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Fatty Acid Composition of Plaque and Tissue Lipids from Pigeons with Spontaneous Atherosclerosis.* (29652)

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The origin of lipids in the atherosclerotic plaque from aortas of man and animals remains obscure. Studies of the fatty acid composition of various tissues have been made in man(1-3), chicken(4,5), rabbit(6), rat, dog, goose, pig, and guinea pig(7). In most of the laboratory animal species, arterial lesions could be induced only after drastic alteration of diets(8-11). Clarkson *et al*(12) reported that the White Carneau pigeon, even when fed a diet of grains, develops atherosclerosis having many of the morphological characteristics of the human disease. The study reported here was conducted to determine the fatty acid composition of plaques and other tissues of these pigeons when the birds were fed a "natural" diet.

Materials and methods. Sixty White Carneau pigeons, 5-8 years of age, were obtained from the Palmetto Pigeon Plant, Sumter, S. C. The pigeons were divided into 6 groups of 10 each. They had been fed corn, wheat, milo, and peas *ad libitum*. At necropsy all of the birds had grossly visible atherosclerotic lesions of the aorta. The tissues collected and pooled according to groups were: livers, sera, excised atherosclerotic plaques from aortas, and non-diseased portions of aortas. Special care was taken to remove all of the extraneous tissue from the adventitia of the aortas prior to the excision of lesions. Since the lesions almost always occurred at the distal segment of the thoracic aorta near the coeliac bifurcation(12), the segment from the aortic arch to a few millimeters proximal to the diseased area was taken as the non-diseased aorta. For serum and liver, duplicate samples were taken from each pool.

Lipids were extracted from the tissues according to Folch *et al*(13) with modifications. All solvents except ethanol and diethyl ether were redistilled. The tissue was homogenized in chloroform:methanol mixture, 2:1 (v/v), in the proportion of at least 20 ml of mixture per gram of tissue. For serum, 5 ml of the sample were slowly added to 100 ml chloroform:methanol mixture with con-

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stant stirring. The extract was allowed to stand in a covered beaker for at least one hour before being filtered through Whatman No. 2 filter paper pre-washed with hot ethanol and ether. The filtrate was collected in a 250-ml beaker, then placed in a 4-liter beaker. The 2 beakers were then slowly filled with distilled water, slow enough to avoid any unnecessary turbulence. The system was covered and allowed to stand overnight. On the following day the clear water-methanol phase was siphoned off leaving a layer about 4 mm in depth over an interphase and the lower chloroform phase. About 25 ml methanol was added to the washed extract to form a single phase, which was then evaporated to dryness under a stream of nitrogen at a water bath temperature of 40°C. The dried extract was immediately dissolved in hexane (Skellysolve B†) to a concentration of 10 mg/ml. The lipid extract was passed through a silicic acid-celite (1:1) column and 3 fractions (sterol esters, glycerides and free sterols, and phospholipids) were obtained by elution from the column with 15 ml of chloroform:petroleum ether, 1:9 (v/v), 20 ml of chloroform, and 20 ml of methanol respectively(14). To check for purity of the fractions, duplicate strips of glass-fiber paper impregnated with silicic acid(15) were spotted with each eluate and developed in 3% diethyl ether in petroleum ether. One chromatogram was dipped in 5% sulfuric acid and allowed to char on a hot plate for detection of sterol esters, glycerides, and free sterols; the other was dipped in 0.001% Rhodamine 6G in distilled water for 2 minutes and then viewed under ultraviolet light to detect phospholipids. Methyl esters of the component fatty acids of the 3 fractions were prepared(16) and analyzed in a gas-liquid chromatograph using a 6-foot, ⅛-inch column packed with 16% (w/w) diethylene glycol succinate polyester on Chromosorb W. The column was operated at a temperature of 195°C. Helium, at a flow rate of 68 ml/min, was the carrier gas. A hydrogen flame detector was used. The recorder was equipped with a disc integrator whereby the area under each fatty acid peak could be estimated as the percentage of the sum of the areas of all the peaks in the sample. Duplicate chromatograms were run on each sample. Student's t-test(17) was used to evaluate the significance of differences among the values for the fatty acids so obtained.

† Skelly Oil Co., Kansas City, Mo.

Results and discussion. The data of Table I show the major fatty acid composition of the 3 lipid fractions from the 4 tissues studied. In this Table, brackets connect the values which differ significantly from each other, and the probability values obtained by statistical comparison are also shown. Values not bracketed indicate that the differences between them are not significant at these levels.

1. *Sterol esters.* It can be seen (Table I) that 18:1 fatty acid comprises the major fatty acid of this fraction in diseased aorta (43.9% of the total fatty acid). This value is markedly higher than that for 18:1 fatty acid in the non-diseased aorta (24.2%), or in the serum (31.8%). Liver sterol esters, on the other hand, contained about the same amount of 18:1 fatty acid (44.4%) as that from plaque lipid. In general each of the sources of lipid studied (plaques, non-diseased aorta, liver, and serum) appears to yield sterol esters having its own distinctive pattern of fatty acids. While quantitative differences exist among the fatty acids derived from the glycerides and phospholipids of these 4 sources, these classes of lipid seem to have a rather uniform pattern of fatty acid distribution among the tissues studied.

It can be seen from Table I that no values are reported for fatty acids more unsaturated or of greater chain length than 18:2 fatty acid. In pigeons, such fatty acids, including arachidonic acid, are in low concentration and their quantitation has been too erratic to be meaningful, owing to the limitations of the instrument available.

Liver is considered to be the major source of sterol esters in the body(18). Thus most of the sterol esters present in the plaque and non-diseased aorta would have to be transported by and deposited at the sites from plasma. Since the fatty acid composition of

TABLE I. Major Fatty Acid Composition of Sterol Esters, Glycerides, and Phospholipids of Various Tissues.†

	Percent of the Total Fatty Acids (No. of Carbons:Double Bonds)				
	16:0	16:1	18:0	18:1	18:2
Sterol Esters					
Plaques	*12.8	7.0	3.0	43.9	31.2
Non-Diseased Aorta	19.1	9.0	9.4	24.2	19.1
Liver	21.5	5.2	14.0	44.4	14.0
Serum	10.1	3.8	4.0	31.8	49.6
Glycerides					
Plaques	23.7	8.6	10.4	42.2	12.4
Non-Diseased Aorta	24.2	7.3	12.3	41.5	10.8
Liver	20.6	5.0	13.3	42.3	17.3
Serum	21.4	5.0	9.5	46.0	18.1
Phospholipids					
Plaques	31.8	4.2	26.1	21.1	9.0
Non-Diseased Aorta	22.4	3.1	27.0	22.7	8.6
Liver	13.8	1.2	34.9	15.4	25.8
Serum	15.7	1.3	33.9	16.6	25.9

* $P < .001$ ---- $P < .005$ ——

† Values for plaques and non-diseased aorta represent a mean of 6 determinations while values for liver and serum represent a mean of 12 determinations.

the serum sterol esters is vastly different from those of other tissues, and also the composition of sterol esters of plaque and non-diseased aorta are distinctly different from each other, one could suppose that (1) the sterol esters synthesized in the liver are selectively mobilized into the plasma for transport; and (2) the deposition and/or exchange of sterol esters between the plasma and the plaque and non-diseased aorta are also selective rather than at random, and that the selectivity of the plaque is different from that of the non-diseased aorta. On the other hand, with the use of C^{14}-labeled acetate, fatty acids have been shown to be synthesized *in vitro* by pigeon aorta and especially in diseased aorta the esterification of sterols with newly synthesized fatty acids accounts for as much as 50% of the total radioactivity(19).

The similarity of the sterol ester fatty acid composition observed in this study to that reported by Swell *et al*(20) for man is remarkable (Table II). The fatty acids, 16:0, 18:1, and 18:2 comprised 88% of total sterol ester fatty acids of the plaques from both

TABLE II. Comparison of Sterol Ester Fatty Acid Composition (%) of Various Tissues of Pigeon and Human.

		Fatty acids						
		A 16:0	B 18:1	C 18:2	Sum of A, B, and C	D Saturated	E Monoenoics	Sum of D and E
Plaque	Pigeon	12.8	43.9	31.2	87.9	15.8	50.9	66.7
	Human*	25.1	37.8	25.1	88.0	26.6	44.9	71.5
Liver	Pigeon	21.5	44.4	14.0	79.9	35.5	49.6	85.1
	Human	24.1	37.3	16.3	77.7	35.3	44.7	80.0
Serum	Pigeon	10.1	31.8	49.6	91.5	14.1	35.6	49.7
	Human	13.7	25.8	43.3	82.8	16.6	30.2	46.8

* Swell *et al*(20).

TABLE III. Comparison of Phospholipid Fatty Acid Composition (%) of Various Tissues of Pigeon and Human.

		Fatty acids						
		A 16:0	B 18:1	C 18:2	Sum of A, B, and C	D Saturated	E Monoenoics	Sum of D and E
Plaque	Pigeon	31.8	21.1	9.0	61.9	57.9	25.3	83.2
	Human*	31.3	22.9	8.7	62.9	50.4	27.8	78.2
Serum	Pigeon	15.7	16.6	25.9	58.2	49.6	17.9	67.5
	Human	39.2	24.5	11.4	75.1	53.6	27.1	80.7

* Swell *et al*(26).

pigeon and man. The major acid is 18:1 in both plaque and liver while 18:2 is the major acid in serum. The plaque and liver contained considerably more saturated and monoenoic acids than serum. No explanation could be given for the differences between the percentages of 16:0 fatty acid and total saturated acids in plaque of pigeon and human aorta but it should be pointed out that the plaque material from pigeon aorta included the media and adventitia whereas the human sample did not.

2. *Glycerides* (Table I). Except for the slight dissimilarity of the relative percentages of 18:2 fatty acid from plaque and non-diseased aorta when compared to those of liver and serum, the fatty acid compositions of all 4 tissues are quite similar. Because of the uniformity of the fatty acid compositions of this fraction, no conclusion can be reached concerning the origin of the glycerides in the plaques, or the amounts deposited, synthesized or exchanged. This observation agrees with that made by Zilversmit *et al*(21) for rabbits.

3. *Phospholipids* (Table I). Except for the difference in the relative percentages of 16:0 fatty acid from the phospholipids of plaque and non-diseased aorta, there are no significant differences in the percentages of other fatty acids in these 2 tissues. Bottcher and van Gent(1) have reported that with increasing degree of atherosclerosis, there are increasing percentages of saturated fatty acids, especially 16:0, in the phospholipid of human aortic wall. The higher value of 16:0 acid in the plaque compared to that of the non-diseased aorta in this study also could be due to the atherosclerotic condition, assuming that the above reported observation holds true for pigeon as well as human. The fatty acid compositions of the phospholipids of liver and serum are essentially the same. However, the fatty acid compositions of phospholipids from plaque and non-diseased aorta were quite different from those of liver and serum. It is conceivable that the fatty acid compositions of phospholipids of liver and serum resemble each other because serum phospholipids originate from liver(22). The similarity in fatty acid composition of phospholipids in plaque and non-diseased aorta would suggest that the majority of the phospholipids present in both these tissues originate from the same source. It has been shown in our laboratory that phospholipids can be synthesized *in vitro* by pigeon aorta (19). This is also true for arterial tissue from human beings(23) and from rabbits(24,25). If the phospholipids present in the plaque and non-diseased aorta originated by synthesis *in situ,* then the differences in fatty acid compositions of the phospholipids of these tissues and those of the liver and serum could be expected.

The major fatty acid compositions of phospholipids of atherosclerotic plaques from pigeon and human(26) are very similar (Table III) while those of the serum are quite different. This may suggest that the sera of these 2 species contain classes of phospholipids in diverse proportions, each having a specific fatty acid composition of its own.

In this study the size of samples available did not permit determination of the quantitative distribution of ester sterols, free sterols, glycerides, and phospholipids. However, such values are available from other studies in this laboratory(27). The data in Table IV show the typical quantitative distribution of

TABLE IV. Lipid Contents of Aortas of Diseased and Non-Diseased Pigeons.*

Lipid fraction	Non-diseased, μM/g	% of total lipid	Diseased, μM/g	% of total lipid
Cholesterol ester sterol	.4	.6	10.8	6.0
Free cholesterol	5.6	7.8	19.1	10.6
Triglycerides	46.7	65.0	115.1	63.9
Phospholipids	18.7	26.0	24.4	13.6

* Hoffman(27).

these 4 classes of lipid studied in non-diseased and diseased arteries of pigeons. These values were obtained from pooled arteries of White Carneau pigeons and are typical of values obtained from pigeons of the same age group in the present study. It can be seen that the sterol ester fraction shows the greatest difference when diseased and non-diseased arteries are compared (27-fold increase). Free sterol, triglycerides and phospholipids are less strikingly different.

Therefore, as in other species studied(3, 28), the sterol esters of pigeon aortas show the greatest increase in quantity when diseased aortas are compared to non-diseased tissue. Also, the fatty acid distribution of this fraction indicates different patterns among various tissues. It seems logical that special attention should be paid to the changes of fatty acid patterns of this fraction in the study of the development of atherosclerosis.

The data obtained in this study have been derived from an animal which develops severe atherosclerosis naturally, without stimulation by dietary cholesterol. A study is now in progress in this laboratory comparing the fatty acid composition of tissues from White Carneau and Show Racer pigeons, the latter being relatively more resistant to spontaneous atherosclerosis.

Summary. Atherosclerotic plaque, non-diseased aorta, liver and serum were collected from 5-8 year old White Carneau pigeons fed a grain diet. Lipids extracted from these tissues were fractionated into sterol esters, glycerides and phospholipids. Methyl esters of the component fatty acids of these fractions were prepared and quantified by gas-liquid chromatography. Unique fatty acid patterns of sterol esters were found in each of the various tissues. There is no difference in the fatty acid compositions of glycerides from the tissues studied. Phospholipids of plaques and non-diseased aorta exhibit similar fatty acid patterns as do those of liver and serum. However, the fatty acid compositions of phospholipids of plaque and non-diseased aorta are distinctly different from those of liver and serum. There is a remarkable resemblance between the fatty acid compositions of sterol esters and phospholipids of various tissues of pigeon and man.

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Collagen Synthesis and Turnover in the Growing Rat Under the Influence of Methyl Prednisolone.* (29653)

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The physical as well as the chemical properties of collagen have been shown to change with age. The content of soluble collagen of skin decreases rapidly with age whereas its tensile strength becomes progressively greater. The literature in this connection has been reviewed by Harkness(1).

Following injection of labeled glycine, the specific activity of skin collagen was greater in young animals than in older ones(2,3,4), reflecting the increased synthesis during the period of rapid growth. Growth retardation induced by dietary as well as hormonal imbalance were significant factors in reducing the rate of skin collagen synthesis as judged by a decreased radioactive uptake(3,5).

Experiments following administration of C^{14}-proline indicate a turnover time of more than 150 days for the insoluble fraction of skin collagen and 25 days for the citrate soluble fraction(6). Values reported by Harkness(1) reflect a turnover of the order of hundreds of days. This relative inertness of collagen is further supported by the findings of Popenoe and Van Slyke using C^{14}-lysine as a tracer(7). On the other hand experiments also using C^{14}-lysine rendered half life values of 28 days for the insoluble collagen and 17 days for the soluble fraction (8).

In most of these experiments only a few animals were used, and observations were made over relatively short periods of time, thus making extrapolation of values corresponding to long half lives inaccurate. Because of this lack of quantitative information, the effects of added variables, such as age, hormones, diet and others become hard to interpret. The following experiments were attempted to try to overcome some of these problems. The incidental variation was minimized by performing small skin biopsies from the same animals at the different time intervals considered.

Experimental procedure. At initiation of the experiment 21 male rats of the Holtzman strain weighing in the range of 142 to

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