A comparison of the turnover and metabolism of cholesterol in normal and atherosclerotic monkey aortas

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Abstract Rhesus monkeys were fed high cholesterol and cholesterol-free diets for 21–24 months. The animals were then given isotopic cholesterol intravenously and autopsied from 1 to 51 weeks later. The plasma and aortic cholesterol contents were 633 ± 130 mg/dl and 35.6 ± 11.4 mg/g dried tissue (45.5% in ester form) for atherosclerotic monkeys and 135 ± 25 mg/dl and 9.9 ± 3.6 mg/g (15.0% in ester form) for control monkeys, respectively. The minimal influx rate of cholesterol from plasma into the aorta was much greater for atherosclerotic animals, 0.470 ± 0.20 mg/g per day versus 0.088 ± 0.031 for control monkeys. There was a rapid turnover of both free and esterified cholesterol in the atherosclerotic aortas, greater than for normal aortas. These studies of cholesteryl ester metabolism indicated a likely origin of aortic cholesteryl ester from the plasma cholesteryl esters. Our data indicated a dynamic cholesterol metabolism and turnover in the aorta during atherogenesis.—**Lin, D. S., W. E. Connor, R. W. Wissler, D. Vesselinovitch, and R. Hughes.** A comparison of the turnover and metabolism of cholesterol in normal and atherosclerotic monkey aortas. *J. Lipid Res.* 1980. **21:** 192–201.

Supplementary key words free cholesterol · esterified cholesterol · rhesus monkey

Atherosclerotic lesions in both humans and experimental animals are largely composed of cholesterol, cholesteryl esters, other lipids, and connective tissues. The cholesterol content of the arteries of humans and experimental animals is greatly increased during the process of atherogenesis. On the other hand, in nonhuman primate studies, when severely atherosclerotic lesions underwent regression from dietary manipulation (1–3), the arterial content of cholesterol and cholesteryl ester diminished greatly. Therefore, information about the metabolism and turnover of cholesterol in the diseased and normal arterial wall is crucial to an understanding of both atherogenesis and regression.

In our previous isotopic studies of cholesterol turnover in man, the plasma cholesterol exchanged with both the free and esterified cholesterol of severely atherosclerotic human arteries (4). The turnover of cholesterol in the atheromatous lesions of the abnormal aorta was calculated to be 8.2 mg/g of dry tissue per month. The turnover time of this arterial cholesterol was estimated at 442 days. Other extensive atheromatous lesions of the common iliacs, femorals, carotid, and coronaries had similar values. These data indicated the dynamic character of even severe human atherosclerotic lesions.

Because of the impossibility of studying cholesterol metabolism in normal and less severely atherosclerotic human arteries with these techniques, we turned to an experimental animal and measured the turnover of free and esterified cholesterol in normal and atherosclerotic arteries of adult male rhesus monkeys. Such studies have not been performed previously. In the work to be described, we fed some adult male rhesus monkeys an atherogenic diet; others were given normal monkey chow. After 21 months of cholesterol feeding, hypercholesterolemia and considerable atherosclerosis resulted. All animals (normal as well as atherosclerotic) were then injected with isotopic cholesterol and the aortas were removed at subsequent intervals of time. The mass of total cholesterol, free and esterified cholesterol, and their radioactivities in both plasma and aortas were measured. From these data, the influx rates of plasma cholesterol and the turnovers of cholesterol and cholesteryl ester in normal and atherosclerotic arteries were examined and compared.

MATERIALS AND METHODS

Twenty-two adult male rhesus monkeys were paired according to their body weights and initial plasma lipid levels. One-half of the animals were fed an atherogenic diet which consisted of a low-fat, cholesterol-free Purina® primate chow to which was added

TABLE 1. The composition of the control and atherogenic diets

Ingredients	Control Diet	Atherogenic Diet
Cholesterol	0	2.0 g
Butter oil[a]	0	12.5 g
Coconut oil	0	12.5 g
Corn oil	25.0 g	0
Vitamin mix[b]	1.0 g	1.0 g
Gelatin	1.5 g	1.5 g
Orange juice	15.0 ml	15.0 ml
Purina monkey chow	72.5 g	70.0 g

[a] 12.5 g butter oil contains about 38 mg cholesterol.
[b] 10 Kg of mix includes the following gram-amounts of vitamins and sucrose: thiamin, 2.00; riboflavin, 2.00; pyridoxine, 2.00; niacin, 10.00; Ca-pantothenate, 6.00; folic acid, 0.20; biotin, 0.04; ascorbic acid, 50.00; sucrose, 9927.76.

coconut oil (12.5%), butter oil (12.5%), and cholesterol (2%), all by weight. The other eleven control "normal" animals were given isocaloric quantities of the cholesterol-free Purina® monkey ration and 25% added corn oil (**Table 1**). The pre-experimental body weights were 10.6 ± 2.1 (SD) kg for control monkeys and 10.8 ± 2.3 kg for atherosclerotic monkeys. The plasma cholesterol levels of the two groups were similar: 165.5 ± 41.6 mg/dl and 155.7 ± 25.0 mg/dl, respectively.

After 21 months of consuming the specific diets, each monkey received 18 μCi of [4-^{14}C]cholesterol intravenously. The isotope was dissolved in a small amount of alcohol and sterilized by passing through 0.2 μm millipore filter. Before use, this alcoholic solution was mixed with 5% dextrose at a 1:9 ratio in a sterile bottle. Five milliliters of this solution containing 18 μCi of [4-^{14}C]cholesterol was injected slowly into the arm vein of each monkey. Monkeys were then killed in pairs (atherosclerotic and normal) by exsanguination at different times from 1 to 51 weeks after the isotopic administration. This sequence is shown in **Table 2.** Ten weeks after the first injection, each of the remaining monkeys (eight pairs) was given another injection of 41 μCi of [1,2-^{3}H]cholesterol. This second and different isotopic injection provided a second time point for the calculation of cholesterol turnover.

The [4-^{14}C]cholesterol (sp act 52 mCi/mmol) was purchased from New England Nuclear Corp., Boston, MA, and [1,2-^{3}H]cholesterol (sp act 39 Ci/mmol) from Amersham Searle Corp., Arlington Heights, IL. Before use, the purity of the isotopes was verified to be above 97% by thin-layer chromatography (5).

Blood samples were collected twice weekly for the first 3 weeks and then once every 1–3 weeks for the duration of the experiment. The cholesterol content of the plasma was determined by the method of Abell et al. (6). For the estimation of radioactivity, the plasma lipids were saponified with alcoholic KOH.

TABLE 2. The cholesterol content of the plasmas and aortas of control and atherosclerotic monkeys at the time of autopsy

Months of Feeding the Specific Diet	Weeks After Isotope[a]		Control Monkeys				Atherosclerotic Monkeys			
			Monkey #	Plasma[b] Chol.	Aortic Atherosclerosis	Aortic Chol.	Monkey #	Plasma Chol.	Aortic Atherosclerosis	Aortic Chol.
	^{14}C	^{3}H		*mg/dl*	*Grade 0–4+*	*mg/g dried tissue*		*mg/dl*	*Grade 0–4+*	*mg/g dried tissue*
21	1		1[c]	114	0	7.5	2	442	2.00	17.1
22	5		3	114	0	6.5	4	562	3.00	57.0
22	7		5	145	0	6.9	6	781	2.75	46.7
23	13	3	7	164	0	9.2	8	516	1.25	28.0
25	19	9	9	185	1.0	17.7	10	823	1.13	37.4
25	20	10	11	139	0	7.9	12	575	2.25	47.1
30	41	31	13	146			14	753	1.35	31.3
30	42	32	15	100	0	11.4	16	766	1.75	39.5
30	43	33	17	128	0	8.1	18	591	1.50	32.2
31	45	35	19	115	0.25	9.9	20	664	1.85	30.5
32	51	41	21	132	0	14.3	22	497	0.50	25.1
Mean				134.7[d]	0.13[d]	9.9[d]		633.6[d]	1.76[d]	35.6[d]
SD				± 24.8		± 3.6		± 130.3	± 0.73	± 11.4

[a] In this study, all monkeys but #1–6 received [4-^{14}C]cholesterol and [1,2-^{3}H]cholesterol injections at 10 weeks apart.
[b] Represents the cholesterol concentration at the time the animal was killed.
[c] Odd number monkeys in the control group were paired with corresponding even number monkeys in the atherosclerotic group according to their pre-experiment body weights and cholesterol levels.
[d] The values for plasma and aortic cholesterol and the amount of aortic atherosclerosis were statistically different for the control and atherosclerotic monkeys, $P < 0.001$.

The nonsaponifiable residue was extracted with hexane, dried, and dissolved in 10 ml of scintillation mixture (4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis(2-(5-phenylozazolyl))benzene in 1 liter of toluene). These samples were counted in a Packard Tri-Carb liquid scintillation spectrometer with an efficiency of 87% for ^{14}C and 41% for ^{3}H. The results were expressed as specific activity, dpm/mg of cholesterol.

At autopsy, the entire aortas were removed and the extent of aortic atherosclerosis was evaluated by microscopic examination of standard pre-determined samples (7), and by gross evaluation of the intimal surface. Immediately after removal and careful stripping of the adventitia, the aortas were opened and graded for the severity of disease. The aortic surface area involved with atherosclerosis grossly was expressed on a scale of 0 to 4+, (1+, 25%, 2+, 50%, 3+, 75% and 4+, 100%). After gross grading, two samples were taken for microscopic evaluation from three standard sites (the arch, the lower thoracic, and the abdominal regions) and stained for fat with oil red, hematoxylin and eosin, and with the Gomori trichrome aldehyde fuchsin stain for fibrous tissue elements (elastin and collagen).

The aortas were dried under vacuum at 100°C, ground to a powder, and extracted with chloroform–methanol 2:1 (8). The cholesterol and radioactive contents of the tissue extracts were then determined by the above methods. For measurement of the specific activities of free and esterified cholesterol, the lipid extracts of the aortas were subjected to thin-layer chromatography. The lipids were chromatographed on a glass plate (20 × 20 cm) coated with Silica gel-G 0.5 mm thick. The solvent system was hexane–chloroform–ethyl ether–acetic acid 80:10:10:1. The bands of free and esterified cholesterol were scraped and the lipids were eluted with ethyl ether. The mass and radioactivity of the free and esterified cholesterol were then determined.

From the results of plasma and tissue analysis, we have estimated the minimal rate of influx of plasma cholesterol into the aorta and turnover time with the same formulations as reported in our previous work (4). The following relationship was used for the calculations:

$$\text{Total influx (mg)} = \frac{\text{radioactivity (dpm) per unit dry weight of aorta}}{\text{an expression of the average SA of plasma cholesterol over the period since labeling}}$$

$$= \frac{\text{dpm/g dry aorta}}{\text{area under the plasma cholesterol SA-time curve/no. of days}} \quad (1)$$

$$\text{Influx rate (mg cholesterol/g dry aorta per day)} = \frac{\text{dpm/g aortic tissue}}{\text{area under the plasma cholesterol SA decay curve}} \quad (2)$$

$$\text{Accumulation in days} = \frac{\text{cholesterol content of atheromatous artery (mg/g dry tissue)}}{\text{influx rate (mg cholesterol/g dry tissue per day)}} \quad (3)$$

$$\text{Turnover time} = \frac{1}{\text{fractional turnover rate}} = \frac{\text{Area under the plasma cholesterol sp act decay curve}}{\text{sp act of atheroma cholesterol}} \quad (4)$$

RESULTS

Plasma and aortic cholesterol levels and atherosclerosis

The plasma cholesterol levels of the control and atherosclerotic diet groups at the time of autopsy were very dissimilar: 134.7 ± 24.8 and 633.6 ± 130.3 mg/dl, respectively (Table 2). Likewise, the monkeys fed the atherogenic diet on the average had moderately severe atherosclerotic aortas (mean grade 1.76), versus the almost completely normal aortas (grade 0.13) in the control group of monkeys. Microscopically, the average frequency of aortic lesions in animals fed the atherogenic diets was 98% with a range of 75–100%, while monkeys fed the control diet had a very low frequency rating of 42% with a range of 0–100%. The standard sections of the aortas showed many raised lesions with necrotic centers and well developed fibrous caps. These advanced lesions were more common in the abdominal aorta. In general, they were correlated with the presence of severe coronary artery luminal narrowing by similar advanced lesions in the coronary arteries.

The mean cholesterol content of the aortas of atherosclerotic monkeys was 35.6 ± 11.4 mg/g of dried tissue as compared with only 9.9 ± 3.6 mg for control animals. From the eleven atherosclerotic monkeys studied, a positive correlation between the aortic cholesterol level and the grade of atherosclerosis was observed ($r = 0.6925$, $P < 0.05$).

TABLE 3. Specific radioactivities (SA)[a] of the plasmas and aortas of the control and atherosclerotic monkeys after the intravenous administration of labeled cholesterol

Weeks After Isotope	Isotopic Cholesterol Given	Control Monkeys				Atherosclerotic Monkeys			
		Monkey #	Plasma SA	Aorta SA	Aortic SA / Plasma SA	Monkey #	Plasma SA	Aorta SA	Aortic SA / Plasma SA
			dpm/mg		%		*dpm/mg*		%
1	^{14}C	1A[b]	5588	893	16	2A	2439	542	22
3	^{3}H	7B	4616	1867	40	8B	2719	1187	44
5	^{14}C	3A	1395	843	60	4A	749	597	80
7	^{14}C	5A	1132	946	84	6A	607	552	91
9	^{3}H	9B	1556	1278	82	10B	679	851	125
10	^{3}H	11B	1554	1392	90	12B	650	907	140
13	^{14}C	7A	768	772	101	8A	339	488	144
19	^{14}C	9A	434	492	115	10A	175	321	183
20	^{14}C	11A	295	345	117	12A	151	296	196
31	^{3}H	13B	535			14B	96	339	353
32	^{3}H	15B	500	823	165	16B	158	412	261
33	^{3}H	17B	484	706	146	18B	140	387	276
35	^{3}H	19B	258	485	188	20B	92	344	374
41	^{3}H	21B	553	854	154	22B	68	402	591
41	^{14}C	13A	233			14A	25	147	588
42	^{14}C	15A	220	358	163	16A	38	130	342
43	^{14}C	17A	132	271	205	18A	51	166	326
45	^{14}C	19A	94	218	232	20A	27	133	493
51	^{14}C	21A	174	387	222	22A	18	139	772

[a] dpm/mg cholesterol.

[b] In this study all monkeys but #1–6 received [4-^{14}C]cholesterol and [1,2-^{3}H]cholesterol injection at 10 weeks apart. The "A" and "B" series represent ^{14}C and ^{3}H values respectively.

Equilibration between plasma and aortic cholesterol

The specific radioactivities of the plasma and aortic cholesterol of both control and atherosclerotic monkeys were measured at various intervals from 1 to 51 weeks after administration of the labeled cholesterol (**Table 3**). The amount of equilibration was determined by comparing the specific activity of aortic cholesterol with that of plasma. The ratio of aortic cholesterol specific activity to that of plasma cholesterol of atherosclerotic monkeys reached unity between 7 and 9 weeks after the intravenous injection of isotope (**Fig. 1**). Control monkeys attained equilibration much later, 13 weeks after the injection of isotope. Thus, the aortic cholesterol of atherosclerotic monkeys equilibrated with the plasma cholesterol faster than that of control monkeys. After the "crossover" point of two specific activity curves, the specific activity of aortic cholesterol remained above that of plasma cholesterol for both groups. However, the curve was much higher for the atherosclerotic monkeys. At the end of the study, 51 weeks after isotope administration, the ratio of aortic cholesterol to plasma cholesterol specific activities was 2.2 for control monkeys and 7.7 for atherosclerotic monkeys.

In order to show the time course of decay of plasma and aortic cholesterol specific activities in control and experimental monkeys, these specific activities were normalized by dose (dpm/g cholesterol per dose × 100). A composite curve was thus constructed (**Fig. 2A, 2B**). Precursor–product relationships were observed for both control and experimental monkeys between plasma and aorta.

The aortic content of free and esterified cholesterol and their specific radioactivities in normal and atherosclerotic monkeys

The free and esterified cholesterol contents and specific activities of the aortas of six representative pairs of monkeys indicated an active cholesteryl ester

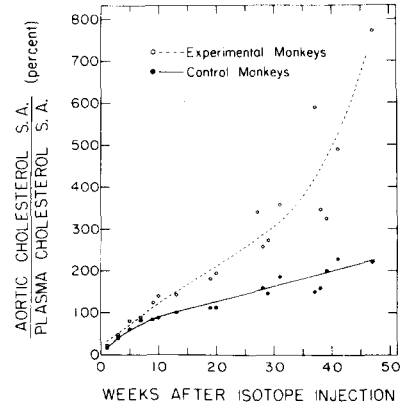

Fig. 1. Equilibration between plasma and aortic cholesterol of control and atherosclerotic monkeys after the administration of isotopic cholesterol.

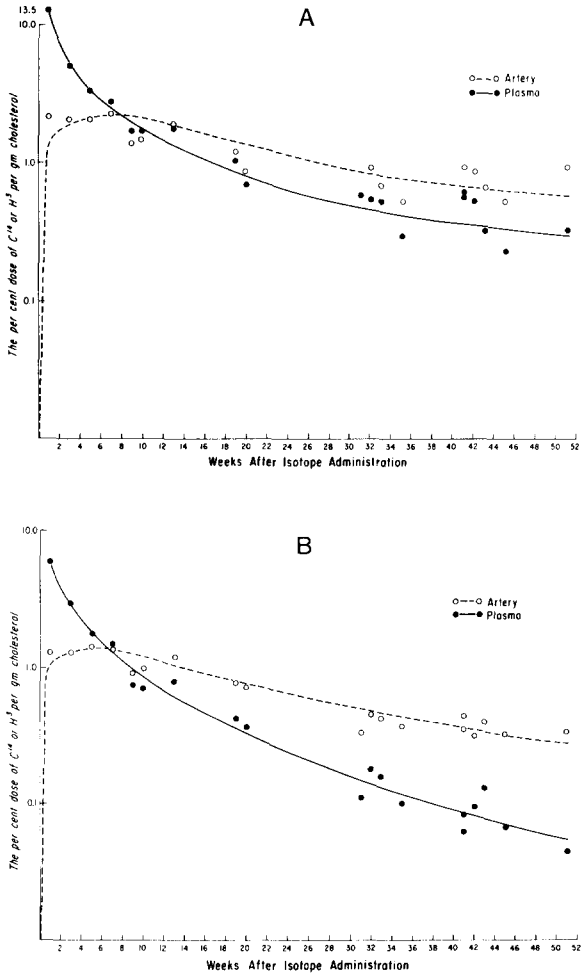

Fig. 2. A. The composite decay curves of the plasma and aortic cholesterol specific activities in control monkeys.

B. The composite decay curves of plasma and aortic cholesterol specific activities in atherosclerotic monkeys.

metabolism (**Table 4**). These data suggested the possibilities of the movement of cholesteryl ester into the arterial wall or else an active cholesteryl ester synthesis in the arterial wall. Inasmuch as some of the monkeys received two doses of isotopes, there were nine points of radiospecific activities (from 1 to 45 weeks after isotope administration) obtained from the analysis of their aortas. The specific activity of the free and ester cholesterol of the atherosclerotic aortas remained similar throughout the period of 45 weeks after isotopic injection. On the other hand, the specific activity of ester cholesterol of normal aortas became higher than that of free cholesterol some 3 weeks after isotope administration and remained higher subsequently.

Most of the cholesterol in the control aortas was in the free form, 6.8 ± 0.99 mg/g dried tissue, and only 1.2 ± 0.67 mg/g was present as esterified cholesterol. For the atherosclerotic aortas, both free and ester cholesterol increased greatly to 20.6 ± 6.08 and 17.2 ± 9.04 mg/g dried tissue, respectively, with ester cholesterol showing the greatest increase, about 1400%.

The content of isotopic total, free and esterified aortic cholesterol of monkeys receiving the same dose of [4-^{14}C]cholesterol

The total [4-^{14}C]cholesterol content in the aortas of a group of paired monkeys (control and atherosclerotic) that were given the same amount of isotope is depicted in **Table 5.** For example, in control aortas the radioactivities were 6500 and 7100 dpm/g of aorta at 7 and 13 weeks after isotopic administration. The radioactivity decreased to 3900 and 4600 dpm/g at 45 and 51 weeks, respectively. For atherosclerotic aortas,

TABLE 4. Free and esterified cholesterol content and specific activities (SA) of the aortas of control and experimental monkeys

Weeks after Isotope Administration	Control Monkeys					Atherosclerotic Monkeys				
		Cholesterol		SA			Cholesterol		SA	
	#	Free	Esterified	Free	Esterified	#	Free	Esterified	Free	Esterified
		mg/g		*dpm/mg*			*mg/g*		*dpm/mg*	
1	1A	5.7	1.8	976	927	2B	12.0	5.1	531	458
3	7B	7.1	2.1	2018	2787	8B	15.0	13.0	1199	1197
5	3A	5.9	0.6	850	1667	4A	27.2	29.8	623	619
7	5A	6.2	0.7	1053	1761	6A	24.8	21.9	593	705
10	11B	7.3	0.6	1579	5500	12B	24.8	22.3	1032	897
13	7A[a]			743	960	8A[a]			599	479
20	11A[a]			407	611	12A[a]			323	278
35	19B	8.3	1.6	573	1112	20B	19.5	11.0	447	262
45	19A[a]			282	460	20A[a]			196	135
Mean		6.8	1.2				20.6	17.2		
SD		± 0.99	± 0.67				± 6.08	± 9.04		

[a] Same monkey had two doses of isotopes at ten weeks apart. The "A" and "B" series represent ^{14}C and ^{3}H values, respectively.

TABLE 5. The radioactivity of total, free and esterified cholesterol in the aortas of control and atherosclerotic monkeys (dpm/g of aorta) after the same dose of [4-^{14}C]cholesterol intravenously

Weeks after Isotope	Control Monkeys				Atherosclerotic Monkeys			
	#	Cholesterol Radioactivities			#	Cholesterol Radioactivities		
		Total	Free	Esterified		Total	Free	Esterified
		dpm/mg				*dpm/mg*		
1	1A	6,689	5,084	1,605	2A	9,269	6,766	2,503
5	3A	5,509	4,903	606	4A	34,001	16,320	17,681
7	5A	6,538	5,230	1,308	6A	25,789	12,378	13,411
13	7A	7,106	5,400	1,706	8A	13,624	8,038	5,586
19	9A	8,786			10A	12,023		
20	11A	2,739	2,410	329	12A	13,947	7,950	5,997
41	13A				14A	4,810		
42	15A	4,076			16A	5,129		
43	17A	2,204			18A	5,347		
45	19A	3,954	2,966	988	20A	4,917	3,540	1,377
51	21A	4,649			22A	3,485		

the radioactivities were 34,000 and 25,000 dpm/g of aorta at 5 and 7 weeks after isotopic injection, respectively. They decreased to 4900 and 3400 at 45 and 51 weeks, respectively.

If we imagine each group as a single monkey (control and atherosclerotic), sampled at different time points after the same dose of isotope, the rise and fall of radioactivity in the aorta after isotopic cholesterol injection into the plasma demonstrated an active turnover of cholesterol in the aortas of both groups. The amounts of both labeled free and esterified cholesterol in normal and atherosclerotic aorta also decreased in time after an initial increase. The isotopic free and esterified cholesterol in control aortas were 5400 and 1700 dpm/g of aorta, respectively, at 13 weeks after isotope administration and these decreased to 2900 and 988 at 45 weeks. In atherosclerotic aortas, the changes were from 16,000 and 17,000 dpm/g of aorta at 5 weeks to 3500 and 6000 at 45 weeks. These data demonstrated that esterified cholesterol in the monkey aorta was as mobile as free cholesterol. In the control aortas there was a limited uptake of radioactivity from plasma into its esterified cholesterol pool (only 19% of total aortic radioactivity). On the other hand, the radioactivity was roughly similar for both free and esterified cholesterol in atherosclerotic aortas.

Influx rate of plasma cholesterol into total, free and esterified aortic cholesterol pools

The influx rate and turnover of aortic cholesterol were calculated under the same assumption and by the same equations used previously (4). For calculating the influx rate, it was assumed that there was minimal loss of radioactive cholesterol from the aorta during the period of study. As indicated in Table 5, there was some loss of isotope from aorta with time, especially after 10 weeks, in the atherosclerotic group. We, therefore, arbitrarily selected the cut-off point 10 weeks after isotope administration, a time before the specific activity of the aorta crossed over the specific activity curve of the plasma cholesterol, and then estimated the minimal influx rate of the two groups of monkeys (control and atherosclerotic) (**Table 6**). The minimal estimate of influx rate 1–10 weeks after pulse labeling was 0.088 ± 0.031 mg/g dried tissue per day for control monkeys and much higher, 0.470 ± 0.200 mg/g dried tissue per day, for atherosclerotic monkeys. With the assumption that the atheroma is in a steady state (when influx equals efflux), the maximal estimates of turnover times were 113.5 ± 50.7 days for control monkeys, and 90.7 ± 42.3 days for atherosclerotic monkeys.

The influx rates of plasma cholesterol into the pools of both free and esterified cholesterol of the normal and atherosclerotic aortas were calculated for five pairs of monkeys based on the same rationale as that used for total cholesterol influx presented previously. The minimal estimate of influx rate 1 to 10 weeks after isotope administration was 0.067 ± 0.022 mg/g tissue per day for free cholesterol and 0.019 ± 0.01 for esterified cholesterol in control monkeys. The influx rate was 0.275 ± 0.079 for free cholesterol and 0.234 ± 0.129 for esterified cholesterol in the atherosclerotic monkeys. The calculated influx rate of esterified cholesterol represents the aortic esterified cholesterol originating from plasma cholesterol whether it is derived by direct transport or via in situ esterification. The data again show the limited uptake of esterified cholesterol from plasma by normal aorta. The in-

TABLE 6. The minimal influx rate of plasma cholesterol into the total, free and esterified cholesterol pools of normal and atherosclerotic monkey aortas and the approximation of accumulation or turnover time of cholesterol in the respective aortas

	Control Monkeys					Atherosclerotic Monkeys				
		Influx Rate to					Influx Rate to			
Weeks After Isotope	#	Total Cholesterol Pool	Free Cholesterol Pool	Esterified Cholesterol Pool	Accumulation[a] or Turnover[b] Time	#	Total Cholesterol Pool	Free Cholesterol Pool	Esterified Cholesterol Pool	Accumulation[a] or Turnover[b] Time
		mg/g/day			*days*		*mg/g/day*			*days*
1	1A	0.124	0.095	0.029	60	2A	0.404	0.296	0.108	42
3	7B	0.118	0.084	0.034	78	8B	0.403	0.216	0.187	70
5	3A	0.063	0.053	0.010	103	4A	0.792	0.379	0.413	72
7	5A	0.079	0.066	0.013	87	6A	0.632	0.308	0.324	74
9	9B	0.100			177	10B	0.271			138
10	11B	0.051	0.041	0.010	155	12B	0.318	0.179	0.139	148
Mean		0.088	0.067	0.019	113.5		0.470	0.275	0.234	90.7
SD		± 0.031	± 0.022	± 0.011	± 50.7		± 0.200	± 0.079	± 0.129	± 42.3

[a] If the efflux of atheroma cholesterol was negligible, this would be the maximal period of accumulation.
[b] If atheroma cholesterol was in a steady state (when influx equals efflux), this would also be maximal turnover time.

flux rates for both free and esterified cholesterol were 4 and 12 times greater, respectively, in the atherosclerotic aortas than in the normal aortas.

DISCUSSION

The plasma cholesterol levels of the atherosclerotic monkeys rose 500% from baseline as a result of the high cholesterol diet. Consequently, moderately severe atherosclerosis resulted in these monkeys over the period of 21 months of feeding. The cholesterol content of the aortas proved to be a crucial index of atherosclerosis. The data from the present study, therefore, demonstrated the direct relationship between the level of aortic cholesterol and the degree of atherosclerosis in rhesus monkeys. In three species of New World monkeys, Portman and Andrus (9) also found a correlation between cholesterol levels and the aortic sudanophilia.

A comparison of the specific activity of plasma and aortic cholesterol in atherosclerotic and control monkeys indicated that more rapid equilibration occurred in the atherosclerotic aortas. The faster equilibration and the higher aortic to plasma specific activity ratio found in the atherosclerotic monkeys must have resulted from the greater influx rate of plasma cholesterol into aorta when the plasma cholesterol levels were higher. Another possibility would be the somewhat faster plasma cholesterol turnover which occurred in the atherosclerotic monkeys, aortic cholesterol turnover being then a tissue pool component of this generally more rapid cholesterol turnover. We believe that this latter is not the major explanation of the phenomenon. Furthermore, the total radioactivities in the atherosclerotic aortas were much higher than those of the control monkeys, despite the fact that the plasma cholesterol specific activity was lower in the atherosclerotic monkeys (greater dilution by a larger plasma cholesterol pool). Indeed, the influx rate in atherosclerotic monkeys was found to be five times higher than that in control monkeys.

An elevated influx rate in atherosclerotic arteries has also been observed in rabbits and pigeons (10–12). A direct correlation between influx rate and the plasma and aortic cholesterol concentrations was demonstrated by Newman and Zilversmit (11) and Zilversmit and Hughes (13) in rabbit experiments. Lofland and Clarkson reported an efflux rate that was higher than the influx rate of both free and esterified cholesterol in normal aortas of pigeons (10). These relationships were reversed in the atherosclerotic pigeon aorta. Certainly, the increased influx rate of cholesterol in the atherosclerotic aorta could be an important factor responsible for the accumulation of cholesterol in this tissue.

In contrast to atherosclerotic aorta, there was a limited uptake of plasma cholesterol into the cholesterol pool of the normal aorta, especially for esterified cholesterol. It has been suggested that the normal arterial intima is a barrier to the passage of plasma constituents into arterial tissue (12, 14, 15). However, under the conditions of the profoundly elevated plasma low density lipoprotein and cholesterol levels, whatever protective mechanism was present in the normal artery to prevent the entry of plasma cholesterol was abolished. The influx of plasma cholesterol into the aortic cholesterol pool, especially the esterified cholesterol pool, increased drastically in the atherosclerotic monkeys. In the present study, the uptake

of plasma cholesterol into the aortic esterified pool in atherosclerotic aorta was 12 times higher than that in normal aorta. The mechanisms behind these phenomena are still not completely understood.

Free and esterified cholesterol in the atherosclerotic aorta rapidly equilibrated and maintained similar specific activities throughout the study. Two explanations of this equality come to mind. First, this could be indicative of active esterification occurring in the aorta. Esterification has been observed in the atherosclerotic aorta of many species (16–19). On the other hand, since free and esterified cholesterol have similar specific activities in plasma (20), another possibility could be the direct transfer of esterified cholesterol from plasma. Serum lipoproteins have been found in the arterial wall (21–29). In cells of the tissue culture system, we have found that intact esterified cholesterol from the media is taken up (30, 31). Newman and Zilversmit (11) also proposed this possibility in the rabbit. From their study of human aorta, Smith and Slater (32) have suggested that in fatty streaks the lipid is within fat-filled cells and cholesterol ester was derived by esterification in situ. These cholesteryl esters were characterized by a very high proportion of oleic acid. The fatty acids were composed of 50% oleic acid and 14% linoleic acid. In fibrous lesions, however, the lipid takes the form of fine, extracellular droplets with the cholesteryl ester probably derived directly from the plasma. The fatty acids of these cholesteryl esters were composed of 26% oleic acid and 43% linoleic acid. In the atherosclerotic aortas of the present monkey study, we found that 32% of the cholesteryl ester fatty acids was oleate and 44% was linoleate. Furthermore, the distribution of these two fatty acids in cholesteryl esters in the atherosclerotic aorta was similar to that in the plasma.[1] This evidence seems to suggest that the direct uptake of cholesteryl ester from the plasma might have had an important role in the accumulation of cholesterol in the atherosclerotic plaque.

The cholesterol efflux rate could not be measured directly in the present experiments. However, a consideration of the efflux rate of cholesterol from the aortas of control and atheromatous monkeys led to a further impression of a dynamic state. In control aortas, there was a continuous steady state with no net accumulation of cholesterol. Therefore, the efflux rate must have then equalled the influx rate, which was calculated to be 0.088 mg/g per day. In the atherosclerotic monkeys, the influx rate was estimated to be 0.470 mg/g per day. If their efflux rate had remained at the control level, 0.088 mg/g per day, the net accumulation rate would be 0.470–0.088 which would equal 0.382 mg/g per day or 2.67 mg/g per week. With an accumulation rate of this magnitude, monkeys fed a high cholesterol diet for different periods of time must then have a significant difference in aortic cholesterol content. Analyzing our data, however, we found that because of the schedule of the killings, monkeys in this study had been fed a cholesterol diet for various periods of time (Table 2). Some had as much as 50 weeks more of the cholesterol feeding than the others. Yet, there was no clear-cut difference in the aortic cholesterol content observed in the animals fed longer or shorter times beyond the basic 21 months of feeding. In view of this information, it seems likely that the atheromatous aortas ultimately attained a relative steady state.

Although the quantitative efflux rates could not be obtained from this study, the disappearance of isotopic cholesterol from the aorta with time (Table 5) indicated that there was indeed a flux of cholesterol into the plasma from both normal and atherosclerotic aortas with a greater efflux probably occurring in atherosclerotic aortas. Both free and esterified labeled cholesterol in normal and atherosclerotic aortas decreased in time. In the atherosclerotic aorta, both the free and esterified cholesterol changed in parallel. This indicated that esterified cholesterol was as labile as free cholesterol.

From the six pairs of monkeys killed 10 weeks after isotope administration, we found that the fractional turnover rate and turnover time was 6.1% per week and 114 days for control aortas, and 7.7% per week and 91 days for atherosclerotic aortas, respectively (Table 6). For comparison, we also calculated these parameters from composite curves (Fig. 2A and 2B) by another method developed by Samuel and coworkers (33). From the data obtained from monkeys killed 10 weeks after isotope injection, the fractional turnover rate and turnover time were calculated to be 5.0% per week and 140 days for control monkeys and 7.0% per week and 101 days for the atherosclerotic monkeys, respectively. From the data of total 51 weeks, the fractional turnover rate and turnover time was 1.8% per week and 350 days for the control monkeys, and 9.6% per week and 70 days for the atherosclerotic monkeys, respectively. Therefore, the fractional turnover rate and turnover time calculated by two independent methods emphasizes the point that there was faster turnover of cholesterol in the aortas of the atherosclerotic monkeys.

This active turnover of cholesterol in the aortas of these monkeys suggested that these atherosclerotic lesions are potentially regressible. This conclusion coincides with the results from the previous study with

[1] Lin, Don S., W. E. Connor, and R. W. Wissler. The fatty acid composition of atherosclerotic plaques. Unpublished data.

mass determinations that the atherosclerotic lesions in this non-human primate will regress when their plasma cholesterol levels were lowered by withdrawing the cholesterol from their diet (1, 2).

Because of the similarity in experimental designs between our previous study of the cholesterol turnover in human arteries (4) and the present study, it is interesting to compare the parameters measured from these two experiments. The influx rate of plasma cholesterol into the severely diseased human abdominal aorta was 0.274 ± 0.027 mg/g per day (4). For the less severe atherosclerotic monkey aorta, it was 0.470 ± 0.200 mg/g per day. The turnover time was 442 days and 91 days for atherosclerotic human and monkey aortas, respectively. The ratio of aortic cholesterol specific activity to plasma cholesterol specific activities reached unity in the aortas of atherosclerotic monkeys 7–9 weeks after isotopic cholesterol injection. In the diseased human abdominal aortas, unity was not reached by 9–14 weeks after the administration of labeled cholesterol. The human aortic-plasma ratios ranged from 10 to 83%. These data clearly point to great differences in cholesterol metabolism and turnover in atherosclerotic lesions of varying severity. Less severe lesions of the aorta had a more rapid cholesterol turnover than both normal aortas and severely atherosclerotic aortas. The stage of the atherosclerotic process appeared to be the compelling factor in cholesterol turnover. While the monkey atherosclerotic lesions had many of the characteristics of human atherosclerosis, they were, in general, less complicated. The human atherosclerotic lesions had reached the ultimate in the atherosclerotic process: they were obstructed, more fibrotic and calcified, and far more necrotic. Thus, it is not surprising that they had a much slower turnover of cholesterol. The monkey lesions were more cellular and represented an earlier stage in the development of atherosclerosis, perhaps analogous to younger, non-obstructive human lesions. Their more rapid turnover of cholesterol indicated a greater propensity to regression should the greater influx rate of cholesterol be lessened by the reestablishment of normal plasma cholesterol levels.

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REFERENCES

1. Armstrong, M. L., E. D. Warner, and W. E. Connor. 1970. Regression of coronary atherosclerosis in rhesus monkeys. *Circ. Res.* **27:** 59–67.
2. Armstrong, M. L., and M. B. Megan. 1972. Lipid depletion in atheromatous coronary arteries in rhesus monkeys after regression diets. *Circ. Res.* **30:** 675–683.
3. Vesselinovitch, D., R. W. Wissler, R. Hughes, and J. Borensztajn. 1976. Reversal of advanced atherosclerosis in rhesus monkeys. I. Light microscopic studies. *Atherosclerosis.* **23:** 155–176.
4. Jagannathan, S. N., W. E. Connor, W. H. Baker, and A. K. Bhattacharyya. 1974. The turnover of cholesterol in human atherosclerotic arteries. *J. Clin. Invest.* **54:** 366–377.
5. Mongold, H. L. 1961. Thin-layer chromatography of lipids. *J. Am. Oil Chem. Soc.* **38:** 708–727.
6. Abell, L. L., B. B. Levy, B. B. Brodie, and F. E. Kendall. 1952. A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. *J. Biol. Chem.* **195:** 357–366.
7. Vesselinovitch, D., G. S. Getz, R. H. Hughes, and R. W. Wissler. 1974. Atherosclerosis in the rhesus monkey fed three food fats. *Atherosclerosis.* **20:** 303–321.
8. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
9. Portman, O. W., and S. B. Andrus. 1965. Evaluation of three species of New World monkeys for studies of dietary factors, tissue lipids and atherogenesis. *J. Nutr.* **87:** 429–437.
10. Lofland, H. B., and T. B. Clarkson. 1970. The bidirectional transfer of cholesterol in normal aorta, fatty streaks, and atheromatous plaques. *Proc. Soc. Exp. Biol. Med.* **133:** 1–8.
11. Newman, H. A. I., and D. B. Zilversmit. 1966. Uptake and release of cholesterol by rabbit atheromatous lesions. *Circ. Res.* **18:** 293–302.
12. Zilversmit, D. B., and L. B. Hughes. 1973. Incorporation in vivo of labeled plasma cholesterol into aortas of young and old rabbits. *Atherosclerosis.* **18:** 141–152.
13. Newman, H. A. I., and D. B. Zilversmit. 1962. Quantitative aspects of cholesterol flux in rabbit atheromatous lesions. *J. Biol. Chem.* **237:** 2078–2084.
14. Zilversmit, D. B. 1968. Cholesterol influx in the atherosclerosis plaque. *Ann. N.Y. Acad. Sci.* **149:** 710–724.
15. Zilversmit, D. B. 1975. Mechanism of cholesterol accumulation in arterial wall. *Am. J. Cardiol.* **35:** 559–571.
16. Hashimoto, S., and S. Dayton. 1966. Transfer of cholesterol and cholesteryl esters into the wall of rat aorta in vitro. *J. Atheroscler. Res.* **6:** 580–590.
17. Whereat, A. F. 1964. Lipid synthesis in aortic intima from normal and cholesterol-fed rabbits. *J. Atheroscler. Res.* **4:** 272–282.
18. Lofland, H. B., D. M. Mowry, C. W. Hoffman, and T. B. Clarkson. 1965. Lipid metabolism in pigeon aorta during atherogenesis. *J. Lipid Res.* **6:** 112–118.
19. St. Clair, R. W., T. B. Clarkson, and H. B. Lofland. 1972. Effects of regression of atherosclerotic lesions on the content and esterification of cholesterol by cell-free preparation of pigeon aorta. Circ. Res. **31:** 664–671.
20. Frantz, I. D., I. B. Carey, R. Moss, J. F. Eckert, M. Goldfork, and H. I. Katz. 1958. Observations on the turnover of cholesterol in human subjects. *Minn. Med.* **41:** 157–161.
21. Kao, V. C. Y., and R. W. Wissler. 1965. A study of the immunohistochemical localization of serum lipopro-

teins and other plasma proteins in human atherosclerotic lesions. *Exp. Mol. Pathol.* **4:** 465–479.
22. Scott, P. J., and P. J. Hurley. 1970. The distribution of radioiodinated serum albumin and low-density lipoprotein in tissues and arterial wall. *Atherosclerosis.* **11:** 77–103.
23. Kniereim, H. J., V. C. Y. Kao, and R. W. Wissler. 1967. Actomyosin and myosin and the deposition of lipids and serum lipoproteins. *Arch. Pathol.* **84:** 118–129.
24. Tracy, R., K. Dzoga, and R. W. Wissler. 1965. Sequestration of serum low-density lipoproteins in the arterial intima by complex formation. *Proc. Soc. Exp. Biol. Med.* **118:** 1095–1098.
25. Tracy, R. E., E. B. Merchant, and V. Kao. 1961. On the antigenic identity of human serum beta and alpha-a lipoproteins and their identification in the aortic intima. *Circ. Res.* **9:** 472–478.
26. Walton, K. W., and N. Williamson. 1968. Histological and immunofluorescent studies in the evaluation of the human atheromatous plaques. *J. Atheroscler. Res.* **8:** 599–624.
27. Watts, H. F. 1963. Role of lipoproteins in the formation of atherosclerotic lesions. *In* Jones Evolution of the Atherosclerotic Plaque. University of Chicago Press, Chicago. 117–138.
28. Watts, H. F. 1963. The mechanism of arterial lipid accumulation in human coronary artery atherosclerosis. *In* Coronary Heart Disease. W. Lickoff and J. H. Moyer, editors. Grune & Stratton, New York. 98.
29. Watts, H. F. 1971. Basic aspects of the pathogenesis of human atherosclerosis. *Hum. Pathol.* **2:** 31–55.
30. Maca, R. D., and W. E. Connor. 1971. The accumulation of serum lipoprotein cholesterol by tissue culture cells. *Proc. Soc. Exp. Biol. Med.* **138:** 913–919.
31. Chen, R. M. Y. Effects of hyperlipemic rabbit serum and its lipoproteins on proliferation and lipid metabolism of rabbit aortic medial cells in vitro. Ph.D. thesis, University of Chicago, 1973.
32. Smith, E. B., and R. S. Slater. 1973. Lipids and low-density lipoproteins in intima in relation to its morphological characteristics. *In* Atherosclerosis: Initiating Factors. Associated Scientific Publishers. 39–62.
33. Samuel, P., W. Perl, C. M. Holtzman, N. D. Rochman, and S. Lieberman. 1972. Long-term kinetics of serum and xanthoma cholesterol radioactivity in patients with hypercholesterolemia. *J. Clin. Invest.* **51:** 266–278.