THE INCORPORATION OF ACETATE-1-C^{14} INTO CHOLESTEROL AND FATTY ACIDS BY SURVIVING TISSUES OF NORMAL AND SCORBUTIC GUINEA PIGS

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(Received for publication, October 7, 1955)

The advanced lesions of atherosclerosis are a complex summation of many secondary changes, superimposed upon, and tending to obscure, a basic primary process. These secondary phenomena include phagocytosis of lipide by macrophages, vascularization of the intima, intimal hemorrhage, and thrombosis. It is to the earliest lesion of atherosclerosis that attention must be turned if the fundamental underlying process in this disease is to be unravelled.

Aschoff (1) considered that the earliest demonstrable lesion of human atherosclerosis is a deposit of stainable lipide along the arterial internal elastic membrane. He believed that the mechanical stress imposed upon the artery caused a change in the properties of the intimal ground substance rendering it more permeable to the plasma lipides. The finding that cholesterol feeding produces atherosclerosis associated with hypercholesterolemia in experimental animals seemed to lend support to this idea. However, Duff (2) has pointed out that the hypercholesterolemia and the deposit of lipide in the reticulo-endothelial system of these animals have no counterpart in the atherosclerotic human. Nevertheless cholesterol feeding has remained the method of producing experimental atherosclerosis (3). Consequently, to a large extent attention has been focused on the plasma lipides as the causative agent in the pathogenesis of atherosclerosis in man.

Recently, however, the possibility that the artery itself may contribute to the accumulation of lipides and sterols has been considered. This notion is supported by the observation that the aorta *in vitro* synthesizes these compounds. Chernick, Srere, and Chaikoff (4) have shown that surviving rat aorta tissue is capable of incorporating C^{14}-acetate into fatty acids and P^{32} into phospholipides. Similarly, Siperstein, Chaikoff, and Chernick (5) have demonstrated the incorporation of C^{14}-acetate into cholesterol by the aorta of rabbit and chicken; the highest specific activity of cholesterol isolated from arterial tissue was found to be approximately one-quarter that observed with liver slices, under the same conditions. Also, Eisley and Pritham (6) have shown the synthesis of cholesterol from labelled acetate by the aortas of hogs and turkeys and have found the aorta of hog to be more active than turkey aorta in this regard. Zilversmit, Shore, and Ackerman (7) have concluded from results of experiments in which rabbits were injected with P^{32}, that the phospholipides

in the atheromatous aortas of the cholesterol group were synthesized five times as fast as in the aortas of the control group. On the other hand, Biggs and Kritchevsky (8), using labelled cholesterol, have obtained evidence that while most of the cholesterol in the tissue deposits of cholesterol-fed rabbits is of exogenous origin, a small amount of endogenous cholesterol appears in the aorta.

It remained to determine what factors might alter the metabolism of arterial tissue so that lipide accumulation would ensue in the absence of cholesterol feeding. Studies to this end have been made possible through the medium of experimental scurvy; in the scorbutic guinea pig the histological lesions of atherosclerosis are manifested rapidly, but are unaccompanied by the extreme hypercholesterolemia and the deposition of lipide in the reticulo-endothelial system which occur in the cholesterol-fed animal (9). Some impetus has been added to this work by the findings of Becker *et al.* (10) who showed that severely scorbutic guinea pigs, compared with normal animals fed *ad lib.*, incorporated 6 times as much C^{14} from acetate-1-C^{14} into adrenal cholesterol and 1.8 times as much into liver cholesterol. Thus ascorbic acid deficiency exerts a stimulatory effect on the *in vivo* synthesis of this sterol.

The study reported in this paper was undertaken in an attempt to throw light on the effect of scurvy on the *in vitro* synthesis of cholesterol and fatty acids by the isolated adrenal, aorta, and liver. We also wished to study the ability of the aorta, which in scurvy is atherosclerotic, to accumulate lipides independent of extra-arterial sources. The concurrent study of these three tissues would permit an assessment of their relative metabolic activities.

EXPERIMENTAL AND RESULTS

Sodium Acetate-1-C^{14}.—Sodium acetate-1-C^{14} was prepared from $Ba(C^{14}O_3)_2$, using the Grignard reagent, methyl magnesium iodide. A sterile aqueous solution of sodium acetate-1-C^{14} containing 1.97×10^6 C.P.M. per ml. was used in the incubations; calculated as anhydrous sodium acetate the concentration of the radioactive solution was 4 mg. per ml. of solution.

Radioactive Analyses.—Radioactivity determinations were done in a windowless gas flow counter. The cholesterol samples were counted as infinitely thin plates; the fatty acid fractions and the non-saponifiable matter were assayed for C^{14} by the differential method of Heard *et al.* (11).

Colorimetric Determination of Cholesterol, Hexosamine, and Ascorbic Acid.—Serum cholesterol was analyzed by a modification of the method of Bloor (12). Liver and spleen ascorbic acid was analyzed by the method of Willis and Fishman (13). Serum hexosamine was analyzed by a modification of the method of Boas (14).

Experimental Animals.—The guinea pigs were divided into five series, each consisting of a scorbutic group and a pair-fed control group. The feed was rabbit pellets produced by Ogilvie Flour Mills, Montreal, and is known to be effective in producing scurvy (9). The pair-fed control groups received a liberal amount of crystalline ascorbic acid mixed daily with their diet. The scorbutic groups of series A, B, and C were deprived of ascorbic acid for 28 days, those of series D and E were deprived for 20 days.

Preparation of Tissues.—The animals were guillotined (series A, B, and C) or stunned by a blow on the head (series D and E) and the following organs were removed: liver, adrenals, and aorta (all series), blood (series A, B, and C) and spleen (series D and E). Liver slices of 0.5

mm. thickness, prepared with a Stadie-Riggs microtome and ranging from 1.19 to 1.97 gm. per animal, quartered adrenals, and longitudinally cut aortas were incubated in a Krebs-Ringer bicarbonate buffer, pH 7.4. The gas phase was 95 per cent O_2–5 per cent CO_2, the bath temperature 37°C., and the time of incubation 3 hours. The liver slices from each animal were

TABLE I

Incorporation of Sodium Acetate-1-C^{14} into Tissue Cholesterol

Series	No. of animals		Liver cholesterol		Adrenal cholesterol		Aorta non-saponifiable	
	Control	Scorbutic	c.p.m./mg. Control	c.p.m./mg. Scorbutic	c.p.m./mg. Control	c.p.m./mg. Scorbutic	c.p.m./mg. Control	c.p.m./mg. Scorbutic
A	6	5	176	108	146	206	29	67
B	7	5	56	132	129	120	41	34
C	5	9	—	119	92	126	58	34
D	6	5	296	1,029	87	178	28	25
E	6	5	909	483	198	556	14	11
Mean			359	374	130	237	34	34
Standard deviation			379	399	45	182	16	21

TABLE II

Incorporation of Sodium Acetate-1-C^{14} into Tissue Fatty Acids

Series	Liver fatty acids		Adrenal fatty acids		Aorta fatty acids	
	c.p.m./mg. Control	c.p.m./mg. Scorbutic	c.p.m./mg. Control	c.p.m./mg. Scorbutic	c.p.m./mg. Control	c.p.m./mg. Scorbutic
A	97	179	44	62	37	48
B	188	151	59	56	70	55
C	105	201	39	24	55	30
D	150	360	33	71	60	142
E	403	191	87	59	79	30
Mean	189	216	52	54	60	61
Standard deviation	125	82	22	18	16	46

incubated in a 125 ml. Erlenmeyer flask containing 10 ml. of buffer and 4 mg. of sodium acetate-1-C^{14}; the pooled adrenals and aortas from each group were incubated under similar conditions.

Extraction and Fractionation of Lipides.—The contents of the liver slice incubation flasks from each group of animals were pooled and hydrolyzed by refluxing for 6 hours in 200 ml. of 95 per cent ethanol and 25 gm. of potassium hydroxide. The contents of each incubation flask of adrenals and aortas were hydrolyzed by refluxing for 6 hours in 40 ml. of 95 per cent ethanol and 5 gm. of potassium hydroxide. Each cooled saponification mixture was diluted

with an equal volume of water and extracted 5 times with petroleum ether (b.p. 30–60°C.). The combined petroleum ether extract was washed with water to neutrality, dried over anhydrous sodium sulfate, filtered, and distilled to dryness to yield the non-saponifiable fraction. The aqueous alkaline phase and the water washings of the non-saponifiable fraction were combined, acidified to pH 1 with dilute hydrochloric acid, and extracted 5 times with petroleum ether. The combined petroleum ether extract was washed with water to neutrality, dried over anhydrous sodium sulfate, filtered, and distilled to dryness to yield the fatty acid fraction.

TABLE III

Analyses of Liver Ascorbate, Serum Cholesterol, and Serum Hexosamine

Series	Liver ascorbate				Serum cholesterol				Serum hexosamine			
	mg./100 gm. Control		*mg./100 gm.* Scorbutic		*mg./100 ml.* Control		*mg./100 ml.* Scorbutic		*mg./100 ml.* Control		*mg./100 ml.* Scorbutic	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
A	25.0	6.1	2.0	0.6	47.0	11.4	55.0	13.2	111.8	7.3	130.8	20.9
B	18.1	8.1	1.9	0.6	46.9	9.4	62.2	13.1	119.0	32.9	122.4	8.4
C	25.2	4.2	1.5	0.4	63.3	14.6	70.8	18.2	112.3	7.9	128.2	11.5
Over-all mean and S.D.					52.1	13.6	64.4	16.4	114.6	20.1	127.4	13.4

Serum cholesterol, $P < 0.02$; 36 degrees of freedom.
Serum hexosamine, $P < 0.05$; 36 degrees of freedom.

TABLE IV

Analyses of Spleen Ascorbate

Series	Spleen Ascorbate			
	mg./100 gm. Control		*mg./100 gm.* Scorbutic	
	Mean	S.D.	Mean	S.D.
D	66.2	11.0	1.2	0.5
E	55.0	8.0	2.1	1.6

Cholesterol was obtained from the liver and adrenal non-saponifiable fractions by the addition of 1 per cent digitonin solution in 80 per cent ethanol. The digitonides were washed twice with acetone-ether (2:1) and twice with ether and then dried at 78°C. *in vacuo* over phosphorous pentoxide. Cholesterol was isolated from the digitonides by the method of Bergmann (15).

Results of radioactivity determinations are given in Tables I and II. The results of the colorimetric determinations of serum cholesterol and hexosamine, and liver and spleen ascorbate are given in Tables III and IV.

DISCUSSION

From the results of the experiment it is apparent that there is no significant difference in the rate of incorporation of C^{14} from acetate-l-C^{14} into

cholesterol and fatty acids by the liver, adrenal, and aorta of scorbutic guinea pigs compared to those of pair-fed control animals. Thus under the conditions of the experiment no effect of ascorbic acid deficiency upon cholesterogenesis and lipogenesis is detectable. It is clear that this *in vitro* finding does not rule out entirely the possibility of a dependence, however indirect, of cholesterol and fatty acid synthesis upon ascorbic acid. Indeed, Becker *et al.* (10) have found that *in vivo* the scorbutic animal exhibits an increased rate of incorporation of C^{14}-acetate into liver and adrenal cholesterol. While our investigation was in progress, Becker *et al.* (16) reported that on incubation of liver slices of normal and scorbutic animals with acetate-l-C^{14}, the specific activities of the cholesterol and fatty acids from the ascorbic acid–deficient livers were about double those found in the control tissues in 1 hour incubation; opposite results were found in 2 and 3 hour experiments. It is noted that the duration of our incubations was 3 hours. At present no explanation can be offered for the discrepancy between our results and those of Becker *et al.*

It is evident that, under conditions of cholesterol feeding, part of the lipide in the arterial intima is synthesized *in situ* and the remainder is derived from the hypercholesterolemic plasma (8). From the results of the experiment it is impossible to determine the source of the extra lipide accumulated in the artery of the scorbutic animal. The possibilities of an accelerated rate of synthesis of cholesterol and fatty acids in the aorta, or of a normal rate of synthesis accompanied by a diminished rate of degradation, or, of an increased permeability of the artery to the plasma lipides are all plausible but in the light of our results the first possibility appears unlikely.

Despite the failure to ascertain the origin of intimal lipide in atherosclerosis, certain interesting points have appeared. It is noted that the specific activities of the fatty acids of the aorta are approximately equal to those of the adrenal fatty acids and almost one-third those of the liver fatty acids. Also the specific activities of the aorta non-saponifiable matter are as high as one-quarter those of the adrenal cholesterol and about one-tenth those of the liver cholesterol. The paucity of aorta tissue precluded the isolation of cholesterol in crystalline state from this tissue, and hence a direct comparison of the specific activities of the cholesterol of the three tissues cannot be made. It may be concluded that arterial tissue is quite active in lipide synthesis. Moreover, in regard to the metabolic activity of the aorta, other investigators have found that rat thoracic aorta tissue respires at a rate approximately one-tenth that of liver slices prepared from the same animal (17).

The finding of an elevation in serum hexosamine in scurvy (Table III) confirms the results of Pirani and Catchpole (18). A similar observation has been made in diabetes mellitus associated with atherosclerosis (19) in the human but it cannot be considered to imply any more than depolymerization of the polysaccharides which form the ground substance. This phenomenon

occurs in a wide range of conditions in which such a breakdown is known to exist (20).

The serum cholesterol was significantly elevated in the scorbutic animals compared with the controls (Table III). The over-all mean in the control animals was 52.1 mg. per cent, whereas in the scorbutic animals it was 64.4 mg. per cent. This degree of elevation of serum cholesterol has been noted by Maddoch (21) and also has been observed in a group of guinea pigs subjected to chronic scurvy in our study of the histological lesions of atherosclerosis.

SUMMARY

The synthesis of cholesterol and fatty acids from acetate-l-C^{14} by the isolated liver, adrenal, and aorta of scorbutic and pair-fed control guinea pigs has been studied. It was found that ascorbic acid deficiency does not affect the rate of incorporation of C^{14}-acetate into cholesterol and fatty acids by the tissues investigated, under our experimental conditions. The relatively high metabolic activity of the artery with regard to cholesterogenesis and lipogenesis was noted. The elevation of serum cholesterol and hexosamine in scurvy has been confirmed.

We are grateful to the Medical Research Division of the National Research Council, Ottawa, and the National Cancer Institute, United States Public Health Service, for financial support of the investigation. We extend our thanks to Dr. H. M. Slatis for advice and assistance concerning the statistical analysis of the data. We are indebted to Dr. M. Francoeur for assistance in the preparation of the liver slices. We wish to express our appreciation to Dr. A. Malkin for his valuable suggestions and advice.

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