Deposition of Cholesterol in Experimental Rabbit Atherosclerosis.* (25613)

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The following investigations were made to study the fate of cholesterol administered to rabbits in amounts known to effect atherosclerotic lesions.

Methods. Several groups of rabbits were fed commercial cholesterol (Wilson), each animal receiving 900 mg/day for 12 weeks. In each group the commercial material was replaced for 6 days as outlined below by one or the other of 2 types of radioactive material. Cholesterol-C^{14}, obtained from livers of rats injected with sodium acetate-1-C^{14} for various purposes, was purified through the digitonide and the dibromide. Cholesterol-H^3 was prepared by Wilzbach's method at New England Nuclear Corp. from cholesterol acetate and tritium and was stabilized in this laboratory by boiling several times with methanolic sodium hydroxide until the last operations showed a nearly constant count of 3.196×10^6 min./mg. This material was dissolved in boiling methanol with calculated amounts of commercial cholesterol (Wilson) and left to crystallize in the cold. It could not be purified further through the dibromide because repeated treatment of a sample with bromine and afterwards with zinc and acid showed a continuous loss of radioactivity. Cholesterol was dissolved in ether and poured together with 5% cotton oil over the rabbit chow and ether was eliminated by heating under stirring or shaking on a water bath. Feeding of radioactive and commercial material was done every day except on Sunday. Young healthy male and female Dutch belted rabbits weighing 3 to 4 lb were arranged in groups of 10 to 13 animals so that each group contained an equal number of males and females. One group was fed cholesterol-H^3 during the first week, another during seventh week and one during twelfth week. In another series cholesterol-C^{14} was fed during the first week and to 3 animals for 2 days in the twelfth week. At end of the week of radioactive cholesterol feeding, 3 or 4 animals were killed and worked up, while remainder of group was continued on ordinary cholesterol, until at end of 12 weeks all animals were killed.

The animals were killed by heart puncture and blood was taken for cholesterol determinations. The animals were then dissected into aortae, livers, small intestines, adrenals, brains, kidneys and blood; the corresponding tissues for each group were combined, weighed and hydrolyzed with 30% KOH and the cholesterol isolated as digitonide. In the C^{14} experiments this material was further purified by bromination. All these operations have been described(1). Cholesterol-H^3 was counted in a scintillation counter, while for cholesterol-C^{14} an end window or scintillation counter was employed.

All animals showed atherosclerotic lesions of varying degrees on macroscopic inspection but no histological evaluation was made. Cholesterol determination in blood serum at time of killing showed usual considerable rise of serum cholesterol in most animals.

Results. The results are presented in Tables I and II. Radioactive cholesterol was found at end of 1 week feeding in each tissue but in different amounts. The brain showed lowest deposition. Even after only 2 days feeding of cholesterol-C^{14} as in Exp. 3 of Table I the substance isolated from aortae contained radioactivity. From the count of this material and total radioactivity fed, one can calculate the amount of cholesterol laid down during feeding and relate it to weight of tissue. It is thus found that the amount of cholesterol deposited in the aorta/feeding day/animal/g of wet aorta weight was 0.03 mg (0.029-0.032) for the 3 experiments carried out with cholesterol-C^{14}. Exp. 2 showed that after deposition of radioactive substance in the aorta, further feeding of ordinary cholesterol not only covered the radioactive layer but replaced the radioactive substance and a nearly

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complete loss of it took place during the following 11 weeks. This disappearance of radioactivity cannot be explained by mere dilution of the active substance *in situ* because at the end of, for example Exp. 2, the aortae had increased their cholesterol content from 2.5 mg to 5.4 mg/g wet tissue, while radioactivity decreased from 130 counts to a trace. A similar picture was obtained for all other tissues. In almost all tissues purification of the

TABLE I. Feeding of Cholesterol-C^{14} and of Commercial Cholesterol to Rabbits.

Exp. No.	Cholesterol-C^{14} Fed week	Killed week	Aorta		Liver		Small intestine		Adrenal		Kidney		Blood		Brain		Counts/min./mg in cholesterol-C^{14} fed
1	1	1	1.9	702	6.7	1152	1.28	937	33.3	536	3.3	578	1.5	1078	9.4	14	1768
				604		1126		1011		368		636		1127		12	
2	1	1	2.5	130	2.7	365	2.3	373	43.1	217	2.9	185	1.0	312	13	3	473
				246		297		317		270		138		308		3	
	1	12	5.4	trace	7.7	trace	.6	trace	45.9	trace	2.6	trace	1.8	trace	5.0		
3	*	12	6.4	39		57		355									1392
				25		40		359									

In each column figure on left side represents mg cholesterol-C^{14} isolated/g wet tissue. The upper figure on right side gives counts/min./mg in isolated digitonin cholesterol-C^{14} and the lower figure the corresponding counts after bromination treatment.

* In this experiment cholesterol-C^{14} was fed for last 2 days only.

TABLE II. Feeding of Cholesterol-H^3 and of Commercial Cholesterol to Rabbits.

Exp. No.	Radioactive cholesterol Fed week	Killed week	Aorta		Liver		Small intestine		Adrenal		Kidney		Blood		Brain		Counts/min./mg in cholesterol-H^3 fed
1	1	1	1.5	2410	2.8	16880	2.4	18960	35.6	10805	2.8	6266	.4	11550	13.4	0	29500
	1	12	3.0	208	7.4	141	3.0	68	32.1	190	4.3	122	2.8	105	8.3	102	
2	7	7	2.9	1250	8.9	6313	.8	5475	39.7	1472	2.3	2038	1.4	4094	18.2	56	11800
	7	12	2.5	1695*	4.4	1152	.6	803	109	2434*	1.3	1369	2.1	981	8.0	127	
3	7	7	3.0	960	6.7	2057	.95	5203	24.2	1515	3.7	1382	3.3	2969	10.9	39	9155
	7	12	1.63	862	4.95	1039	1.19	756	57.2	1291	2.21	898	.31	865	9.92	56	
4	12	12	3.1	2900	15.4	4610	1.2	15030	62.8	1372	2.2	3615	2.5	8160	9.0	72	29500

In each column figure on left represents mg cholesterol-H^3 isolated/g wet tissue. Figure on right gives counts/min./mg in the isolated cholesterol-H^3.

* These figures were assumed to be due to an error and the experiment was repeated as Exp. 3.

digitonin precipitate by bromination showed some formation of cholesterol derivatives, which were still precipitable.

Much the same situation was found in Exp. 1-4 of Table II with cholesterol-H^3. When this substance was fed in the first or seventh week and ordinary cholesterol was given during remaining part of experiments, radioactivity at the end of 12 weeks decreased considerably in all tissues. As in experiments with C^{14} this decrease was greater than a possible dilution caused by increase of total cholesterol in the tissues. For example, in Exp. 1 the aorta cholesterol doubled from 1.5 to 3.0 mg/g wet tissue whereas the count dropped from 2410 to 208. In other tissues such as liver, small intestine or adrenal, this discrepancy, possibly because of their specific metabolism, was more pronounced. Only in the brain was a definite increase of radioactive material observed at end of experiments, although these counts remained quite low. This may be so because the brain has apparently a very low cholesterol turnover—adult brain does not synthesize cholesterol from acetate(*2*)—and exchange of cholesterol between blood and brain may be very limited(3). The decrease in counts of cholesterol in all tissues is therefore determined not only by dilution with non-radioactive cholesterol present in the tissue prior to feeding, or by subsequent deposition of non-radioactive substance fed after the radioactive feeding period, but also by the specific metabolism including biosynthesis of cholesterol in each tissue. It is further interesting to note that even after one week's feeding whether it was the first, seventh or twelfth week in the experiment, the counts of cholesterol-H^3 used were diluted in each tissue at a different rate. Thus for example the relation of the count in starting cholesterol to the count in cholesterol isolated from the aortae in Table II is 8.2, 10.6, 10.5 and 9.8 after 1 week of feeding of radioactive material. The same calculation however gives 36.4 and 25.1 in Exp. 1 and 2 (Table I) with cholesterol-C^{14}, which were carried on for 1 week. It was pointed out above that amount of cholesterol laid down in the aortae/day/animal, and /g of wet aorta weight can be calculated as 0.03 mg. The same calculation extended to experiments with cholesterol-H^3 in Table II, gives considerably lower values, namely 0.011 (0.005-0.017). Similar differences are found between experiments of Tables I and II for other tissues investigated, with exception of the brain where the figures are too small to allow evaluation. Dilution of the original count was always greater for cholesterol-H^3 than for cholesterol-C^{14}. In both types of experiments however the amount of radioactive material fed was the same, namely 900 mg/day/animal and it is therefore evident that the amount of deposited radioactive material must be the same for each corresponding pair of tissues of Tables I and II. The explanation of the discrepancies seems to be that in the C^{14}-material the radiocarbon is part of the skeleton of the molecule, while the radioactive hydrogen is on its periphery, exposed to attack of oxygen, and therefore can be detached from the molecule without destroying its integrity. Borgstroem and Wintersteiner(4) have indeed shown that cholesterol in colloidal suspension, under conditions similar to those in blood, is very easily oxidized by molecular oxygen, giving 7-keto- and 7α- or 7β-hydroxycholesterol. Katienagar and Morton(5) found in plaques of human aortae Δ^3,Δ^5-diene-7-one and other substances derived from cholesterol by oxidation. These findings were confirmed by Henderson(6) who by paper partition chromatography demonstrated in human aortae the presence of Δ^5-cholestene-3β,7β-diol, Δ^5-cholestene-3β,7α-diol, cholestane-3β,5β,6β-triol and Δ^5-cholestene-3β, 24 (or 25)-diol. All these substances can only be formed from cholesterol by loss of hydrogen which in cholesterol-H^3 causes the observed decrease of radioactivity. A further loss of hydrogen and therefore tritium may be effected by dehydrogenation. Festenstein and Morton(7) made probable the presence in tissues of a specific dehydrogenase, the normal substrate of which is cholesterol.

This peripheral oxidation of cholesterol may account for the striking differences between experiments with the 2 types of radioactive cholesterol (*i.e.*, C^{14} or H^3). However the almost complete disappearance of radio-

activity when one week of radioactive cholesterol is followed by 7 or 11 weeks of ordinary cholesterol, is explained by degradation of the cholesterol skeleton itself in formation of bile acids and by elimination of cholesterol through the feces.

The figures for cholesterol isolated from blood suggest that a large part of oxidative degeneration must occur in blood, in agreement with experiments of Borgstroem and Wintersteiner(4). The small differences between the digitonin and dibromo-cholesterol figures in Table I showed that in the course of 1 week during which radioactive material was fed, little degradation of the cholesterol skeleton itself occurred.

Continuation of experiments with both types of radioactive cholesterol by feeding non-radioactive substance resulted in considerably lower counts in most tissues. This shows that there is a continual exchange and subsequent degradation taking place between cholesterol of tissues and blood. Such reaction has been observed before(8) but it is surprising that in the aortae, where cholesterol is deposited in plaques, such an exchange is also functional. While it is not possible to decide how much of these changes occur in blood before and how much in the tissue after deposition of cholesterol, it is quite probable that almost all of it is finally broken down in the liver and so disposed of, thus explaining disappearance of radioactivity at end of experiments.

The experiments here reported are in agreement with Biggs and Kritchevsky(9) who fed cholesterol-H^3 to rabbits and established that exogenous cholesterol is deposited in tissues, and of Dayton(10) who fed cholesterol-C^{14} to chicks. The present work however shows that cholesterol, once deposited, is continuously exchanged between tissues including aortae and blood and that the amounts involved in these shifts are extremely small. The smallness of the deposit in the aortae—only 1/3000 of 1 percent of cholesterol administered—explains why the accumulation of deposits in the aortae takes as long as known from the course of human disease, and supports the view that cholesterol deposition is not the primary damaging factor in atherosclerosis.

Summary. Cholesterol-C^{14} and cholesterol-H^3 were fed to rabbits for 1 week and cholesterol was isolated from aortae, livers, small intestines, adrenals, kidneys, brains and blood. Isolated material from all tissues had a lower count than the material fed. It was surprising that cholesterol-H^3 counts were always comparatively lower than cholesterol-C^{14} counts. This discrepancy may be explained by a peripheral oxidation of the cholesterol-H^3 molecule, in which it loses radioactive hydrogen and therefore radioactivity. This peripheral oxidation is apparently mainly operative in blood. If after feeding of radioactive material non-radioactive cholesterol is fed for 7 or 11 weeks, radioactivity in both types of experiments is reduced to a small fraction of original values. From these data one can calculate that about 0.03 mg cholesterol are laid down in the aorta/animal/day/g of wet tissue, a very small part of the 900 mg fed/day/animal. The experiments show that there is a continuous exchange of radioactive cholesterol between tissues and blood in which even the plaques, formed in the aortae, must take part.

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1. Schwenk, E., Alexander, G. J., Fish, C. A., *Arch. Biochem. and Biophys.*, 1955, v58, 37.

2. Waelsch, H., Sperry, W. M., Stoyanoff, V. A., *J. Biol. Chem.*, 1940, v135, 297.

3. Nicholas, H. J., Thomas, B. E., *J. Neurochem.*, 1959, v4, 42.

4. Borgstroem, S., Wintersteiner, O., *J. Biol. Chem.*, 1941, v141, 597.

5. Katienagar, N. L., Morton, R. A., *Biochem. J.*, 1955, v60, 25.

6. Henderson, A. E., *J. Histochem. and Cytochem.*, 1956, v4, 153.

7. Festenstein, G. N., Morton, R. A., *Biochem. J.*, 1955, v60, 22.

8. Gould, R. G., *Am. J. Med.*, 1951, v11, 209.

9. Biggs, M. W., Kritchevsky, D., *Circulation*, 1951, v4, 34.

10. Dayton, S., *Circulation Res.*, 1959, v7, 468.

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