Uptake and Esterification of Exogenous Cholesterol by Organ Cultures of Normal and Atherosclerotic Pigeon Aorta[1] (35956)

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It is now well established that arterial tissue can esterify cholesterol with fatty acids and that esterification is enhanced in atherosclerotic arteries (1–3). Most of these studies have involved the esterification of added radioactive fatty acids, or those synthesized *de novo*, with cholesterol preexisting in the artery. With the exception of a report by Felt and Benes (4), most workers have had little success in demonstrating esterification by arterial tissue of cholesterol added *in vitro* (1, 2, 5).

In this report we describe an organ culture system for the relatively long-term maintenance of arterial tissue in a metabolically active state. Using this technique the esterification by arterial tissue of radioactive cholesterol added to the culture medium, and the effect of atherosclerosis on cholesterol esterification, can be readily demonstrated.

Materials and Methods. Culture medium preparation. Serum from White Carneau pigeons and squirrel monkeys (*Saimiri sciureus,* Brazilian type) was heated at 56° for 30 min to inactivate lecithin-cholesterol fatty acyl transferase. Cholesterol-1,2-^{3}H (New England Nuclear Corp., Boston, MA) and oleic acid-1-^{14}C (Amersham/Searle, Des Plaines, IL) were purified by thin-layer chromatography (TLC) and dissolved in redistilled ethanol. Approximately 40 μl of this ethanolic solution were added to the heat inactivated serum and the serum was incubated for 2 hr at 37° with constant shaking. The ethanol was removed from the serum containing the cholesterol-1,2-^{3}H by filtration of the serum through a membrane filter that retained molecules of a molecular weight greater than 50,000 (Centriflo Membrane Ultrafilter, Amicon Corp., Lexington, MA). The concentrated protein solution was then made up to its original volume by addition of a solution of 0.9% NaCl containing 1 mg/ml of glucose. By this technique more than 99% of the added cholesterol-1,2-^{3}H was bound to lipoprotein. Upon electrophoresis 74% of the radioactivity was found in the β-lipoprotein fraction, 18% in α-lipoproteins, and 8% remained at the origin.

The oleic acid-1-^{14}C was also added to the serum in ethanol as described for cholesterol-1,2-^{3}H. Most of the ethanol was then removed from the serum containing oleic acid-1-^{14}C by evaporating the serum solution at room temperature to a small volume under a vigorous stream of N_2. The protein solution was then made up to its original volume with 0.9% NaCl solution.

The final culture medium was made up of Eagle's minimum essential medium with Earle's balanced salt solution (Microbiological Associates, Bethesda, Md.), 20–50% serum, to which had been added the desired radioactive lipids, penicillin (100 units/ml), streptomycin sulfate (50 μg/ml) and L-glutamine (292 μg/ml). The complete culture medium was gassed with 95% air and 5% CO_2 and then sterilized by passing it through a 0.43 μ Millipore filter and finally refrigerated.

Culture procedure. White Carneau pigeons were obtained from our stock colony and fed either a control or a cholesterol-supplemented diet for a period of 2 months as described previously (2). Aortas were removed aseptically, cleaned of periadventitial tissue, and

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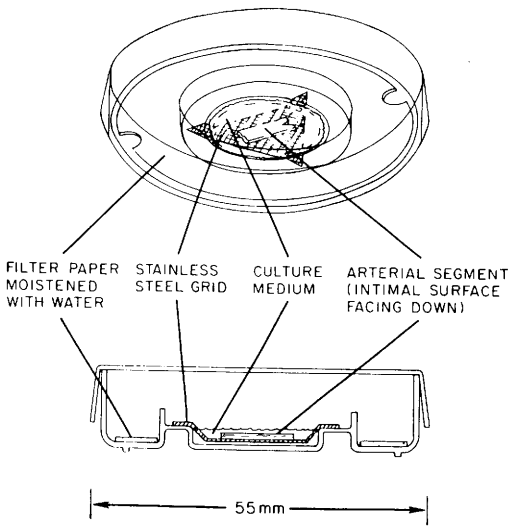

FIG. 1. Graphic representation of the organ culture system. Enough culture medium was placed in the center well to just cover the arterial segment (approximately 1 ml).

cut into rectangular segments weighing approximately 25 mg. These segments were placed with the intimal surface down, on stainless steel grids in 60 × 15 mm organ culture dishes (Falcon Plastics, Los Angeles, CA.). For all experiments, we used controls consisting of arterial segments that had been submerged in boiling water for 10 min. Approximately 1 ml of the culture medium was added to the center well of the organ culture dish (Fig. 1) and the organ culture dish was placed in a gastight Plexiglas chamber. The chamber was then flushed with 95% air and 5% CO_2, sealed, and kept in an incubator at 38°.

The culture medium was changed daily, since, after 24 hr of incubation, a 25 mg segment of pigeon aorta consumed approximately one-half of the glucose of the culture medium (1 mg/ml). For each arterial segment in culture we measured the utilization of glucose from the culture medium as a monitor of tissue viability. The rate of glucose utilization by the arterial segments remained relatively constant over a 9-day culture period. Glucose was determined by the glucose oxidase method (Glucostat, Worthington Biochemical Corp., Freehold, NJ).

In these experiments the cultures were maintained for up to 9 days, at which time the arterial segments were removed and the culture medium from each organ culture dish was checked for bacterial contamination by incubation for 72 hr on blood-agar plates. Bacterial contamination was not detected in any of the cultures.

It has been our experience that there are no major qualitative differences in results using the organ culture system when serum from several different species is used as a carrier for the lipid substrates. In these studies we used serum from either pigeons or squirrel monkeys while in other experiments we have used commercially available bovine serum. For the specific serum used in each experiment, refer to the footnotes of Tables I and II.

After removal from culture, arterial segments were rinsed twice in a 0.9% saline solution, once in blood serum, and twice more in saline. Segments were then blotted and weighed, homogenized in chloroform and methanol (2:1) and the residue was separated by centrifugation. The residue was extracted twice more with chloroform and methanol (2:1); the lipid extracts were combined, then made up to a known volume. An aliquot was taken for analysis of the concentrations of free and esterified cholesterol and the remainder of the lipid extract was taken to dryness at 45° under a stream of N_2. Exactly 200 μl of chloroform and methanol (2:1) containing 200 μg each of tripalmitin, oleic acid, cholesterol, and cholesteryl oleate were added as carrier for TLC. The tubes were stoppered, the contents were mixed rapidly, and 50 μl were taken for determination of total radioactivity. An additional 50 μl aliquot was separated into the various lipid fractions by TLC using Skellysolve B–diethyl ether–glacial acetic acid (146:50:4). Lipid-containing areas on TLC plates were stained with iodine, the iodine was allowed to sublime, and the lipid fractions then scraped directly into counting vials. By this method, essentially 100% of the radioactivity placed on the thin-layer plates could be recovered. All samples were counted in a Beckman DPM-100 liquid scintillation counter to a 2-sigma error of 2%.

The content of free and esterified cholesterol of individual arterial segments was de-

termined by gas–liquid chromatography following separation on TLC. Free cholesterol and cholesterol liberated after saponification of cholesteryl esters were converted to the trimethylchlorosilane (TMS) derivative (Sil-Prep, Applied Science Labs., State College, PA) and separated at 230° on 5 ft, 1/8 in. stainless steel columns of 3% DC-560 on 100/120 Gas Chrom Q using a MicroTek GC-2000R chromatograph. Results were quantified by means of an Infotronics CRS-108 digital integrator using 5α-cholestane as an internal standard.

Results. The distribution of radioactivity in segments of normal pigeon aorta after 144 hr in culture is shown in Table I. As was true for cell-free preparations of pigeon aorta (2), the organ cultures readily incorporated oleic acid-1-^{14}C into phospholipid, triglyceride, and cholesteryl ester. Little radioactivity was found in these fractions from the boiled controls. Only about 3% of the total radioactivity was present in the free fatty acid fraction, with the amount of radioactivity in this fraction similar in both boiled and viable segments.

The uptake of free cholesterol from the culture medium was rapid and did not require a viable arterial segment since uptake was similar for boiled control segments (Tables I and II). From the results presented in Table I, it is also apparent that substantial amounts of cholesterol-1,2-^{3}H were esterified by the arterial segments in culture. Boiled controls had only trace amounts of radioactivity in the cholesteryl ester fraction.

Figure 2 shows the uptake of free cholesterol by segments of normal pigeon aorta with respect to the duration of the culture period. The uptake of free cholesterol was similar in viable and boiled control segments at all time intervals studied, from 30 min to 216 hr. After 8 hr of culture the specific activity of the free cholesterol isolated from the arterial segment was 2% of that of the culture medium while after 216 hr incubation the specific activity was 25% of that of the culture medium.

The effect of atherosclerosis on uptake and esterification of cholesterol-1,2-^{3}H by segments of pigeon aorta in organ culture is

TABLE I. Distribution of Radioactivity in Lipid Extracts from Segments of Normal Pigeon Aorta after 144 hr in Organ Culture.[a]

Substrate	*N*	(dpm/25 mg aorta segment)				
		Phospholipid	Free fatty acid	Triglyceride	Cholesteryl ester	Free cholesterol
Oleate-1-^{14}C	8	38,177[b] ±3871	4360 ±422	79,798 ±8462	17,328 ±2023	—
boiled controls	2[c]	219 100	4444 4984	14 33	0 0	— —
Cholesterol-1,2-^{3}H	14	—	—	—	6026 ±399	113,246 ±3489
boiled controls	4	—	—	—	245 19	167,222 3956

[a] There were 1,652,200 dpm cholesterol-1,2-^{3}H or 731,100 dpm oleate-1-^{14}C/ml of culture medium. The culture medium contained 50% pigeon serum to which the substrate had been added as described in the materials and methods section.

[b] Results expressed as the mean ± the standard error of the mean.

[c] Results from both boiled control experiments are presented. Standard errors were not calculated because of the small sample size.

TABLE II. Effect of Atherosclerosis on Esterification of Cholesterol-1,2-^{3}H by Segments of Pigeon Aorta in Organ Culture.[a]

	Cholesterol radioactivity (dpm/25 mg aorta segment)		Cholesterol content (μg/25 mg aorta segment)		
	Cholesteryl ester	Free cholesterol	Free cholesterol	Cholesteryl ester	Total
Controls	3335	*[b]	32	—[c]	—
	10,554	*	43	—	—
	2848	*	44	—	—
	5127	124,385	45	2.8	47.8
	4834	115,144	48	3.3	51.3
	3991	112,758	52	4.2	56.2
(boiled segment)	270	130,042	41	1.5	42.5
Cholesterol-fed	2433	*	36	—	—
	4241	*	44	2.0	46.0
	7108	*	71	21.5	92.5
	17,039	111,370	74	67.0	139.0
	9648	140,626	63	95.8	158.8
	73,818	130,965	162	403.0	565.0
(boiled segment)	351	111,017	41	4.4	45.4

[a] Aortic segments were maintained in culture for 9 days. The specific activity of the culture medium was 15,478 dpm/μg free cholesterol and 101 dpm/μg cholesteryl ester. There were 1,501,760 dpm cholesterol-1,2-^{3}H/ml of culture medium. The culture contained 20% squirrel monkey serum to which the substrate had been added as described in the materials and methods section.

[b] Uptake of free cholesterol was not measured in these segments.

[c] The quality of the chromatograms was not sufficient to allow accurate quantification.

shown in Table II. The concentrations of free and esterified cholesterol were relatively constant among aortic segments from control animals but varied considerably in segments from cholesterol-fed animals. This wide variation was to be expected since the extent of atherosclerosis in arterial segments from cholesterol-fed pigeons ranged from those with no grossly visible disease to segments with fatty streaks and others with well-defined atherosclerotic plaques. The uptake of free cholesterol was similar in normal and atherosclerotic arterial segments, while in those segments with increased concentrations of free and esterified cholesterol, the rate of cholesterol esterification was increased to 30-fold. The correlation coefficient for rate of cholesterol esterification versus the total cholesterol content of the aortic segments (as a measure of severity of atherosclerosis) was $r = 0.9$ ($p < .001$).

Discussion. In this report we describe an organ culture technique for the long-term maintenance of arterial tissue. Using this technique we have maintained arterial segments in a metabolically active state for up to 9 days; and hopefully, further refinements in methodology will allow the maintenance of cultures for even longer periods of time. Studies by Higginbotham and Higginbotham (6), in which segments of rabbit aorta were implanted in the anterior chamber of the eye, suggest that organ cultures, if properly prepared, may remain viable for several months.

Jarmolych and co-workers (7) have described a technique for the long-term maintenance of arterial tissue *in vitro*. Their technique differs considerably, however, from that reported here in that they used medial tissue from swine aorta cut into approximately 1 mm cubes and incubated for up to 21 days by submerging them in nutrient medium. With this technique there was extensive peripheral outgrowth of "fibroblast-like" cells from the medial explants.

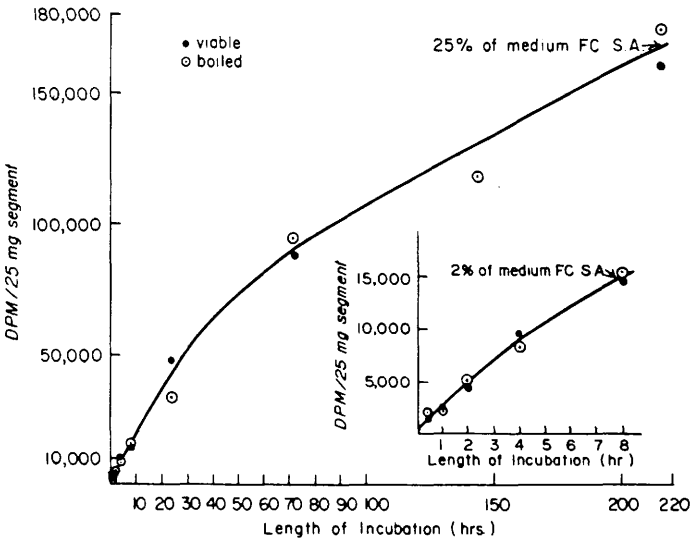

FIG. 2. The effect of the duration of culture on the uptake of free cholesterol by viable and boiled segments of normal pigeon aorta. The conditions of culture are the same as those described in Table II. (inset) an expanded view of the results after the first 8 hr of culture.

Although we have not performed a detailed morphologic study of arterial segments from organ culture, light microscopic examination showed very clearly that peripheral outgrowth of cells did not take place in the organ cultures and that there was not a major loss of cells from the arterial segment as occurs with the medial explant technique (7). We cannot explain the lack of peripheral outgrowth of cells with the organ culture technique but it probably involves the fact that with the medial explants the tunica intima is removed and the explant has a relatively large area of exposed cut surfaces, from which cells can be lost. In the organ culture technique, however, the tunica intima is left intact and there is a much smaller area of cut surface exposed to the medium. The fact that the rate of glucose utilization by arterial segments did not change with the duration of culture is further evidence that the organ culture technique maintains the arterial tissue in a relatively stable and biochemically active state.

Arterial segments readily take up free cholesterol from the culture medium. As reported by others, the uptake of free cholesterol by arterial tissue (8, 9) as well as by cells in tissue culture (10) is apparently by means of a physical chemical process as seen by the fact that uptake is similar for both boiled and viable segments. Surprisingly, however, the uptake of free cholesterol was not substantially different in atherosclerotic segments than in controls. This was true even though the free cholesterol content of the atherosclerotic aortic segments was as much as four times greater than in controls.

Using cell-free preparations of arterial tissue, we (2) and others (1, 5) have been unable to demonstrate esterification of added cholesterol-1,2-^{3}H; whereas fatty acids are readily esterified to cholesterol preexisting in the artery. Newman and co-workers (1) have suggested that the failure of arterial tissue to esterify added cholesterol may be because cholesterol does not penetrate to the esterifying site. This may partially explain the failure to demonstrate esterification of added cholesterol, particularly when intact arterial segments are used. An additional explanation, however, is that there is more than one pool of cholesterol in the artery (11), and that it is from only one of these pools that cholesterol is esterified. Support of this concept comes from the fact that in cell-free preparations of aorta, added fatty acids are readily esterified to cholesterol preexisting in the ar-

tery; whereas cholesterol added *in vitro* is not esterified (2). In the organ culture studies we could demonstrate esterification of cholesterol only after about 24 hr of culture, suggesting that exogenous cholesterol equilibrates slowly with the pool of cholesterol available for esterification. The metabolic characteristics of this slowly equilibrating pool of cholesterol are not known but it is conceivable that it is necessary for cholesterol to become bound to specific tissue carrier proteins, perhaps similar to those described for cholesterol biosynthesis (12), or even involve the actual incorporation of the cholesterol into intracellular membranes such as the endoplasmic reticulum.

Esterification of cholesterol by atherosclerotic arterial segments was increased in direct proportion to the severity of disease. The results are similar to those obtained with the esterification of cholesterol with fatty acids added *in vitro* (2, 3) or synthesized *de novo* from acetate (13, 14).

Knowing the number of disintegrations per minute (dpm) of cholesteryl ester synthesized and the specific activity of the free cholesterol isolated from the arterial segments, it is possible to estimate the micrograms of cholesteryl ester synthesized during the 9 days of culture. Results ranged from 1–2 μg for control segments to 54 μg for the most severely diseased arterial segment (565 μg total cholesterol). Although it is difficult to extrapolate data from *in vitro* experiments to the situation *in vivo*, it is obvious that the esterification of cholesterol by the arterial wall could contribute significantly to the accumulation of cholesteryl esters by the atherosclerotic artery.

Summary. An organ culture system is described in which segments of pigeon aorta have been maintained in a metabolically active state for up to 9 days. Using this system, we have studied the uptake and metabolism of cholesterol-1,2-^{3}H and oleic acid-1-^{14}C by normal and atherosclerotic arterial tissue. The uptake of cholesterol-1,2-^{3}H by arterial tissue was rapid and was similar in both boiled and viable arterial segments. Results suggest a physical chemical mechanism for uptake of free cholesterol. Arterial segments esterified oleic acid-1-^{14}C to phospholipid, triglyceride, and cholesteryl ester. Cholesterol-1,2-^{3}H was also esterified with a significantly increased rate of esterification seen in arterial segments from atherosclerotic aortas. Uptake of free cholesterol was not significantly different in normal and atherosclerotic arterial segments.

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