The Bi-directional Transfer of Cholesterol in Normal Aorta, Fatty Streaks, and Atheromatous Plaques[1] (34394)

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There is abundant evidence that the arteries of man and many experimental animals are capable, to some extent, of synthesizing cholesterol (1–3). On the other hand, there is little to suggest that synthesis of cholesterol *in situ* is of quantitative importance in the development of atheromatous lesions. The results of studies from this laboratory have shown that local (arterial) synthesis of fatty acids, including those esterified to cholesterol, is accelerated in developing atheromata in pigeons and some nonhuman primates (4, 5). Likewise, the synthesis of phospholipids by the artery has been shown to contribute significantly to the lipids accumulating in lesions of the rabbit aorta (6). It appears, however, that plasma cholesterol is the source of most arterial free cholesterol, and perhaps also of cholesteryl esters.

The mechanisms by which cholesterol and its esters enter and accumulate in atheromatous lesions are far from clear. The work of Watts (7), and of Kao and Wissler (8) showed the presence of cholesterol-bearing low-density lipoproteins within the intimal layer. Jensen suggested that a pinocytotic process is involved (9). On the other hand, Newman and Zilversmit showed that cholesterol flux occurs *in vitro* even in arteries which have been boiled, or poisoned with cyanide (10), a finding which suggests that metabolic activity of the artery may not be involved in the uptake of cholesterol from plasma by arteries. In contrast, Rothblat and Kritchevsky (11) showed that certain cell types grown in tissue culture are highly selective in the uptake of cholesterol, in that the free form is more readily transported than are cholesteryl esters. Zilversmit recently reviewed current knowledge regarding cholesterol flux in arteries (12), in which, as was suggested previously (13), the concept of a metabolic barrier to the penetration of plasma cholesterol is presented. The present studies were designed to gain information on this aspect of sterol metabolism in pigeon arteries.

The White Carneau pigeon is ideally suited for studies of the type described herein. In this animal, atherosclerotic lesions of the aorta develop naturally even when the diet is devoid of cholesterol (14); the inclusion of cholesterol in the diet markedly exacerbates the disease, and hastens its onset (15). Of more importance, the lesions have a chemical composition not unlike that of human atheromata. They are, furthermore, focal in distribution, and commonly in a single aorta one finds, and can excise, areas of normal tissue, fatty streaks, and complicated plaques. It seemed logical to assume that metabolic differences might exist in regard to the exchange of cholesterol between plasma and these 3 types of tissues. We were especially interested in whether metabolic differences could be detected in the early (fatty streak) stages of atherosclerosis.

Materials and Methods. One hundred and fifty White Carneau pigeons were selected from our farm colony of birds which had been maintained on a cholesterol-containing diet.[2] In order to obtain a broad spectrum of

[1] Aided by grants from the USPHS (H-4371, H-4722, and H-4352).

[2] Purina Pigeon Pellets, Ralston-Purina Co., St. Louis, Mo. The pellets were coated with cholesterol and lard to give a final concentration of 0.25 cholesterol and 5% lard.

lesion types, birds were chosen which had been on the diets for periods ranging from 3 to 17 months. During the acute phase of the experiment, the birds were housed in growing batteries in the vivarium in a temperature and humidity-regulated environment, lighted from 7:00 a.m. to 4:30 p.m.

A solution of isotopic cholesterol was prepared by dissolving cholesterol-1, 2-^{3}H[3] in 30% ethanol containing a few drops of Tween 20. For the first 30 days of the experiment, each bird received, by stomach tube, 0.5 ml (1 μCi, 70 μg) of the emulsion of isotopic cholesterol. During the entire 136 days of the experiment, all birds continued to consume the cholesterol-containing diet.

Beginning with the third day after initiation of isotope feeding, at weekly intervals, blood samples were drawn from a subgroup of 8 birds, which were then killed; aortas were quickly removed and treated as described below. During the final days of the experiment, the sacrifice intervals were extended to 2–3 weeks apart.

At the time of sacrifice, each aorta was removed, cleaned of adhering tissue, opened longitudinally, and washed in several changes of saline. By visual inspection, the presence and types of atherosclerotic lesions were noted. The size of the plaques, when present was measured, and each plaque was carefully excised; one plaque from each affected aorta was placed on the chuck of a cryostat, frozen at —20° and three to four 5 μ sections were cut transversely, to be used later for observations on lesion morphology and, in some cases, autoradiography. The remainder of that plaque, plus any others present in a given aorta, were weighed and quickly frozen. Visualization of both plaques and fatty streaks in pigeons is relatively easy, due to the intense yellow staining of the aorta by the large amounts of carotenoid contained in the grain diets. Thus we were able to distinguish clearly between grossly unaffected tissue (referred to hereafter as "normal"), and areas containing fatty streaks. Both of the latter were excised, weighed, and preserved by freezing. Some aortas contained only normal tissue and fatty streaks; others, only plaques and fatty streaks, etc.

The specific activity of serum free and esterified cholesterol was determined by preparing an isopropanol extract of serum; a portion of each extract was placed on thin-layer chromatographic plates coated with silica gel-G (activated at 100° before use), then developed in a solvent consisting of Skellysolve B, diethyl ether and glacial acetic acid in a volume ratio of 146:50:4. Bands of free and esterified cholesterol were eluted, and the cholesterol concentration was determined by an automated procedure (16). Radioactivity measurements were made using a Beckman DPM-100 liquid scintillation spectrometer.[4] Counting was carried out in a solvent consisting of 6 g/liter of diphenyloxazole in toluene, to a two-sigma error of 3%. Quenching, when present, was corrected for by the external standard channels-ratio method.

Likewise, the specific activity of aorta free and esterified cholesterol was determined. The weighed portion of normal tissue, fatty streak or plaque from each aorta was placed in an all-glass homogenizer containing 2:1 chloroform:methanol. At this point, approximately 400 dpm each of cholesterol-4-^{14}C (free) and cholesterol-4-^{14}C-oleate were added as markers to monitor the completeness of recovery of free and esterified cholesterol from thin-layer plates. Each aorta segment was ground and the tissue was extracted repeatedly with chloroform:methanol, then brought to a volume. A portion of this extract was used for the determination of total cholesterol and radioactivity as described above. The remainder of the extract was applied to thin-layer plates. After the determination of cholesterol content and radioactivity of the fractions, the results were corrected for recovery on the basis of the carbon-14 marker added, then calculated as dpm/g of wet tissue, or as specific activity (dpm/mg of cholesterol) of each portion of normal tissue, fatty streak, or plaque from the aorta of a single bird.

Morphologic observations. Two cryostat sections obtained from each plaque were

[3] New England Nuclear Corp., Boston, Mass.

[4] Beckman Instruments Corp., Fullerton, Calif.

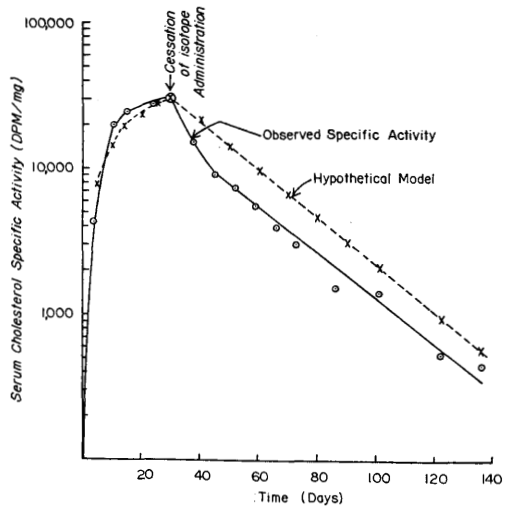

FIG. 1. Change in specific activity of serum cholesterol following oral administration of cholesterol-1, 2-^{3}H to pigeons.

stained with Sudan IV–hematoxylin and two were stained with hematoxylin and eosin. The aorta sections were graded using a method we have described previously (17). On a subjective basis of 0–3 plus, a score was recorded for cholesterol clefts, calcification, ossification, "fibrous" cap formation, ulceration, thrombosis, hemorrhage into the plaque, medial thinning, adventitial collections of cells associated with the plaque, and vascularization of the plaque. In addition, a subjective estimate of the percentage of the plaque that was lipid was recorded. The purpose of such grading was to determine whether any relationship existed between variations in plaque composition, metabolic characteristics of the plaque and the histologic characteristics of the plaque. From the cryostat sections cut on day 30 and representative sections during the remainder of the experiment, using a Wratten No. 2 safelight, unstained sections were dipped in liquid photographic emulsion[5] which was maintained in a waterbath at 43° and placed on a cold glass plate to dry for 45 min. After drying, the slides were placed in a Joftis exposure chamber[6] along with a small amount of dried silica gel and the chamber was filled with carbon dioxide. After 3 weeks of exposure the emulsion was developed and the sections were examined with the light microscope for silver particles.

Results. Figure 1 shows the results of plotting on a semilogarithmic scale the appearance and disappearance of the isotope (change in sp act) in the serum of birds receiving tritiated cholesterol for 30 consecutive days. Each point represents the mean value for the 8 birds sacrificed at each of the time intervals shown. At the end of 30 days, the curve approached a plateau, then declined in an essentially linear fashion for the next 100 days after cessation of isotope feeding. The line shown for the linear (disappearance) portion of the curve is the regression line derived from the logarithms of the observed specific activity values at the various time intervals. It appeared that at the time of cessation of isotope administration, all body pools of cholesterol had still not completely equilibrated with plasma, since there is a brief rapid decline in radioactivity for the first few days, then a linear relationship for the remainder of the experimental period. In theory, the appearance and disappearance of the isotope, following administration of diet of constant specific activity should resemble the broken line shown in Fig. 1.[7] The experimental values agree reasonably well with the theoretical curve, and allowed calculation of the average plasma specific activity during the early part of the experiment. From the latter values, as shown later, rates of influx of cholesterol into the artery can be calculated.

At every time interval studied, the specific activities of free and esterified cholesterol in plasma were essentially the same

[5] Eastman-Kodak NTB-2, Eastman-Kodak Co., Rochester, N. Y.

[6] Controls for Radiation, Cambridge, Mass.

[7] This curve was derived from the formula $S_b = c(1 - e^{-k_2 t})$ for the increase in specific activity, and $S_b = S_{bo}(e^{-k_2 t})$ for the disappearance curve. S_b = plasma specific activity; c = specific activity of the precursor (diet), in this case 35,000 dpm/mg, a value obtained from the dpm of isotope administered, and the known cholesterol intake, and assuming complete mixing; k_2 = fractional turnover rate (found to be 0.038); S_{bo} = specific activity at zero time, t = time (18).

TABLE 1. Contents of Free Cholesterol and Cholesterol Esters from Normal Artery, Fatty Streaks, and Plaques of Pigeon Aortas.[a]

	No. of observations	Free cholesterol	Cholesteryl esters
Normal	66	2.2 ± 0.08	0.84 ± 0.05
Fatty streaks	67	5.1 ± 0.34	2.30 ± 0.23
Plaques	96	25.1 ± 1.30	20.30 ± 0.13

[a] Mean values (mg/g of wet tissue) followed by the standard errors of the means.

(the mean analytical ratio of these substances in plasma was 0.496).

The analytical amounts of cholesterol in normal aorta fatty streaks and plaques are shown in Table I. It is apparent that in normal tissue the quantity of free cholesterol exceeds that of the esterified form by a factor of almost 3. In fatty streaks the free cholesterol content was more than doubled, and cholesteryl esters had increased almost 3-fold. Still more dramatic changes are seen in plaques, which compared to normal aorta, had roughly 12 times as much free and 25 times as much esterified cholesterol. It is obvious that at some time during cholesterol feeding certain areas of these aortas were in marked positive cholesterol balance, especially in regard to the accumulation of sterol esters.

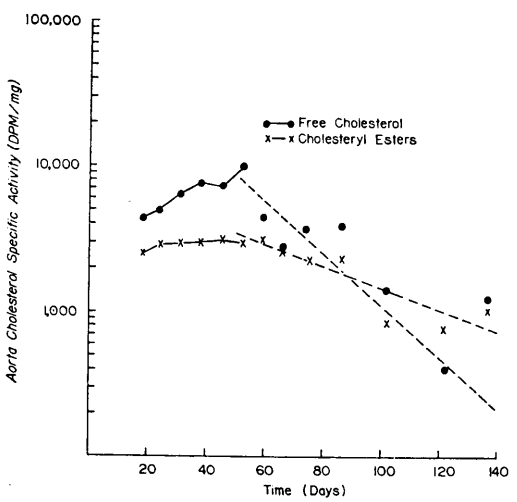

FIG. 2. Change in specific activity of cholesterol in normal areas of pigeon aorta following oral administration of cholesterol-1, 2-^{3}H.

Figure 2–4 show the changes in free and ester cholesterol specific activity in normal aorta, fatty streaks, and plaques, respectively, from days 18 through 136 of the experiment. Subgroups were also sacrificed at days 3 and 10; at these early time intervals, however, the radioactivity values recovered from the aortas were very low and in our opinion, were unreliable. Hence data for these two time intervals are not shown.

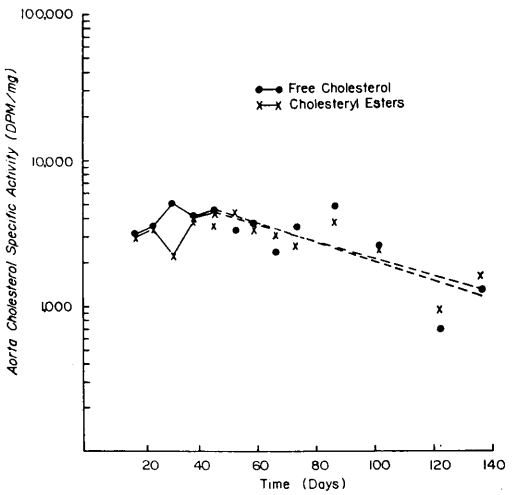

FIG. 3. Change in specific activity of cholesterol in fatty streaks from pigeon aorta following oral administration of cholesterol-1, 2-^{3}H.

Figure 2 shows that the specific activity of both the free and esterified fractions increase until about day 50 of the experiment (isotope administration was stopped on day 30). After day 50 the decline in specific activity is essentially linear (the line shown for the decline in specific activity is the linear regression line derived from the logarithms of the specific activity values). The rather wide scatter in the points is due to the fact that different groups of birds, with varying degrees of atherosclerotic involvement, were used at each time interval. The free cholesterol fraction becomes labeled considerably more rapidly than does the ester fraction; likewise, the specific activity of the free fraction declines more rapidly. These findings are consonant with the concept that the normal aorta permits the penetration of free cholesterol at a much greater rate than esterified.

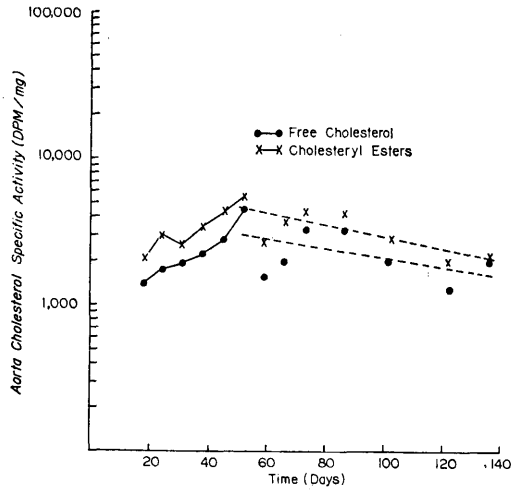

FIG. 4. Change in specific activity of cholesterol in atheromatous plaques from pigeon aorta following oral administration of cholesterol-1, 2-H^3.

The difference in rate is even more striking when pool sizes are considered (see Table I). While there is a significant rate of influx of cholesterol into normal pigeon aorta, this rate is not sufficient to allow complete equilibration with plasma cholesterol during the 30-day period (the maximum specific activity was only one third of that of plasma). It is of interest that at the 30-day time interval, the cholesterol present in muscle had almost reached the same specific activity as blood (24,500 dpm/mg for muscle, compared to 30,000 dpm/mg for blood).

A different pattern of labeling was seen for fatty streaks from the aortas of these same animals (Fig. 3). At almost every time interval studied, the specific activities of free and esterified cholesterol were essentially identical, and the maximum specific activity was less than that seen in normal aorta. A still different pattern of labeling is seen in plaques (Fig. 4), in which the specific activity of cholesteryl esters exceeded that of free cholesterol throughout the entire experiment.

It is apparent that the three types of arterial tissue exhibit striking differences in the rates at which they accumulate radioactive cholesterol from plasma. In a sense, however, specific activity data may be misleading, especially where sizable accumulations of cholesterol occur, as in plaques. There is no

TABLE II. Changes in Free and Ester Cholesterol Radioactivity in Normal Artery, Fatty Streaks, and Plaques at Various Time Intervals.[a]

	Normal artery		Fatty streaks		Plaques	
Day	Free cholesterol	Cholesteryl esters	Free cholesterol	Cholesteryl esters	Free cholesterol	Cholesteryl esters
18	16,727 ± 1081	2781 ± 492	24,970 ± 2096	11,478 ± 2787	54,734 ± 11,680	59,100 ± 21,003
24	17,948 ± 1024	4859 ± 1118	39,310 ±11,835	31,702 ±15,361	78,917 ± 4567	125,628 ± 10,391
31	20,722 ± 3122	3932 ± 1614	27,526 ± 5178	8037 ± 2684	87,075 ± 27,544	111,325 ± 52,636
38	27,456 ± 2010	5740 ± 1228	36,535 ± 5719	16,414 ± 3161	93,201 ± 16,882	134,870 ± 39,021
52	43,603 ±26,566	3172 ± 1548	37,935 ± 6043	18,442 ± 4424	123,891 ± 25,581	178,639 ± 64,723
66	14,204 ± 569	8826 ± 1750	27,074 ± 4151	14,808 ± 3479	86,000 ± 10,132	118,635 ± 50,337
86	8901 ± 1796	1597 ± 252	20,274 ± 5949	8214 ± 4755	74,858 ± 17,598	89,098 ± 25,869
122	2072 ± 622	2067 ± 1242	11,572 ± 5025	4235 ± 2076	86,660 ± 19,571	103,732 ± 24,445

[a] Mean values (dpm/g of wet tissue) for 8 birds, at each time interval, followed by the standard errors of the means.

TABLE III. Influx and Efflux of Free Cholesterol and Cholesteryl Esters in Normal Artery, Fatty Streaks, and Plaques.[a]

	Free cholesterol			Cholesteryl esters		
	Influx	Efflux	Difference	Influx	Efflux	Difference
Normal aorta	71.3	86.4	−15.1	11.9	14.5	−2.6
Fatty streaks	106.5	75.7	+25.8	49.0	29.6	+19.4
Plaques	198.0	167.0	+31.0	259.0	183.0	+76.0

[a] Values for influx and efflux represent μg of cholesterol/g of aorta/day.

assurance that, and these experiments shed no light on whether, all of the cholesterol present in an artery represents a metabolically exchangeable pool. The difference in influx rates in the three types of tissue, however, is clear, as is shown in Table II, in which the total radioactivity (dpm/g of tissue) for the three sites at selected time intervals is shown. It is apparent that the relative rate of sterol influx in plaque> fatty streak>normal aorta.

Absolute values for influx and efflux of cholesterol were calculated and are shown in Table III. Influx was calculated at the earliest possible time interval (day 18), by dividing the observed radioactivity (dpm/g of tissue) by average plasma specific activity obtained from the curve shown in Fig. 1. Efflux values were obtained from the fractional turnover rates calculated for the disappearance of isotopic cholesterol from the aortas during the later time intervals, and the known pool sizes of free and ester cholesterol. The assumption is made that during the time interval over which efflux was calculated, the size of the pools did not change significantly. It is apparent from these data that both the rates of influx and efflux of free cholesterol in normal aorta are considerably greater than are those of cholesteryl esters. For either fraction, however, influx and efflux values are about the same, suggesting that in normal aorta there is no net difference in the direction of cholesterol accumulation. On the other hand, a different situation prevails in fatty streaks and plaques. For both free and ester cholesterol, influx values obtained exceeded those for efflux, a situation which would result in a net positive cholesterol balance for those areas of the aorta. The differences in influx and efflux values of total cholesterol for plaques was 107 μg/day (31 μg/day of free and 76 μg/day of ester). In a 1-year period this would result in the accumulation of 365 $\times$ 107 or 39 mg of cholesterol. On the average, normal aorta contains about 3.0 mg/g of total cholesterol, while plaques contain about 45 mg/g, a difference of 42 mg/g. These data suggest that the values obtained for the differences in rates of influx and efflux are of reasonable magnitude.

Morphologic observations. The histologic characteristics of the plaques varied considerably among the birds in this experiment. Some of the plaques were small and raised only slightly above the intimal surface while others were large and often complicated by ulceration and hemorrhage into the plaque. The smaller lesions were most often intimal accumulations of sudanophilic material with little in the way of cellular reaction. Crystalline sterol was not seen in the small lesions. The large lesions, however, were usually associated with variable amounts of crystalline sterol, calcification, "fibrous" cap formation and adventitial reaction. No relationship could be found between the morphologic and metabolic characteristics of the plaques. Such an observation is not unexpected, however, since flux rates were calculated from pools of plaque material from several birds, while morphologic observations were made on sections from individual birds.

The data were examined to determine if plaques with different histologic characteristics had different amounts of free and ester cholesterol (Table IV). Plaques in which no crystalline sterol was seen had less free and ester cholesterol than did lesions with cholesterol clefts. Among plaques that were subjec-

TABLE IV. Cholesterol Content of Plaques with Different Histologic Characteristics.[a]

Cholesterol clefts				"Fibrous" cap				Adventitial reaction		
Grade	No. of birds	Free cholesterol	Cholesteryl esters	Grade	No. of birds	Free cholesterol	Cholesteryl esters	Grade	No. of birds	Free cholesterol	Cholesteryl esters
0	18	12.5 ± 1.5	12.3 ± 2.2	0	50	24.0 ± 2.0	20.2 ± 2.0	0	19	18.0 ± 2.7	12.9 ± 2.3
1	26	30.2 ± 2.7	23.8 ± 3.1	1	14	32.9 ± 3.9	26.3 ± 4.1	1	31	25.2 ± 2.2	22.6 ± 2.6
2	23	25.1 ± 1.9	19.9 ± 2.2	2	8	23.8 ± 3.2	18.0 ± 3.2	2	25	25.8 ± 2.1	19.7 ± 2.1
3	27	29.3 ± 2.6	22.7 ± 2.6	3	22	24.1 ± 2.1	17.7 ± 2.1	3	19	32.2 ± 3.7	24.8 ± 3.3

[a] Expressed as the mean (mg/g of wet wt) followed by the standard error of the mean.

tively classified as having grade 1, 2, or 3 clefts, however, no differences could be seen in the cholesterol contents. There appeared to be no differences in cholesterol content among plaques with different degrees of "fibrous" cap formation. Plaques having some amount of adventitial reaction had higher contents of both free and ester cholesterol than did those with no adventitial reaction; however, no relationship could be seen between the amount of either free or ester cholesterol and the degree of the reaction.

Autoradiography. Exposure of the emulsion by the isotopic cholesterol was seen most often deep within the plaques and was more extensive in large than in small plaques. On the basis of these autoradiograms the isotope appeared to accumulate in the portion of the plaque having the most crystalline sterol. In many instances the adventitia accumulated considerable isotope although exposure of the emulsion over the media was infrequent and when present to a much lesser extent than in the intima and adventitia.

Discussion. From these data it appears that clear-cut differences exist in regard to the rates at which isotopic cholesterol and its esters from plasma appear in normal aortas, fatty streaks, and atherosclerotic plaques. Such differences in rate apparently are related to the amounts of cholesterol already present in the artery, and the rates change as the analytical composition of the artery changes. In our opinion, these findings support the concept presented by Zilversmit, that the normal arterial wall represents a metabolic barrier to the entrance of cholesterol from plasma. It is of interest that those areas of the aorta which remain "normal" do so even though they are continuously exposed to plasma having a cholesterol concentration which may be in excess of 2000 mg/100 ml. Very early in lesion formation (fatty streak stage) certain areas of the aorta appear to lose the ability to regulate influx and efflux, and tend to develop a net positive cholesterol balance. From these studies, it is not possible to state with certainty that the fatty streaks observed were newly-formed and were actively progressing toward becoming plaques. They may equally well represent a

nearly static stage of a lesion which will progress little further. This would imply that in such a lesion the rate of influx will eventually be balanced by an equal rate of efflux. In our opinion, however, the findings suggest that future studies should be directed toward understanding the very early changes in the arterial wall which allow the enhanced entrance of plasma cholesterol and cholesteryl esters.

Summary. Isotopic cholesterol was administered per os for 30 days to cholesterol-fed White Carneau pigeons. At weekly intervals and for 100 days after cessation of isotope administration, subgroups were killed and their aortas were divided into normal tissue, fatty streaks, and atherosclerotic plaques. Cryostat sections from each plaque were examined for morphologic features, and from the different areas of the aorta cholesterol concentration and radioactivity were determined. From the values obtained, rates of influx and efflux of plasma cholesterol were calculated. The results indicated that in normal aorta the influx of free cholesterol greatly exceeds that of cholesteryl esters. There appeared to be no net positive cholesterol balance. In fatty streaks, the influx of both free and esterified cholesterol was greater than for normal tissue, and influx rates exceeded those for efflux. In plaques, still greater rates of influx and efflux were seen, and the influx of cholesteryl esters frequently exceeded that of free cholesterol, and like fatty streaks, plaques appeared to be in positive cholesterol balance. No relationship was observed between the morphologic and metabolic characteristics of the plaques.

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