraction was collected for approximately 10 min at 190°C following the 20:4 peak. Further collection resulted in no significant additional radioactivity.

TABLE 6 DISTRIBUTION OF RADIOACTIVITY AMONG FATTY ACIDS FROM THE AORTAS OF CHOLESTEROL-FED AND CONTROL SHOW RACER PIGEONS AFTER PERFUSION WITH ACETATE-1-^{14}C*

Fatty Acids†	Phospholipids		Glycerides		Cholesteryl Esters	
	Control	Chol-Fed‡	Control	Chol-Fed	Control	Chol-Fed
			% of total radioactivity			
<16:0§	2 ± 1.3	2	3 ± 1.2	5	12 ± 8.0	0
16:0	26 ± 1.3	41	36 ± 2.4	45	26 ± 4.1	44
16:1	1 ± 0.5	3	2 ± 1.0	3	4 ± 2.1	7
>16:1 < 18:0	1 ± 0.2	1	1 ± 1.0	1	2 ± 1.2	4
18:0	28 ± 2.4	24	14 ± 1.0	10	9 ± 3.2	2
18:1	12 ± 1.5	10	14 ± 2.2	10	12 ± 3.2	37
18:2¶	3 ± 0.7	2	2 ± 1.3	2	6 ± 4.2	2
18:3 + 20:0	3 ± 0.8	4	9 ± 1.6	8	3 ± 2.2	1
>18:3 < 20:4‖	11 ± 1.6	6	11 ± 1.4	10	12 ± 4.3	0
20:4 + 22:1	6 ± 0.6	7	3 ± 0.4	3	12 ± 7.8	4
>20:4**	6 ± 4.8	1	4 ± 2.2	3	4 ± 2.7	0
Number of experiments	5	2	4	2	3	2
Number of birds	36	12	30	12	30	12

Mean values ± SEM (n given at bottom of table). Chol, cholesterol.

* All counting, including background, was carried out for a sufficient length of time to exceed a 2-sigma counting error of 3%. Actual activity ranged from a low of 41 dpm to a high of 10,787 dpm.

† Fatty acids are designated by chain length: number of double bonds.

‡ Results from the two experiments did not differ by more than 5% for any one fatty acid. Because of the small sample size, standard errors were not calculated.

§ Collected from the time of injection up to the appearance of the 16:0 peak.

¶ Radioactivity in the 18:2 area is probably due to incomplete separation from 18:1.

This fraction includes 20:1, 20:2, 20:3, and 22:0.

** This fraction was collected for approximately 10 min at 190°C following the 20:4 peak. Further collection resulted in no significant additional radioactivity.

The composition of the cholesteryl ester fatty acids from aortas of control WC pigeons was similar to that reported by Young and Middleton (15), except that in the present study more oleic acid and less linoleic acid was observed. This difference may be due to the fact that our birds were slightly older and would be expected to have some spontaneous disease. With cholesterol feeding, the proportion of oleic acid in the cholesteryl ester

TABLE 7 INCORPORATION OF ACETATE-1-^{14}C INTO ALKYL AND CARBOXYL CARBONS OF FATTY ACIDS FROM AORTAS OF CHOLESTEROL-FED WHITE CARNEAU PIGEONS*

Fatty Acids	Total Radioactivity:Carboxyl Radioactivity		
	Phospho-lipids	Glycerides	Cholesteryl Esters
Palmitic acid	7.5:1	7.3:1	8.5:1
Palmitoleic acid	4.4:1	4.3:1	4.8:1
Stearic acid	1.6:1	1.6:1	1.6:1
Oleic acid	1.3:1	1.5:1	1.8:1

* Values are for cholesterol-fed WC pigeons, but similar results were obtained for control WC pigeons, as well as for SR pigeons. Radioactivity in several fractions was so low that it was not always possible to run decarboxylations in duplicate; those that were run in duplicate, however, did not differ by more than 10%. Twelve decarboxylations of palmitate-1-^{14}C gave total:carboxyl ratios ranging from 1.2:1 to 1.0:1. The theoretical ratio is 1.0:1 for such a compound in which the radioactivity is entirely in the carboxyl carbon.

fraction rose from 45% to 62%—substantially higher than the percentage (43.9%) reported in WC pigeons with spontaneous atherosclerosis (16). This discrepancy may have resulted from the fact that the cholesterol-fed birds had more extensive atherosclerosis than birds with spontaneous disease or from some fundamental differences in the pathogenesis of the lesions. On the other hand, severity of disease may not be an obligatory requirement for high levels of cholesteryl oleate, as evidenced by the 60–70% oleic acid of the cholesteryl esters of cholesterol-fed SR pigeons (Table 3).

Although in the atherosclerotic arteries the *absolute* content of all major fatty acids of the three lipid classes was increased (Table 4), the *relative* increase in cholesteryl oleate was disproportionately large, resulting in a percentage increase in oleic acid at the expense of all other fatty acids.

The content of phospholipid and glyceride fatty acids was approximately twice as great in the aortas of cholesterol-fed pigeons as in the controls (Table 4). This increase compares very closely with that for the synthesis of lipids from acetate (Table 1). These data suggest that the additional fatty acids present in atherosclerotic aortas could arise largely from synthesis, as has been shown to be true for phospholipids in the aorta of the rabbit (1).

Fatty Acid Synthesis by Pigeon Aortas

That atherosclerotic arteries synthesize more fatty acids than do nonatherosclerotic arteries (3, 6) is again demonstrated by the data presented in Table 1. Synthesis of the sterol precursor, squalene, shows a similar correlation with the extent of atherosclerosis (17).

Under the conditions of the present study, the greatest incorporation of acetate-1-^{14}C into fatty acid was found in palmitic acid, and was increased in aortas from cholesterol-fed birds. Substantial radioactivity was also demonstrated in palmitoleic acid, stearic acid, and oleic acid, as well as in those fractions containing various satu-

TABLE 8 CONCENTRATION OF NEWLY SYNTHESIZED AND ESTERIFIED FATTY ACIDS*

	Control				Total Fatty Acid Synthesis‡	Cholesterol-Fed								Total Fatty Acid Synthesis
	16:0†	16:1	18:0	18:1		16:0		16:1		18:0		18:1		
	mμmoles/g of tissue (wet wt)													
White Carneau														
Phospholipids	0.46	0.05	2.00	0.82		1.75	(3.8)§	0.32	(6.4)	3.52	(1.8)	1.95	(2.4)	
Glycerides	0.41	0.05	0.64	0.53		1.22	(3.0)	0.27	(5.4)	0.83	(1.3)	0.88	(1.7)	
Cholesteryl esters	0.02	0.01	0.06	0.10		0.42	(21.0)	0.31	(31.0)	0.21	(3.5)	0.79	(7.9)	
					5.15 [12.2]¶									12.4 [23.8]
Show Racer														
Phospholipids	0.50	0.03	2.54	1.35		1.10	(1.2)	0.14	(4.7)	3.03	(1.2)	1.55	(1.1)	
Glycerides	0.44	0.04	0.77	0.83		0.76	(1.7)	0.09	(2.2)	0.77	(1.0)	0.82	(1.0)	
Cholesteryl esters	0.02	0.01	0.04	0.04		0.11	(5.5)	0.03	(3.0)	0.02	(0.5)	0.42	(8.4)	
					6.62 [14.8]									8.84 [17.4]

* The amount of newly synthesized and esterified fatty acids was calculated in the following manner. Total mμmoles acetate incorporated (Table 1) × percentage of total label per fatty acid (Tables 5 and 6) = mμmoles acetate incorporated into each fatty acid × molecular ratio of acetate utilization (Table 7) = mμmoles of newly synthesized and esterified fatty acids.

† Fatty acids are designated by chain length:number of double bonds. The four fatty acids shown in this table accounted for 51–90% of the total fatty acid radioactivity.

‡ Values represent net fatty acid synthesis if one includes only the four fatty acids shown.

§ Values in parentheses are the *relative* increases in newly synthesized and esterified fatty acids of the cholesterol-fed group over those of the controls.

¶ Values in brackets indicate net fatty acid synthesis if one includes radioactive fatty acids eluted from the GLC column after 18:1 and assumes that these fatty acids are synthesized via chain elongation.

rated acids (20:0 and longer) and polyunsaturated acids (18:3 and longer).

Calculation of the amount of fatty acid synthesis indicates a substantial difference between percentage incorporation of acetate radioactivity into fatty acids (Tables 5 and 6) and actual amounts of fatty acid synthesized (Table 8). It is obvious that although palmitic acid contained the most radioactivity, it was not necessarily synthesized to the greatest extent.

In the phospholipid fraction stearic acid was the main fatty acid synthesized while in the glyceride fraction palmitic, stearic, and oleic acids were the major fatty acids synthesized. Oleic acid was the principal newly synthesized fatty acid of the cholesteryl ester fraction.

Total fatty acid synthesis was stimulated in aortas from cholesterol-fed birds. This stimulation was slight for the SR pigeons but amounted to an approximate doubling of synthesis in aortas from cholesterol-fed WC birds. Although this increase in fatty acid synthesis occurred in all lipid classes, the greatest *relative* increase was seen in the cholesteryl ester fraction.

The four fatty acids shown in Table 8 accounted for 51–90% (average 72%) of the total fatty acid radioactivity. The remaining 28% was found in fatty acids eluted from the GLC column after oleic acid. If, as reported by Howard (8) and by Whereat (9), these fatty acids are synthesized primarily by chain elongation, then considerably more net fatty acid synthesis would result (see values in brackets in Table 8).

It must be remembered that this study actually shows the result of two processes: synthesis and esterification. Calculation of total fatty acid synthesis by summation of only those newly synthesized fatty acids that are esterified would result in the calculation of a minimum value for total fatty acid synthesis, since it does not take into account newly synthesized but not esterified fatty acids. In our experience, however, this is only a small percentage of the total fatty acids synthesized by this system.

When one compares the concentrations of newly synthesized fatty acids (Table 8) with the actual fatty acid composition of the aorta (Tables 2 and 3) the similarity is evident. Of particular interest is the amount of newly synthesized oleic acid that is esterified to cholesterol and the stimulation of this process by cholesterol feeding.

Day and Wilkinson (7), utilizing aortas from cholesterol-fed rabbits, recently demonstrated that of the newly synthesized fatty acids esterified to phospholipid, 58.9% of the radioactivity was found in saturated fatty acids while in the cholesteryl ester fraction monoenoic fatty acids predominated (37.5% of the total radioactivity). Although these authors did not identify which fatty acids were synthesized, their results on the distribution of radioactive fatty acids agree quite closely with ours and are in sharp contrast to Whereat's findings with subcellular fractions from rabbit aortas (9). In his study, most of the radioactivity was recovered in the fractions containing the fatty acids 20:0 + 18:3, 20:2, and >20:4. It is difficult to explain these very marked discrepancies, although it is possible that Whereat's use of subcellular fractions rather than whole tissue may account for the differences in results. Howard (8), however, has demonstrated chain elongation as well as de novo synthesis in subcellular fractions from aortas of squirrel monkeys. In these animals, maintained on a "stock ration" without cholesterol supplementation, elongation occurred primarily in particulate fractions; the supernatant fraction synthesized palmitic acid and stearic acid de novo.

Like the aorta of the squirrel monkey and probably that of the rabbit, the aorta of the pigeon appears to possess the complete metabolic potential for the synthesis of fatty acids: de novo synthesis, chain elongation, and desaturation. From the radioactivity in the carboxyl and alkyl carbons of the newly synthesized fatty acids, it is obvious that palmitic acid arises completely from de novo synthesis, while the stearic acid and oleic acid appear to result primarily from chain elongation.

From both the analytical and the radiochemical data, it appears that esterification of specific fatty acids occurs selectively among the various lipid classes. Cholesterol ingestion has little influence on the relative composition of fatty acids esterified to phospholipids and glycerides, but produces a disproportionately large increase in the amount of oleic acid esterified to cholesterol. Such differences in the handling of certain fatty acids, particularly oleic acid, have been reported to occur in serum (18). Perhaps hypercholesterolemia stimulates the selective utilization of oleic acid for cholesterol esterification in the arterial wall, as Morin (19) has suggested it does in liver mitochondria from cholesterol-fed rats.

As with all in vitro studies, it is difficult to apply the data directly to processes in vivo. The perfusion technique, however, utilizes intact arteries and it seems reasonable that extrapolation of results from such a preparation to in vivo processes would be more valid than if preparations such as minces or homogenates were used. In order to evaluate the possible contribution of fatty acid synthesis to the total amount of fatty acids present in the atherosclerotic aorta, we calculated from the fatty acids synthesized in 4 hr the theoretical yearly rate of fatty acid synthesis (52.0 μmoles) by the aortas of cholesterol-fed WC pigeons. When this figure is compared with the total increase in fatty acid content of the atherosclerotic aorta following 1 yr of cholesterol feeding (157.5 μmoles), it becomes apparent that synthesis might account for 33% of this increase (52.0/157.5 × 100). Such a figure must certainly represent only a crude estimate of

fatty acid synthesis since it is impossible to know whether the rate studied over the 4 hr experimental period is indicative of the rate occurring during the 6 month–1 yr development of the atherosclerotic lesion. Also, differences in the preexisting pool of acetate would cause unknown dilutions of the acetate-1-^{14}C. Nevertheless, it is obvious that local synthesis and esterification of fatty acids by the artery may be of great significance in the accumulation of lipids in the atherosclerotic lesion.

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References

1. Shore, M. L., D. B. Zilversmit, and R. F. Ackerman. 1955. *Am. J. Physiol.* **181:** 527.
2. Newman, H. A. I., A. J. Day, and D. B. Zilversmit. 1966. *Circulation Res.* **19:** 132.
3. Whereat, A. F. 1964. *J. Atherosclerosis Res.* **4:** 272.
4. Newman, H. A. I., E. L. McCandless, and D. B. Zilversmit. 1961. *J. Biol. Chem.* **236:** 1264.
5. Lofland, H. B., Jr., D. M. Moury, C. W. Hoffman, and T. B. Clarkson. 1965. *J. Lipid Res.* **6:** 112.
6. Lofland, H. B., Jr., and T. B. Clarkson. 1965. *Arch. Pathol.* **80:** 291.
7. Day, A. J., and G. K. Wilkinson. 1967. *Circulation Res.* **21:** 593.
8. Howard, C. F., Jr. 1968. *J. Lipid Res.* **9:** 254.
9. Whereat, A. F. 1966. *J. Lipid Res.* **7:** 671.
10. Lofland, H. B., Jr., and T. B. Clarkson. 1959. *Circulation Res.* **7:** 234.
11. Stoffel, W., F. Chu, and E. H. Ahrens, Jr. 1959. *Anal. Chem.* **31:** 307.
12. Karmen, A., I. McCaffrey, J. W. Winkelman, and R. L. Bowman. 1963. *Anal. Chem.* **35:** 536.
13. Howard, C. F., Jr., and G. W. Kittinger. 1967. *Lipids.* **2:** 438.
14. Swell, L., and C. R. Treadwell. 1963. *In* Atherosclerosis and Its Origin. M. Sandler and G. H. Bourne, editors. Academic Press, Inc., New York. 301–347.
15. Young, F., and C. C. Middleton. 1966. *Proc. Soc. Exptl. Biol. Med.* **123:** 816.
16. Young, F., C. C. Middleton, and H. B. Lofland, Jr. 1964. *Proc. Soc. Exptl. Med.* **117:** 613.
17. St. Clair, R. W., H. B. Lofland, Jr., R. W. Prichard, and T. B. Clarkson. 1968. *Exptl. Mol. Pathol.* **8:** 201.
18. Goodman, DeW. S., and T. Shiratori. 1964. *Biochim. Biophys. Acta.* **84:** 104.
19. Morin, R. J. 1967. *Biochim. Biophys. Acta.* **144:** 594.