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Electron Microscopic Autoradiographic Study of Cholesterol Passage across Arterial and Capillary Endothelium

P. Constantinides and K. D. Wiggers*
Pathology Department, University of British Columbia, Vancouver, Canada

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Summary. The objective of this study was to compare for the first time, by means of electron microscopic autoradiography, the movements of orally administered labelled cholesterol across (a) normal arterial and capillary endothelium, and (b) normal and pathological arterial endothelium.

For this purpose, 15 mCi/kg ^{3}H-Cholesterol, spread in 5 daily doses, was given by stomach tube to (a) normal, (b) chronically hyperlipemic-atherosclerotic, (c) serum sick, and (d) desoxycorticosterone (DOCA) treated rabbits. The animals were killed 6 hours after the last ^{3}H-Cholesterol feeding and thick unstained sections from their glutaraldehyde-digitonin fixed aortae, coronaries, myocardium and liver were then coated with a photographic emulsion, exposed for 2 or 6 months and examined electron-microscopically.

It was found that in normal animals only very few labelled cholesterol molecules crossed the arterial endothelium, whereas large numbers of them crossed the capillary endothelium to enter the muscle cells of the heart and the hepatocytes of the liver. In contrast to the normal arterial endothelium, however, the arterial endothelium of hyperlipemic-atherosclerotic, serum sick and DOCA treated rabbits proved highly permeable to the labelled cholesterol molecules, allowing them to pour in great numbers into the arterial wall. The labelled lipid crossed the endothelium mainly trans-cytoplasmically, but in animals exposed to protracted hyperlipemia it also entered the arterial wall through opened interendothelial junctions that were occasionally observed in this condition.

These results show that the normal arterial endothelium, unlike that of normal capillaries, has a very low permeability for physiologically presented cholesterol (or lipoprotein) molecules, but that certain pathological conditions such as protracted hyperlipemia, hypertension and immune insults change it and make it highly permeable to these molecules.

Introduction

A tacit assumption behind most atherosclerosis research of the last two decades has been the idea that the giant lipoprotein molecules of the blood—or their lipid derivatives—percolate continuously through the wall of arteries (moving from the plasma through the intima and media into the adventitial lymphatics) and that when there is hyperlipemia these molecules enter the arterial wall faster than they leave it, thus gradually accumulating in it (Page, 1954; Doerr, 1963, 1970; Constantinides, 1965).

However, in view of strong evidence from hyperlipemia experiments with animals that the normal arterial endothelium resists penetration of lipids for a long time whereas arterial injury of any kind promotes extremely rapid invasion of the arterial wall by such substances (Constantinides, 1965, 1968), we postulated a different model of atherogenesis in recent years (Constantinides, 1969, 1970, 1971), as a working hypothesis for further experimentation. We proposed that under normal conditions the endothelium of arteries has a much lower permeability for lipoprotein (or lipid) molecules than the endothelium of capillaries, but when the

* Present Address: Animal Science Dept., Iowa State University, Ames, Iowa 50010, USA.

arterial lining is exposed to certain pathological conditions (including protracted hyperlipemia) it changes and becomes much more permeable to these molecules. In other words, far from being continuously traversed by lipoproteins, the normal arterial endothelium would tend to resist the penetration of these molecules into the arterial walls.

Partial support for the above concept has been provided by the recent studies of Adams *et al.* (1970), Adams (1971), Scott and Hurley (1970) and Gore *et al.* (1972). These workers injected intravenously as a single shot labelled cholesterol or lipoprotein labelled in its protein component and, using purely quantitative measurements of tissue radioactivity, found an increased entry of both these materials into the atherosclerotic—as compared to the normal—arterial wall of rabbits (Adams, 1971; Gore *et al.*, 1972), as well as minimal entry of lipoprotein into the aorta—as compared to the liver—of human terminal patients injected before death (Scott and Hurley, 1970).

Significant though they were, however, these findings left a number of questions unanswered: Does cholesterol which is introduced into the blood stream in an unphysiological form, i.e., intravenously, behave in the same manner as cholesterol that circulates in a physiological form, i.e., as a lipoprotein? Do intravenously injected, protein-labelled lipoproteins exhibit the same behaviour as lipoproteins manufactured by the organism itself from physiologically (orally) administered labelled cholesterol? How do labelled lipoproteins (or lipids) cross the endothelium and enter the arterial wall?

It appeared that one way of answering some of these questions and of testing the new hypothesis more directly would be to attempt to visualize by means of electron microscopic (E/M) autoradiography the transendothelial movements of labelled lipoproteins manufactured from orally administered ^{3}H-Cholesterol in normal rabbits and in animals subjected to various arterial insults (such as chronic hyperlipemia, serum sickness and desoxycorticosterone treatment). E/M autoradiography had not yet been applied successfully to the exploration of transendothelial movements of lipoproteins labelled in this manner apparently because (a) the enormous dilution over the inner surface of the whole vascular tree necessitated vast amounts of oral label, and (b) because most of the labelled cholesterol was extracted out of the tissues by the lipid solvents used during processing for electron microscopy. It was possible in the present study to overcome the first difficulty by giving unusually large amounts of ^{3}H-Cholesterol orally, and the second one by preserving free cholesterol in the tissues with the help of digitonin, a compound that forms an insoluble precipitate with this steroid and has been recently used to demonstrate it electron microscopically in liver (Ökrös, 1968; Scallen and Dietert, 1969; Williamson 1969), adrenal cortex (Frühling *et al.*, 1969) and myelin (Napolitano *et al.*, 1972).

Materials and Methods

A. General Procedure

^{3}H Cholesterol (NET-139 Cholesterol-1, 2-^{3}H, Specific Activity 50 Ci/mM, from New England Nuclear Co.) was given by stomach tube at the dosage level of 15 millicuries per kilogram body weight to 5 normal, 5 hyperlipemic, 1 desoxycorticosterone-treated and 2 serum sick New Zealand white male rabbits weighing 3–4 kilograms. The total dose for each animal (45–60 mCi) was divided into 5 daily doses given on 5 successive days, each dose being dissolved in 9–12 ml maize oil. Six hours after the last labelled cholesterol feeding, the animals were killed, their aorta was quickly perfused first with buffer and then with glutaraldehyde,

and multiple samples of their aorta, myocardium and liver were fixed in a mixture of buffered glutaraldehyde and digitonin for 18 hours, under agitation. Following fixation, small blocks from the tissue samples were speedily dehydrated and embedded in epon, in order to minimize the exposure of tissues to lipid solvents. To increase the radioactive cholesterol content of the sections, the latter were cut unusually thick (at approximately 2500 Å), and they were left unstained in order to eliminate even the slightest possible quenching of their radioactivity by an overlying heavy metal film of uranium or lead. The sections were then coated with an Ilford L-4 photographic emulsion, exposed in the dark for 2 or 6 months at 5° C in the presence of desiccants, developed, and examined in a Phillips EM 300 electron microscope. For control purposes, sections were also examined from duplicate tissue specimens treated exactly as above but (a) extracted with a 2:1 (v:v) mixture of $CHCl_3$ and CH_3OH for 1 hour prior to fixation, or (b) fixed without digitonin. The main endpoint of this study was the morphological (qualitative) assessment of the label localisation pattern in tissues, but in order to obtain a rough quantitative idea of the label content of various areas, grain counts were also undertaken in most sections.

B. Specific Procedural Details

1. Preparation of Labelled Cholesterol for Oral Administration

The labelled cholesterol was supplied by New England Nuclear Co. in vials containing lots of 25 millicuries, each lot representing 0.193 mg of the labelled steroid and dissolved in 6.25 ml benzene. To prepare the solution for gavage, the benzene solvent was evaporated through gentle heating (under a stream of nitrogen) in a water bath at around 60° C leaving a dry steroid residue adhering to the walls of the lower part of the vial; the dry residue was dissolved in 1 ml absolute ethyl alcohol (added to the vial) which, in turn, was thoroughly mixed with 25 ml maize oil; to prevent diarrhea, this maize oil solution of alcoholic labelled cholesterol was enriched with finely powdered cellulose (alphacel), in a proportion of 5 g cellulose/100 ml maize oil, before being force-fed to the rabbits by means of a No. 8 French rubber catheter.

2. Induction of Chronic Alimentary Hyperlipemia

Pronounced hyperlipemia and atherosclerosis was produced by means of 1% cholesterol feeding for 7 months. To avoid the excessive toxicity and mortality caused by continuous cholesterol alimentation, four 1-week rest periods on normal diet were inserted into the high cholesterol regime of the last 5 months. The labelled cholesterol was force-fed for 5 consecutive days to these hyperlipemic-atherosclerotic animals at the end of the seventh month of the high cholesterol diet.

3. Induction of Serum Sickness

250 mg bovine serum albumin (BSA) was given i.v. as a single shot to each of 2 rabbits on day 1. Labelled cholesterol was then force-fed to these animals on days 5, 6, 7, 8, 9, i.e., during a period when soluble antigen-antibody complexes are known to arise and circulate in their bloodstream in response to the foreign antigen BSA (Humphrey and White, 1965).

4. DOCA Treatment

Three rabbits were unilaterally nephrectomised and placed on 1% NaCl as drinking water ad lib. for the duration of the experiment. Two weeks after nephrec-

tomy and placement on 1% NaCl they started receiving 5 mg/kg of desoxycorticosterone acetate subcutaneously (suspended in 2 ml buffer) 3 times weekly for 4 weeks. At the end of this period, 2 animals died and the one surviving rabbit was given the labelled cholesterol by mouth on 5 successive days. The above treatment is known to produce hypertension in most laboratory animals but in the rabbit it also induces severe hypopotassemia and changes resembling those found in toxemia of pregnancy (Masson *et al.*, 1953). Blood pressure was not measured in the NaCl-DOCA treated animal but its greatly hypertrophied heart offered presumptive evidence that it did develop hypertension, since the mean heart weight of the 5 normal rabbits was 2.17 g/kg body weight (range: 1.96–2.32) whereas that of the NaCl-DOCA treated animal was 2.92 g/kg body weight.

5. Aortic Perfusion, Specimen Taking and Processing

Six hours after the last labelled cholesterol feeding, at a time of maximal intestinal absorption of the oily steroid solution (as evidenced by their chyle-filled milk-white lacteals), all rabbits were deeply anesthetized with 1 g/kg Urethane intraperitoneally, followed by 10 mg/kg sodium pentobarbital, intravenously. A mid-line incision from the pubic symphysis to the thoracic inlet was then made (cutting through the cartilaginous sternal ends of the ribs) to expose all thoracic and abdominal viscera. With the help of hemostats, the origins of the ascending aorta, celiac, mesenteric and 2 renal arteries were then clamped, the distal ends of the 2 external iliac arteries transsected, and the tip of a No. 8 French catheter inserted into the middle of the aortic arch through a small transverse incision and fastened in place with a ligature. Finally, with the help of a large syringe attached to the catheter, the aorta was perfused first with 20 ml of Millonig's phosphate buffer within about 20 seconds (in order to thoroughly clear it of blood) and then with 20 ml 2.5% buffered glutaraldehyde (in order to initiate endothelial fixation). Immediately after the end of the aortic perfusion, the following stock samples were removed and placed in vials containing a mixed solution of 2.5% glutaraldehyde and 1.5% digitonin in Millonig's buffer, for 18 hours, under continuous gentle agitation: (a) 2 samples of thoracic aorta; (b) 2 samples of abdominal aorta; (c) one sample of external iliac artery; (d) one slice from the base of the heart, going through both ventricles and the stems of the main coronary arteries; (e) a sample from the arterior margin of the right lobe of the liver. Care was taken during the removal of the arterial specimens from the body (and during all subsequent manipulations) to avoid any damaging exposure of arterial endothelium to air by keeping the arterial samples immersed in fluids at all times.

After the end of their exposure to glutaraldehyde-digitonin, blocks were cut from the specimens, rinsed thoroughly in 3 changes of Millonig's buffer and subjected to a greatly accelerated alcoholic dehydration and propylene oxide infiltration procedure (1/5 of the usual times) before being embedded in epon. All sections cut from artery blocks were transverse.

6. Coating of Sections with Photographic Emulsion

The 2500 Å thick, unstained sections were mounted on 100 mesh, parlodion coated grids which were, in turn, mounted on glass slides by means of double-

faced adhesive celluloid tape, 3 grids to a slide. After transfer to a darkroom, in the presence of red light, the sections were then coated with an ultrathin film of 50% aqueous Ilford L-4 Nuclear Research Emulsion (grain size ca. 1400 Å) by means of the wire loop technique of Caro and Van Tubergen, (1962), dried and placed together with a desiccant (magnesium silicate) in a dark box that was sealed with tape and stored at 5°C. One set of sections was exposed in this manner for 2, another duplicate set for 6 months. At the end of their exposure period the sections were developed in the darkroom in Microdol-X for 1.5 minutes, rinsed in 3 changes of water for a few seconds each time, fixed in acid fix solution for 3 minutes, rinsed terminally in 8 changes of water for a total 20–30 minutes, and air dried for electron microscopic examination.

Post-staining of the developed sections with lead citrate, or post-development removal of the emulsion from them by means of alkaline solutions was avoided because test counts of reduced grains before and after such procedures showed us that the latter cause an irregular and often great loss of reduced grain from sections.

7. Counting of Reduced Grains in the Sections

To obtain a rough idea of how many labelled cholesterol molecules had crossed through the endothelium into the subendothelial space of aortic, coronary and capillary walls, the number of reduced grains in 20 contiguous fields at the 8000 X magnification level was counted in 2 sections per grid and 2 grids per specimen in (a) 2 aortic specimens from every normal, hyperlipemic and DOCA-treated rabbit, (b) 1 myocardial and 1 hepatic specimen from every normal animal, and (c) 1 coronary artery specimen from 2 normal, 2 hyperlipemic and the 2 serum sick rabbits. In the case of the aortic and coronary artery wall, the counts represented all reduced grain found within a row of 20 consecutive fields along the inner wall of the vessel, encompassing the endothelium and the upper layers of the media; in the case of the myocardial and hepatic parenchyma, the counts represented all grain found within a circle of 20 contiguous fields around a capillary (or arteriole), encompassing its endothelium and pericapillary tissues (heart muscle or hepatocytes, respectively); to make up 20 fields in the case of the heart and the liver, it was usually necessary to pool the pericapillary counts of 2 or more capillaries, because of the small diameter of the latter. Every count of label-containing tissue was accompanied by a "background count", i.e. by a count of the number of reduced grains in 20 consecutive fields of an adjacent tissue-free area of the section (e.g. the vessel lumen). These background counts yielded, without exception, very low values (ranging from 0–4 grains per 20 fields) and were subtracted from the tissue counts to give the "true" counts presented in table 1 of this paper. Of the two duplicate sets of coated sections counted, the set exposed for 6 months revealed higher counts than the one exposed for 2 months and was therefore chosen as the source for the values in the table.

Results

Very little label crossed through the endothelium into the aortic or coronary wall of normal animals, under our conditions. Since the average grain count for the inner arterial wall of normal rabbits was only 1.2–3.5 grains per 20 fields

Table 1. Grain Counts. The values listed in this table are the mean grain counts of the groups concerned, with their ranges presented in brackets and "n" signifying the number of animals from which the counts were obtained. In computing these values, the grain count of each individual animal represented the mean count of 2 different tissue specimens, while the value of each specimen represented, in turn, the mean of 4 different section counts

	Aorta Endothelium and inner media	*Coronary Arteries* Endothelium and inner media	*Coronary Arterioles* Endothelium and media	*Capillaries of Heart* Endothelium and pericapillary myocardium	*Sinusoids of Liver* Endothelium and perisinusoidal hepatocytes
Normal controls	1.2 (0–3) $n=5$	3.5 (2–5) $n=2$	15 (13–17) $n=2$	41 (32–52) $n=4$	112 (48–240) $n=4$
Hyperlipemic-atherosclerotic	104.8 (60–151) $n=5$	37 (34–40) $n=2$			
DOCA-treated	20.5 $n=1$				
Serum sick		32 (28–36) $n=2$			

(Table 1), the typical field in the normal arterial endothelium and subendothelial space was free of reduced grain (Figs. 1–2). By contrast, much label crossed the normal capillary endothelium to reach the pericapillary space in the heart and in the liver (Fig 3).

When, however, the same amount of labelled cholesterol was given to chronicaly hyperlipemic rabbits, large amounts of label poured through the severely altered, highly vacuolated arterial endothelium of these animals into the underlying atherosclerotic wall (Fig. 4). Most of this label seemed to go through the cytoplasm of the endothelial cells (Fig. 4), but some of it also entered—either free or carried within the cytoplasm of immigrating monocytes—through the opened intercellular junctions that were occasionally observed in the endothelium of the hyperlipemic rabbits (Figs. 5–7). Upon reaching the subendothelial space, some of the labelled molecules moved into smooth muscle cells caught in various stages of transformation into foam cells (Figs. 8–9), while others remained extracellular (Fig. 10).

Similarly, much more label penetrated through the aortic endothelium of the DOCA treated and the coronary endothelium of serum sick (Fig. 11) than that of normal animals. No vacuolation or opened junctions were, however, observed in the arterial endothelium of the latter two groups under the present conditions.

The means and ranges of grain counts found in the various tissues studied are contained in Table 1. In this table it can also be seen that the permeability of the normal endothelium for label increased gradually as one moved from the aorta through the coronaries to the arterioles and the capillaries.

No reduced grain was found in any of the sections from duplicate specimens that were extracted with chloroform-methanol, and only little grain was seen in the tissues that were fixed in the absence of digitonin.

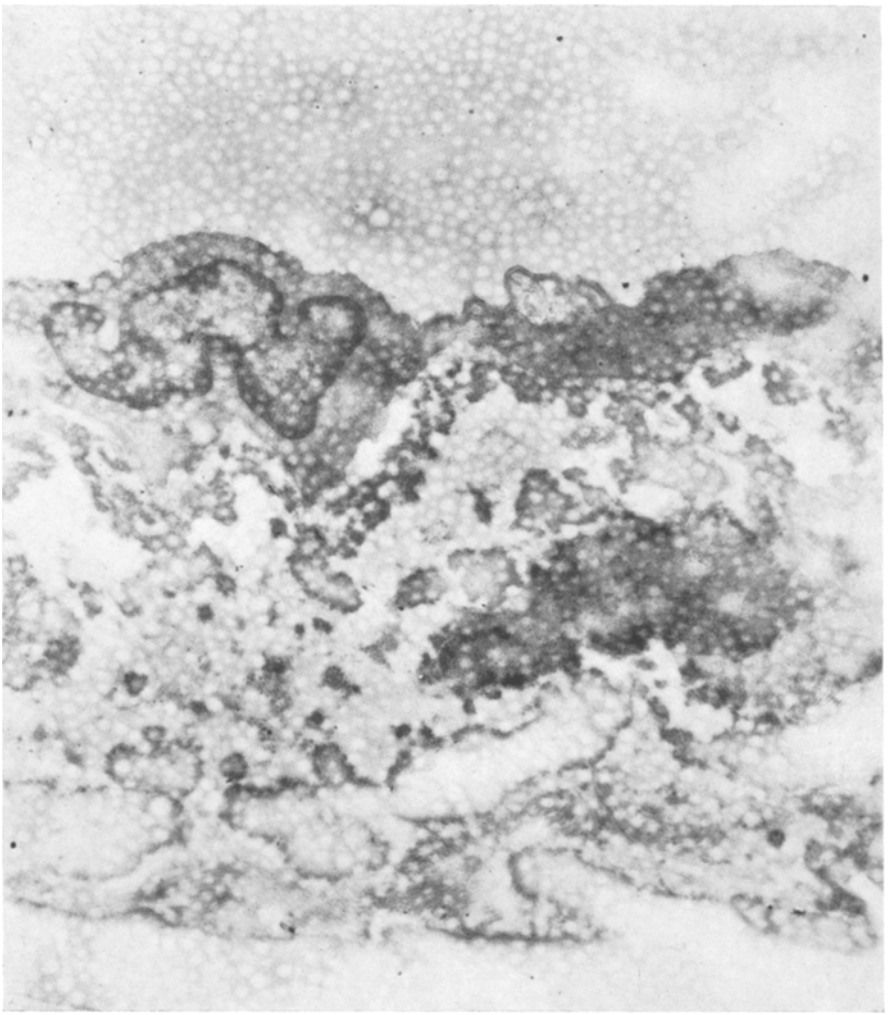

Fig. 1. Endothelium and subendothelial space of the upper thoracic aorta of a normal rabbit fed labelled cholesterol, × 9370. No label is present in the intima. The sieve-like pattern in this and subsequent figures represents the thin photographic emulsion film that overlies the section; the holes in it are caused by the loss of all the unreduced spherical emulsion grains during fixation and washing. The fuzziness and low resolution of this and all other figures is due to the fact that the sections of this study were cut unusually thick and left unstained

Discussion

The results of this study show that the endothelium of normal arteries has a much lower permeability for orally administered cholesterol than that of normal capillaries, but that certain pathological conditions—such as protracted hyperlipemia, serum sickness and desoxy-corticosterone (DOCA) treatment—change it and make it much more permeable to this lipid.

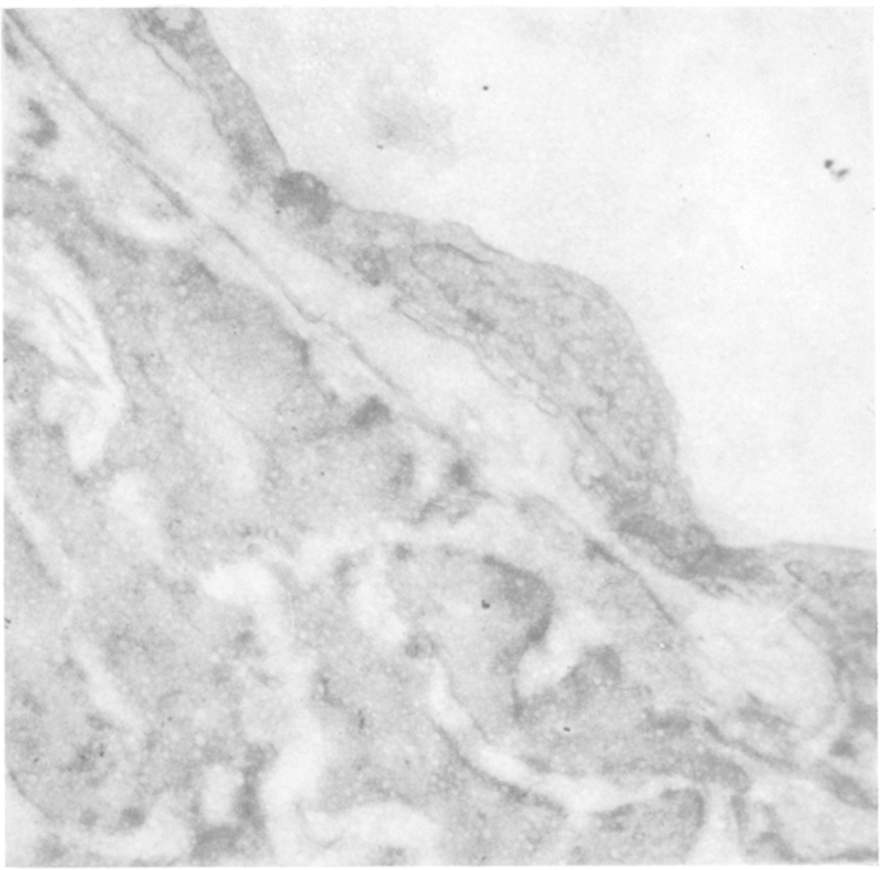

Fig. 2. Endothelium, internal elastic lamella and inner media myocytes of a coronary artery of a normal rabbit fed labelled cholesterol, × 7920. No label is present in the inner arterial wall

Our finding that the capillary endothelium is much more permeable to cholesterol than the arterial endothelium supports the hypothesis this study set out to test. It suggests that most of the giant lipoprotein molecules leave the vascular tree at its finest twigs, to get to the tissues in which they are needed and consumed as raw materials for the building of cell membranes or as high energy fuel. It is evident that organs with a very high mitosis rate (such as the gastrointestinal tract and the hemopoetic organs) or with a very high production of membrane-bound protein (such as the liver) have an enormous turnover of unit membranes that must be continuously replaced. It is also known that tissues such as the heart muscle have an insatiable energy need. By contrast, the cells of the normal arterial wall have a very low mitotic rate (Constantinides, 1968) and a much lower energy demand than the cardiac muscle. It would thus be very inefficient if the lipoprotein molecules were to be lost in the walls of the arterial canals that carry them—where they are not needed—before they reach their destination in the tissues—where they are urgently needed. Indeed, the very fact that the

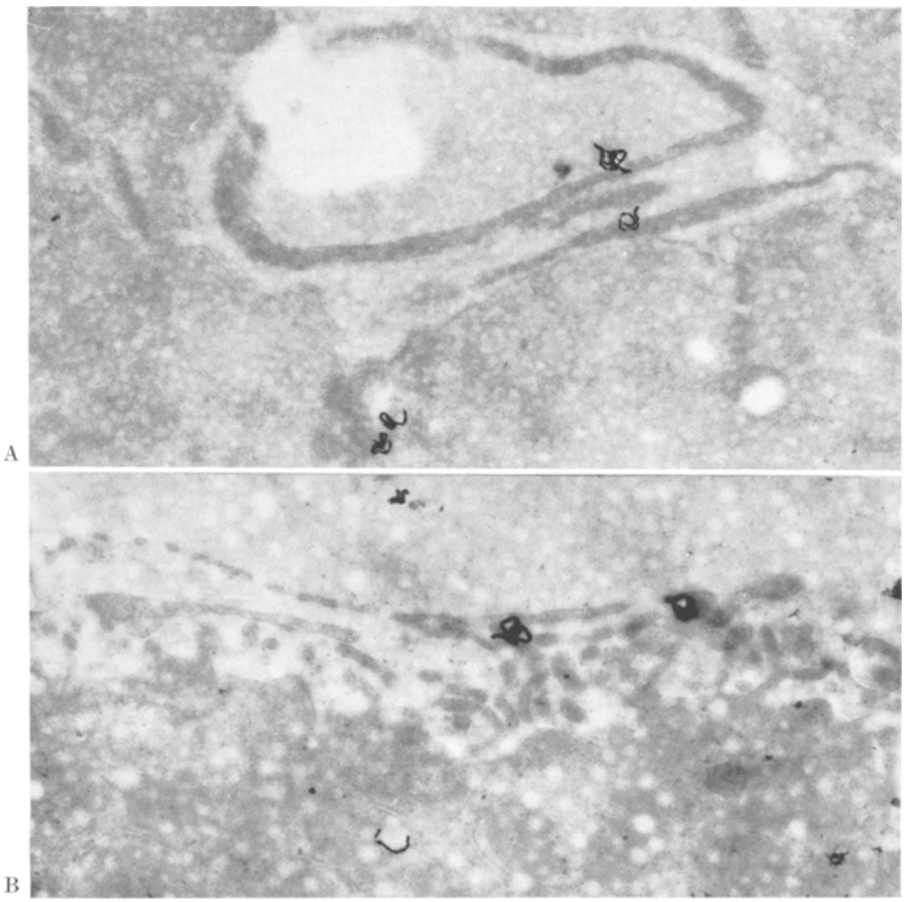

Fig. 3. A. Myocardium of a normal rabbit fed labelled cholesterol, × 11880. One labelled molecule is in a capillary at the top, another has crossed into the pericapillary space and two molecules have just entered a heart muscle fiber at the bottom. B. Wall of a liver sinusoid, space of Dissé and part of an adjacent hepatocyte in a normal rabbit fed labelled cholesterol, × 14520. Two labelled molecules have just crossed the thin, perforated sinusoid wall and have entered the perisinusoidal space while a third molecule has penetrated into the underlying hepatocyte cytoplasm

capillary endothelium of certain organs is perforated, with or without ultrathin diaphragms (as in the liver, the gastrointestinal tract and the hemopoetic tissues), or extremely thin (as in the myocardium) could be taken to indicate that it is *there* that the exit of the giant lipoprotein molecules is programmed by the organism.

The above considerations do not, of course, deny the existence of a constant transarterial perfusing stream of fluid that may carry smaller than lipoprotein molecules (e.g. water, oxygen, electrolytes, glucose, aminoacids, small proteins) in abundant amounts from the blood through the intima-media into the adventitial venules and lymphatics, to service the metabolic needs of the normal arterial wall. That such a perfusion process operates in human arteries is indicated, among

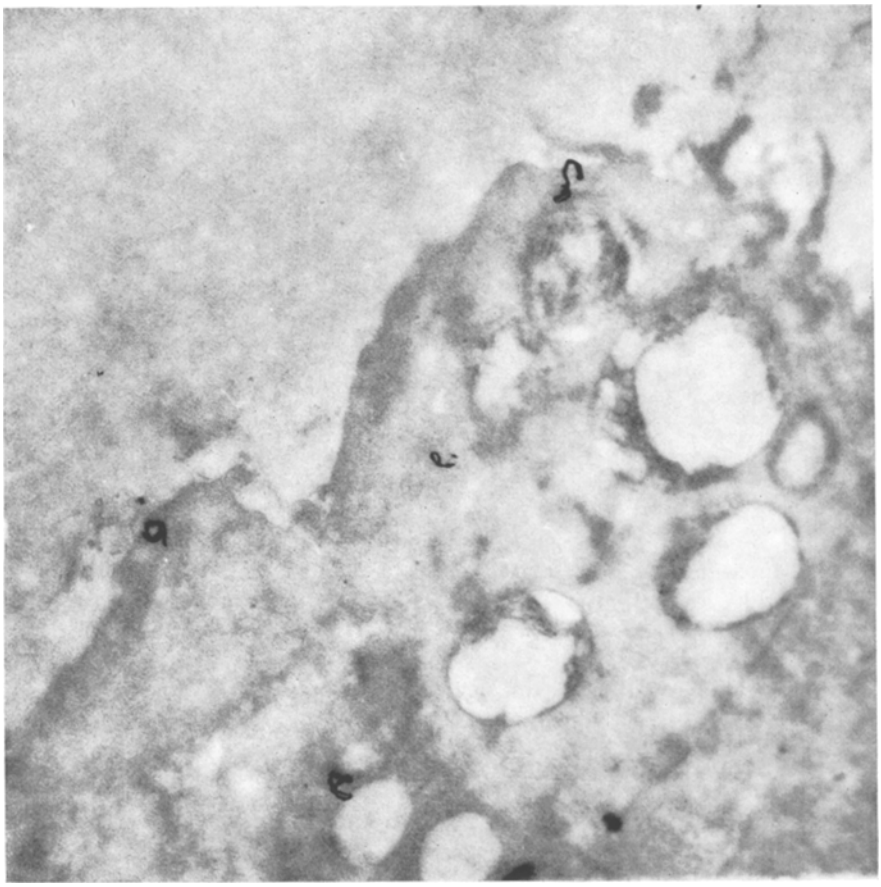

Fig. 4. Thoracic aorta endothelium of a hyperlipemic rabbit that was fed labelled cholesterol, × 14520. The endothelium is highly vacuolated and its cytoplasm is being traversed by four labelled molecules. The complete absence of labelled molecules from the lumen of this and all other arteries is due to their perfusion by buffer and fixative solution that has washed away all "hot" blood; it is also a testimony to the extremely low level of spontaneous grain reduction (background fogging) that was encountered in the present study

other things, by the elegant autopsy studies of Doerr (1970) who demonstrated that conditions which block the adventitial outflow channels cause marked aortic wall edema.

Our autoradiographic finding that the pathological conditions of protracted hyperlipemia-atherosclerosis, serum sickness and DOCA treatment greatly increased the cholesterol permeability of the arterial endothelium is in general agreement with related findings of other workers obtained through different methods. Thus, Adams *et al.* (1970), Adams (1971), and Gore *et al.* (1972), using quantitative measurements of arterial radioactivity, found that protracted hyperlipemia-atherosclerosis increased the permeability of the aortic intima for intravenous cholesterol or lipoprotein; Hartmann *et al.* (1966) reported in a light

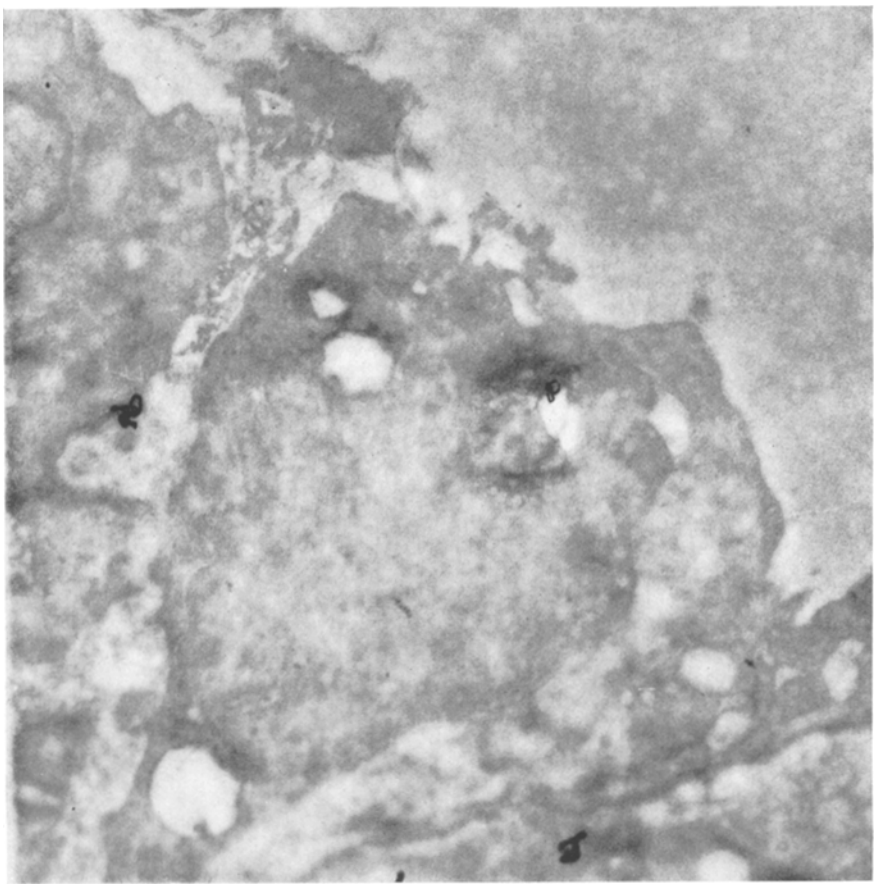

Fig. 5. Thoracic aorta endothelium of a hyperlipemic rabbit that was fed labelled cholesterol, × 14520. A gap has developed between endothelial cells in the left and a labelled molecule is apparently entering the subendothelial space through this gap

microscopic study that 6 months of high cholesterol feeding caused increased entry of water (edema) in the aortic wall of rats; Hess and Stäubli (1969), and Veress *et al.* (1972) presented ultrastructural evidence in rats that a diet inducing hypercholesterolemia increased the aortic wall entry of intravenously injected thorium dioxide or colloidal iron, respectively; Levy (1967) found that serum sickness greatly increased the deposition of cholesterol in the coronaries of cholesterol fed rabbits; Hüttner *et al* (1970) found that an aortic-ligation-induced blood pressure rise increased the transendothelial transport of ferritin, and the marked augmenting effect of experimental and spontaneous hypertension on arterial cholesterol and protein deposition has long been established (Constantinides, 1965; Hüttner *et al.*, 1970).

How did the above pathological conditions increase the entry of labelled cholesterol into the arterial wall ?

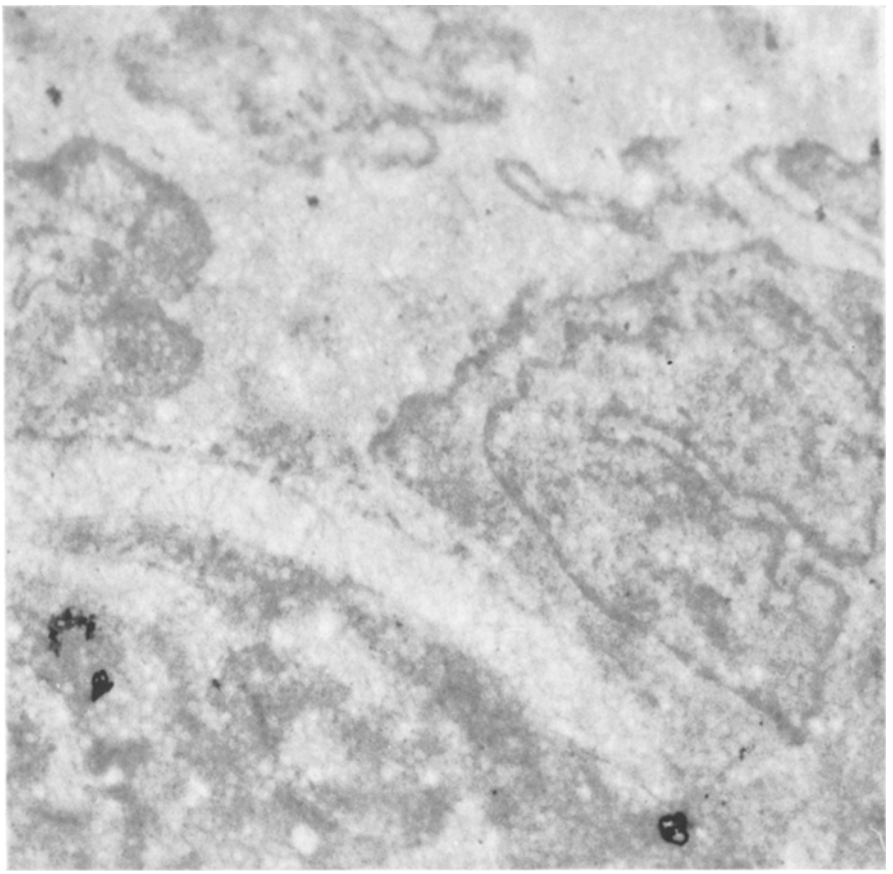

Fig. 6. Coronary artery endothelium and subendothelial space of a hyperlipemic rabbit that was fed labelled cholesterol, × 14520. A wide gap is visible between two endothelial cells overlying the internal elastic lamina. Labelled molecules such as the two that have entered the subelastic space apparently pass very easily into the arterial wall (together with plasma) through a gap of this type. The molecule in the lower right has just penetrated through a *pore in the internal elastic lamina*

In the case of the rabbits with protracted hyperlipemia, the answer must lie in the profound structural changes we found in the arterial endothelium of these animals (i.e. in the extensive macrovacuolation and the opened interendothelial junctions) as well as in the transjunctional migration of lipid-carrying monocytes. Of these changes, extensive macrovacuolation is evident in the electron micrographs of most previous morphological investigators of experimental atherosclerosis, even though its nature is still obscure. Opened endothelial junctions have not, however, yet been described, to our knowledge, in hyperlipemic animals, perhaps because most previous ultrastructural studies have been concerned with shorter cholesterol feeding experiments than those of the present study and because, in scanning multiple sections in multiple samples we surveyed a larger than usual area of aortic endothelium. Future work will show whether the opening of junctions was

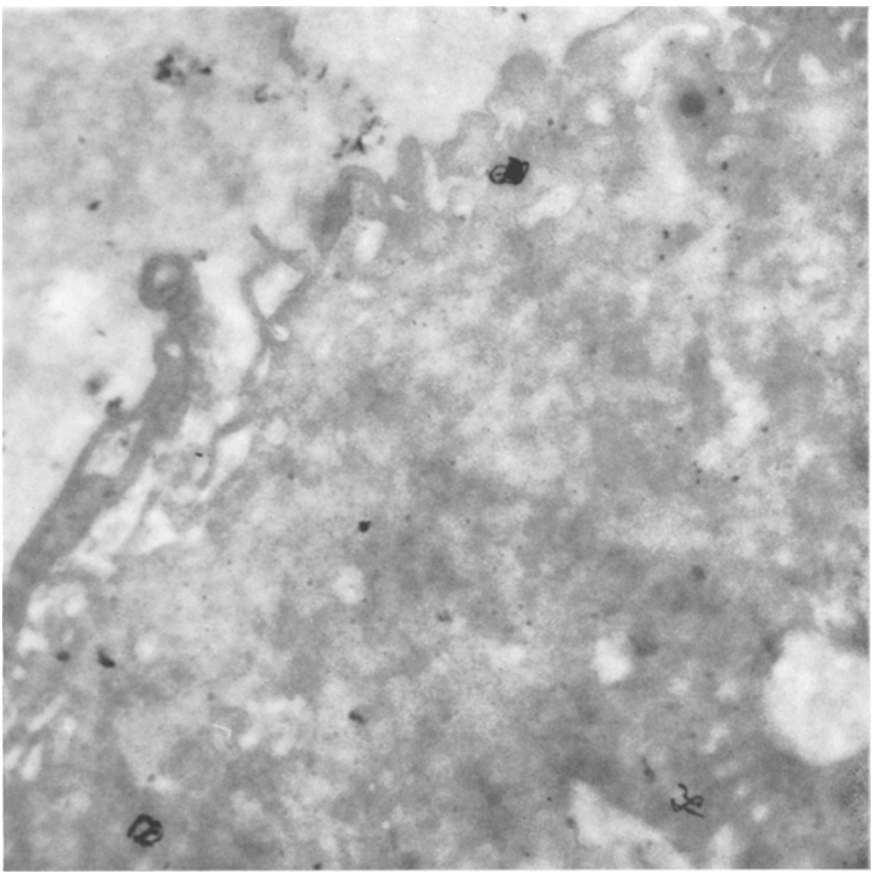

Fig. 7. Abdominal aorta endothelium and subendothelial space of a hyperlipemic rabbit that was fed labelled cholesterol, × 18480. The left lip of a wide endothelial gap is formed by a thin endothelial process, visible in the upper left, whereas the right lip is outside the present field. A large phagocytic monocyte fringed by numerous tentacles has apparently entered the subendothelial space through this gap from the blood and is transporting in its cytoplasm three labelled molecules

related to the loss of the electronegative *sialic acid coat* of endothelial cells that was recently observed by Weber (1973) in hyperlipemic rabbits, and (or) the loss of the electropositive ion *calcium* (Constantinides, 1972), both of which seem to be important structural components of arterial endothelial junctions. As to the immigration of lipid-laden monocytes, this has been documented before in hyperlipemic animals (Leary, 1941; Poole and Florey, 1958).

It was obvious that labelled cholesterol moved into the arterial wall of the hyperlipemic rabbits (a) through the cytoplasm of the markedly altered endothelial cells ("transcytoplasmically"), (b) as a free particle through the gaps of the opened endothelial junctions ("transjunctionally), and (c) as an inclusion in the cytoplasm of monocytes that penetrated through endothelial junctions ("transmonocytically"). Although this study was not designed to compare these

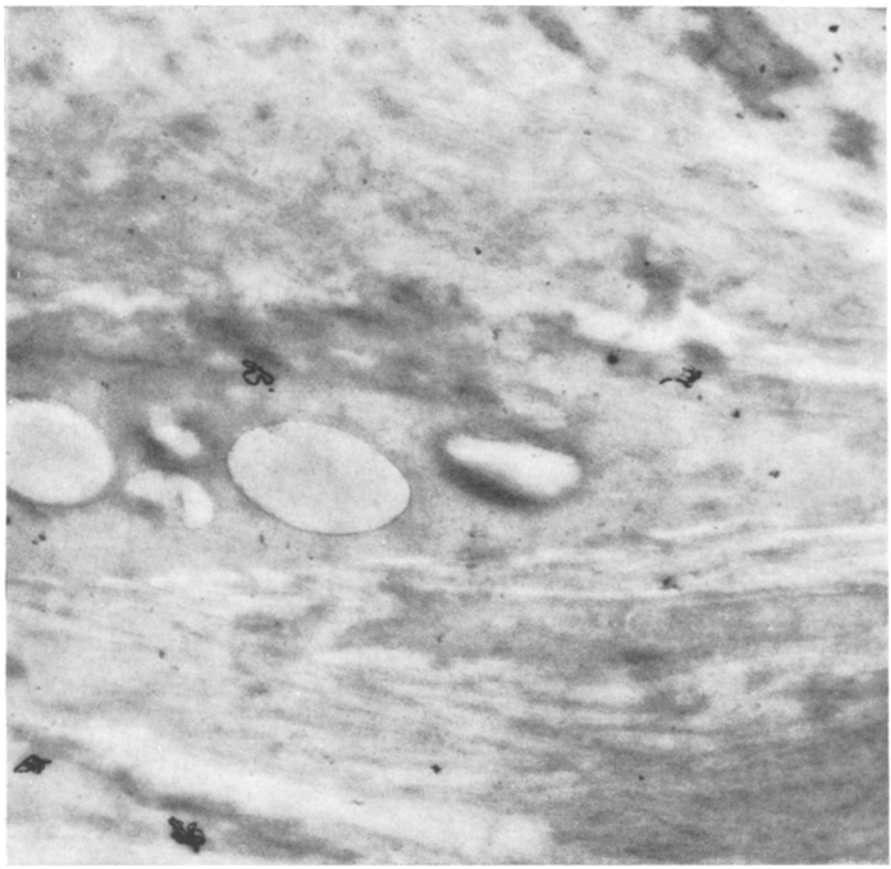

Fig. 8. Superficial region of an atheroma in the thoracic aorta of a hyperlipemic rabbit fed labelled cholesterol, × 14520. Labelled molecules are entering spindle-shaped smooth muscle cells that begin to turn into foam cells

three mechanisms of entry quantitatively, and although even the relatively few opened junctions observed could promote considerable label entry with time, it appeared that most of the label crossed into the wall through the *cytoplasm* of the altered endothelial cells. Our present experiments do not allow us to determine whether cholesterol crossed the endothelial cytoplasm through pinocytosis or through direct penetration of the endothelial plasma membrane and cytoplasmic matrix, because the sections were too thick and the emulsion grain too large for precise localisation of the label in relation to the pinocytic vesicles. Since the recent electron microscopic autoradiographic study of Stein *et al.* (1973) with aortic perfusions of protein-labelled lipoprotein in normal rats (and the same size of emulsion grain) has not provided any proof of pinocytic lipid transfer either, it will be up to future work with thinner sections and smaller emulsion grain to resolve this problem.

In what form did the label cross the endothelium ?

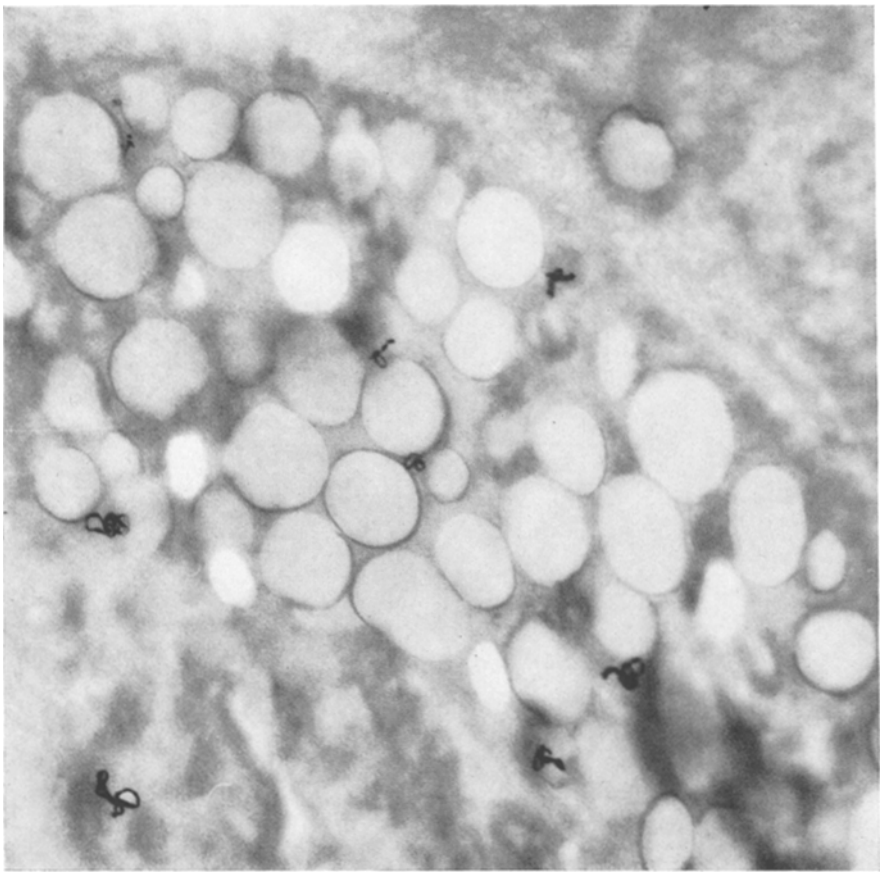

Fig. 9. Superficial region of an atheroma in the thoracic aorta of a hyperlipemic rabbit fed labelled cholesterol, × 14520. Several labelled molecules have entered the cytoplasm of a fully developed foam cell

It seems certain that the label represented lipid (since it disappeared from the tissues following chloroform-methanol extraction), and that most of it was free cholesterol (since most of the label disappeared from tissues fixed in the absence of digitonin). We have no proof that the labelled cholesterol that moved through the *cytoplasm* of endothelial and other cells was still coupled to protein although recent findings of protein-labelled lipoprotein within the endothelium of perfused normal rat aortas (Stein *et al.*, 1973) make this a likely possibility. We can be sure, however, that the cholesterol which—together with plasma—crossed through the gaps of the opened endothelial junctions entered the sub-endothelial space in the same form in which it was circulating in the blood after its absorption from the gut, i.e. as an intact lipoprotein molecule.

While the foregoing discussion has brought out the fact that prolonged hyperlipemia caused severe endothelial changes in arteries and promoted the entry of labelled cholesterol molecules through three different mechanisms, the situation

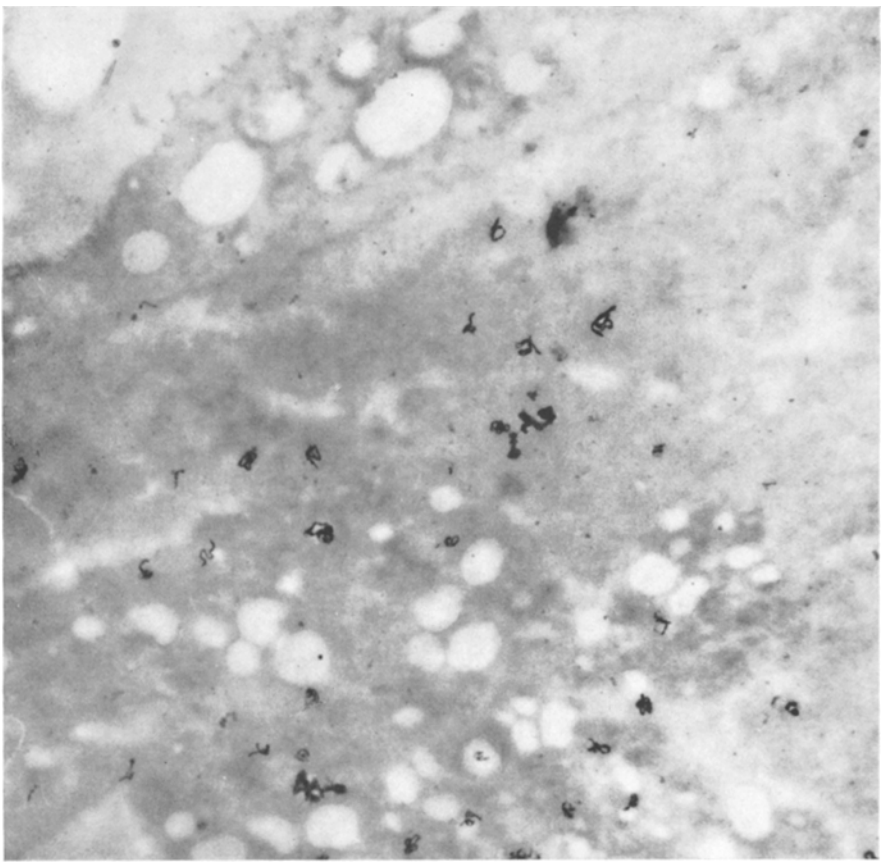

Fig. 10. Endothelium overlying superficial region of an atheroma in the thoracic aorta of a hyperlipemic rabbit fed labelled cholesterol, × 7920. The lumen is visible in the upper left. Large numbers of labelled molecules have crossed the vacuolated endothelium into the underlying superficial atheroma region, where they lie partly extracellular and partly intracellular within foam cells

was different in the serum sick and DOCA-treated animals. Here, due to the relatively short duration of these treatments, no dramatic endothelial changes—such as the opening of junctions described by Holle (1973) in serum sickness and by Suzuki *et al.* (1971) in hypertension—had yet developed, and cholesterol entry appeared to be exclusively transcytoplasmic. It would thus seem that serum sickness and DOCA hypertension can promote cholesterol entry by increasing pinocytosis and (or) plasma membrane permeability in endothelial cells, even before they produce advanced damage such as physical gaps in the endothelial lining.

How do our findings of very low cholesterol permeability of the normal arterial endothelium relate to the results of other workers who reported a fair aortic entry of intravenously injected peroxidase and ferritin (Hüttner *et al.*, 1970) or labelled cholesterol (Somer and Schwartz, 1972) and of perfused protein-labelled lipoprotein (Stein *et al.*, 1973) in normal animals?

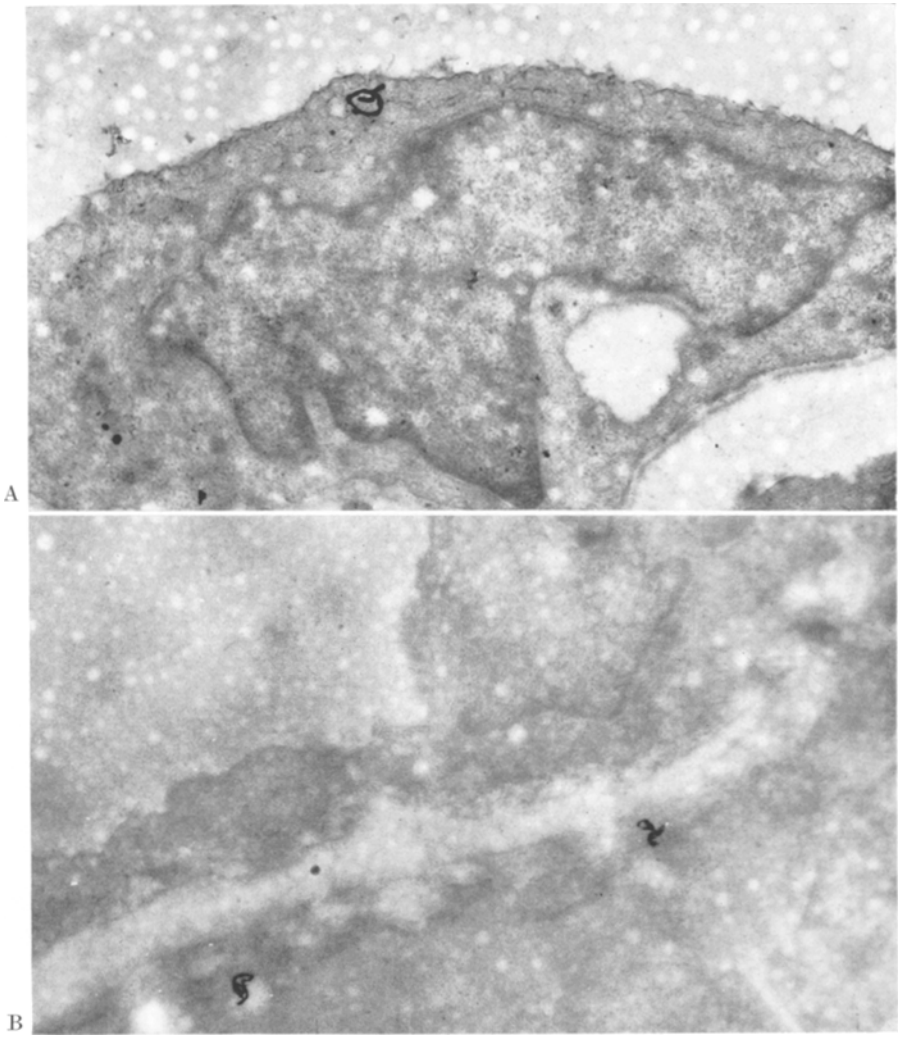

Fig. 11. A. Typical thoracic aorta endothelium of a DOCA-treated rabbit fed labelled cholesterol, × 18480. A labelled molecule has entered the cytoplasm of an endothelial cell. B. Coronary artery endothelium and inner media of a serum sick rabbit fed labelled cholesterol, × 14520. Two labelled molecules have penetrated through endothelium and internal elastic lamina into the subelastic space

As far as the results of Hüttner *et al.* (1970) are concerned, the difference between our findings and theirs probably means that the arterial endothelium is much more permeable to the relatively smaller protein molecules of peroxidase and ferritin than to the large cholesterol-carrying lipoprotein molecules we studied. It is reasonable to expect that the normal endothelium would handle molecules of different size, composition and properties in a different manner. Furthermore, there are indications that tracers such as ferritin and peroxidase may not be inert passenger molecules, but may have effects of their own on the endothelial lining;

thus, Becker (1972) recently reported that ferritin increases endothelial permeability, and it is conceivable that the oxidative action of peroxidase could affect the structural components of endothelial junctions.

As far as the difference between the findings of Stein *et al.* (1973) and our own results is concerned, this could well be due to the vastly different experimental conditions of the two studies. In the study of Stein *et al.* foreign lipoproteins were given to rats by aortic perfusion as a massive pulse; this could have created within a short time a much higher concentration of such molecules in the plasma (and a correspondingly higher arterial entry) than in our experiments, where the lipoproteins were generated by rabbits in vivo and were released into their own plasma slowly as a result of the physiological absorption of cholesterol from the gut over several hours.

Lastly, the finding by Somer and Schwartz (1972) of measurable aortic entry of intravenously injected cholesterol is quite compatible with our own observation of only one or two labelled cholesterol molecules per 20 electron microscope fields of inner aortic tissue. It is conceivable that what these authors found to be a measurable aortic uptake of label with liquid scintillation counting of relatively large aortic tissue samples would show in our electron microscopic survey of 2000–3000 Å thick aortic sections as only very few molecules per section. Furthermore, it might be expected that the relatively small "naked" cholesterol molecules that Somer and Schwartz introduced into the plasma intravenously would penetrate more easily into endothelial, blood and other cells than our orally administered cholesterol molecules that entered the plasma coupled to proteins, phospholipids and triglycerides to form the much larger and more complex lipoprotein particles.

It should be kept in mind that the main thrust of our study was not to assess the absolute amount of cholesterol that crossed any vessel lining, but to *compare* the cholesterol permeability of normal arterial versus normal capillary endothelium, as well as of normal arterial versus pathological arterial endothelium. It is the existence of enormous *differences* of cholesterol permeability between these various types of endothelia that constituted the main conclusion of our experiments and this conclusion is not opposed by any relevant findings of other workers.

We finally have to deal with the possible role in our results of molecular exchange between the labelled cholesterol we administered and the unlabelled structural cholesterol of the cells of the recipient animals—a subject that has been the focus of some attention in recent years, particularly in in vitro experiments or in acute (short-term) experiments involving parenterally injected free molecular cholesterol (Somer and Schwartz, 1972; Bell *et al.*, 1972).

We did not obtain much evidence of exchange between the "hot" cholesterol we administered and the "cold" cholesterol of blood cells and blood vessel wall cells in our studies. Had such an exchange occurred on an appreciable scale, we would have expected a random, indiscriminate and approximately equal uptake of label from the plasma by all cells that came in contact with it, e.g. by the endothelial cells of the entire vascular tree (arteries as well as capillaries) and by the erythrocytes—which was not the case. We did not observe any red cell labelling and the endothelial—indeed, all vascular—labelling proved highly selective: much capillary versus very little arterial labelling, and much pathological

versus very little normal arterial labelling. This suggests that exchange phenomena were minimal under our experimental conditions, perhaps because (a) the orally administered cholesterol molecules which enter the plasma bound to and surrounded by shells of proteins and phospholipids as lipoprotein molecules (Scanu, 1972) are not as readily available for exchange with the cholesterol of neighbouring cell membranes as are "naked" cholesterol molecules added to blood in vitro or injected intravenously (i.e. introduced into the body in unphysiological form), and (b) our cholesterol administration was relatively chronic, whereas exchange phenomena occur mainly in acute, single-pulse type of experiments (Scanu, 1972; Schwartz, 1973). But even if some exchange had occurred in our study, it could not change our basic conclusion that there are great functional—and, therefore, physicochemical—differences between different types of endothelia. One would, in that case, have to postulate that any exchange that may have occurred was controlled and directed by these biological differences, since one type of endothelial lining allowed 100 times more cholesterol to cross it and exchange with the subendothelial tissue steroid than another.

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Dr. P. Constantinides, M. D., Ph. D.
Pathology Department
University of British Columbia
Vancouver/Canada