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Endothelial Integrity and Cholesterol Transfer in the Aorta of the Rabbit

Cholesterol content, accumulation and elimination in morphologically defined experimental atherosclerotic lesions and normal tissue

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AKADEMISK AVHANDLING

SOM FÖR VINNANDE AV MEDICINE DOKTORSGRAD,
MED VEDERBÖRLIGT TILLSTÅND AV MEDICINSKA FAKULTETEN
VID UNIVERSITETET I GÖTEBORG, KOMMER ATT OFFENTLIGEN
FÖRSVARAS Å AULAN, SAHLGRENSKA SJUKHUSET,
ONSDAGEN DEN 24 MAJ 1972 KL. 9 F.M.

Av

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GÖTEBORG 1972
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The present thesis is based upon the following research papers:

I. BONDJERS, G., and S. BJÖRKERUD: Fluorometric determination of cholesterol and cholesteryl ester in tissue on the nanogram level. Anal. Biochem. 42: 363 (1971).

II. BJÖRKERUD, S., and G. BONDJERS: Endothelial integrity and viability in the aorta of the normal rabbit and rat as evaluated with dye exclusion tests and interference contrast microscopy. Atherosclerosis 15: 285 (1972).

III. BONDJERS, G., and S. BJÖRKERUD: Cholesterol accumulation and content in regions with defined endothelial integrity in the normal rabbit aorta. (1972). Accepted for publication in Atherosclerosis.

IV. BJÖRKERUD, S., and G. BONDJERS: Arterial repair and atherosclerosis after mechanical injury. Part 1. Permeability and light microscopic characteristics of endothelium in non-atherosclerotic and atherosclerotic lesions. Atherosclerosis 13: 355 (1971).

V. BONDJERS, G., and S. BJÖRKERUD: Arterial repair and atherosclerosis after mechanical injury. Part 3. Cholesterol accumulation and removal in morphologically defined regions of aortic atherosclerotic lesions in the rabbit. (1972). Accepted for publication in Atherosclerosis.

VI. BONDJERS, G., and S. BJÖRKERUD: Arterial repair and atherosclerosis after mechanical injury. Part 4. Uptake and composition of cholesteryl ester in morphologically defined regions of atherosclerotic lesions. Atherosclerosis 15: 273 (1972).

These papers will be referred to in the text by their roman numerals.

Abbreviations: CEFA - cholesteryl ester fatty acid
LCAT - lecithin cholesteryl acyl transferase
TLC - thin layer chromatography

CONTENTS.

1. INTRODUCTION

1.1 Atherogenesis

Atherosclerotic changes are now generally considered to be of multifactorial origin (reviews: see 1, 2, 3, 4). However, most research on atherosclerosis has been influenced by the filtration hypothesis of atherogenesis. In its first form, as presented by Virchow 1856 (5), this hypothesis attributed the formation of atherosclerotic deposits to imbibition of the arterial wall with blood constituents, whereas the localization of these deposits was supposed to be determined by tearing and stretching due to the "force of the blood", i.e. by hemodynamic factors.

The filtration hypothesis gained much support from the studies of Anitschkow, who 1912 succedded in producing experimental atherosclerotic lesions by feeding rabbits cholesterol (6), and subsequently identified cholesterol crystals in the lesions (7). Anitschkow presumed that a rather constant stream of nutrient liquid containing "lipoid" material was filtered through the arterial wall from the blood (8). Deposition of cholesterol was attributed to "flocculent precipitation of lipoids". This view has directed the interest of many researchers to the relationship between serum lipids, especially serum cholesterol, and atherosclerosis. On the other hand, there are many observations which are difficult to account for only by increased cholesterol content in filtered serum passing through the arterial wall (see 1, 2, 3); the preferential localization of atherosclerotic lesions (8, 9, 10) cannot be explained by filtration through a passive filter as outlined above, and the induction of atherosclerotic changes in experimental animals has involved increments in blood cholesterol levels to highly unphysiological levels, i.e. 2000 mg/100 ml (3). Therefore, many investigators have questioned the filtration hypothesis (reviews: 1, 2, 3) and emphasized the importance of other factors, such as hemodynamic strain (11, 12, 13), encrustation of surface thrombi (14, 15, 16) local metabolic changes (4, 17, 18, 19), or immunological factors (20).

1.2 Deposition of cholesterol

The deposition of free and esterified cholesterol is one of the most characteristic features of the atherosclerotic lesion (2, 21, 22, 23). In 1951, Biggs and Kritchevsky demonstrated that labelled cholesterol given per os could be recovered in the arterial tissue of normal and hypercholesterolaemic rabbits (24). This finding has been confirmed by many investigators, both in humans and in experimental animals, in normal arterial tissue as well as in athero-

sclerotic tissue (reviews: 2, 25, 26, 27). These results have been considered to be a strong argument in favour of the hypothesis that filtration of plasma is the primary factor causing cholesterol deposition in atherosclerotic lesions (2, 25, 27).

1.3 The endothelium and atherogenesis

The endothelial cell layer forms the boundary between the blood and the subendothelial arterial tissue (review: 28). Thus, all substances which enter the arterial tissue from the lumen, must pass through the endothelium (28). By virtue of their anatomical position, the endothelial cells are also connected to the formation of mural thrombi (28, 29). In addition, it seems very likely that the endothelial cell layer should be the first structure influenced by increased hemodynamic force (29), for example due to turbulance. It follows that hypotheses on atherogenesis, such as the filtration hypothesis, the encrustation hypothesis, and the hemodynamic stress hypothesis, must take the properties of the endothelium into account. However, the morphology of the arterial endothelium is difficult to study by means of histological sections, as it is arranged in cylinders. Studies on en face preparations may, on the other hand, provide more representative information on the structure of the endothelium in normal and atherosclerotic arteries. The elaboration of new, simple techniques for the preparation of such material (30), with decreased risks for artifacts, may, therefore, be of great potential significance for the advancement of our knowledge of atherosclerosis and its origin.

1.4 Experimental atherosclerosis induced by mechanical trauma

Much evidence has been presented to support Virchow's view that hemodynamic trauma is one important factor in atherogenesis (reviews: 1, 2, 3, 12). To imitate the effect of hemodynamic injury, Björkerud constructed a microsurgical instrument for the induction of defined mechanical trauma to the lumenal surface of rabbit aortae (31). With this instrument it was possible to show that different types of mechanical trauma elicit different tissue responses in the arterial wall: (i) A superficial mechanical injury with small area (longitudinal injury) induces a tissue reaction suggestive of progressive intimal thickening (31); (ii) A superficial mechanical injury with large area (transverse injury) initiates changes, morphologically similar to those in early human atherosclerosis (32); (iii) A total, local necrosis (deep injury with small area) causes cell death, calcification, encapsulation and capillarization (33), i.e. changes similar to those in media sclerosis or advanced atherosclerotic lesions. The sequential morphological changes during the progression and regression of these lesions have been systematically studied. In the lesions, different regions

with characteristic and reproducible morphological properties have been identified by light microscopy (34),transmission electron microscopy (35), and scanning electron microscopy (36). These results indicate that experimental arterial lesions with predictable morphological properties and of known age, may be produced at will by defined mechanical injury.

The selection of material may exert a determining influence on the relevance of results obtained from morphologically heterogeneous or undefined tissues. Variable composition of the tissue samples investigated may obscure true characteristics (27, 37), and at the same time produce artifactual results (37). These considerations may be especially relevant for studies on atherosclerotic lesions which are focally distributed, structurally variable, of differing age, and have a marked intrinsic heterogeneity (reviews: 2, 3). Therefore, the possibility of inducing experimental atherosclerotic lesions with defined morphological properties by means of mechanical injury may allow workers in this field to obtain particularly relevant information about biochemical and other properties of atherosclerosis.

1. 5 Purpose of the study

The general aim of the present investigation was to study two factors intimately related to current concepts on atherogenesis, as discussed above: (i) structural and functional properties of the endothelium in normal and atherosclerotic tissue; and (ii) local transfer mechanisms for free and esterified cholesterol in normal arteries and in atherosclerosis. For this purpose it was necessary to develop new techniques for the study of endothelial cell viability (II), and more sensitive methods for the determination of cholesterol in tissue (I). These techniques were combined and applied to normal aorta and morphologically defined atherosclerotic lesions induced by mechanical trauma in normo-lipidemic rabbits, in an attempt to contribute to the knowledge concerning the following more specific problems:

1) Is the normal aortic endothelium of rabbit and rat structurally and functionally homogeneous? (II, III).

2) What is the nature of the local mechanisms involved in the transfer of cholesterol into the normal rabbit aorta? (III).

3) Are the structural and permeability characteristics of the endothelium related to morphological properties of different aortic lesions induced by defined mechanical trauma in the rabbit? (IV).

4) What local mechanisms may be involved in the accumulation of cholesterol in experimental atherosclerotic lesions induced by defined mechanical injury in the rabbit? (V).

5) Are the characteristics of accumulation, removal and composition of free and esterified cholesterol related to selected morphological properties of mechanically induced atherosclerotic lesions in the rabbit? (V, VI).

2. SUMMARY OF THE RESULTS

2.1 Structural and functional aspects of the normal aortic endothelium

2.1.1 Structure of the normal aortic endothelium

II: Cell viability in rabbit and rat aorta was evaluated by means of dye exclusion tests, which resulted in a differential staining of the aortic surface. Unstained areas were covered with normal, intact endothelial cells. Stained areas were covered with defective endothelium, containing endothelial cells with structural properties, indicating irreversible injury or cell death. Microthrombi were frequently seen adhering to injured endothelial cells. Medial smooth muscle cells underlying defective endothelium were also injured, as judged from their morphological and staining characteristics. The presence of injured endothelium and media cells in regions, where increased hemodynamic strain is likely to occur, and which constitute predilection sites for atherosclerosis, indicate that localized areas in the normal artery may be preconditioned for additional injury leading to atherosclerosis.

2.1.2 Permeability of the normal aortic endothelium

III: To study the significance of endothelial integrity for the transfer of cholesterol, the concentration and uptake *in vivo* of free and esterified cholesterol was determined in regions with intact and defective endothelium in the aorta of normal rabbits. The cholesterol content and the uptake of labelled free and esterified cholesterol was higher in regions with defective endothelium. The higher uptake of labelled esterified cholesterol suggests filtration of lipoproteins in regions with defective endothelium. The results indicate that the intact endothelial lining may represent a structural barrier against excessive transfer and deposition of cholesterol in arterial tissue.

2.2 Mechanisms for the transfer of cholesterol into the normal aortic tissue

III: In aortic regions with intact endothelium, an inverse relationship was found between cholesterol content and uptake of labelled cholesterol from plasma, suggesting an adjustment of cholesterol transfer to local requirements in the aortic wall. In addition, in the same regions a direct relationship was found between the uptake of labelled free and esterified cholesterol, with a ratio between the fractions of 20:1. This is consistent with active transport of cholesterol, involving hydrolysis of cholesteryl ester, a mechanism suggested by others (38) on the basis of in vitro studies. In regions with defective endothelium none of these relationships were observed, suggesting deranged transfer of cholesterol into the arterial tissue.

2. 3 Structure of the endothelium in different aortic lesions induced by mechanical injury in the rabbit

IV: Atherosclerotic and non-atherosclerotic arterial lesions were induced by different types of defined mechanical injury in the aorta of rabbits. The permeability characteristics of the lesions were studied with Evans blue, administered in vivo, and the morphology of the endothelium was investigated with light microscopy after perfusion staining with silver salts. In contrast to non-atherosclerotic lesions, the atherosclerotic lesions had a markedly greater permeability than uninjured tissue, as judged from filtration of the Evans blue albumin complex. The non-atherosclerotic lesions were completely re-endothelialized after 4 weeks, whereas the atherosclerotic lesions were partially devoid of lining cells, and partially covered with monocytes.

2.4 Mechanisms for the accumulation of cholesterol in atherosclerotic lesions induced by mechanical injury in the rabbit

V: Transfer of labelled cholesterol and cholesterol content in morphologically defined samples of experimental atherosclerotic lesions in normo-lipidemic rabbits was investigated. The uptake of ^{3}H-cholesterol from the blood was 15 times higher in atherosclerotic arterial tissue with defective endothelium than in endothelialized arterial tissue. In addition, the cholesterol content in the former was ca. 3 times that in plasma or in endothelialized arterial tissue. As atherosclerotic arterial tissue with defective endothelium was also characterized by increased permeability to plasma proteins (IV), these results suggest that cholesterol in plasma lipoproteins may filter into the atherosclerotic lesions through the defective endothelium and accumulate in the tissue underlying the endothelial defects.

2.5 Cholesterol transfer in morphologically defined regions of aortic experimental atherosclerotic lesions induced by superficial mechanical injury

2.5.1 Cholesterol transfer and cholesterol content

V: Different regions having characteristic and reproducible morphological properties may be recognized in atherosclerotic lesions induced by superficial mechanical injury with large area. The cholesterol content in regions with defective endothelium was 3 times that of the controls, while, on the other hand, the cholesterol content in regions, where the original arterial wall structure had been restored, was equal to that of the controls. This indicates that cholesterol is eliminated during repair and re-endothelialization of the lesions. In regions with defective endothelium, the specific activity of cholesteryl ester was lower than that of free cholesterol, suggesting that far more cholesterol enters the arterial wall than is deposited in it. These results further support suggestions that cholesterol may be rapidly eliminated from the aortic tissue.

2.5.2 Esterified cholesterol

V: The ratio of esterified cholesterol to total cholesterol was increased in regions with defective endothelium, suggesting a relationship between this parameter and endothelial integrity.

2.5.3 Cholesteryl ester composition

VI: In all regions of the lesions, regardless of the morphological distribution of lipids, cholesteryl esters with mono-unsaturated fatty acids dominated, whereas cholesteryl esters with di-unsaturated fatty acids dominated in plasma. This suggests that the cholesteryl ester composition of the lesions is primarily determined by local cellular activity, and is independent of plasma influx. The specific activities in the three more saturated cholesteryl esters were rather similar, suggesting that both differential hydrolysis and differential esterification might contribute to the specific cholesteryl ester composition of the lesions.

2.5.4 Mechanisms for cholesterol elimination

VI: The specific activities of cholesteryl esters with fatty acids containing three or more double bonds was lower than that of the more saturated esters, and the proportion of such esters increased during restoration of the arterial wall structure. Therefore, these results do not support suggestions that esterification of cholesterol with fatty acids, containing three or more double bonds, is involved at least in the primary stages of cholesterol elimination.

3. METHODOLOGY

3.1 Experimental animals

3.1.1 Rabbits

In a systematic study involving a large number of animals, it was found that albino rabbits of the Danish country strain have a very low incidence of arteriosclerotic changes in the descending thoracic and abdominal aorta (39). Male rabbits of this strain, obtained from one breeder, fed 125 gms daily of low-calorie, high fiber-content rabbit pellets, and weighing 2.4-3.5 kg, were used in II, III, V and VI. Serum cholesterol levels of the animals used for cholesterol transfer experiments were 27-55 mg/100 ml. In IV, female, albino rabbits of the Swedish country strain, obtained from one breeder, fed ad libitum on ordinary rabbit pellets, and weighing 3.6-4.3 kg, were used. The results from IV have been confirmed by studies on male, albino rabbits of the Danish country strain, both with light microscopy (34), transmission electron microscopy (35), and scanning electron microscopy (36).

3.1.2 Rats

Male, Sprague-Dawley rats, fed ordinary rat pellets ad libitum, and weighing between 400 and 500 gms, were used in I and II. They were free from chronic respiratory disease, as judged by inspection post mortem.

3.2 Experimental atherosclerosis

Atherosclerotic lesions were induced (IV, V, VI) by means of a superficial mechanical injury with large area (transverse injury) with the microsurgical instrument described by Björkerud (31). In a study on the morphology of this type of atherosclerotic lesion at different times after the operation, we found that a large segment of the aortic surface was initially denuded of endothelial lining by the trauma (34). During re-endothelialization of this defect, regions with different, specific morphological properties developed in the initially injured segment (34) (see schematic illustrations; V: Fig. 1 and VI: Fig. 1). These regions constitute consecutive stages in the repair of the tissue(34).

i. The central region of the lesions is moderately elevated, still devoid of endothelial lining, and contains abundant, finely dispersed, extracellular lipid (34). This region was designated YARD (34).

ii.The surrounding, heavily thickened region is partly covered with endothelium, and partly devoid of endothelial lining (34). In endothelialized parts, both

the intima and the media show a varying degree of necrosis, with intracellular lipid in shoals of foam cells (34). The morphology of such parts is very similar to that of fatty streaks (34). The whole regions were designated BANKS (34).

iii. In some portions of the initially injured segment, the normal arterial wall structure has been more or less completely restored. The endothelial lining is continuous, and the intimal thickening has regressed in such regions (34). They were designated STAGES (34).

In the cholesterol transfer studies (V, VI) defined samples of YARD, non-endothelialized BANK, endothelialized BANK and STAGE were cut out with microscissors, freed from advential tissue with microdissection, and analyzed as described below.

3.3 Cholesterol determination and fractionation

3.3.1 Cholesterol determination

Previous methods for the determination of cholesterol were either not sensitive enough (for review: see e.g. 40) or too laborious (discussion: see I), taking account for the large number of very small tissue samples. Therefore, a simple ultramicro-method for the determination of free and esterified cholesterol in the nanogram range was developed. It is described in detail in I. Optimal conditions for the reaction and possible interference by a number of other sterols were investigated. The recovery of cholesterol added to an arterial tissue lipid extract was 101.5±2.1 % (S.D.) (I).

3.3.2 Fractionation of free and esterified cholesterol

Free and esterified cholesterol were separated (IV, V, VI) by thin layer chromatography on a 250μ layer of silica gel H, as described by Gloster and Fletcher (41), with the slightly modified solvent: n-hexane: diethyl ether: acetic acid (80:20:2)(42). The recovery of added free cholesterol was 99.0±1.9 %(S.D.), and the recovery of added cholesteryl ester was 100.0±2.7 % (S.D.) after TLC (I).

3.3.3 Fractionation of different cholesteryl ester classes

Different cholesteryl ester fractions were separated (VI) by thin layer chromatography on a 250μ layer of silica gel G as described by Alling et al.(43). They characterized the different cholesteryl ester fractions in serum lipids by gas chromatography (44). Fraction 1 contained almost exclusively cholesteryl esters with saturated fatty acids; fraction 2 cholesteryl esters with mono-unsaturated fatty acids; fraction 3 cholesteryl esters with di-unsaturated fatty acids; fraction 4 cholesteryl esters with tri- and tetra-unsaturated

fatty acids; and fraction 5 consisted mainly (ca. 90 %) of cholesteryl esters with penta- and hexa-unsaturated fatty acids. The recovery of total cholesteryl ester after this fractionation procedure was in our hands 93.2±5.2 % (S.D.) (VI), as determined by comparison with the total cholesteryl ester fraction obtained after TLC according to Gloster and Fletcher (41), modified as described above.

3.3.4 Recovery of cholesterol

In the assay of material containing radioactive cholesterol, the radioactivity in an aliquot of the original washed tissue lipid extract was determined. The recovery of total radioactive cholesterol after fractionation, extraction from the silica gel, and washing, was 98.0±4.2 % (S.D.) (II) and 96.1±6.8 % (S.D.) (V).

3.4 Morphology

3.4.1 Cell viability tests

Cell viability in the aortic endothelium was evaluated (II, III, V, VI) by dye exclusion tests as described in II. In preliminary experiments we tested a number of dyes used by others for cell viability tests (for reference: see 45). The dyes were: lissamine green, erythrosine A, erythrosine B, water-soluble eosin, trypan blue and nigrosin. Only nigrosin and trypan blue yielded satisfactory contrast and staining intensity. The tissue distribution of these stains on the light microscopical level was studied in detail, as well as that of the non-toxic structural isomer of trypan blue, Evans blue. The general staining pattern of these compounds was similar, and those of trypan blue and Evans blue were almost identical. Uncomplexed Evans blue under these conditions has entirely different staining properties compared to Evans blue used *in vivo* (IV). Under *in vivo* conditions Evans blue is complexed to albumin (46) and may be used as a marker for increased permeability for albumin through the endothelium (review:46).

3.4.2 Silver staining and permeability for plasma proteins

The endothelium of experimental atherosclerotic lesions, induced by superficial mechanical injury with large area (transverse injury) (see above), and of non-atherosclerotic lesions, induced by superficial injury with small area (longitudinal injury) was studied after silver staining by a method slightly modified from that described by Poole *et al.*(47) (IV). The permeability for serum albumin in these lesions was studied after injection of Evans blue on 2 successive days before killing the animals (IV).

3.4.3 Preparation and microscopy of arterial tissue

Whole segments of arterial tissue were mounted in a purified starch hydrolysate with a high proportion of polymeric sacharides (II, IV), as described by Björkerud (30). In this mounting medium even whole-thickness preparations of rabbit aorta are transparent and allow microscopy at the theoretical resolution limit of the light microscope. The tissue samples were studied by interference contrast microscopy according to Nomarski (48), using a Zeiss interference contrast microscope, equipped with large working distance immersion objectives.

3.5 Studies with labelled cholesterol

3.5.1 Radiochemical purity

^{3}H-(G)-cholesterol was obtained from the Radiochemical Centre, Amersham, England. According to specifications the isotope was 98 % radiochemically pure. Thin layer chromatography, according to Gloster and Fletcher (41) (see above), of an aliquot of the isotope with an intima lipid extract as carrier showed that more than 99 % of the radioactivity was in the free cholesterol fraction.

3.5.2 Injection of labelled cholesterol

Labelled cholesterol was incorporated into serum lipoproteins in vitro, as described by Whereat and Staple (49), and injected intravenously into the rabbits (III, V, VI). Blood samples were taken from five rabbits at regular intervals following the injection, for determination of specific activities in free cholesterol (III, VI), total cholesteryl ester (III) and individual cholesteryl ester fractions (VI). Aliquots were also taken for electrophoresis of serum lipoproteins on agar gel plates (50). The labelled cholesterol was rather evenly distributed between the α and the combined β-, pre-β fractions at all time intervals after the injection (51).

The specific activity of free cholesterol decreased from an initial high value one hour after the injection of the isotope and levelled off after 12 hours (III: Fig. 1; VI: Fig. 3). The specific activity of cholesteryl ester reached a maximum after 4-8 hours, and then decreased and levelled off at values close to those of free cholesterol (III: Fig. 1). The same pattern was found in the different sub-fractions of cholesteryl ester, with some minor quantitative differences (VI: Fig. 3). Exchange of tritium label between cholesterol and other lipid classes was negligible, as indicated by the recovery after separation by thin layer chromatography (III, V).

3.5.3. The evaluation of studies with labelled cholesterol

Quantitative investigations on the metabolism of cholesterol in the arterial wall are both difficult to perform and to evaluate. This is reflected in the difficulty to interpret many of the results obtained from isotope studies with cholesterol (see reviews: 25, 26, 27). At present transfer rates for the movement of cholesterol from the blood into the arterial tissue cannot be determined from isotope studies. Thus, the labelled cholesterol found in the arterial wall may have accumulated both by the actual transfer of cholesterol and by physical exchange of labelled cholesterol between the blood pool and the tissue pool (see: 25, 26, 27, III). It has so far not been possible to determine the relative contribution of each of these two processes in vivo or in vitro (discussion: see III). However, as physical exchange is probably restricted to those tissue lipoproteins at the lumenal surface of the artery, which have direct contact with serum lipoproteins (see III and below), it seems reasonable to assume that this factor may not vary too much when the results are expressed as radioactivity per unit of surface area. Thus, more extensive variations between different aortic regions may be related to different rates for the net transfer of cholesterol into the arterial tissue. In addition, if the methods used are sensitive enough to allow investigations on the accumulation of esterified cholesterol, the results concerning this cholesterol fraction may be more easily interpreted, as it is not influenced by physical exchange (52, 53, 54; discussion: see III and V).

3.5.4 Transfer of labelled cholesterol

Several investigators have observed that the uptake of labelled cholesterol per time unit is rather constant up to 24 hours after the administration of the isotope (55, 56, 57, 58). In dietarily induced atherosclerotic lesions, or after oral administration of the isotope, this constancy may last for even longer time periods (25, 55, 56, 58). Thus, Zilversmit emphasized "the absence of a relationship between the calculated rate of influx and the time of exposure" (to the isotope/G.B.) (25). The constant rate of accumulation of the isotope may reflect large differences in specific activity between the blood cholesterol pool and arterial tissue cholesterol pools. On the other hand, Felt et al. (58) presented results, indicating that the rate of acccmulation of labelled cholesterol decreased rapidly after 24 hours. This may imply that a state of isotopic equilibrium between the blood cholesterol pool and an arterial tissue cholesterol pool was approaching.

In view of the results discussed above, it was inferred that observations at more than one time would not contribute significantly to the information obtained from only one time period after the injection of the isotope. In order to obtain maximal radioactivity in the tissue, before equilibrium specific activities between the tissue cholesterol pools and the blood cholesterol pool, the animals were killed 24 hours after the injection of the isotope. However, due to the difficulty in defining in precise, absolute terms the contributions of actual transfer and exchange to the amount of labelled cholesterol, we refrained from expressing the radioactivity measurements in terms of cholesterol amounts or serum equivalents. The radioactivity in the tissue samples after 24 hr of exposure to labelled cholesterol was designated UPTAKE of ^{3}H-cholesterol, and was expressed as radioactivity per unit surface area.

3.5.5 Liquid scintillation counting

The radioactivity of the samples was determined in aliquots of total, free and esterified cholesterol in a Packard Tri-Carb Liquid Scintillation spectrophotometer, with a scintillation mixture of 0.5 % butyl-PBD (w/v; CIBA Ltd., Basle, Switzerland) in toluene. The samples were counted for at least ten minutes or in low-activity samples to a theoretical standard deviation of less than 2.5 % (more than 2000 registered counts)(59). Quenching was corrected for by means of an external standard.

3.6 Statistical methods

3.6.1 Distribution of the data

There were no a priori criteria indicating that the numerical data of our studies were normally distributed. In some instances, there was even strong evidence indicating that there was a skewed distribution (see for example III: Fig. 2). In view of the questionable validity of the significance limits of the student´s test and other tests for normally distributed populations in populations with unknown distribution (60), distribution-free (non-parametric) tests were used throughout the study.

3.6.2 Significance tests

For significance tests, Wilcoxon´s test for paired observations (61) was used. This test is almost as efficient as the student´s test in normally distributed populations (60), and more reliable and efficient in other distributions (60). In III, samples from regions with decreased endothelial integrity were paired with samples from regions with intact endothelium from the same part of the aorta.

If more than one sample of either type was present, all possible differences were calculated and ranked for Wilcoxon´s test. In V, tissue samples from the same lesions were paired.

3.6.3 Regression analysis

Correlations were tested with Spearman´s rank correlation coefficient (62).

3.6.4 Considerations on the number of samples

A large number of samples was investigated in III and V, although the total number of animals was restricted. In addition, the samples were evenly distributed between the animals, and no single animal deviated from the general trend of the observations. Therefore, the possible uncertainty introduced by the small number of animals does not decrease the validity of the conclusions in the studies.

4. ENDOTHELIAL INTEGRITY

4.1 Endothelial integrity in the normal aorta

4.1.1 Cell viability tests

Although there is strong evidence for endothelial changes leading to increased endothelial permeability as one central factor in atherogenesis (28, 63), only little information has been available on the structural integrity of normal arterial endothelium. The results from the application of dye exclusion tests to test endothelial cell viability, indicated a marked structural heterogeneity in the normal aortic endothelium (II). Areas stained with nigrosin, trypan blue and Evans blue were present in the aortae of rabbits and rats, most frequently at branching points and in the aortic arch, i.e. in regions where increased hemodynamic strain may be expected.

4.1.2 Intact endothelium

Unstained areas were covered with cells with a structure conforming to current concepts of normal endothelial cells (II) (review: 28). Such cells are attenuated and completely flat, in some cases with a slightly bulging nuclear region. They are oblong and longitudinally oriented in the aorta. It has been suggested that endothelial cells are held together by intercellular junctions, to form a continuous layer completely covering the lumenal surface of the arteries (28). Most evidence suggests that a basement membrane is absent or rudimentary

in the rabbit aorta and other arteries of similar size (reference: 28). Studies of the incorporation of labelled thymidine have indicated that the turnover of endothelial cells in the normal aorta is very low (67), and it has even been suggested that these cells are replaced only a few times in an animal's lifespan (67).

4.1.3 Defective endothelium

The concept of a continuous endothelial cell layer, completely covering the lumenal surface, may seriously be questioned against the back-ground of the results of II. The existence of regions with aberrant structural or functional properties is suggested also by the recent reports of isolated areas with an increased rate of cell divisions in the aorta of guinea-pigs (68, 69, 70). Such areas were localized to the aortic arch and the mouths of aortic branches (68, 69, 70), and, after local experimental injury, to the damaged regions (70).

The intracellular uptake of anionic dyes, such as nigrosin, trypan blue, or Evans blue, is probably related to a decreased integrity of the cell membrane during cellular injury or death (45, 71). In the interference contrast microscope, stained cells in transparent samples of normal rabbit aorta were characterized by cytoplasmic and nuclear oedema, intracellular vacuolization, and changed surface properties (II). In a systematic study of the morphology of injured and dead cells of other types in tissue culture, Bessis found that these characteristics are reproducible components of pre- and perimortal changes in a variety of different cells (71). The induction of a local mechanical injury, or chemical injury, is followed by similar alterations in vivo in endothelial cells, as indicated by light microscopy of sectioned samples and transmission electron microscopy (review and references: 63). Furthermore, swelling and cytolysis have been described as components of spontaneous cell death in arterial endothelium (72). All this evidence indicates that increased uptake of supravital stain in specific regions of the aortic endothelium is due to an increased incidence of damaged or dead cells in these regions.

4.1.4 Endothelial integrity and hemodynamic strain

It has been observed that experimentally-increased hemodynamic stress may cause cell injury and death in the aortae of dogs (73, 74). As discussed above, several studies have indicated that the aortic arch and branching points, i.e. the regions where stained cells were most frequent (II), are subject to increased hemodynamic strain also in unmanipulated animals (9, 64, 65, 66). Consequently , it seems reasonable to suggest that injury from such strain may be the cause of increased cell death in such areas. On the other hand, the increased rate of mitoses at the mouth of aortic branches (68, 69, 70) indicates

reparative changes in response to the injury. The repair reactions after different types of experimental mechanical injury were characterized by specific morphological alterations in the arterial wall (review: 75). Superficial mechanical injury with small area (longitudinal injury) was followed by a rapidly regressive repair reaction (31), involving rapid re-endothelialization (IV). This reaction was morphologically suggestive of progressive intimal thickening (31). Superficial mechanical injury with large area was followed by a protracted reaction (32, 34), involving delayed re-endothelialization (IV). The morphological features of this reaction were suggestive of early atherosclerosis (32, 34). An increased incidence of progressive intimal thickening and atherosclerosis in man (8, 9, 10, 12), and of dietarily induced atherosclerotic lesions in animals (18), has been observed at branching points and other areas, characterized by more or less constantly heavy hemodynamic stress. Therefore, the observation of injured endothelium and media in areas with a similar distribution (II) may suggest that cell injury due to locally increased hemodynamic strain, and the tissue reaction to such injury, may be important factors in the development of atherosclerotic lesions.

4.1.5 Endothelial integrity and thrombotization

The occurrence of mural microthrombi in regions with injured endothelium (II) confirms results presented by others (29, 76) and establish that such aggregates are present in the aorta of normal rabbits. During thrombotization, thrombocytes and leukocytes may release various pharmacologically active agents (29, 77) which, in turn, may increase endothelial permeability (29, 77) and even initiate degenerative changes in the endothelial cells (77). However, from the present results it appears difficult to assess, whether formation of microthrombi or endothelial cell injury may be the causative factor in the relationship between decreased endothelial integrity and thrombotization.

4.2 Permeability characteristics of normal aortic endothelium

4.2.1 Heterogeneity in permeability characteristics

Structural heterogeneity in the endothelium of the rabbit aorta corresponded to heterogeneous permeability characteristics. It was observed that the anionic dyes used in the dye exclusion tests penetrated the endothelial lining exclusively in regions with decreased endothelial viability (II). In addition, uptake of labelled cholesteryl ester was twice as high in such regions as in regions with intact endothelium (III). This suggests increased uptake of plasma lipoproteins, as there is substantial evidence that cholesteryl ester enters

the arterial wall mainly in intact lipoproteins (78)(discussion: see below and III). This suggestion is supported by an analogy with the demonstration of localized accumulations of ^{131}I- or Evans blue-labelled albumin in the aortic wall of pigs (79) and rabbits(76),with a distribution reminiscent of that of the defective endothelium in rabbits and rats (II). The correspondance between the results of the former study and those of the latter is emphasized by the demonstration of similar permeability characteristics *in vivo* for ^{131}I-albumin and ^{131}I-labelled serum lipoproteins (80). Furthermore, autoradiographic studies on *en face* preparations of rabbit aorta indicated the presence of isolated areas with increased permeability to ^{32}P-labelled plasma constituents (81). These areas were localized to branching-points and to the aortic arch (81), i.e. to regions where decreased endothelial integrity is present (II).

4.2.2 Filtration of plasma proteins through the intact aortic endothelium

Most investigations on endothelial permeability concern the situation in the capillaries, but there is evidence suggesting that the results from such studies may be relevant also for arteries (28). In the capillaries a high permeability to plasma proteins and other macromolecules is associated with discontinuities in the endothelial lining (review and references: 28, 82). Such discontinuities are normally present in the capillaries of certain regions, as for example in the renal glomeruli and in the intestinal villi (28, 82). However, no evidence for the existence of such discontinuities in the normal arterial endothelium has previously been presented. Therefore, it has been assumed that the passage of macromolecules through the normal arterial endothelium may be restricted (28). This idea received support from the results presented by Adams *et al.*, demonstrating a gradient of radioactivity in the aortic wall of normal rabbits, from the adventitial side and inwards, after the injection of ^{125}I-labelled plasma proteins (83). The direction of the gradient may be consistent with a smaller penetration of plasma proteins through the lumenal endothelium than that through the endothelium of the adventitial vessels (83, 84). However, Duncan *et al.* have presented evidence for a gradient of the distribution of ^{131}I-albumin in the opposite direction in the aorta of the dog (85). The discrepancy between the two studies may be difficult to explain, but it may, of course, as suggested by Adams *et al.*, be attributed to species differences (83). Duncan *et al.* have also presented results from studies on the passage of ^{131}I-albumin into the normal aortic wall of the dog *in vitro*, indicating that increased filtration pressure without coincident stretching did not increase the passage of label into the aortic tissue (86). These results indicate that passive filtration of plasma proteins into the

aortic wall was restricted under these conditions. On the other hand, stretching of the aorta considerably increased the passage of labelled albumin into the tissue (86), indicating derangement of a barrier against filtration of plasma constituents. There is considerable evidence that stretching of the aorta may decrease endothelial integrity (e.g. 87), and therefore these results are well compatible with the idea that the maintenance of endothelial continuity may prevent excessive influx of plasma constituents into the aortic tissue.

4.2.3 Increased endothelial permeability

As suggested by Duncan's study on the passage of ^{131}I-albumin into the normal aortic tissue of the dog (87, see above), the formation of discontinuities in the aortic endothelium may lead to increased permeability for plasma proteins. In accordance with such a mechanism increased permeability to the Evans blue albumin complex was confined to regions of aortic lesions devoid of endothelial lining, after the induction of a defined mechanical injury to the aorta of normal rabbits (IV). Not only local mechanical injury but also generally increased hemodynamic stress due to the development of hypertension, may induce discontinuity in the arterial endothelium (88, 89, 90). Also such discontinuities were associated with increased permeability to colloidal markers from the blood (88, 89), further supporting the relationship between endothelial discontinuity and increased permeability. However, there is also evidence for increased permeability through the arterial endothelium without relation to any type of trauma. Thus, the penetration of plasma proteins through the aortic endothelium of new-born rabbits was larger than that of older animals (91). Therefore, the possibility should not be disregarded that increased endothelial permeability for plasma constituents in the arteries may also be of physiological significance during growth, and possibly also during repair of arterial tissue.

4.2.4 Arterial tissue ground substance and permeability

The evidence presented above conforms with the concept that the endothelial integrity may be of primary importance for the regulation of permeability through the arterial wall. However, other investigators have emphasized the role of the ground substance in filtration of various substances through the arterial tissue (e.g. 92, 93, 94). In one study, the penetration of trypan blue and hemoglobin yielded a differential staining of the pig aorta (95). The staining pattern was very similar to the distribution of fatty streaks and spots (95). Treatment of the aorta with glycosaminoglycan hydrolysing enzymes increased the filtration of the markers considerably (95). From these results the authors concluded that the aggregation state of the ground substance might determine the

filtration from plasma through the arterial wall. Similar conclusions were reached in studies of acute hypertension in rats, in which a drastic increase in sulfate incorporation preceded increased filtration of lipoprotein constituents (94). All these results clearly demonstrate the important role of the ground substance in the filtration of various substances through arterial tissue. However, in the first of the above studies (95), the aortae were taken from the slaughter-house and used two hours after the death of the animal, without control of the viability characteristics in the samples. Consequently, the results may demonstrate only the permeability characteristics of the dead, or damaged, arterial wall with a defective endothelium. The second of the related studies (96) involved the induction of hypertension, which has been observed to alter endothelial integrity. Consequently, the increased incorporation of labelled sulfate may perhaps reflect different rates of penetrations for the radioactive precursor through the arterial endothelium. The time lag between sulfate incorporation and lipoprotein penetration may only reflect different permeability due to differences in size of the substances (see 94). On the other hand, the results of the present study are difficult to explain in terms of changes in the ground substance. It is unlikely that hemodynamic stress primarily affects the ground substance under the endothelium, and secondarily the boundary layer, subject to the stress. Uni-directional gradients, in the distribution of labelled plasma protein (83) are also difficult to explain in terms of changes in the glycosaminoglycans, unless there also is a gradient in the aggregation state of these substances within the arterial wall. Therefore, most evidence points to the endothelial cell layer as the initial selective barrier against infiltration of plasma components into the arterial wall. Within the limits of the permeability characteristics of this barrier, however, the ground substance may regulate diffusion of various substances within the arterial tissue.

5. CHOLESTEROL TRANSFER

5.1 The origin of cholesterol in the normal arterial tissue

5.1.1 Filtration of serum lipoproteins

The identification of cholesterol crystals in experimental atherosclerotic lesions induced by cholesterol-rich diets (7), has stimulated research on cholesterol transfer in the normal arterial tissue as well. In conformance with Anitschkow's version of the filtration hypothesis, many investigators have regarded filtered serum lipoproteins as a source of cholesterol (review and references: 2, 25, 26, 27). This idea received support from the immunochemical demonstration of different serum lipoproteins in arterial tissue (e.g. 96). It was also supported by studies indicating that the entry rate for a number of different plasma constituents was equivalent to the calculated entry rate of a constant volume of plasma into the arterial wall (57, 97, 98). Observations of transfer of labelled cholesterol from plasma (review and references: 2, 25, 26, 27), and the autoradiographic demonstration of labelled cholesterol on the lumenal side of the medial elastic membranes (99, 100) also were compatible with a stream of "nutrient liquid" (8), with cholesterol-containing serum lipoproteins passing through the arterial wall towards the adventitial lymph vessels.

5.1.2 Arguments against filtration of serum lipoproteins

However, certain observations threw doubt on the concept of filtration of serum lipoproteins as a mechanism for cholesterol transfer into the normal arterial tissue. Thus, in III as well as in studies *in vivo* (101) and *in vitro* (38, 102, 103), described by others, the uptake of labelled free cholesterol far exceeded that of labelled cholesteryl ester. The ratio between the two fractions was different from that of free and esterified cholesterol in serum, and this has been taken as evidence against the filtration hypothesis (25, 26, 27). Furthermore, studies with immunohistochemical methods on the lipoprotein content of apparently normal arterial tissue from young humans have demonstrated the virtual absence of serum lipoproteins from such tissue (104, 105, 106, 107). In addition, results presented by Adams *et al.* suggest differing distributions of ^{3}H-labelled plasma cholesterol and ^{125}I-labelled plasma proteins in the normal rabbit aorta (83). Finally, transfer of lipoprotein-bound labelled cholesterol *in vitro* was not affected by a drastic increase in the cholesterol content of the serum-containing incubation medium above a plateau value (101, 102). This evidence taken together indicates that filtration of plasma lipoproteins cannot

be the sole mechanism of transfer of labelled cholesterol into the normal arterial wall.

5.1.3 Transfer of labelled cholesterol

An alternative mechanism for the transfer of labelled cholesterol into the arterial tissue may be physical exchange between serum lipoproteins and membraneous lipids of the arterial wall. Such exchange has been observed in the transfer of labelled free cholesterol between different serum lipoprotein classes (52), and between serum lipoproteins and membraneous lipids of erythrocytes (108, 109) and tissue culture cells (110, 111, 112). However, rapid transfer of labelled free cholesterol by this mechanism does not necessarily involve actual net transfer of cholesterol (110, 111). Therefore, the existence of actual transfer of plasma cholesterol into the arterial tissue has been questioned (25, 26, 102, 113). On the other hand, physical exchange reactions are restricted to labelled cholesterol in the free form (52, 53), and consequently observations of transfer of labelled cholesteryl ester into the arterial tissue (III) strongly indicate actual transfer from plasma. The exchange of labelled free cholesterol may occur during rapidly transient collisions between different lipoprotein complexes, and direct contact between the different cholesterol compartments probably is a prerequisite for it (54). Uptake of labelled cholesterol by this mechanism would consequently be restricted to the membraneous lipids of endothelial cells and cells in direct contact with these. Therefore, the autoradiographic demonstration of labelled cholesterol, in layers of the aortic wall not in contact with endothelial cells (2, 100, 114, 115) , constitutes further evidence for actual transfer of cholesterol from plasma into the arterial tissue.

5.1.4 Cholesterol biosynthesis

In addition to evidence for actual transfer of cholesterol from the blood into the arterial tissue, there is also evidence for biosynthesis of cholesterol in the arterial wall (reviews: 2, 26). Furthermore, squalene and sterols other than cholesterol may be synthesized at even higher rates than cholesterol (e.g. 116). Attempts have been made to determine the relative contributions of plasma cholesterol and local cholesterol biosynthesis to the cholesterol content of normal arterial wall, by isotope dilution techniques (55, 117). However, the differing exchangeability of labelled free and esterified cholesterol (see above), the possibility of more than one cholesterol pool in the tissue, and the heterogeneous permeability characteristics of the normal arterial wall (II, III) make

the evaluation of such experiments uncertain. Therefore, it seems wise to refrain from quantitating the contributions of local synthesis and transfer from plasma, to the cholesterol content in the normal arterial wall, at the present state of knowledge.

5.2 The origin of excess cholesterol in atherosclerotic lesions

5.2.1 Cholesterol content and atherogenesis

The first observations of cholesterol and cholesteryl ester as constituents of atherosclerotic lesions date from as early as 1857(118). Later, the demonstration that cholesterol was in fact the principal lipid of advanced atherosclerotic lesions, and the observation of a correlation between coronary heart disease and serum cholesterol levels, stimulated more specific interest in cholesterol and its functions in atherogenesis (review and references: e.g. 119). Systematic studies on the lipid composition of human arterial tissue with age and atherosclerosis have indicated that increased concentrations of free and esterified cholesterol are characteristic features of aging , and of the formation of atherosclerotic lesions (22, 120). Thus, it now appears to be firmly established that the cholesterol content of the arterial wall increases during atherogenesis.

5.2.2 Filtration of serum lipoproteins

Whereas the significance of serum lipoprotein filtration for cholesterol transfer into the normal arterial wall is doubtful, there is much evidence for such filtration in atherosclerotic arterial tissue. Thus, the presence of serum lipoproteins in atherosclerotic lesions has been demonstrated with immunochemical (104, 121), immunohistochemical (93, 106, 107, 122, 123, 124), and electrophoretic (124) techniques. These investigations provide strong support for the concept of lipoprotein filtration as an important mechanism of transfer of cholesterol into atherosclerotic tissue, but they do not provide any information concerning the mechanism of cholesterol deposition.

5.2.3 Deposition of cholesterol

Some investigators have presented results which have been interpreted to mean that the filtration volume, i.e. the amount of plasma entering the arterial wall per unit of surface area, does not increase during atherogenesis (57, 58, 97). Consequently they have concluded that the main factor in the deposition of cholesterol may be increased serum cholesterol levels. However, the fact that atherosclerosis can develop at moderate cholesterol levels in humans (see 1, 3), the focal distribution of atherosclerotic lesions (8, 9, 10, 12), and the demon-

stration that cholesterol may accumulate in experimental atherosclerotic lesions at serum cholesterol levels below 50 mg/100 ml (V), provide strong evidence for the participation of other factors as well. Other investigators have suggested that deposition of cholesterol may be due to changes in the aggregation state of acid glycosaminoglycans in the arterial tissue (92). In addition, the formation of insoluble complexes between acid glycosaminoglycans and serum lipoproteins has been suggested to be an important mechanism for the deposition of cholesterol (93). On the other hand, results presented by Hollander et al., indicating partial independence between deposition of labelled cholesterol and acid glycosaminoglycan metabolism (125), appear difficult to combine with such a hypothesis. In addition, results presented in V, indicating that the deposition of cholesterol in experimental atherosclerotic lesions is related to increased filtration of labelled cholesterol from plasma, emphasize that not only the deposition but also the rate of transfer of cholesterol may be changed in atherosclerotic lesions.

5.2.4 Increased filtration of serum lipoproteins

The transfer of labelled cholesterol into atherosclerotic lesions is considerably higher than that into normal arterial tissue of the same experimental animal, as indicated by results of V and those of other investigators (e.g. 99, 125). In addition, while the distribution of ^{125}I-labelled plasma proteins and ^{3}H-cholesterol was dissimilar in normal aortic tissue (see above), it was similar in severely atherosclerotic aortic tissue (83). This has been interpreted as evidence for leakage of plasma lipoproteins through the lumenal arterial endothelium into the atherosclerotic tissue (83, 84). This interpretation gains support from our results, indicating that drastically increased transfer of labelled cholesterol into atherosclerotic tissue (V) parallelled excessive filtration of Evans blue-albumin complex (IV). Therefore, it appears probable that increased filtration of plasma lipoproteins may play a significant role in the deposition of cholesterol during atherogenesis.

5.2.5 Cholesterol biosynthesis

As discussed above, it is not possible at the present state of knowledge to estimate the relative contributions of cholesterol transfer from plasma and local biosynthesis, to the cholesterol content in the normal arterial wall. These considerations, which are based on the restrictions imposed by the available methods, are valid also for the total cholesterol content in the atherosclerotic arterial tissue. However, the capacity for cholesterol biosynthesis in vitro is low also in atherosclerotic arterial tissue (126), while cholesterol transfer from plasma is increased concurrently with deposition of cholesterol

in experimental atherosclerotic lesions induced by mechanical injury (V). In addition, the filtration of plasma proteins was increased in the lesions (IV), and the presence of plasma constituents may inhibit cholesterol biosynthesis, as suggested by in vitro studies (127, 128). Therefore, it does not seem unreasonable to suggest that cholesterol biosynthesis is of minor importance, at least for the increments of the cholesterol content in experimental atherosclerotic lesions induced by mechanical injury.

5.3 Cholesterol transfer in arterial tissue with intact endothelium

5.3.1 The role of cholesterol in normal arterial tissue

Cholesterol is an important constituent of membrane lipoproteins in all mammalian cells (54). The cholesterol content may be of considerable significance for the normal structural and functional properties of the cell memmbrane. For example, decreased cholesterol content in the plasma membrane of erythrocytes decreased their ability to withstand osmotic strain (129, 130). No doubt, cholesterol is also important for the functional and structural integrity of the cells in the arterial wall. Furthermore, it has also been suggested that increased cholesterol content is not always related to atherosclerotic changes in the arterial wall, but may also for example reflect increments of cell membrane material (27).

5.3.2 Cholesterol transfer and metabolic activity

From in vitro studies on the biosynthesis of cholesterol in calf aortae, Werthessen has reported a direct relationship between glucose consumption and increase in cholesterol content (131). In analogy, Jensen observed that the transfer of labelled cholesterol into the normal rabbit aorta in vitro was related to the metabolic activity (132). Certain metabolic inhibitors decreased the transfer of labelled cholesterol, whereas others increased it (132). On the other hand, these data conflict with those presented by others, suggesting no effect of boiling (102, 103, 113) or metabolic inhibitors (113) on the transfer of labelled cholesterol from serum-containing incubation media into arterial tissue. However, boiling the arterial tissue, or administration of potent metabolic inhibitors, may decrease endothelial integrity. That this was actually the case is indicated by Jensen's note that the tissue integrity appeared decreased after incubation with certain inhibitors (132). As the formation of endothelial defects is followed by excessive influx of serum lipoproteins (V, 27), filtration of lipoprotein cholesterol may have replaced metabolically dependent transfer of cholesterol, leaving the total uptake of labelled cholesterol unaf-

fected. Therefore, these studies do not contradict the concept that the cholesterol content and transfer of plasma cholesterol may be dependent on the metabolic activity in the arterial wall.

5.3.3 Active transport of cholesterol

On the basis of his *in vitro* studies, Jensen suggested that transfer of labelled cholesterol from plasma into the arterial tissue was due to active transport involving primary, non energy-requiring binding of serum lipoproteins at the lumenal surface of the endothelial cells (133) and subsequent hydrolysis of esterified cholesterol in them (38). He suggested that such transport might be mediated by pinocytotic activity (133). Alternatively, by analogy with cholesterol transport mechanisms in tissue culture cells, active transport could also involve binding of free and esterified cholesterol at the cell membrane, rapid hydrolysis of esterified cholesterol and intracellular transport of cholesterol in the free form (111, 128, 134, 135). Subsequently, cholesterol might be released from the cells, primarily in the free form (111, 127, 128, 136). Both these mechanisms are consistent with the results of III which suggest a direct relationship in regions with intact endothelium between free cholesterol radioactivity and cholesteryl ester radioactivity, with a ratio between the fractions of 20:1.

5.3.4 Control of cholesterol content

The cholesterol content in the arterial wall might conceivably be determined by both local biosynthesis and cholesterol transfer from plasma. The inverse relationship between transfer of labelled free cholesterol and free cholesterol content (III) in aortic tissue with intact endothelium suggests an adjustment of cholesterol transfer to the local requirements in the arterial wall. Werthessen and collaborators reported a similar inverse relationship between net increase of unlabelled cholesterol and initial cholesterol content, from *in vitro* studies on whole calf aortae perfused with a serum-containing medium at high pressure (200 mm mercury) (131, 136). In another study from the same laboratory, the data presented show a similar inverse relationship ($R = -0.54$; $p<0.05$ calculated as Spearman´s rank correlation coefficient) after perfusion of bovine aorta with a medium containing serum (137). As suggested above, these results may be explained both by adjustment of the biosynthesis and by adjustment of cholesterol transfer from serum lipoproteins in the medium. However, the fact that the rate of incorporation of ^{14}C-acetate into digitonin-precipitated material was not related to the net increase of unlabelled cholesterol during the study ($R = -0.18$; n.s. calculated as Spearman´s rank correlation coefficient from the data presented by Werthessen *et al.*) supports the latter of the explanations.

In any case these results, taken together with those presented in III, constitute strong evidence for Werthessen´s hypothesis "that the aortic content of cholesterol .... is subject to control" (136). Furthermore, the results of III indicate that control of the cholesterol content may involve adjustment of cholesterol transfer from plasma in response to the local requirements.

5.4 The cholesterol barrier

5.4.1 Earlier evidence for a cholesterol barrier

Some investigators have hypothetically postulated the existence of a barrier against the influx and deposition of cholesterol in arterial tissue (25, 132, 138). It has been suggested that the gradual break-down of this barrier during atherogenesis may allow the accumulation of serum cholesterol in the arterial wall. This concept is consistent with a number of interesting observations, for example the exponentially increasing rate of cholesterol accumulation (139), and the rapidly increasing influx of labelled cholesterol during the development of dietarily induced atherosclerotic lesions (discussion and references: 25, 138).

5.4.2 Endothelial integrity and filtration of serum lipoprotein cholesterol

As discussed in detail above, there is much evidence that increased filtration of serum lipoproteins is the most important mechanism for the accumulation of cholesterol during atherogenesis. Such filtration probably is restricted to arterial tissue with endothelium of decreased integrity (see above). The observation of increased transfer of labelled free and esterified cholesterol in regions of both normal (III) and atherosclerotic (V) tissue having defective endothelium indicates that the rate of transfer of cholesterol by filtration of serum lipoproteins may exceed that by active transport even in experimental animals with serum cholesterol levels below 50 mg/100 ml. It may be inferred that in individuals with higher serum cholesterol levels, this difference would be even larger. Hence, it may be suggested that the maintenance of endothelial integrity might be a very important factor in preventing excessive influx of serum lipoprotein cholesterol into arterial tissue.

5.4.3 Endothelial integrity and deposition of cholesterol

Not only transfer of plasma proteins (and, accordingly, transfer of serum cholesterol), but also deposition of cholesterol may be influenced by endothelial integrity. Thus, in normal as well as in atherosclerotic arterial tissue of normo-lipidemic rabbits, the cholesterol content of tissue devoid of endothelial lining was higher than that of corresponding tissue with intact endothelial lining (III, V). Obviously, this accumulation of cholesterol might be related

to imbibition of the arterial wall with serum lipoproteins. However, as (i) the cholesterol content of the atherosclerotic tissue was three times that of plasma, calculated on a dry weight basis (V), (ii) only a minor proportion (5 % in normal tissue, 15 % in atherosclerotic tissue) of the cholesterol in the tissue was esterified (III, V), whereas 75% of serum cholesterol is esterified, and (iii) the cholesteryl ester composition at least in atherosclerotic tissue differed markedly from that in plasma (VI), it seems unlikely that the increased cholesterol content merely was due to infiltration with serum lipoproteins. As discussed above, it has been suggested that cholesterol in cellular membranes may contribute to increments of the cholesterol content in the arterial wall (27). In support of this concept, an increase in cell membrane material has been observed during maturation and aging of aortic smooth muscle cells in the rat (140). Furthermore, formation of myelin figures (141), and increased pinocytotic activity (142), have been observed in dietarily induced atherosclerotic lesions. The demonstration of augmented synthesis of sphingomyelin (which is a characteristic component of plasma membranes) in atherosclerotic lesions induced by superficial mechanical injury with large area (143) suggests that accumulation of cell membrane material may account for an appreciable portion of the increased cholesterol content in the experimental atherosclerotic lesions of V as well.

5.4.4 Deposition of cholesterol in dietarily induced atherosclerosis

During the rise of the aortic cholesterol content in dietarily induced atherosclerosis, the relative proportions of free and esterified cholesterol were different at different total cholesterol concentrations (144). In aortae with moderately increased cholesterol content, most of the rise was accounted for by free cholesterol. In aortae with higher cholesterol content, cholesteryl ester contributed more to the total rise. Atherosclerosis was apparent only in aortae with more markedly increased cholesterol content, and characterized by a higher relative contribution of esterified cholesterol (144). As indicated by previously discussed observations, atherogenic diets may have important effects on endothelial integrity both in the initial stages of atherogenesis and during the propagation of atherosclerotic lesions. Therefore, a comparison with the results of III and V might be appropriate. In these studies, increased content of free cholesterol was characteristic of regions with decreased endothelial integrity, in normal (III) as well as in atherosclerotic arterial tissue (V). However, increased proportion of esterified cholesterol was observed only in the tissue underlying larger endothelial defects, and characterized by atherosclerotic changes(V). Therefore, it may speculatively be suggested that the early changes in the chol-

esterol composition of dietarily induced atherosclerotic lesions may be related to changed endothelial integrity and tissue responses following endothelial defects, rather than being strict reflections of changes in serum cholesterol.

5.4.5 Deposition of cholesterol and formation of atherosclerotic lesions

In conformity with the filtration hypothesis of atherogenesis, the formation of fatty streaks has been attributed to the attainment of a critical cholesterol concentration in the arterial wall (23, 27). However, the present results indicate heterogeneity in cholesterol content both in normal and atherosclerotic arterial tissue (III, V). Deposition of cholesterol is almost exclusively confined to tissue with decreased endothelial integrity. In regions with intact endothelium, cholesterol levels appear to be subject to sophisticated control mechanisms. Thus, the endothelialized BANKS, which have several structural characteristics in common with fatty streaks, have cholesterol concentrations which are not significantly different from those of control aortic tissue. Therefore, the concept that the arterial tissue may adopt certain of the morphological characteristics of early atherosclerotic lesions in response to generally increased cholesterol content in the arterial tissue may be questioned.

5.4.6 The cholesterol barrier-conclusions

Plasma protein filtration is restricted by the intact arterial endothelium (see above. In addition, the cholesterol levels of the tissue underlying intact endothelium appears to be subject to control, probably involving an adjustment of the cholesterol transfer from plasma (III). On the other hand, when endothelial integrity decreases, (i) increased filtration of plasma proteins, (ii) increased transfer of plasma cholesterol into the tissue, and (iii) deposition of cholesterol in the arterial tissue may occur (III, V). These results provide strong evidence for the existence of a barrier against excessive cholesterol influx and deposition, as hypothetically suggested by others. In addition, the results indicate that the structural correlate of this barrier may be the continuous arterial endothelium.

5.5 Cholesteryl ester composition in atherosclerosis

5.5.1 Cholesteryl ester composition in plasma and atherosclerotic lesions

The cholesteryl ester fatty acid (CEFA)composition of the experimental atherosclerotic lesions of the present study was very similar to that described in fatty streaks and so-called "fatty plaques" [x] in humans (21, 37, 120, 121, 145, 146, 147, 148, 149, 150) and dietarily induced experimental atherosclerotic lesions (151, 152, 153, 154, 155), This very characteristic CEFA pattern is markedly

x) Definition: see ref. (120).

different from that in plasma of humans and experimental animals (VI; review and references: 53)and in more advanced atherosclerotic lesions in man (120, 149, 56 , 157). Mono-unsaturated cholesteryl ester fatty acids constituted the largest fraction in the experimental lesions (VI), fatty streaks and "fatty plaques", whereas the fraction with di-unsaturated fatty acids was higher in plasma (VI) and other types of atherosclerotic lesions. In addition, cholesteryl esters with fatty acids containing three or more double bonds were more frequent in the lesions than in plasma (VI). Increased amounts of such cholesteryl esters have also been observed by other investigators in atherosclerotic lesions of humans (146, 147, 150, 156, 158, 159). It has been demonstrated that these esters contain fatty acids characteristic of essential fatty acid deficiency (158). High content of cholesteryl esters with mono-unsaturated fatty acids may also be induced by essential fatty acid deficiency (160). However, the present results from experimental animals wfthout signs of systemic essential fatty acid deficiency indicate that, at least in this study, this factor does not induce the specific CEFA pattern observed. It also seems unlikely that local essential fatty acid deficiency is of importance, as the CEFA pattern is similar in regions with very different rates of filtration of albumin (VI) and consequent different influx of free fatty acids from plasma.

5.5.2 Local arterial tissue factors

The CEFA pattern of macroscopically normal intima from human changes with age. In children between 0 and 10 years old, cholesteryl oleate was the predominating fraction (150). However, during growth and aging the CEFA composition in the arterial tissue gradually became more similar to that in plasma. Thus, in the intima of humans over 30, the cholesteryl ester composition was rather similar to that in plasma (120, 161). These observations support suggestions that infiltration of the arterial wall with plasma cholesteryl esters may contribute very significantly to the increase of total cholesteryl esters with age. However, the characteristic CEFA pattern in intima of children suggests that local mechanisms may also be important in determining the cholesteryl ester composition in normal arterial tissue.

The CEFA distribution was rather similar in all regions of the experimental atherosclerotic lesions of the present study, despite the large differences in filtration of plasma constituents (VI). This emphasizes the importance of local arterial tissue mechanisms for the cholesteryl ester composition in atherosclerotic lesions as well. Further evidence for the importance of such mechanisms was provided by studies, indicating a relationship between cellularity and CEFA composition in different types of atherosclerotic lesions in humans (159).

The cholesteryl ester composition was similar, although the morphological distribution of lipids was different in different regions of the lesion (VI). These results are not consistent with those of other studies which demonstrate significantly different CEFA composition in intracellular and extracellular lipids (161, 162). The aberrant results of the present study may of course be due to species differences, as rabbits were used in the present study, whereas the other studies have been performed on autopsy material, derived from adult or elderly human populations. However, by analogy with the results discussed above, they could conceivably also be related to age differences. Thus, it may be speculated that the intracellular lipid composition may not change much from that during childhood, whereas the extracellular cholesteryl ester composition may conform to that in plasma during growth and aging, not only in normal tissue, but also in atherosclerotic tissue. However, the validity of such an interpretation is difficult to assess because of the species differences.

5.5.3 Cholesteryl ester metabolism

Cholesteryl ester metabolism in the arterial wall was studied by isotope techniques in vivo (VI). To our knowledge, such studies have not previously been performed with normo-lipidemic experimental animals. The evaluation of the results from these studies may be subject to some limitations, besides those discussed above for all studies with labelled cholesterol. As only one time interval was studied, it was not possible to establish when plateau values for the uptake of radioactivity in the different cholesteryl ester fractions were reached. Therefore, the possible existence of non-homogeneous cholesteryl ester tissue pools might limit the validity of the results. However, the observations were made at a time point well before the attainment of equilibrium specific activities between the total cholesterol pool in plasma, and that (or those) in the arterial tissue. Results presented by Swell et al. indicate that this holds true also for different cholesteryl ester sub-fractions (163), at least in dietarily induced atherosclerosis. Finally, we found that the cholesteryl ester specific activities in plasma exceeded the final specific activity in the arterial wall during the time studied (VI), and that the specific activities in different cholesteryl ester sub-fractions did not differ much (VI). Therefore, differences in specific activities of different cholesteryl ester sub-fractions probably are related to differences in turn-over between the fractions.

Different mechanisms have been suggested for the characteristic increase in cholesteryl oleate in experimental atherosclerotic lesions, human fatty streaks and so-called "fatty plaques". Some investigators have presented evidence for enhanced esterification of cholesterol with oleic acid as compared to other fatty acids (164, 165, 166) indicating a mechanism of selective esterifi-

cation. Other investigators have observed a decreased hydrolysis of cholesteryl oleate, as compared to other cholesteryl esters (167, 168), indicating a mechanism of selective hydrolysis. Finally, it is conceivable that interconversion of other cholesteryl esters to cholesteryl oleate, and more pronounced retention of cholesteryl oleate than of other cholesteryl esters (37), may be responsible for the increment of this particular cholesteryl ester sub-fraction. Higher rate of esterification of cholesterol with oleic acid than with other fatty acids in the arterial tissue would give higher specific activity in this fraction, as the free cholesterol specific activity was higher than the cholesteryl ester specific activity. On the other hand, the other mechanisms discussed above would give lower specific activity in cholesteryl oleate than in the other cholesteryl ester sub-fractions. Therefore, the absence of consistent differences in specific activities, between the three main, more saturated cholesteryl ester sub-fractions, may indicate that no single mechanism is responsible for the characteristic cholesteryl ester composition of the lesions. It may also support suggestions that both selective hydrolysis and selective esterification may be significant for the cholesteryl ester composition in atherosclerotic lesions (169).

The specific activity of the three main, more saturated cholesteryl ester fractions was considerably higher than that of cholesteryl esters with fatty acids containing three or more double bonds (VI), suggesting a low turnover of the latter. This suggestion is supported by the observation that the relative concentration of such cholesteryl esters increased, after removal of cholesterol during the restoration of the arterial wall structure (VI). Preferential deposition of these specific cholesteryl esters may reflect a protective response against the sclerogenic effect of other forms of cholesterol, as hypothetized by Abdullah et al. from observations on the effect of different forms of cholesterol in subcutaneous tissue implants in rats (170).

5.6 Cholesterol removal and atherosclerosis

5.6.1 Decreased cholesterol removal and atherosclerosis

Deposition of cholesterol in arterial tissue may be related to decreased outflow as well as increased influx of cholesterol. During aging, progressive intimal thickening continuously increases the distance for diffusion of various nutrients into the central parts of the arterial wall (1, 2, 17). This process may promote hypoxic damage to parts of the tunica media of the arteries (17). Damage and necrosis to the tissue may impair the capacity for cholesterol removal in the arterial wall, and consequently induce deposition of cholesterol in the ischaemic zone and in regions on the lumenal side of this zone (for review and discussion, see 2).

5.6.2 Regression of atherosclerotic changes

In the experimental atherosclerotic lesions investigated in the present study, the cholesterol concentration in regions which were still devoid of endothelial lining was 3 times as high as in re-endothelialized regions (V). This discrepancy between different regions which had all been denuded of endothelial lining by the initial trauma, indicates that cholesterol is readily eliminated during repair and re-endothelialization of the lesion. In the non-endothelialized regions, the discrepancy in uptake of labelled free and esterified cholesterol may suggest that far more cholesterol enters the arterial wall than is deposited in it (V). This suggestion is supported by the fact that, during the 4 weeks following the trauma, ca. 10 mg/g dry tissue weight of cholesterol has been deposited in the YARD, whereas the uptake of radioactivity in the same regions suggests that 15 mg may have passed through the lesion during the 24 hours of exposure to radioactive cholesterol (V). The low rate of deposition, in spite of the rapid transfer of cholesterol, may be related to high capacity for removal of cholesterol in atherosclerotic tissue of normo-lipidemic rabbits (V).

The question, whether dietarily induced experimental atherosclerotic lesions may regress or not, has been the subject of some controversy. Some investigators have been unable to show any change in the lesions after withdrawal of a cholesterol-rich diet (171). Others have observed that atherosclerotic changes decreased after the cessation of cholesterol feeding, while on the other hand the cholesterol content in the arterial wall did not change (172). Finally, some investigators have been able to demonstrate that the incidence of atherosclerotic changes, as well as the concentration of cholesterol in the arterial wall decreased significantly after long periods on cholesterol-free diets (173). Unpublished results from our laboratory indicate that regress of experimental atherosclerotic lesions induced by mechanical trauma in normo-lipidemic rabbits, was related to the restoration of an intact endothelial lining (34). In addition, the present results suggest that cholesterol may be eliminated during re-endothelialization of atherosclerotic tissue (V). Therefore endothelial integrity appears to be an important factor, not only for the maintenance of the normal cholesterol content in the arterial wall, but also for the removal of cholesterol deposits during regression of atherosclerotic changes.

5.6.3 Cholesterol removal-possible mechanisms

A variety of different mechanisms have been proposed for the removal of cholesterol from normal and atherosclerotic arterial tissue. The incorporation of cholesterol into lipoproteins (174, 175), dispersal of cholesterol by phospholipids (176, 177, 178), esterification of cholesterol with poly-unsaturated fatty acids

(178, 179), and cholesteryl ester hydrolysis (173) have all been considered as possible mechanisms of elimination of cholesterol. The question, whether cholesterol is initially mobilised mainly in its free form, or if it is esterified with poly-unsaturated fatty acids, has been subject to debate (e.g. 169, 178). However, the present results indicate that the turnover of poly-unsaturated cholesteryl esters is low. Therefore the formation of such cholesteryl esters does not seem to be involved, at least in the initial steps of cholesterol removal from the tissue. On the other hand, cholesteryl esters are important constituents of lipoproteins, and lecithin cholesteryl acyl transferase (LCAT) activity in the blood is considered to be a prerequisite for the incorporation of tissue cholesterol into serum lipoproteins (review: 180). Consequently, cholesteryl ester formation may be an important factor in the removal of cholesterol from atherosclerotic tissue, even though pre-existing cholesteryl esters may be hydrolysed during the initial stages of the elimination process. Recently, Rutenberg and Soloff presented results which appear to be consistent with such a mechanism (181). They studied the movement of cholesterol from arterial tissue into serum in vitro, and found that cholesterol was mobilised from arterial tissue in its free form, concurrently with esterification of free cholesterol by LCAT activity in the serum. Similarily, Murphy observed that esterification of cholesterol in serum promoted transfer of free cholesterol from erythrocyte cell membrane lipoproteins to serum lipoproteins (129). The extent of this phenomenon was such that the functional integrity of the erythrocyte cell membrane was impaired due to decreased cholesterol content (129). An observation in the former study which may appear confusing, was that if the arterial tissue was incubated in serum, in which the enzyme activities had been inactivated by heating, mainly esterified cholesterol was transferred from the tissue into the incubation medium (181). However, this observation may be related to the recently demonstrated presence of LCAT activity in the arterial wall (179), exerting its effects on the incubation medium. This idea is supported by the observation that the final distribution of cholesterol between the free and esterified fractions in serum was similar in serum incubated alone, serum incubated with the tissue, and heat-inactivated serum incubated with the tissue (181). These studies, and those indicating little or no efflux of labelled cholesterol from arterial tissue into a serum-free medium (102, 182), emphasize that plasma factors may also be important for the removal of cholesterol from the arterial wall. It seems reasonable to assume that the elimination of cholesterol may be a process involving local tissue factors as well as plasma factors as necessary components.

6. ENDOTHELIAL INTEGRITY AND EXPERIMENTAL ATHEROSCLEROSIS

6.1 Experimental atherosclerosis induced by mechanical trauma

The development of lipid-rich arterial lesions was recently reported following certain types of defined mechanical injury in normo-lipidemic rabbits (32) and rats (183) These observations indicate that certain types of mechanical injury may incite tissue responses related to those in atherosclerosis (33), and, therefore, must be considered as one possible initiating factor in atherogenesis. It was suggested that the characteristic accumulation of lipids in atherosclerosis is related to defective endothelium (33). This suggestion receives strong support from the results of V, indicating that deposition of cholesterol was confined to regions with defective endothelium in experimental atherosclerotic lesions induced by superficial mechanical injury with large area. The absence of endothelial lining in these regions may allow the influx of plasma lipoprotein cholesterol in quantities exceeding the capacity for removal, leading to deposition of cholesterol.

6.2. Dietarily induced experimental atherosclerosis

Not only experimental mechanical injury, but also pre-existing endothelial defects in regions subject to increased hemodynamic stress, may be related to the localization of atherosclerotic lesions. Thus, after the induction of hypercholesterolemia, the incidence of such lesions was increased in regions, distributed similarily as regions with defective endothelium in the normal rabbit (8, see II and above). However, atherosclerotic lesions with lipid deposition develop in these areas only if the blood lipid levels of the experimental animals are increased(3), but at very low levels in areas with larger, experimentally induced endothelial defects (32). Therefore, it may be hypothetized that changes either in blood lipid levels or in endothelial integrity, as well as concurrent changes in both factors, may be significant for the formation of atherosclerotic lesions with deposition of lipids.

The effects of hypercholesterolaemia are not restricted to increased cholesterol concentration in the serum filtrate passing through areas of defective endothelium into the arterial tissue. Thus, within 1-3 days after the commencement of cholesterol-rich diets, increased mitotic activity and other morphological changes in the arterial endothelium have been observed (184, 185, 186). At such early times the serum cholesterol levels of the experimental animals were not significantly different from those of controls (186), suggesting that endothelial changes preceded more significant increments in blood lipid levels. The permeability characteristics of the arterial wall were also changed very early.

and during the first three weeks of cholesterol feeding, the filtration of ^{131}I-labelled plasma proteins increased drastically (187, 188, 189). Taken together the evidence suggest that the induction of structural and functional alterations in endothelial cells may be an important component of the early changes in dietarily induced atherogenesis.

After the formation of dietarily induced atherosclerotic lesions, evidence of endothelial cell injury, such as cytoplasmic oedema and formation of intercellular gaps, have been observed (154, 190). In addition the gradient of radioactivity from labelled plasma proteins in the arterial wall reverted in severely atherosclerotic lesions (83, 191), suggesting that "the endothelium or some other structural component of the intima is sufficiently damaged to allow direct leakage of plasma constituents from the lumen into the inner arterial wall" (84). The permeability to Evans blue-albumin complex (91) and thorotrast (192) was increased, especially in areas adjacent to more normal tissue. Some investigators have regarded these results as evidence that the induction of increased permeability through the endothelium surrounding the lesions is one possible mechanism for the dissemination of atherosclerotic lesions (91). Therefore, there is evidence that endothelial changes involving increased permeability for plasma proteins may be important not only in the initial stages of dietarily induced atherosclerosis, but also in the propagation of the lesions.

6.3. Endothelial integrity and the filtration hypothesis

The results summarized in the present work constitute strong support for the hypothesis that the formation of atherosclerotic deposits, containing cholesterol, may be related to imbibition of the arterial wall with plasma. However, they also indicate that the existence of a continuous endothelial cell layer may prevent excessive influx of cholesterol-containing lipoproteins, and deposition of cholesterol. Therefore, it is possible that the endothelial structural and functional integrity may be a very significant factor in the maintenance of the normal arterial wall structure.

7. ABSTRACT

Structural and functional properties of the aortic endothelium, and local transfer mechanisms for free and esterified cholesterol in normal aorta and in aortic experimental atherosclerotic lesions have been studied. For the investigations it was necessary to develop new techniques for the study of endothelial cell viability and structure (II) and a method for the ultra-micro assay of cholesterol in tissue (I). These techiques have been combined, and together with isotope techiques applied to normal aorta and experimental atherosclerotic lesions induced by superficial mechanical injury with large area in normolipidemic rabbits.

The endothelium of the normal aortic surface was structurally and functionally heterogeneous (II). Defective endothelium was present where increased hemodynamic strain occurs (II). The increased incidence of atherosclerotic changes in the same regions suggests that decreased endothelial integrity may be an important factor in the interrelationship between hemodynamic strain and atherosclerosis. Mural microthrombi were observed in regions with defective endothelium (II), suggesting that decreased endothelial integrity may also be related to thrombotization.

Structural heterogeneity was parallelled by heterogeneous permeability characteristics (II, III). The results indicate that filtration of plasma proteins probably is confined to regions with defective endothelium (III, IV, V).

In normal arterial tissue covered with intact endothelium, an inverse relationship was observed between cholesterol content and transfer of labelled cholesterol from plasma (III), suggesting an adjustment of cholesterol transfer to local requirements of cholesterol in different regions of the normal aortic tissue. In addition, a direct relationship was found between the transfer of labelled free cholesterol and that of labelled esterified cholesterol, with a ratio between the fractions of 20:1 (III). This is consistent with the concept of active transport of cholesterol, involving rapid hydrolysis of cholesteryl ester, a mechanism suggested by others.

In normal and experimental atherosclerotic arterial tissue with defective endothelium, increased cholesterol transfer and deposition of cholesterol was observed (III, V). In addition, the control of cholesterol transfer appeared to be deficient (III). These results are consistent with the break-down of a barrier against excessive influx of cholesterol from plasma and deposition of cholesterol in the tissue. In addition, they indicate that the structural

correlate of this barrier is the continuous arterial endothelium.

Cholesteryl esters with mono-unsaturated fatty acids dominated in the experimental atherosclerotic lesions, whereas cholesteryl esters with di-unsaturated fatty acids dominated in plasma (VI). In the lesions, the cholesteryl ester composition appeared to be independent from the rate of plasma protein influx (VI), suggesting that local tissue factors may determine the cholesteryl ester composition in the tissue. Isotope studies indicated that both preferential esterification of cholesterol with mono-unsaturated fatty acids, and preferential hydrolysis of other cholesteryl esters may be important for the increased proportion of cholesteryl esters with mono-unsaturated fatty acids (VI). Cholesteryl esters with fatty acids containing three or more double bonds appeared to be deposited in the tissue, as indicated by low specific activity, and the increased proportion of such cholesteryl esters after elimination of deposited cholesterol (VI).

The results suggest a high capacity for removal of cholesterol in the experimental atherosclerotic lesions (V). The low specific activity in and the retention of cholesteryl ester with fatty acids, containing three or more double bonds, indirectly indicate that hydrolysis of cholesteryl ester may be a prerequisite for the elimination of cholesterol deposits from atherosclerotic lesions (VI).

The properties of the endothelium of experimental atherosclerotic lesions induced by superficial mechanical injury suggest that both the presence and the localization of lipid deposits were influenced by endothelial integrity (IV). Similarily, decreased endothelial integrity may be important both in the initial stages of dietarily induced experimental atherosclerosis and in the propagation of such lesions.

The results of the present work constitute strong support for the hypothesis that the formation of atherosclerotic deposits may be related to imbibition of the arterial wall with plasma. However, they also indicate that endothelial structural and functional integrity may be a significant factor in the maintenance of the normal arterial wall structure.

8. ACKNOWLEDGEMENTS

I wish to express my gratitude to:

Professor Holger Hydén, the prefect of the Department of Histology, where laboratory facilities for the Geriatric Research group, sponsored by the Swedish Medical Research Council, have been provided.

Professor Lars Werkö for his interest and support throughout the studies summarized in the present thesis.

Dr. Sören Björkerud, my scientific teacher and my collaborator throughout the study, for generous support and constructive criticism.

Mrs. Ingeli Andreasson, Mrs. Lena Hjalmarsson, Mrs. Gun Olsson and Miss Ann-Marie Wasshede for excellent technical assistance.

Miss Gull Grönstedt for careful secretarial work.

Dr. Irwin Levitan for critical reading and correction of the manuscript.

Dr. Tore Scherstén for valuable criticism of the manuscript.

The investigations summarized in this thesis were supported by grants from the Swedish National Association against Heart and Chest Diseases, the Swedish Medical Research Council (Project No. 19X - 2589) and Anna Bäwer´s Foundation.

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Printed in offset
ELANDERS BOKTRYCKERI AKTIEBOLAG
GÖTEBORG 1972