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AN *IN VITRO* METHOD FOR THE STUDY OF CHOLESTEROL UPTAKE AT THE ENDOTHELIAL CELL SURFACE OF THE RABBIT AORTA

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SUMMARY

In order to study the mechanism for the uptake of cholesterol at the intimal surface of the thoracic aorta of normal rabbits an *in vitro* method was developed.

1. The metabolic activity of the aortic segments similar to those used in the cholesterol uptake experiments was estimated from the determination of 2 parameters, *viz.* the oxygen consumption rate and the rate of lactic acid production. At least 85% of catabolized glucose was converted to lactic acid.

2. The serum used in the experiments on the uptake of [^{14}C]cholesterol by aortic segments was labelled with [4-^{14}C]cholesterol by an *in vivo* method. Polyvinyl chloride block electrophoresis showed the labelled cholesterol to be distributed almost exclusively in the α- and β-globulin regions. The specific activities of free and esterified cholesterol in the serum were approximately equal.

3. Following incubation of aortic segments in serum containing labelled cholesterol, the preparation of an intima-media layer from the central part of the incubated segments made it possible to measure the endothelial uptake of labelled cholesterol.

4. Among the animals, some variation was found in the 4-h uptake of labelled cholesterol by intima-media, but the intima-media of 2 hemisegments taken from the same aorta at the same level were shown to take up fairly equal amounts of labelled cholesterol when incubated in the same serum. Hence control conditions can be easily established.

INTRODUCTION

An uptake of plasma lipoprotein or lipoprotein components by the arterial wall has been established in a number of studies, *in vitro*[1–5] as well as *in vivo*[6–17]. Some of these studies[6,7,11,13,15,17] seem to indicate that the rate of entrance into the arterial wall of plasma lipoprotein components, in particular cholesterol and phospholipids, is sufficiently high to explain the accumulation of these substances in the arterial wall during experimental atherogenesis associated with hyperlipemia. The rate of entrance of labelled cholesterol, labelled albumin and lipoprotein at various sites of the normal

dog aorta has been shown to correlate with the accumulation of cholesterol at the same sites during the early stages of atherogenesis[9,10,14,16]. Further, in the hyperlipemic cockerel the entrance rates for plasma phospholipids and cholesterol correlated with the aortic concentrations of these substances[17] and in the hypercholesterolemic rabbit it has been shown that larger quantities of cholesterol in the atheromatous lesion were associated with higher influxes[15]. All these studies provide some quantitative evidence for the significance of the plasma lipoprotein components in the development of experimental atherosclerosis.

As to the mechanism of the transfer of lipids into the arterial wall it has been widely held that such transfer depends on a plasma filtration[18,19]. Recent electronmicroscopic studies[20–22], however, suggest that a pinocytotic activity by the endothelial cells of the arterial intima might provide the explanation for such transfer. If plasma lipoprotein components—or lipoprotein molecules as a whole—were to be taken up "actively" by the endothelial cells, *e.g.*, by pinocytosis, it might be expected that variations in the metabolic activities of the cells in question would influence the uptake. In order to elucidate the relationship between metabolism and uptake the *in vitro* method described below was elaborated.

MATERIALS AND METHODS

Animals

The animals were obtained from uniform breeds of white female rabbits (from the Danish State Serum Institute). Before the experiments the rabbits were fed an ordinary vegetable diet. They were used at an age of 4–6 months and their weights ranged from 2.14 to 2.80 kg.

Preparation of aortic segments. The rabbits were anaesthetized with pentobarbital intravenously (40 mg per kg body weight), supplemented, when necessary, with diethyl ether. The chest was opened and the descendent part of the thoracic aorta removed. The thoracic aorta was cleansed under running cold modified Krebs–Ringer solution (for composition, see below). The loose adventitial tissue was then stripped off and the vessel cut open longitudinally between the branches of the intercostal arteries on the posterior wall. After further cleansing from blood, the aorta was cut into 4 segments, 2 upper ones (U_a and U_b) and 2 lower ones (L_a and L_b) of approx-

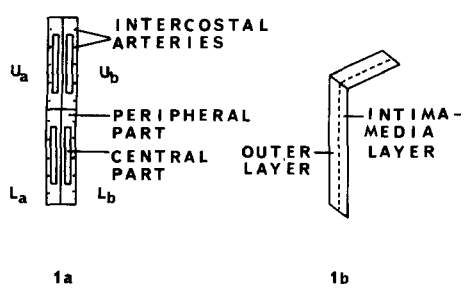

Fig. 1a. Thoracic aorta cut open in longitudinal direction posteriorly between the branches of the intercostal arteries. For further explanation see text. 1b. Division of the aortic wall into intima-media and outer layer.

imately the same surface area (Fig. 1). Each segment was blotted on filter paper and weighed. The surface areas were determined by planimetry. The whole procedure, starting with the pentobarbital injection, took about 35 min and was immediately followed by incubation of the segments either in an artificial medium for the measurement of oxygen uptake and lactic acid production or in serum containing labelled cholesterol for the measurement of cholesterol uptake. The first-mentioned type of incubations served to test the metabolic activity of aortic segments similar to those used in the cholesterol uptake experiments.

Oxygen consumption and lactic acid production by aortic segments. The segments were incubated in standard Warburg flasks each containing 2 ml of a modified Krebs–Ringer solution with 0.2% glucose and buffered with Tris and phosphate. The solution contained per l: Na^+, 140 mequiv; K^+, 4.1 mequiv; Ca^{2+}, 7 mequiv; Mg^{2+}, 2 mequiv; phosphate, 2 mmoles; Tris, 20 mmoles (pH at 38°, 7.4). Oxygen consumption was measured by the direct Warburg technique[23] with atmospheric air as the gas phase. CO_2 was absorbed by filter paper saturated with 0.2 ml 2 M KOH situated in the centre well.

Temperature equilibration (to 38°) prior to the start of the experiments took 10 min. Manometer readings were done at intervals of 15 or 30 min. At the end of the incubations (4 h) the concentration of lactic acid in the medium was determined. The aortic segments were later dried to constant weight in a freeze-dryer (model Gamma, Christ) for 24 h. Their weights were recorded with a Cahn electrobalance weight.

pH measurements. pH was determined at 38°, using a direct-reading pH meter 22 (Radiometer, Copenhagen).

Lactic acid determination. Lactic acid was determined enzymatically[25–27]. The Biochemica test combination TC-B of Boehringer was used. The coefficient of variation calculated from the duplicate determinations was 1.1% ($n = 23$).

Serum-labelling procedure. 10 ml of blood were taken from the lateral ear vein of the donor rabbit into a heparinized test tube. The blood was mixed with 0.2 ml of 96% ethanol containing 0.2 mC of [4-^{14}C]cholesterol (from the Radiochemical Centre, Amersham, England). The specific activity was approx. 50 μC/mg. The blood containing labelled cholesterol was incubated at 37° for 5 h and then re-injected intravenously into the donor rabbit. 24 h after the injection, the donor rabbit was exsanguinated from one of the carotid arteries under pentobarbital–ether anaesthesia. The blood was centrifuged at 1700 × g for 10 min. Serum was stored at 4° and used within about one week.

Electrophoresis of labelled serum. In 2 cases polyvinyl chloride block electrophoresis of serum containing labelled cholesterol was performed. The method of electrophoresis was as described by KEIDING[24], making use of starch as supporting medium. Following the electrophoresis, a filter paper was placed along the wet block and then dyed for proteins with bromophenol blue. The block itself was cut transversally into 1-cm segments. Serum lipids from these segments were in one case extracted with boiling methanol–chloroform (1:1, v/v) for about 2 h, in another case with methanol–chloroform (1:1, v/v) for about 15 min, at room temperature. Cholesterol and ^{14}C activity were determined on these extracts as described below.

Uptake of [^{14}C]cholesterol. Aortic segments were placed in standard Warburg flasks each containing 2 ml serum containing labelled cholesterol. Before incubation the serum was aerated with a gas mixture moistened with water at 38°. The composi-

tion of the dry gas was: CO_2, 5.8%; O_2, 16.4% and N_2, 77.8%. Determination of pH was performed on each vessel before and after incubation. Cholesterol and [^{14}C]lipid activity was determined on serum before and/or after incubation. The incubations were of 4 h duration. The temperature was 38°.

At the end of incubation, the aortic segment was taken up and rinsed for approx. 1.5 min with the above-mentioned modified Krebs–Ringer solution, the surfaces being gently brushed with a soft brush. Each segment was divided into a central and a peripheral part by punching (Fig. 1). Each part was divided into two layers, an inner intima-media layer and an outer layer. Before analysis the layers were dried to constant weight in a freeze-dryer for 24 h and weighed.

Lipid extraction

Serum. The lipids were extracted for at least 30 min at room temperature with 19 or 49 vol. of methanol–chloroform (1:1, v/v) before determination of radioactivity and cholesterol, respectively. The 1:1 mixture was used instead of the usual 1:2 mixture to obtain a specific gravity at which the proteins settled easily. In three experiments serum cholesterol was found to be extracted equally well by the 1:1 and by the 1:2 mixture. Cholesterol determination was performed on the clear supernatant.

Aorta. The freeze-dried aortic tissue was homogenized in 6 ml of methanol–chloroform (1:2, v/v) making use of a Potter–Elvehjem-type of homogenizer[28,29]. The homogenate was applied to a fat-free filter paper. After draining, three washings were performed each with 4 ml methanol–chloroform (1:2, v/v). The methanol–chloroform was then evaporated at low pressure, the lipids were redissolved in 5 ml chloroform and transferred to a 10-ml tube with 2 washings of 2.5 ml chloroform.

In order to check the effectiveness of this extraction procedure 2 lots of filter papers with precipitate from several aortic segments were re-extracted with boiling methanol–chloroform (1:2, v/v) for 2 h under reflux. Only 0.6% and 0.9% of the total activity were found in these additional extracts.

Cholesterol determination. The Tschugaeff reaction was used in the modification of HANEL AND DAM[30]. In the Tschugaeff reaction, free and esterified cholesterol gave approximately the same molar extinction[30]. The molar extinction is about 7 times greater than that obtained with the Liebermann–Burchard reaction.

An aliquot of the lipid extract was taken to dryness under low pressure. The reaction mixture added to the lipids was made up of chloroform, acetyl chloride and zinc chloride (3:1:1). Between the extinction (y) and the concentration of cholesterol (x) no proportionality was found, but a linear relationship was always found between y and x in the range of 20–80 μg cholesterol per 3 ml of the reaction mixture. Therefore 3 standards were run with each group of cholesterol determinations.

The coefficient of variation calculated for the duplicate determinations was 1.6% ($n = 30$).

Separation of free and esterified cholesterol

In 2 cases the specific activity of free and esterified cholesterol was followed in the donor rabbit during the 24-h period between injection of labelled cholesterol and exsanguination. An aliquot of serum methanol–chloroform extract was taken to dryness in an atmosphere of N_2 and the lipids were redissolved in hexane. The separation

of free and esterified cholesterol was carried out on columns of florisil (activated magnesium silicate to which had been added 7% of water[31]). The esterified cholesterol was eluted with diethyl ether–hexane (5:95, v/v), the free cholesterol with diethyl ether–hexane (25:75, v/v).

The elution pattern was followed by activity measurements on successive 2-ml portions of eluate. The recovery of serum total cholesterol added to the column was checked by radioactivity measurements as well as by chemical analysis and was, on the average, found to be 94% and 93%, respectively.

Radioactivity measurements

Serum. Of the lipid extract, 0.2-ml aliquots were plated in duplicate on aluminium planchets. The ^{14}C activity of the "infinitely thin" layers obtained was registered in a window-less methane-flow counter (Frieseke and Hoepfner). The constancy of the activity-registration equipment was checked with a [^{14}C]cholesterol standard for every 2 samples. 10 000 counts were recorded. The net activity was at least 20 times the background. The coefficient of variation calculated for duplicate samples of serum was 2.1% ($n = 23$).

Aorta. The chloroform extract of the aortic lipids was taken to dryness in an atmosphere of N_2 and redissolved in benzene (0.65 ml). The benzene solution was plated with one washing on aluminium planchets. When occasionally necessary, correction for self-absorption was made from a self-absorption reference curve. This curve was based on measurements of the activities on planchets with various amounts of [^{14}C]cholesterol-containing liver lipids. 4000–10 000 counts were recorded. The net activity of the aortic samples was at least equal to the background (13±0.5 counts/min).

The coefficient of variation calculated from duplicate determinations on the lipid extracts from 23 outer layers was 3% (10 000 counts recorded).

Polyvinyl block electrophoresis segments. In the lipid extract used for activity measurements some polyvinyl material was also found. Hence a correction for absorp-

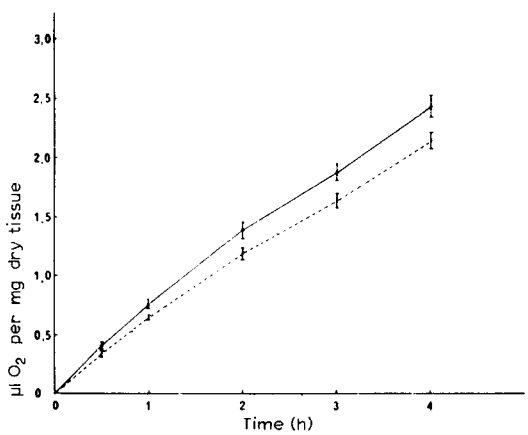

Fig. 2. Oxygen consumption by 12 upper and 11 lower segments of thoracic aortas from 6 normal rabbits. ○—○, upper segments; ○— — —○, lower segments. The points are mean values. The vertical lines indicate the standard errors of the mean values. Abscissa, time in h; ordinate, oxygen consumption in µl per mg dry tissue.

tion of ^{14}C activity was performed. The recovery of [^{14}C]cholesterol present in the serum added to the blocks was 107% and 90% in two experiments.

RESULTS

Aortic oxygen consumption and aerobic glycolysis

When aortic segments were incubated in a Krebs–Ringer solution buffered only with the small amounts of phosphate present in a Ca^{2+}-containing solution the pH changes amounted to 0.6–0.7 unit. As a standard medium for incubation the aforementioned Tris-containing Krebs–Ringer solution was therefore used.

TABLE I

LACTIC ACID PRODUCTION BY AORTIC SEGMENTS UNDER AEROBIC CONDITIONS

The values are expressed in μg per mg dry tissue per h. Mean values $\pm$ S.E.; n, number of segments.

	Lactic acid production (μg/mg per h)
Upper segments (n = 12)	4.23 ± 0.25
Lower segments (n = 11)	4.45 ± 0.35

Even in this medium some changes in pH took place. The mean pH value (at 38°) before incubation was 7.40±0.02 (S.D.). At the end of incubations of upper and lower segments the pH was 7.13±0.11 and 7.17±0.09, respectively.

The oxygen consumption during a 4-h incubation is shown in Fig. 2. The total oxygen consumption was 2.44±0.09 and 2.15±0.07 μl per mg dry tissue for upper and lower segments, respectively (mean values ±S.E.). The Q_{O_2} (*i.e.* μl O_2 consumed per mg dry tissue per h) was 0.76±0.04 and 0.65±0.02 for upper and lower segments, respectively, during the first hour of incubation. The oxygen consumption by upper and lower segments differs significantly ($P<0.02$).

The lactic acid production is shown in Table I.

TABLE II

CONVERSION RATES FOR GLUCOSE → LACTIC ACID (A) AND GLUCOSE → CO_2 + H_2O (B) AND MINIMUM PERCENTAGE OF CATABOLIZED GLUCOSE CONVERTED TO LACTIC ACID (C)

The figures are mean values. In (A) and (B) values are given ± S.E; n, number of segments.

	Upper segments (n = 12)	*Lower segments (n = 11)*
	(μg/mg per h)	
(A) Glucose → lactic acid	4.23 ± 0.25	4.45 ± 0.35
(B) Glucose → CO_2 + H_2O (maximal values)	0.82 ± 0.03	0.72 ± 0.02
(C) $\frac{A}{A+B} \times 100$	84%	86%

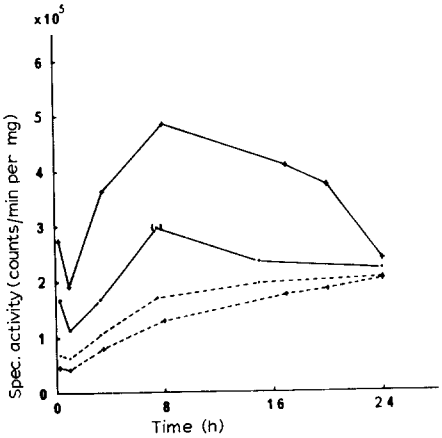

Fig. 3. The specific activity of free (—) and esterified (- - - -) cholesterol after injection of labelled cholesterol into 2 donor rabbits. ○—○ and ○- - - -○, rabbit No. 623 A; + — + and + - - - +, rabbit No. 357 A.

Assuming glucose to be the only source of energy, the maximal rate for glucose conversion to $CO_2 + H_2O$ may be calculated from the oxygen consumption rate. From this maximal value and the rate for lactic acid production the minimum percentage of catabolized glucose converted to lactic acid may be calculated. These values are shown in Table II for upper and lower segments of the thoracic aorta.

Incorporation of [^{14}C]cholesterol into the normal serum cholesterol pools

Fig. 3 shows the specific activity changes for free and esterified cholesterol in the serum of 2 donor rabbits during a 24-h period after injection of blood containing labelled cholesterol.

There is an initial drop in the specific activity, at least for free cholesterol. After 1 h the specific activity of free cholesterol rises for a 7-h period, after which it decreases. The specific activity of esterified cholesterol rises after 1 h throughout the rest of the

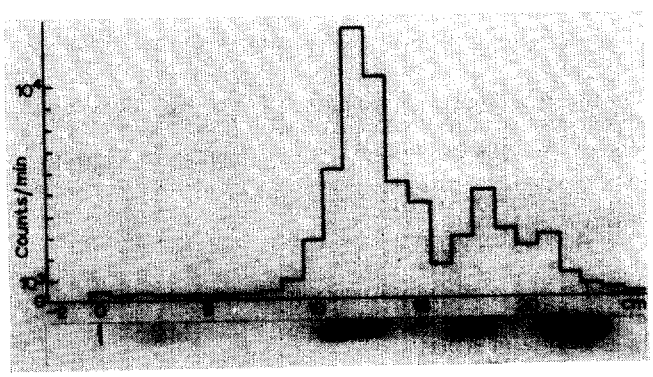

Fig. 4. Electrophoresis on a block of polyvinyl choride of 1 ml serum from a donor rabbit 24 h after injection of labelled cholesterol (rabbit No. 357 A). Electrophoresis was started at the vertical line. The curve shows the ^{14}C activity from 1-cm segments of the block. Below, a strip stained for protein with bromophenol. Albumin at extreme right.

24-h period, at the end of which the specific activities are approximately identical for the two cholesterol fractions.

The electrophoretic pattern of the ^{14}C activity and serum protein fractions is shown in Fig. 4. The activity is highest corresponding to the α- and β-globulin regions.

4-h uptake of labelled cholesterol

The changes in serum pH (at 38°) during the 4-h incubations were insignificant. Thus, the pH change found during incubations of upper segments was from 7.42 ± 0.01 to 7.37 ± 0.04 and for lower segments from 7.42 ± 0.01 to 7.39 ± 0.02 (S.D.).

The total uptake of [^{14}C]cholesterol by an incubated aortic segment represents about 1% of the [^{14}C]cholesterol in the medium, and the changes in the concentration of [^{14}C]lipid activity and cholesterol during the incubation are not significant. The specific activity of cholesterol in the medium therefore remains constant.

The surface area of the central parts of the aortic segments was 0.75 cm^2. The area of the peripheral parts was about 1 cm^2 for upper as well as lower segments.

On a dry-weight basis the intima-media layers from 10 upper and 10 lower segments made up $45 \pm 3\%$ and $49 \pm 2\%$ of the total aortic wall thickness, respectively (mean values $\pm$S.E.).

In this study the uptake of [^{14}C]cholesterol is expressed as the ^{14}C activity found in the aortic preparation per cm^2 surface area after incubation for 4 h. In Table III

TABLE III

UPTAKE OF LABELLED CHOLESTEROL FROM SERUM BY CENTRAL AND PERIPHERAL PARTS OF INTIMA-MEDIA LAYERS AND CENTRAL PARTS OF OUTER LAYERS OF THE THORACIC AORTAS FROM 5 NORMAL RABBITS

The uptake is expressed as counts/min per cm^2 surface area per 4 h. Serum cholesterol concentration, 53 mg per 100 ml. Serum cholesterol specific activity, $2.4 \cdot 10^5$ counts/min per mg. Mean values $\pm$ S.E.

Segments	*[^{14}C]Cholesterol uptake (counts/min per cm^2 per 4 h)*		
	Intima-media layers		*Outer layers*
	Central part	*Peripheral part*	*Central part*
Upper	31 ± 2	93 ± 6	298 ± 61
Lower	27 ± 3	86 ± 6	192 ± 24

are shown mean values for the uptake of [^{14}C]cholesterol by the intima-media layers of central and peripheral parts and by the outer layers of central parts from the thoracic aorta of 5 normal rabbits.

It will be seen that, for the central parts, the outer layers show a 8–10 times greater uptake of labelled cholesterol than the intima-media layers. The uptake of labelled cholesterol by the intima-media is about 3 times greater in the peripheral than in the central parts. Both differences are significant ($P < 0.001$).

The uptake of labelled cholesterol by central intima-media layers varies maximally by a factor of 3 between the individual animals. In contrast, the values for

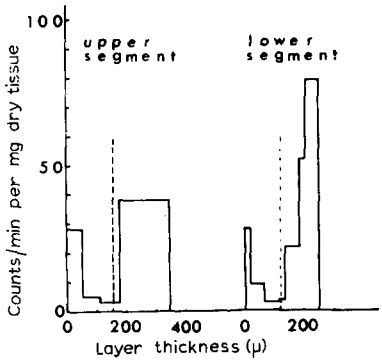

Fig. 5. The distribution of labelled cholesterol in an upper and a lower aortic segment. Abscissa, layer thickness in μ. Ordinate, radioactivity per mg dry tissue. Intimal surfaces to the left. The stippled lines show where the division of 10 upper and 10 lower segments took place.

corresponding, *i.e.* right and left, central intima-media layers from the same animal are much alike. The ratio between the [^{14}C]cholesterol uptake by right and left central intima-media layers was 1.04±0.04 (mean value ±S.E., n = 10).

To localize the labelled cholesterol in the aortic wall some central parts were sectioned parallel to the intimal surface by means of a microtome. The technique was like that described by HVID HANSEN AND ZERAHN[32]. The distribution of activity in successive microtome sections of central parts from an upper and a lower segment is shown in Fig. 5. As the sections were not of equal thickness, the thickness was determined on a dry weight basis. The distribution of ^{14}C activity shows 2 maxima, one at the intimal and one at the outer surface.

DISCUSSION

Oxygen consumption and lactic acid production

The values for Q_{O_2} and lactate production (Table I) represent the rate of oxygen consumption and lactic acid production in segments of the whole arterial wall and give no information about the contributions of the different cell types, especially the endothelial cells.

The Q_{O_2} values found in this study are in close agreement with those reported in other studies on the respiration of the rabbit aorta. MICHELAZZI[33] found values from 0.60 to 1.49 and COSTA, WEBER AND ANTONINI[34] values from 0.62 to 0.93.

The oxygen consumption rate is decreasing, at least during the first 2 h (Fig. 2). HENDERSON AND MACDOUGALL[35] found approximately the same oxygen consumption rate whether air or oxygen was the gas phase (1.6–2.1 and 1.2–2.2 μl of oxygen per mg dry tissue per h, respectively). The maximum fall in oxygen tension during the present 4-h incubations amounted to about 2% of the initial tension. Presumably, therefore, the decrease in oxygen consumption rate during the incubations can scarcely be due to a lack of oxygen.

From the wet weights and surface areas of the aortic segments it can be calculated that the average thickness of the upper segments (which are thicker than the lower segments) is 0.34 mm (0.30–0.36 mm). According to HILL[36], the maximum depth

to which an oxygen-consuming layer can be supplied with oxygen by diffusion from a bathing fluid may be calculated, provided the oxygen consumption rate and the diffusion coefficient of oxygen for the tissue are known. The formula of HILL is as follows: $b = \sqrt{2 \times k\, y_0/a}$, where b is the maximum depth to which oxygen penetrates, k the diffusion coefficient, y_0 the concentration of oxygen in the fluid expressed in ml/ml, and a the rate of oxygen consumption by the tissue expressed in ml per g wet wt. per min. If the tissue layer is exposed to an oxygen-containing medium on both sides, the $2b$ value has to be used.

It can be calculated that, at the end of the incubations, the oxygen concentration in the fluid is approx. 0.0035 ml/ml. Using the mean value of Q_{O_2} found for the first hour of incubations of upper segments, the value for a was calculated to be 0.00405 ml per g wet wt. per min. If, furthermore, the diffusion coefficient found by KIRK AND LAURSEN[37] for human intima-media layers (0.00054 as an average for intimal and media layers) is used in the present calculations, the maximum thickness of an aortic segment which can be supplied with oxygen will be 0.61 mm ($2b$ value). It is therefore reasonable to assume that the oxygen tension in the mid-zone of the aortic segments is above zero during the incubations.

The difference in oxygen consumption rate between upper and lower aortic segments is significant. Perhaps this difference is an expression of a decreasing oxygen consumption rate along the aorta from the arch distally like that found for thoracic and abdominal aortas of rats[38,39].

KIRK, EFFERSØ AND CHIANG[40] have reported that the aerobic glycolysis of human and dog aortic tissue was responsible for 51% and 39% of the energy production by the tissue, provided carbohydrate was the source of energy. If similar considerations are used in this study the corresponding figures would be 32% and 36% for upper and lower segments, respectively.

Incorporation of labelled cholesterol into lipoproteins

24 h after the injection of labelled cholesterol into the donor rabbit an approximately equal specific activity of free and esterified cholesterol was found in the donor serum. The electrophoretic pattern of the serum used showed that the ^{14}C activity was located mainly in the α- and β-globulin regions. This location is consistent with an incorporation of the labelled cholesterol into the α- and β-lipoprotein cholesterol moieties.

Aortic uptake of labelled cholesterol

The higher uptake of [^{14}C]cholesterol by peripheral than by central parts of the intima-media layers, is probably due to adsorption of lipoproteins to the free borders of the peripheral part (see Fig. 1). Furthermore, the branches (intra-aortic part) of the intercostal arteries are found in the peripheral parts. Thus, the peripheral and the central parts are structurally different, and this difference might also well be of significance in the [^{14}C]cholesterol uptake.

However, the uptake of labelled cholesterol by intima-media layers prepared from the central parts of aortic segments must presumably be due to an uptake at the intimal endothelial cell surface. The activity distribution shown in Fig. 5 indicates the validity of this statement.

ADAMS[41] has shown that tritium-labelled cholesterol injected intravenously into

rabbits also entered the aortic wall when the vasa vasorum had been stripped from the vessel. The labelled cholesterol penetrated to the outer surface of the tunica media, but the extent of labelling in this outer zone was less than that in the intact aorta. From these experiments it was concluded that—*in vivo*—most of the cholesterol was transported from the lumen, but that a small part probably entered the aortic wall through the vasa vasorum.

Two corresponding intima-media layers, *i.e.* a right and a left intima-media preparation of the thoracic aorta from the same animal were shown to take up approximately equal amounts of labelled cholesterol. It is thus possible to investigate the influence of various parameters upon the [^{14}C]cholesterol uptake within the same aorta.

The conditions described in this paper seem to be suitable for further investigations because various parameters influencing the cholesterol-entry process at the endothelial cell surface can be altered and controlled *in vitro*.

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