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PHOSPHOGLUCOISOMERASE ACTIVITY IN ATHEROSCLEROTIC AORTA.

T. Anastassiades

PHOSPHOGLUCOISOMERASE ACTIVITY AND CONNECTIVE TISSUE CHANGES IN THE HUMAN AORTA DURING ATHEROSCLEROSIS

by

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Preface

The work presented in this thesis represents, in part, an effort to ascertain some of the biochemical changes that occur in the aorta during the development of atherosclerosis. My interest was first stimulated during the course in pathology in the early part of medical training. The possible significance of local connective tissue changes in the development of the disease also became apparent to me at that time. Later, during clinical work, I was impressed by the preponderance of vascular disease over all other causes of morbidity and mortality, and astonished at the relative lack of basic biochemical work, particularly on the connective tissue aspects of the disease. The emphasis on basic enzymology at the Department of Biochemistry, and the facilities and research activity in the Royal Victoria Hospital, afforded a desirable environment for such an approach to the study of atherosclerosis.

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INTRODUCTION

PART I. Concepts Concerning the Etiology of Atherosclerosis.

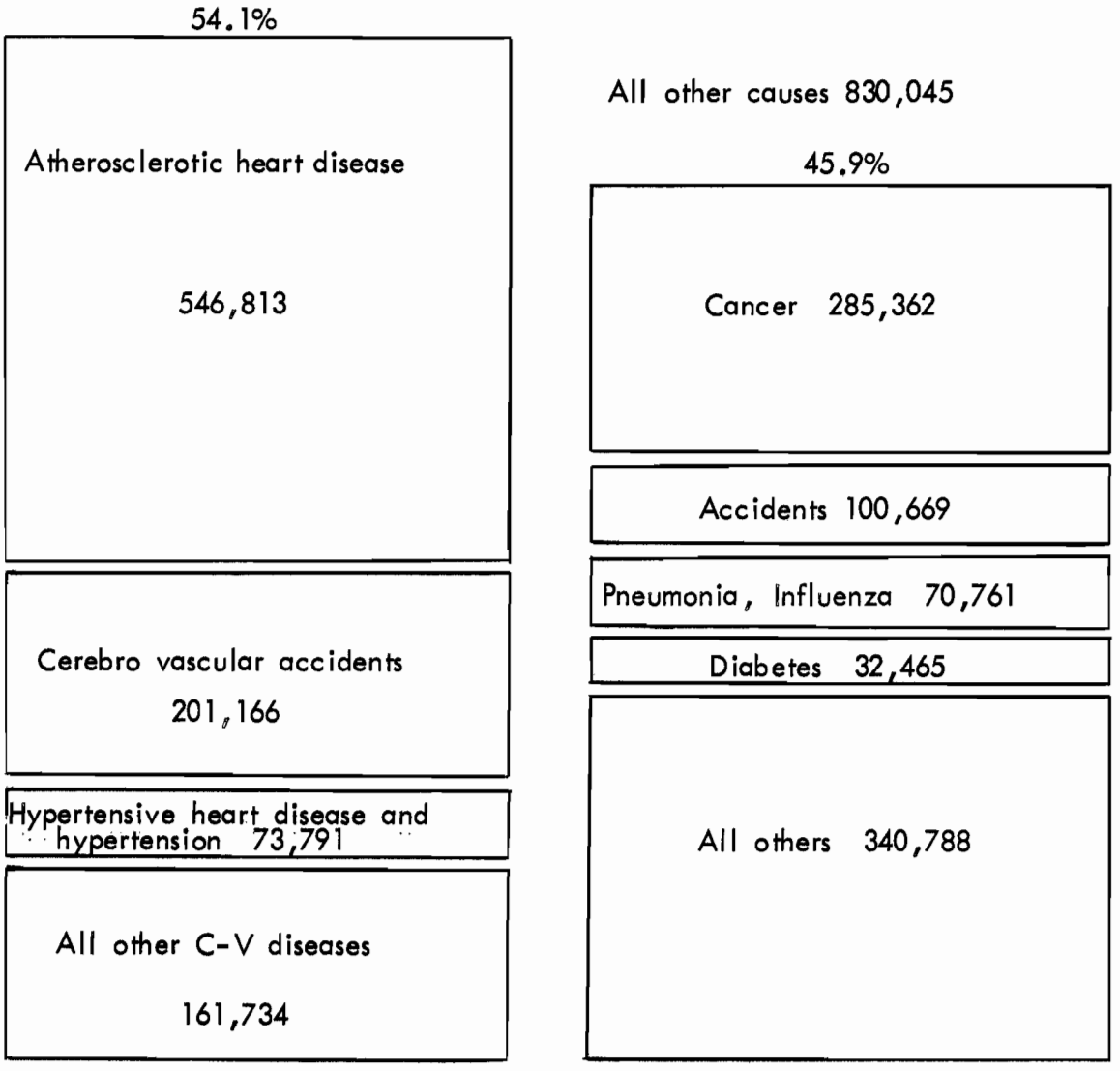
1. Atherosclerosis and Clinical Cardio-Vascular Disease.

Cardio-Vascular (C-V) disease in man is responsible for a greater morbidity and mortality than any other disease (1-5). In Canada it accounts for more than half the deaths from all causes, exclusive of accidents and perinatal mortality, and it is responsible for about three times as many deaths as cancer (1). As indicated in Figure 1 C-V diseases in the United States account for more deaths than all other causes combined (3,5).

It is generally considered that atherosclerosis plays an important part in the etiology of C-V disease. Some authorities hold that atherosclerosis is directly responsible for most cases of coronary occlusive disease, peripheral occlusive disease and cerebrovascular accidents. However, such a direct relationship can not be taken for granted in view of the variety of causes that may precipitate or accentuate the manifestations of these diseases and considering that many people with severe atherosclerosis do not demonstrate clinical C-V disease. It is therefore important briefly to consider the evidence for a relationship between atherosclerosis and the clinical manifestations of occlusive vascular disease. The evidence is twofold:

Figure 1

U.S. Deaths in 1963 from all Causes 1,813,549



From: Public Health Service of the U.S. Department of Health, Education and Welfare (5).

1) Pathological: repeated post mortem observations of developing lesions within a vessel until the vessel is nearly or completely occluded. Concomittent demonstration of ischemic changes in the tissue supplied by the involved vessel, is an important, although not essential, part of this evidence.

2) Statistical: parallelism between the incidence of atherosclerosis, found at autopsy, and the occurrence of clinical C-V disease. Some of the statistical studies have been carried out with a view to comparing this parallelism in a variety of sex, racial and socio-economic groups (6). Other statistical studies (23, 24, 25) have endeavoured to show the close correlation between atherosclerosis and clinical C-V disease in more homogenous population groups that have experienced abrupt changes in economic and particularly nutritional status.

To further clarify the pathological evidence for the relationship between atherosclerosis and C-V disease it is useful to consider, diagramatically, the development of a lesion. It is understood that the following account represents only one pathogenetic concept and it is used simply as an illustrative example:

Lipid deposition begins in the aorta during the first year of life; in the coronary arteries during the second decade and in the intercranial arteries during the third decade (6). Duff and McMillan (7) have suggested that this early accumulation of fatty material is associated with local tissue changes, such as alterations in the composition or content of

the ground substance of the intima. Further accumulation of connective tissue may occur around some of the fatty deposits and lead to formation of a plaque. Plaque formation represents an important stage in the pathogenesis of atherosclerosis. Haemorrhage within the plaque, or formation of thrombus over the plaque, as indicated in Figure 2, may reduce the size of the lumen to a critical point so as to result in partial or complete ischemia. The ischemia may become manifest as angina pectoris, myocardial infarction, cerebral infarction or peripheral gangrene.

Let us now consider in closer detail an example of the statistical studies which relate the incidence and extent of atherosclerotic involvement to the incidence of clinical C-V disease. The picture is complicated by the circumstance that factors, such as thrombosis, may contribute to clinical C-V disease and furthermore a large proportion of individuals with advanced atherosclerosis die without manifest cardiovascular distress. McGill et al. (6) endeavoured to test the correlation between the incidence of lesions in the coronary artery and that of ischemic heart disease, by studying two racial groups of both sexes in Louisiana. The results are summarized in Figure 3. It is apparent that the order of incidence of coronary artery involvement by atherosclerosis in the males of the white and the coloured groups was comparable with the mortality rates from ischemic heart disease. The same applied to the females of each group. The groups arranged in descending order of mortality were: white

Figure 2

Relationships Between Atherosclerosis and Clinical C-V Disease.
A Pathogenetic Scheme.

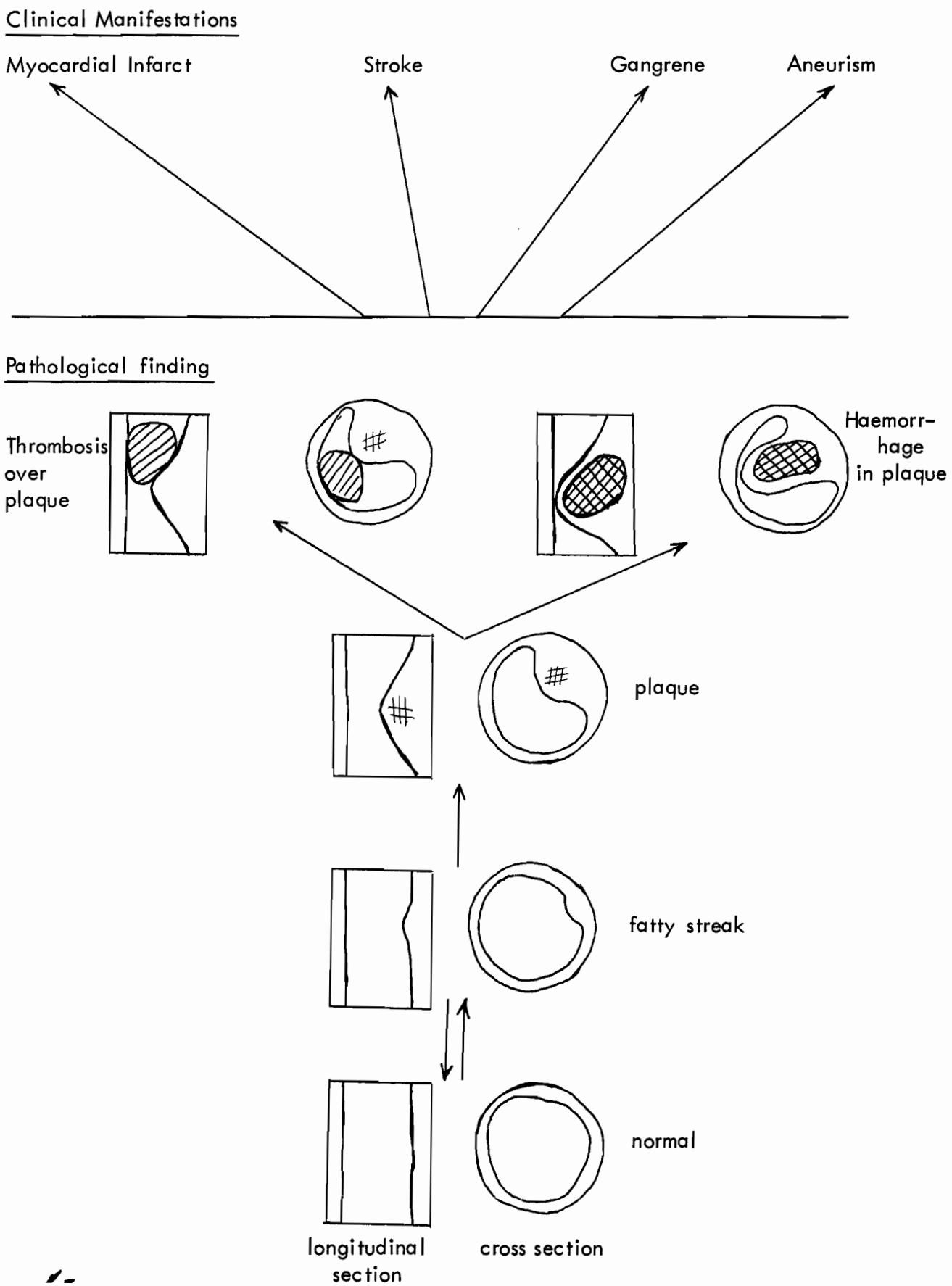
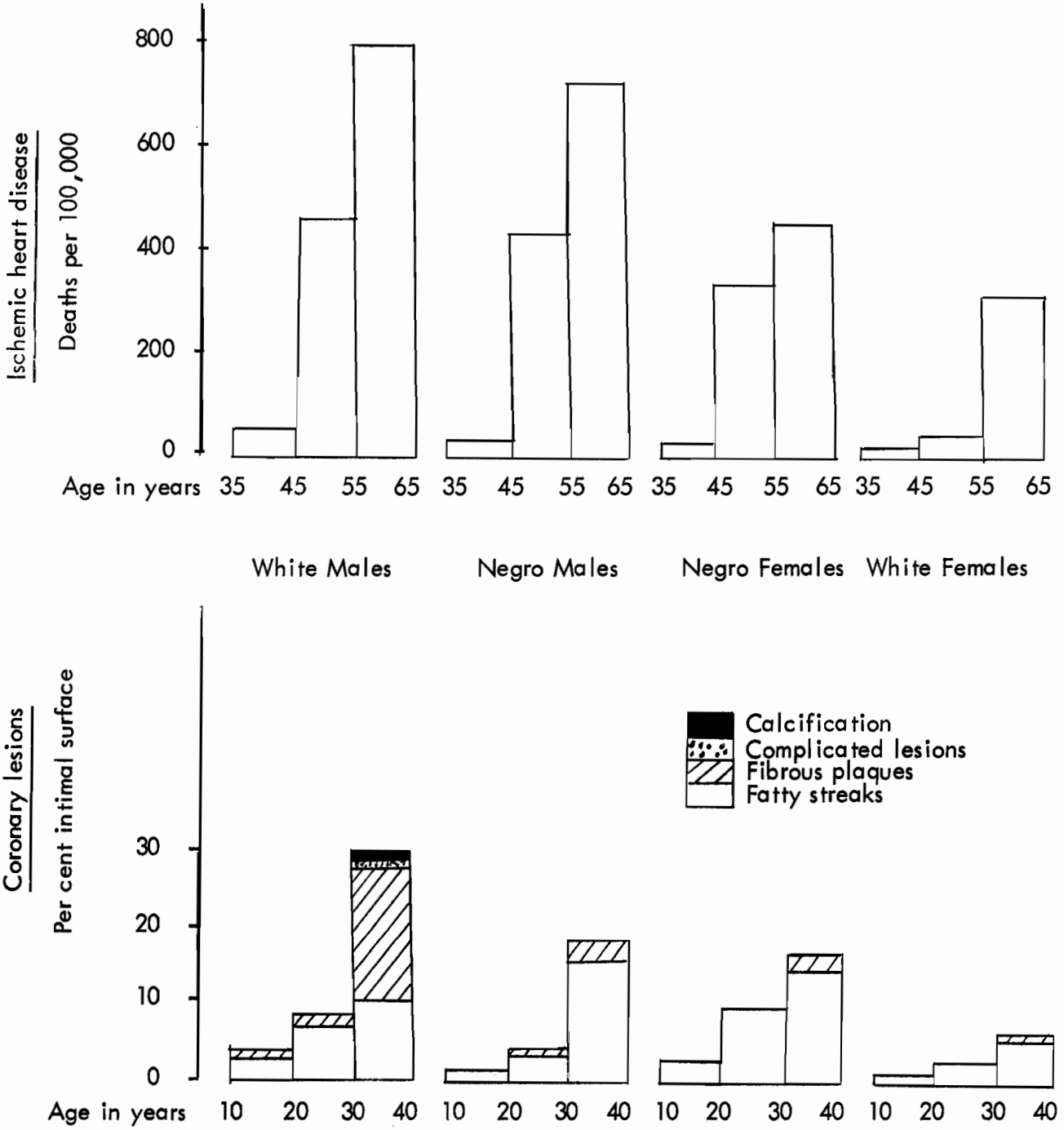


Figure 3

Relationships Between Coronary Atherosclerosis and Ischemic Heart Disease.



Comparison of mortality rates from ischemic heart disease, ages 35-64, and coronary artery lesions, ages 10-39.

From: McGill et al. (6).

males, negro males, negro females, white females. Furthermore, these investigators (6) suggest that the degree of coronary atherosclerotic involvement in individuals between the ages of 10-40 years affords a reliable base for prediction of the relative frequency of ischemic heart disease for each group, 25 years later.

Many investigators also have reported cross-correlations for similar groups (9-12), thus strongly indicating a direct causal relationship between the incidence of atherosclerosis and C-V disease. These and similar studies have been well reviewed by Stamler (8, 53), McGill et al. (6) and Larsen (10).

2. Morphology of Arterial Tissue and Early Pathological Concepts.

Arteries are composed of three coats. The innermost the tunica intima, and intermediate the tunica media and the outer the tunica adventitia. These are clearly distinguishable in the larger arteries but much less well defined in the arterioles.

The tunica intima is composed of a thin layer of endothelial cells supported on subendothelial connective tissue. At birth the tunica intima is quite thin, but subsequently with the growth of the individual, the deposition of subendothelial connective tissue produces progressive thickening of the intima. The outer boundary of the intima is demarcated by a condensation of longitudinally disposed elastic fibres, which form the internal elastic lamina.

The tunica media is composed of transverse layers of smooth muscle and elastic fibres. Condensation of the elastic tissue at the outer limit forms the external elastic lamina.

The tunica adventitia is a poorly defined layer of investing connective tissue in which are dispersed small thin wall nutrient vessels, the vasa vasorum. These vessels are derived mainly from the junctions of larger branches and travel back to the wall of the artery (13). The vasa vasorum ramify into smaller channels which provide a blood supply to about the outer two thirds of the media. The intima and inner third of the media are not vascularized (14). This avascular layer increases in thickness with advancing age largely due to an increase in thickness of the intima (15).

The avascular layer presumably receives oxygen and nutrients by diffusion from the blood within the main vessel.

A large number of hypotheses of the pathogenesis of atherosclerosis have been put forward. Among the earliest and most influentidl was that of Virchow who in 1856, described the accumulation of a "mucous intimal substance" which contained lipid believed to be deposited from the invading plasma (16). He named this affection of the arteries "endarteritis chronica nodosa sive deformans", since he considered the deposition of mucous substance in the intima to be the result of an exudative inflammatory process. According to this view mucous substance accumulations together with fatty deposition constituted the initial pathological changes.

The term 'atherosclerosis' was first introduced by Marchand in 1904 (17) to designate the deposition of lipids in the intima. (ἀθήρη = gruel). However, the main support for the view that atherosclerosis is a disturbance primarily involving deposition of lipids, derived from the studies of Anitschkow and Chalatow (18). Their findings inspired much of the investigation of the Soviet school which promulgated the view that atherosclerosis arises from a disturbance of cholesterol metabolism (19). The Russian workers held that the source of cholesterol could be either dietary or endogenous or both and that fat deposits in the brain might be among the endogenous sources (19). Support of this view derives from experiments in nephrectomized dogs which developed hypercholesterolemia and in which

the source of the cholesterol was shown to be the brain (19).

More recently, however, Anitschkow defined (20) atherosclerosis as "a chronic disease of the large arteries which is characterized by focal thickening of the intima, consisting of connective tissue and containing more or less lipid often complicated with calcification and ulceration".

Aschoff (21) in 1933 suggested that atherosclerotic lesions arise from penetration of plasma lipids into the arterial wall. He refered to this process of lipid uptake as "plasma imbibition" (21).

The views of Rotitansky (22) also deserve mention, particularly as they have received recent support from the works of Duguid (40, 41). Rotitansky (22) in 1852 suggested that the arteriosclerotic deposit is "an endogenous product derived from the blood, and for the most part from the fibrin of the arterial blood". He further felt that the deposit cannot be regarded as a product (excudation) of an inflammation in the arteries. Rotitansky's views were vigorously opposed by Virchow who regarded atheroma as an inflammatory process by the connective tissue cells of the arterial wall (16, 40).

3. Dietary Evidence Relating Increased Triglyceride and Cholesterol Intake to Incidence of Atherosclerosis and C-V Disease.

Statistics on nutrition and health from many countries have been cited to show the relation between triglyceride and cholesterol intake and an increased incidence of atherosclerotic disease. However, in many oversimplified cases the validity of such correlations may be open to criticism on several grounds. The accuracy of the diagnosis given on the death certificates as to the cause of death may be questionable, particularly if the certificate has been made by a medically unqualified official, as for example in France. The cause of death may be stated in ambiguous terms guided merely by custom. In other countries where medically qualified officers are employed, they themselves may have certain biases. For example in the United States sudden unexpected death is usually designated on the certificate as "coronary heart disease"; in Japan it would be probably recorded as "cerebral haemorrhage" (23).

Further, in making assessments of the caloric and fat intake of individuals the data areoften based on mean food consumption of the whole population. Variability of distribution of fat and calories among various sectors of the population is often not considered and even less information is available as to the amount of food eaten or wasted.

Nevertheless certain important points can be derived from these surveys. During World War II a number of European countries showed a reduction in mortality from C-V disease. This decrease occurred during

a period of acute scarcity of food, particularly fats. In Finland, for example, the number of deaths ascribed to atherosclerosis began to decline by 1942, and fell to about one third the pre-war figure during 1943-46 (23). Observations made at autopsies during the same period showed a marked decrease in the occurence of advanced and complicated lesions, particularly in the 30-60 year group, while there was little change in the incidence of fatty streaks on the intima (23).

Of particular interest are the results of socio-nutritional studies on the occurence of atherosclerosis in Israel in individuals who emigrated to that country (25). These studies showed that recently immigrated Yemenites (after 5 years in Israel) as a rule, had low plasma cholesterol values (mean: 158 mg/100 ml). The "early" Yemenites (those who had resided 20 years in Israel) showed higher cholesterol values comparable to the European manual-workers. Europeans of the middle class had the highest values (mean 248 mg/100 ml). The cholesterol levels correlated closely with dietary fat intake. The Yemenites of recent arrival consumed the lowest and the European middle class individuals the highest amount of dietary fat. The serum cholesterol correlated closely also with the incidence of atherosclerosis and the mortality from myocardial infarction, the incidence being highest in the middle class Europeans and lowest in the early Yemenites. The correlations are particularly conspicuous when the incidence of infarcts are compared for males of the various age groups. The incidence was 0.1 for "recent" Yemenites, 1.6 for "early" Yemenites, 5.2 for immigrants of

Eastern origins and 17.7 for those of Western origins (25).

The authors of demographic experiments, such as described, often are inclined to draw definite conclusions from too few data. While the correlations are plausible it is hardly valid to regard the correlations as established on demographic evidence alone. The conclusions must be considered with reservation also in view of the inability of other investigators to demonstrate a rise in blood cholesterol in man from feeding a diet high in cholesterol (23, 26). However, the studies of Beveridge et al. (26) have clarified some aspects of this problem. These workers showed in dietary studies with groups of university students that the intake in the diet of the most volatile fractions from butter oil, which are rich in cholesterol (3.83%), caused a great increase in plasma cholesterol, while cholesterol-poor fractions led to a relatively small increase. When cholesterol was added to these fractions and included in the diet, comparable increases were obtained in the blood cholesterol. However, the addition of medium chain length saturated fatty acids (C_6 - C_{12} fatty acids derived from coconut oil) either with or without added cholesterol to the control diet (without butter oil) did not significantly increase the plasma cholesterol. The addition of coconut oil and cholesterol, on the other hand, significantly elevated plasma cholesterol. These results suggest that the blood cholesterol in man is influenced by dietary cholesterol, but only when the latter is associated with certain types of triglycerides in the diet. The same workers showed also that the effect of dietary cholesterol (in the presence of other tri-

glycerides) in increasing blood cholesterol is observed only with quantities not exceeding 634 mg of cholesterol per day; higher quantities up to 4,500 mg per day produced no further significant increase in blood cholesterol (27).

It is now apparent ffom other studies (28, 29) that saturated fats in the diet raise the serum cholesterol level while polyunsaturated fats tend to lower it. About 2 g of linoleic acid were found to be required to counteract the effect of 1 g of the commonly occurring saturated fat in the human dietary.

In the United States the studies of the Farmingham (52) and the Technical group (28) have shown a high correlation between the occurence of high blood cholesterol and an increased incidence of clinical C-V disease. However, as is pointed out by Keys (23), there are no data directly relating blood cholesterol to atherosclerosis in man.

4. Lipid Metabolism in Relation to Atherosclerosis.

Only the most relevant aspects of this large subject will be discussed in this section.

There is considerable evidence that lipids, particularly cholesterol, participate in the development and probably the etiology of atherosclerosis. Some of the statistical evidence for this in man already has been presented. It is significant also that most, though not all, procedures for the production of atherosclerosis in animals have in common the feeding of added cholesterol in the diet. In many of these studies therefore, the biochemical aim has been to determine the relationship between the lipids of the plasma and those found in the aorta and in the atherosclerotic lesions.

Two views have been put forward more recently regarding the accumulation of lipids in atheromatous lesions. One view postulates that the tissue lipids are derived directly from plasma lipids by a nonselective process. The strongest evidence in man in support of this view comes from the work of Hirsch and Weinhouse (30).

Some of their findings are indicated in Table I.

TABLE I

(From: Hirsch, E.F. and Weinhouse, S. Physiol. Rev. 23, 185, 1943). Comparison of lipid composition of blood plasma and arterial tissues.*

	Blood Plasma	Intima	Early Plaques	Media
Free cholesterol	14.1	14.2	16.2	17.3
Cholesterol esters	38.3	38.6	38.5	16.7
Phospholipids	22.8	20.1	19.0	34.1
Neutral fat	23.3	27.1	26.3	31.9

*Values expressed as per centage of total lipid.

It is of interest that the simple fatty deposits in the atheromatous plaques were of the same lipid composition as that of the blood plasma and the normal intima. The authors concluded that atherosclerosis developes from disturbances in the metabolism of lipid infiltrated into the intima from the plasma, and that the lipid depositions in the intima are of a nonselective nature.

The other view postulates that lipids are synthesized in the aorta wall. This however is an oversimplification as the evidence indicates that various lipids are metabolized differently by arterial tissue.

In man only the free and esterified cholesterol and the phospholipids in the aorta have been studied in any detail. The studies of Rabinowitz et al. (31) and Field et al. (32) have shown that the rate of turnover of cholesterol in the aorta is slower than in most tissues, and that the rate of turnover of cholesterol in early atheromatous plaques is slower than in the intima. The cholesterol in advanced plaques has virtually no turnover. They have been able also to show that the total (free and esterified) cholesterol in normal intima is derived to a large extent from the plasma, and that only 35-40% is synthesized in situ.

Zilversmit et al. (33) have studied the biosynthesis of phospholipids in normal and in the involved aorta, and found that, after injection of P^{32}-phosphate into patients with vascular disease, the specific activity of phospholipids in early plaques exceeded the specific activity of phospholipids in the plasma. These findings suggested to these workers that phospholipids in the plaques are the products of biosynthesis by the arterial wall.

Studies of animals have confirmed that cholesterol and its esters are derived principally from the blood (34). Phospholipids also are synthesized largely by the aorta, the plasma accounting for only about 10% of the total. Studies on the origin of the triglycerides in the rabbit have shown that triglyceride fatty acids of cholesterol-fed rabbits are synthesized largely by the aorta (35).

Relationships of lipid metabolism in some tissues:

The relationship between lipid metabolism in the aorta and that in other tissues and blood is of fundamental importance. This topic is well reviewed by L. Swell and C.R. Treadwell (34) and in the International Conference on Diet, Serum Lipids and Atherosclerosis (34A). Part of the following summary is based on these reviews.

Virtually all tissues have the capacity to synthesize cholesterol from acetate. The most active tissues are the liver, intestine, skin and adrenal. The rate of cholesterol synthesis in the liver appears to be controlled by the cholesterol intake in the diet. Liver tissue slices prepared from rats, dogs, and monkeys previously fed a diet with a high content of cholesterol showed a depression in the incorporation of acetate-1-C^{14} into cholesterol. Furthermore removal of the bile or lymph by fistula and hence removal of a considerable amount of cholesterol greatly increased the rate of cholesterol synthesis.

While many tissues can synthesize cholesterol, only the liver can furnish free cholesterol to the plasma. Moreover the free cholesterol of the liver and plasma readily attain an equilibrium, and thus may be considered to belong to a single pool. While a major portion of the free

cholesterol of the blood plasma is derived from the liver, the exchange of free cholesterol between the extrahepatic tissues and the plasma is relatively slow, but some of the free cholesterol in these tissues may be derived from the plasma.

Esterification of cholesterol can occur in the liver and other tissues. Of particular interest is the esterifying enzyme of the pancreas (36, 37). This esterase has the requisite specificity for the synthesis of cholesterol esters in the proportions in which they occur in other tissues. Cholesterol esterase thus appears to play a role in the intestine absorption of cholesterol from the diet. It has been shown also that the various subcellular fractions (microsomes, mitochondria, the soluble protein) can esterify added cholesterol-C^{14}. At least the microsomal and mitochondrial preparations require the addition of ATP and CoA (38). It is believed that the liver serves as the chief source of cholesterol esters and that the free cholesterol is the precursor of esterified cholesterol fraction in the liver and the serum. Studies on subjects with heart disease and atherosclerosis have shown that they tend to have a lower proportion of linoleic acid and a higher proportion of monounsaturated acids (mostly oleic) in the serum cholesterol esters than normal individuals, and second, similar changes have been observed with ageing in subjects without heart disease.

The fatty acid composition of the triglycerides in the blood was found to be characteristic of the individual species. However, the three fatty acids - palmitic, oleic and linoleic - account for more than

80% of the total fatty acid of the blood triglyceride fraction in man, the rabbit and the rat. The ratio of oleic to linoleic acid tends to be variable, depending on the content of these acids in the diet. Thus, it appears that the composition of the blood triglycerides is influenced by the kind and proportion of fatty acids in the diet. However, no significant difference has been demonstrated between the serum triglyceride level in normal and atherosclerotic subjects (39).

The fatty acid composition of human serum phospholipids is not significantly altered with increase in age, nor in atherosclerosis or in alimentary lipemia (39).

The predominant unesterified fatty acids in the plasma have been shown to be palmitic, stearic, oleic, linoleic and arachidonic, with saturated acids accounting for 30-40% of the total unesterified fraction. Schrade et al. (39) found no significant change in unesterified fatty acid composition with advancing age. However, in subjects with atherosclerosis the proportion of saturated fatty acids in that plasma fraction was higher, and that of linoleic and arachidonic acids lower, compared to that of normal healthy subjects.

Thus it appears that much more information is needed particularly regarding the mechanism of the transport of lipids, before any firm conclusions can be reached regarding their exact role in atherosclerosis. The overall evidence, however, strongly suggests that cholesterol esters play an important role in the development of the atheromatous lesions. However, it is not yet possible to determine whether the derangement of cholesterol ester metabolism in the aorta is a primary or a secondary effect.

5. Some Theories of Etiology of Atherosclerosis.

A large number of theories of etiology of atherosclerosis have been proposed, and the following account summarizes some of the more important ones.

The view of Virchow that atherosclerosis is primarily an "accumulation of mucous substance in the intima", and the view of Anitschkow and Chalatow that the condition is primarily one involving the deposition of lipids, have already been referred to. Among the more recent theories are the following:

Thrombogenic theory:

This has been proposed by J.B. Duguid (40, 41) and postulates the formation, in the artery, of mural thrombi which become covered with endothelium. As the thrombus becomes organized it becomes incorporated in the vessel wall finally producing a fibrous thickening of the intima. Since the new tissue is similar to and continuous with that of the original intima it appears to be an intimal overgrowth. Duguid further suggests that these mural thrombi may undergo fatty change and assume the character of atherosclerotic plaques. Other investigators (42) however suggest that along with the thrombogenic production of plaques, fatty lesions also may be formed by the penetration of foam cells into the subendothelial space. This view is based on observations from electron-microscopic studies of the aortas of rats fed mixtures of corn oil or butter and cholesterol. Fat filled macrophages were claimed to have been shown in the process of

penetrating the endothelium and to become deposited in the intima (43). The prominent accumulation of lipid droplets in the extracellular space in the subendothelial regions was attributed to the disintegration of foam cells.

The role of vascular dynamics:

Texon et al. (44) have put forward the view that the laws of fluid dynamics are the primary factors in the etiology and localization of atherosclerosis, and that other factors such as age, sex, race, diet, lipid metabolism, etc. play only a modifying role. They suggest that the physical and the flow characteristics of the blood and the geometric pattern of the vessels determine the predilection of certain sites, for example the bifurcations and junctions of vessels, for the formation of atherosclerotic lesions. They have carried out experiments also in dogs, in which the left carotid artery was excised and grafted in the left femoral artery which thereby assumed an S-shaped curvature. Histological examination of these curved arteries, compared with control carotids and femorals showed a thickening of the intima by fibroblastic proliferation and the formation of plaques. The thickening occurred in the region of the greatest curvature. Further observations suggested that the atherosclerotic changes are a consequence of disturbances in fluid dynamics of the vessels.

The lipoprotein filtration concept:

This concept originated, at least in part, from the early work of Duff and McMillan (45). They elicited a pronounced rise in the serum cholesterol by administering cholesterol in the diet to alloxan diabetic

rabbits. The animals showed also extreme turbidity in their serum. However, the degree of atherosclerosis was found to be much less pronounced compared to that of the cholesterol fed controls. Pierce (46) confirmed the differences between the cholesterol-fed normal rabbit and the cholesterol-fed alloxan-treated animal. They showed also that the concentrations of the S_f 12-30 and S_f 20-30 classes of lipoproteins correlated well with the occurence of atherosclerosis as observed in the alloxan-treated rabbit, whereas both the free and the total serum cholesterol gave a negative correlation with the occurence of the lesions. Pierce further postulated the existence of a metabolic block in the conversion of S_f 80-100 lipoproteins (the principal carrier of cholesterol in the plasma of the alloxan-diabetic animal) to S_f 40 and less lipoproteins.

Gofman et al. (47) suggested that high blood pressure plays a key role in the development of atherosclerosis by increasing the degree of infiltration of lipoproteins. In support of this view they quote the results of experiments on rabbits with coarctation of the abdominal aorta, accomplished by introduction of a silver constrictor. The rabbits post-operatively were maintained on a diet with added cholesterol. These workers were able to demonstrate a positive correlation between the difference in the degree of atherosclerosis and blood pressure above and below the constrictor. These workers suggested also a mathematical expansion for the relationship between lipoproteins and the degree of atherosclerosis. They feel that the degree of athero-sclerosis, as measured by accumulation of atherosclerotic tissue within the

intima, is a direct function of the integral of the lipoprotein level over time: degree of atherosclerosis $= \int_0^t$ rate dt. They consider also that blood pressure is the main factor in determining the constant of proportionality in the rate equation. Efforts to relate the types of lipoproteins to the production of atherosclerosis gave similar results in the rabbit and in man. Correlation of the lipoproteins of various S_f ranges with the degree of atherosclerosis was as follows: S_f 12-20 > 0-127 > 20-100 > 100-200. The reason for the apparent activity and specificity of the S_f 12-20 lipoproteins in atherogenesis is still not understood. It is of interest, however, that the size of that group (200-800A) corresponds to the range of dimensions of the intracellular spaces of endothelium of capillaries and of the arteries. Gofman et al. (47) suggested that the larger lipoprotein molecules cannot gain entrance into the subendothelial region of the intima. Following the same reasoning, the smallest lipoproteins of the S_f 0-12 should have a greater capability of entering the intima, but may also readily leave it. Thus the postulated mechanisms of high blood pressure and the S_f 12-20 lipoprotein types in atherogenesis have not been established.

Influence of steroid hormones:

Steroid hormones also have been considered to be a factor in the development of atherosclerosis.

Long term epidemiological studies have been carried out to evaluate the therapeutic efficiency of estrogens in coronary disease. Also steroids have been administered to animals with a view to finding out

whether there is any relationship between atherosclerosis and certain blood lipids.

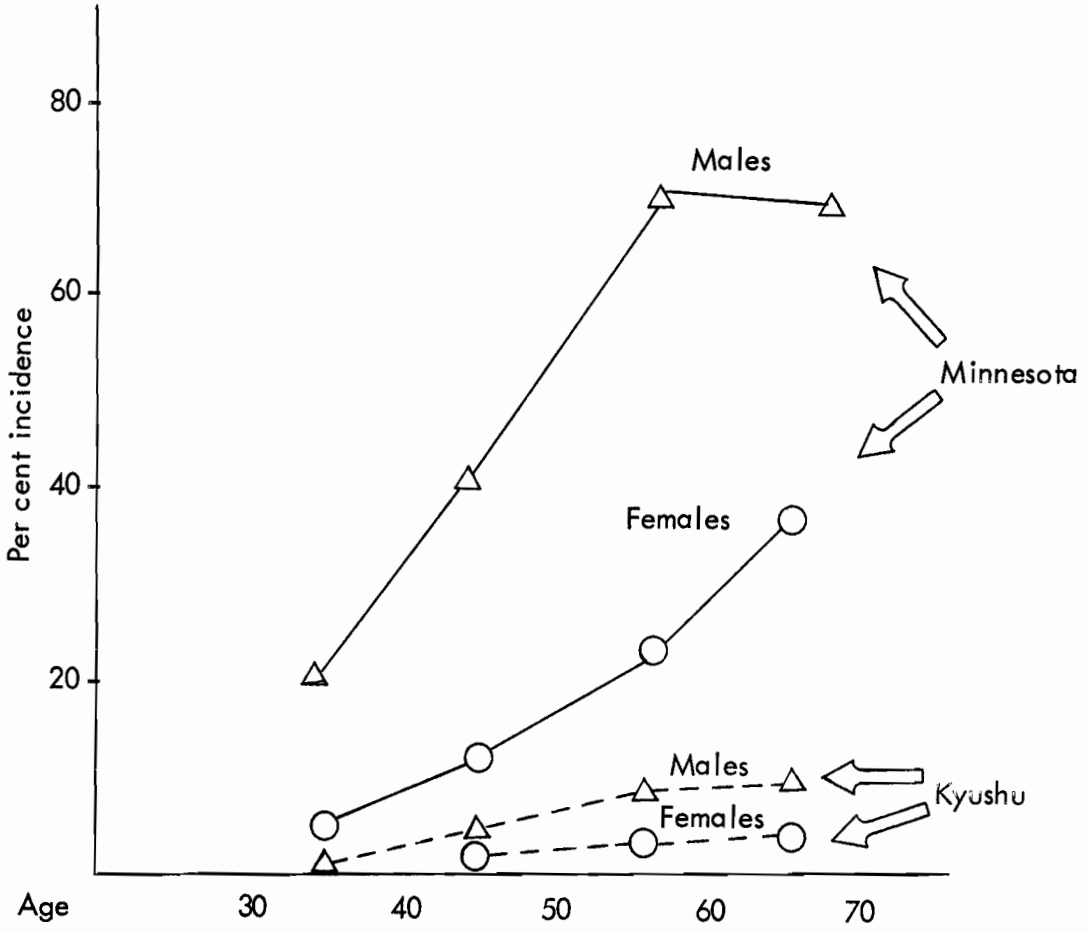
Probably the most significant finding arising from epidemiological studies is that the sex differential in atherosclerotic heart disease, so apparent in middle aged Americans, is absent or insignificant in peoples such as the South Africans, Bantu, Italians and the Japanese (48) (Figure 4). To explain these observations Pick et al. (48) have suggested that for atherogenesis a nutritional prerequisite is essential, i.e. a habitual diet of high caloric content, especially with a high total fat content and particularly rich in saturated fats and cholesterol. This prerequisite is present in the common American dietary. Females, apparently by virtue of protection afforded by oestrogenic secretion, are relatively resistant during the premenopausal years, and thereafter this predilection gradually disappears. In the less affluent countries, where dietary insufficiencies may prevail, the sex differential is less evident.

In a long term study by Stamler et al. (49) the administration of oestrogens, (Premarin, a preparation of mixed conjugated equine oestrogens) compared to a placebo has indicated a marked beneficial effect in terms of both long term survival and mortality rates. This was found with both good risk and poor risk patients. The decrease in mortality was generally more than 50 per cent.

It is noteworthy, however, that in a similar study Oliver and Boyd (50) observed a somewhat higher mortality in the oestrogen-treated

Figure 4

National Incidence of Coronary Sclerosis.



Incidence of high grade coronary sclerosis in consecutive autopsies, United States versus Japan. Note gross sex difference for Americans and its virtual absence for the Japanese. Note also the markedly higher incidence of coronary sclerosis in American males at all ages.

From: Pick, R. et al. (48).

group. It may be significant that ethinyl estradiol was used in his study and that all the patients were ones who had recovered from a single acute myocardial infarction.

In experiments with cockerels Pick et al. (48) demonstrated that the oestrogen-treated birds showed a significantly lower incidence of coronary atherosclerosis than the untreated group. The diets of both groups were supplemented with cholesterol and cottonseed oil. It is noteworthy that no difference was observed in the degree of aortic atherosclerosis between the two groups. No explanation was offered for the difference observed in vascular tissue localization.

There is no satisfactory explanation for the influence of oestrogens on blood and tissue lipids. However, Boyd (50, 51) has offered an explanation for the mode of action of oestrogens on the composition of the plasma ester cholesterol. He observed that the ratio - linoleate to oleate to palmitate - in the cholesterol ester fraction of the plasma of male subjects was 46:25:15, while that in a corresponding group of subjects treated with 200 micrograms ethinyl estradiol per day for over four years, was 30:20:22. He suggests that the rate of the metabolism of the various cholesterol esters by the reticulo endothelial system differs with the type of ester, being highest for cholesterol linoleate. This explanation is implied from the observed drop in the plasma level of cholesterol linoleate in the ethinyl estradiol treated males and from evidence indicating that oestrogens may increase the phagocytic activity of the reticulo endothelial system (51A).

The findings of Boyd, however, suggest an apparent contradiction in the literature: it has been pointed out in section 4, part I of the Introduction that the bulk of evidence indicates that subjects with heart disease and atherosclerosis tend to have a lower percentage of linoleic acid and a higher proportion of monounsaturated (34) and saturated (39) acids in their serum cholesterol esters. It is apparent that if the cholesterol esters play a significant role in the development of the lesions, and if oestrogens produce a protective effect through action on the metabolism of the cholesterol esters (51) there should be an increase in the proportion of linoleic esters to the monosaturated and saturated esters, on prolonged oestrogen administration. The findings of Boyd (51) in fact indicate the reverse type of change. No satisfactory explanation is evident for this contradiction.

Connective tissue changes.

The important role played by connective tissue changes in atherogenesis is discussed in detail in part II of the Introduction.

Thus, there exist many views on atherogenesis. Much of the evidence is contradictory. Some investigators hold to explanations in keeping with particular fields of interest and consider all other factors of secondary importance (41, 44). Others, however, accept a multicausal etiology (7, 13, 48, 49).

6. Experimental Atherosclerosis.

The first successful attempt to produce atherosclerosis experimentally was that of Ignatowski and Antischkow (1908-1912) in the rabbit (53). These workers were the first to establish that the atherogenic material in the diet is cholesterol and fat rather than protein. Since that time atherosclerosis has been produced experimentally in several species and by a variety of means. It appears however that cholesterol must be present in the diet in order to produce atheromas. On the other hand, arteriosclerotic lesions of various kinds can be produced by dietary or non-dietary means, with or without cholesterol.

The two most responsive animals for the production of atherosclerosis are the rabbit and the chick (53, 54). The simple addition of cholesterol to commercial diets or natural foods is adequate for the production of lesions in these species.

The rat used to be considered a resistant animal to the dietary production of atherosclerosis. Now it is probably the most preferred species for study, for many reasons. It is the most economical and easiest to handle, has been used extensively in nutritional studies and particularly in studies on atherosclerosis because the lesions produced closely resemble the early lesions observed in man (55, 56). The object in all the dietary studies is to raise the blood cholesterol to much higher levels than is normally found in the rat and the man. This is accomplished by feeding a high total fat (40%) and cholesterol content (5%) in the diet. In addition it is common

practice to make the animals hypothyroid by including thiouracil (0.3%) in the diet. Among the most effective atherogenic diet devised is the one by Gresham and Howard (56). The writer has used it with success, and its exact composition is described under "Materials and Methods".

The results obtained in the rat with these diets are greatly influenced by the type of fat used. Gresham and Howard (56) showed that when arachis oil alone was used as the fat, atherosclerosis was produced but without arterial thrombosis. However, butter or beef fat, or hydrogenated arachis oil caused arterial thrombosis but without atherosclerosis. Mixtures of butter and arachis oil, or butter with 8 per cent methyl linoleate and maize oil produced both kinds of lesions. The workers concluded that saturated fatty acids are thrombogenic and that linoleic acid, in conjunction with saturated fatty acids, is atherogenic. This conclusion was supported by purely artificial diets of known composition (57) as well as by the work of Thomas and Hartford (58). Further they demonstrated by feeding a diet high in saturated fat a decrease in the bleeding, the coagulation and the prothrombin times and an increase in the number of platelets.

Arteriosclerotic lesions have been produced also by several other means. Administration of ACTH (0.3 μ) three times a week for seven weeks to white rats will cause them to produce an "explosive" type of arteriosclerosis (60). If the animals are previously subjected to unilateral nephrectomy the atherosclerosis is greatly intensified. The diet contained no added cholesterol or other fat and the serum cholesterol did not rise.

The lesions so produced demonstrated large amounts of mucopolysaccharide histochemically.

Constantinides et al. (61) produced focal medial injuries in the aorta in rabbits by treating them for 10 days with toxic amounts of epinephrine and thyroxin. When these animals were exposed for only 20 days to alimentary hypercholesteremia they developed measurable intimal atherosclerosis, whereas the uninjured controls did not. The medial injury focii often fused with overlying intimal plaques and thus lost their initial identity. This author considers that these findings provide support for the view that mural injury plays a role in the pathogenesis of atherosclerosis.

Sellers et al. (62) have shown that in rats, fed on commercial diets and exposed to cold, hyaline material containing blood elements and lipid occurred subintimally in amounts which sometimes occluded the lumen. More severe atherosclerosis could be produced in old rats that had lived in the cold when fed a diet supplemented with 2 per cent cholesterol.

Change in the vitamin intake also can produce arteriosclerosis. Duguid (63) demonstrated that hypervitaminosis-D in the rat produced medial calcification of the aorta. The condition resembles Monckenberg's sclerosis in man. The intima overlying such lesions showed fatty infiltration and some hyalinization. Pyridoxine (B_6) deficiency has been known to produce pronounced lesions in the aorta and coronary arteries in the monkey (64). The lesions took the form of intimal proliferation and accumulation of hyaline material with the staining characteristics of mucopolysaccharide.

No fat deposits were detectable in these lesions. However, it is significant that the diets of the monkeys contained only two per cent corn oil as the fat component.

Results of special significance were obtained by Constantinides et al. (65) at the University of British Columbia. Rabbits were fed diets supplemented with cholesterol over repeated time intervals, thus inducing a "cyclic" hypercholesterolemia. The lesions of the abdominal aorta of the animals were relatively advanced, with ulceration, calcification and true atheroma content. These lesions are considered, by a number of pathologists, to resemble closest the variety of lesions found in man.

Arteriosclerotic lesions have now been produced in most species by widely different means. Prolonged intra-aortic infusions serotonin in the dog (66), pyruvic acid administration intra-abdominally to the rabbit (67), X-irradiation of the rat (68), and traumatization by implantation of wire coils (69) are examples of inducing a vascular tissue response that is morphologically characterized as the "development of arteriosclerosis".

Only a few selected examples have been considered. One observation stands out, namely, that a wide variety of agents and treatments can elicit a cellular response by vascular tissue. This response is often characterized, at least in the initial stages, by the accumulation of a hyaline material which has the staining characteristics of mucopolysaccharide. It seems clear, furthermore, that for the experimental lesions to resemble closely the atheromas found in man, cholesterol and fat must be present in the diet.

PART II. The Role of Mucopolysaccharides in Atherosclerosis.

1. Newer Pathological Concepts and the Applications of Histochemical Techniques to the Study of the Development of Atherosclerotic Lesions.

The widespread interest in the alleged role of lipids as primary etiological agents in atherogenesis, stems largely from the work of Antischkow and Chalatow during the first part of this century. Even yet many questions still remain unanswered particularly as to the factors and conditions that initiate the atherosclerotic process. In recent years attention has turned toward the part played by local tissue factors. The studies of Duff and McMillan (7) have contributed to this trend. They have maintained that local tissue differences determine the distribution of lesions in the vascular intima, and that the response to injury, from whatever cause, also is an important factor.

The presence in the normal human aortic wall of material with staining properties like those of mucin was demonstrated by Bjorling in 1911 (70). This observation was substantiated by many investigators in man and in a variety of animal species. The earlier literature on this subject has been summarized by J.E. Kirk in his review article on mucopolysaccharides (MPS) of the arterial wall (71).

Many workers also have reported increased metachromasia in the arterial wall in spontaneous atherosclerosis in children as well as

in adults (71) and in experimentally produced atherosclerosis in the rabbit (72). These findings were of particular interest in view of observations, on the aortas of monkeys, showing a pronounced and constant increase of metachromatically staining material during the initial stage of atherosclerosis (73). The lesions that developed in the monkeys were found to resemble closely those observed in man (73).

Many new histochemical techniques have now been applied to this problem. Curran and Crane (74) have used a colloidal iron-PAS combination for demonstrating MPS. They showed a clear-cut increase in acid MPS during the development of atherosclerosis. The commonly used PAS stain, which is considered to demonstrate neutral MPS, showed smaller increases in atherosclerosis. The work of Bertelsen and Jensen (75) likewise showed a pronounced increase in PAS-positive fibrils in the fibrous plaques during atherosclerosis. The fibrils were observed to be arranged in parallel bunches and reacted like collagenous fibrils with Van Gieson-Hansen's stain. These workers suggest that the fibrils contain mucoids and glycoproteins in addition to the collagen-like protein (76).

It has long been realized that atherosclerotic changes in the large arteries are not peculiar to the adult. As already mentioned these changes begin early in childhood, and even though macroscopically the aorta may appear normal, microscopic changes are observed. During fetal life a lining of endothelial cells lies directly on the internal elastic lamina. From the first or second year the accumulation of metachromatic

substance may be demonstrated in the subendothelium (76). At the same time there is a proliferation of the fibroblasts. Up to the end of the second decade, metachromatic material dominates the picture and little PAS-positive substance is present. During the third and fourth decades the PAS-positive substance becomes evident, while metachromatic material continues to accumulate. About this time PAS-positive fibrils also appear, and these show more vigorous staining and become more dense with advancing age (76).

In the macroscopically normal media there is evidence also of a uniform increase in the amount of ground substance with ageing (76). This accumulation of metachromatic material (also confirmed with alcian blue and colloidal iron stainings) is largest in the luminal half of the media. It occurs primarily between the second and the fourth decades, but after the age of 50 it is impossible to decide histologically whether more metachromatic material has accumulated.

These changes in ground substance material of the apparently normal aorta with ageing, must be kept in mind when attempting to evaluate changes in MPS metabolism during atherosclerosis. It is clear, however, that intimal changes in aortas with normal gross appearance are identical with intimal changes in the corresponding normal parts of atherosclerotic aortas.

Electron-microscopic examination of the aorta has revealed distinct differences in ultrastructure between the intimal and medial cells. This evidence has a direct bearing on the studies described in this thesis, for it is partially responsible in convincing the author that the intima and media of the aorta should be studied separately.

It will be recalled that the aortic intima is composed of an endothelium and a subendothelial layer. The endothelial cells have a phagocytic capacity that is enhanced by experimental procedures such as the feeding of cholesterol or by arterial ligature (77,78).

Electron-microscopic studies by Buck (79) of the subendothelial smooth muscle cells have revealed a striking similarity between the changes that these cells undergo during experimental injury (arterial ligature and freezing) and during atherosclerosis. The cells show certain features characteristic of fibroblasts, macrophages as well as smooth muscle cells. Prominent among these features is the pronounced development of the ergastoplasm resembling that in active fibroblasts or osteoblasts during production of extracellular materials. These cells are considered to be "intermediate" cells and are thought to be derived from the smooth muscle cells of the media. In addition to their increased capacity for synthesis of extracellular materials, they manifest phagocytosis, particularly for lipids (80). It appears that their acquired additional synthetic apparatus is directed particularly towards the production of MPS, elastin and collagen.

The media, on the other hand, is composed only of one type of cell: the smooth muscle cell (81). It appears therefore, that in the media the smooth muscle cell forms and maintains the elastic and the collagen fibres and the ground substance.

It has therefore been demonstrated that the cells in the subendothelium, during injury or atherosclerosis, tend to show, under the electron-microscope, evidence of increased synthetic activity. These findings and the evidence that atherosclerosis is primarily a disease of the intima have strongly suggested to the author to compare the activity of various enzymes in the intima and media separately, in normal and atherosclerotic tissue.

2. Chemical Constitution of the Ground Substance in the Normal and Arteriosclerotic Aorta.

Much of the recent knowledge of the mucopolysaccharides (MPS) in connective tissue has been contributed by the work of Karl Meyer (82-85). Three groups of MPS are found in connective tissue: mucoproteins, mucoids and glycoproteins. The mucoproteins consist of complexes of acid MPS with protein. The mucoids and glycoproteins are characterized by the existence of a firm covalent bond between the hexosamine units of a neutral MPS and the protein. Meyer (82) makes a distinction between the mucoids and the glycoproteins, in that the former contain more than 4 per cent of hexosamine, while the latter have a lower content. The molecular weights of MPS of connective tissue lie between 15,000 and 10,000,000 and the number of saccharide units in a molecule is between 50 and 50,000.

Mucopolysaccharides are composed of similar disaccharide units, called repeating units or periods. In most cases one of the units is a hexosamine, either D-glucosamine or D-galactosamine. The other saccharide unit usually is a uronic acid, either D-glucuronic or L-iduronic acid, but in one case is D-galactose.

The polysaccharides of connective tissue whose structures are known, at least partially, are listed in Table I (86).

TABLE I

The Components of Some Intercellular Polysaccharides Produced by Connective Tissue Cells.

Polysaccharide	Hexosamine	Hexuronate	Hexose	Sulfates/Period Ester	Amide
Hyaluronate	Glucosamine	Glucuronate	-	-	-
Chondroitin-4-Sulfate	Galactosamine	Glucuronate	-	1.0	-
Chondroitin-6-Sulfate	Galactosamine	Glucuronate	-	1.0	-
Dermatan-4-Sulfate	Galactosamine	Iduronate	-	1.0	-
Heparin	Glucosamine	Glucuronate	-	1.5	1.0
Heparitin Sulfate	Glucosamine	Glucuronate	-	0.5	0.5
Keratan Sulfate	Glucosamine	-	Galactose	1.0	-

From: M. Schubert in Connective Tissue: Intercellular Macromolecules, Proc. of Symp. by New York Heart Assn. (1964), (86).

The amino group of the hexosamine seems never to be free; it is either acetylated or sulfated. The result is that it is never basic, and at the pH of tissues these amide groups do not attach a proton and cannot become positively charged or cationic. On the other hand, each of these polysaccharides has ester groups or anionic groups; carboxylate, or amide sulfate, which at pH of tissues exist as a negatively charged or anionic group, each associated with a small cation such as sodium. These regularly

spaced negative charges along the polysaccharide chain may be one of their most important biological characteristics (86).

The first investigator to isolate chondroitin sulfate was Morner in 1895 in Sweden (87). He was also the first person to report isolation of a MPS chondroitin sulfate-like material from the arterial wall. He isolated the material from the aortic and pulmonary vessels of cattle with a 2 per cent solution of sodium hydroxide followed by acidification with acetic acid. P.A. Levene in 1918 carried more extensive identification studies on a chondroitin sulfate-like material obtained from 100 pounds of aorta. This material (corresponding to 8.8 mg/g of wet tissue) was purified and further characterized as chondroitin sulfate, and by the isolation of galactosamine hydrochloride from an acid hydrolysate of the compound (88).

Jorpes established the presence of heparin in aortic tissue of cattle in 1937, using an electrodialysis technique and succeeded in obtaining the brucine salt of heparin (89).

K. Meyer et al. (84) next succeeded in isolating two kinds of chondroitin sulfate - chondroitin sulfate B and C from the aorta of cattle. These chondroitin sulfates differ through their solubility and optical rotation (84). These workers demonstrated also the occurrence of hyaluronic acid, chondroitin sulfate A and heparitin sulfate in the bovine aorta (82).

Direct determination of hexosamine and hexuronic acid in the human aorta.

Hexosamine

Kirk et al. (90) in 1956 determined the hexosamine content of the whole aortic wall and attempted to correlate the content with the age of the individual and the degree of atherosclerosis. No correlation was found in either case. However, these workers expressed their results only in terms of wet weight of the tissue.

Buddecke (91) analyzed human aortas of normal gross appearance and found the content of hexosamine to be increased with age. Kaplan and Meyer (92), on the other hand, found no change in the relative hexosamine concentrations with the age of the individual. It is noteworthy that in all of these analyses the total intima medial section of the wall was included.

Bertelsen (93,94) in 1961-2 determined the hexosamine content in the medial and the intimal sections separately. With normal tissues he found a significant increase in hexosamine concentrations with age up to the fourth decade. Thereafter the increase was smaller but still significant up to the seventh decade. In the older age groups there was no significant further increase. Analyses of the fibrous plaques (intimal) showed no correlation between the degree of involvement and the content of hexosamine. The mean total concentration of hexosamine was about the same in the fibrous plaques and in the intimal tissue with normal gross appearance. The average content in the medial tissue from an athero-

sclerotic and non-atherosclerotic group was identical.

Some of the results of these investigators are summarized in Table II . It will be noted that there are contradictions among workers regarding hexosamine variations with ageing. This may be in part due to the different ways of expressing the hexosamine content.

Hexuronic Acid

Values for hexuronic acid determined directly in human aortic tissue have not been reported. The only values available have been obtained from analyses of MPS material isolated from aortic tissue. The reason for this lack of data may be the known liability of hexuronic acid on heating the tissue at 118°C with 4N HCl, as used in the standard method. On the other hand, hexuronic acid is readily determined in MPS which have been isolated with relatively mild methods. As there may be important differences in total hexuronic acid content of the tissue and the amount present in extracted MPS, a method for determining hexuronic acid directly in tissues would be welcomed. The method of resin hydrolysis developed by P.A. Anastassiades and R.H. Common at Macdonald College, P.Q., (95), and used in the present work, may fulfill this requirement.

Isolation and characterization of the mucopolysaccharides of human aorta.

Bertelsen (76) reported that both the neutral and acid MPS fractions increase with advancing age. This is evident when the analytical

TABLE II

Variations of Hexosamine(s) in the Aorta with Age and in Atherosclerosis

	Kirk et al. (90)	Bertelsen (93, 94)			Buddecke (91)		Kaplan & Meyer (92)
Age group (decade)	Per cent of wet weight of tissue	Per cent of dry defatted weight of tissue			Per cent of dry weight of tissue		
	Whole intima and media homogenates	Intima (94)	Media (93)	Fibrous plaques (94)	Whole intima and media homogenates		Whole intima and media homogenates
					gluco-samine	galacto-samine	
1st	0.29	1.11-1.28	1.04		(I)		No significant variation with age. (Actual values not reported).
2nd	0.31	1.09-1.44	1.17				
3rd	0.31	-	1.20		1.57	1.08	
4th	0.33	1.30-1.54	1.48				
5th	0.29	1.78	1.46	1.64	(II)		
6th	0.30	1.76	1.55	1.87	2.29	1.66	
7th	0.29	1.63-1.73	1.53	1.44-1.48	(III) 4.5	2.1	
8th and subsequent	0.29	1.44-1.58	1.52	1.20-1.60			
Mean	0.27	1.29* / 1.59**		1.59			

Legend: (I), (II), (III) degree of atherosclerosis involvement; arrows indicate age span. .
*0-40 years
**40-80 years

results are expressed by weight of the dry defatted tissue (Table III). The increase is obscured and may not be shown when the values are expressed on the basis of wet weight as reported for instance by Dyrbye and Kirk (96), (Table III). These differences may be in part due to the increased amount of fat generally found in older age groups and partially due to the difference in extraction procedures (e.g. the material of Dyrbye and Kirk being highly purified).

It is also evident, from Table III, that in the results of Dyrbye and Kirk the mean yield of MPS for all the samples was 33.1 per cent, estimated on the basis of hexosamine analysis, and 60.9 per cent when calculated on the basis of the sulfate content. The authors offer no explanation for this discrepancy. A comparable difference has been reported by Bertelsen (93, 76) who used a different extraction method. He attributes the difference to incompleteness of the alkali extraction method used. An alternative explanation however might be that hexosamine may be present also in short chain carbohydrate components that are not precipitated along with the long chain MPS. There is indirect evidence for the presence of such short chain hexosamine containing carbohydrates in plasma (97).

Changes in composition of MPS fractions from human aorta with ageing and atherosclerosis.

Kaplan and Meyer (92) found that hyaluronic acid constituted approximately one seventh of the extracted material and that the content of this acid decreased sharply with ageing. Chondroitin sulfate C was

TABLE III

Mucopolysaccharides Extracted from Human Aorta.

Dyrbye and Kirk (96)						
Age group (years)	Number of samples	Quantity of tissue (g)	Quantity of MPS obtained		Percentage Yield	
			mg	mg/g wet tissue	I*	II**
0-9	5	2.5	12.2	4.9	34.7	56.0
20-39	5	11.7	61.2	5.2	34.9	71.5
40-49	5	13.2	51.9	3.9	39.3	60.8
50-59	9	16.7	66.0	4.0	33.9	66.7
60-76	6	22.9	54.2	2.4	24.6	47.4

Bertelsen (76)		Fractions expressed in % dry defatted wt.	
Age (years)	Number samples	Acid Fraction (%)	Neutral Fraction (%)
7/12	1	3.3	3.1
2	1	5.3	4.2
13	1	11.3	4.2
21	1	10.0	6.1
30	1	13.6	4.3
37	1	14.7	4.1
45	1	11.4	4.9
58	1	14.1	5.4
60	1	14.6	6.2
70	1	15.1	6.6

Legend: *Calculated on basis of hexosamine analysis
**Calculated on basis of acid-hydrolyzable sulfate

found to make up more than 50 per cent of the MPS and it too was found to decrease with age of the individual. Chondroitin sulfate B and heparitin sulfate account for approximately 10 per cent of the extracted material and these showed a slight tendency to increase with age.

Kirk and his associates (90,96,98) by means of paper electrophoresis separated two MPS fractions from highly purified MPS material from aortas of individuals of various ages. The faster moving fraction, which the authors suggest was chondroitin sulfate A or C, contained galactosamine and uronic acid but no glucosamine. The fraction apparently contained no hyaluronic acid or heparin. The slower moving fraction contained 22 per cent hexosamine (of which 56 per cent was galactosamine and 44 per cent was glucosamine), 28 per cent uronic acid and 16 per cent sulfate. There appeared to be a slight increase in the galactosamine: glucosamine ratio in individuals after the age of 60.

Buddecke (99) isolated the acidic and neutral MPS of the aortic wall. The acid fraction was found to be composed of hyaluronic (15 per cent) chondroitin sulfates A and B (50 per cent), the remainder included heparin and keratosulfate. The proportion of galactosamine to glucosamine appeared to increase slightly with age.

Bertelsen (76,93) showed that the MPS from the aorta of individuals of various ages contained hyaluronic acid and chondroitin sulfates A and/or C. They tried further to determine the change in the proportion of the hyaluronic acid and the sulfated MPS with age. They

found that hyaluronic acid constituted about 40-50 per cent of the MPS from the individuals under 20 years, about 30-35 per cent in the group 20-60 years, and between 10 and 20 per cent in individuals older than 60 years. Bertelsen also found that the proportion of sulfated MPS tends to increase with age.

Smith (93A) correlated amounts of isolated MPS with the type of lesion. It was found that a small but significant ($P < 0.01$) increase occurred in fatty streaks, but the amount of MPS decreased slightly in non-calcified plaques ($P = 0.05$) and fell sharply in calcified plaques.

From the above review a rather complex picture emerges, with some contradictions. Part of the difficulty stems from the variety of extraction procedures and methods of characterization of the MPS. Some workers report their analytical results on the basis of the wet weights of the tissue while others on the basis of the dry defatted weights. Perhaps the most serious difficulty arises from the failure of many investigators to make a clear-cut distinction between the changes of MPS with ageing in macroscopically normal tissue and atherosclerotic tissue from individuals of corresponding age groups. The reason for this is probably the practical one of obtaining a sufficient quantity of tissue for extraction and fractionation. Whole aortas have been used in most cases.

Nevertheless certain tentative conclusions are warranted.

First, the concentration of total MPS tends to increase with advancing age (and at least in the early stages of atherosclerosis) when the results are expressed in terms of dry defatted tissue weight. Second, the concensus is that the proportion of hyaluronic acid tends to decrease with age in relation to the sulfated MPS. Third, there is evidence that the overall content of sulfated MPS tends to be increased with advancing age, while the chondroitin sulfate C is decreased.

Hydroxyproline as a measure of collagen content of the aorta.

Lowery (100) in 1941, by means of a gravimetric method and subjection of the aortic tissue to autoclaving (elastin is not broken down and thus can be removed) was the first to report values for the collagen content of human aorta. He obtained a value of 28 per cent based on the dry tissue weight. Myers and Lang (101) using the same procedure measured the collagen content of aortas from individuals of various ages. They found that the content was virtually constant (15.5 per cent) up to the age of 45 but thereafter tended to increase as much as 18-19 per cent. Their results were expressed in terms of dry tissue weight. Buddecke (91), on the contrary, found no change with age and obtained values between 20-24 per cent of the dry weight of the defatted tissue. Bertelsen (76) analyzed the intima and media separately for collagen. He found that the concentration in the media remains unchanged with age, while that of the intima tends to increase with age, in normal tissue. A more pronounced increase occurs in cases of sclerotic tissue samples. These values were expressed in terms of dry, defatted, decalcified tissue.

3. Intermediary Metabolism of Normal and Atherosclerotic Vascular Tissue.

The most extensive studies on the metabolic aspects of human vascular tissue have been carried out by J.E. Kirk and his associates in the Washington University School of Medicine in St. Louis. The following account is partially based on several reviews by Kirk (71,102,103) and by Zemplenyi (104,105).

The respiratory activity of normal human aortic tissue is comparatively low with a mean Q_{O_2} of 0.26 for specimens obtained two to four hours after death.

There is evidence to suggest that arterial tissue depends largely on glycolysis for its energy requirements. The average respiratory quotient of aortic samples is 0.91, thus indicating principally carbohydrate is utilized by this tissue. The rate of glycolysis is comparatively high and accounts for 51 per cent of the energy production. Further, the aerobic glycolytic rate is only 30 per cent lower than the anerobic value. Thus the Pasteur effect is comparatively low.

The rate of respiration and glycolysis in aortic tissue stored in the cold (4°C) drops rapidly in the first 48 hours but utilization of oxygen is detectible for 25 to 50 days, and in some cases, glycolytic activity can be demonstrated after 100 days.

As pointed out in an earlier section the internal one half to two thirds of the aortic wall does not have a capillary blood supply

and this portion of the artery obtains its nutritional requirements by diffusion of materials from the blood in the lumen. Kirk and his associates determined the diffusion coefficient of nitrogen, oxygen, carbon dioxide, lactate, iodide and glucose in the aorta. The diffusion coefficient is defined as the number of units of a substance that diffuses through one square centimeter of membrane in one minute at a concentration gradient of one unit per millimeter per centimeter. They found that the average diffusion coefficient of oxygen was 0.000505 for human aortic tissue. From the value obtained for the respiratory rate they estimated that the maximum thickness of the aortic wall that could be adequately supplied by means of diffusion was 0.91 millimeter. Since the average thickness of the avascular layer varies from 0.84 millimeter in young adults to 1.10 millimeter in middle aged persons (15), it is evident that the availability of oxygen is limited, especially in the subintimal region.

At the same time there is evidence of the operation of many pathways: the glycolytic pathway, the hexosemonophosphate shunt, the tricarboxylic cycle, the malate shunt and the oxidative chain. Most of the evidence for the activity of these systems is based on the demonstrated presence of the enzymes in crude vascular tissue homogenates. These studies have been discussed in many reviews (71, 102, 103, 104, 105). Suffice to say that very few data are available on the activity of the enzymes involved in the biosynthesis of MPS or their precursors.

Most of the information on the metabolism of arterial MPS

has been obtained from studies with radioactive sulfate. The radioactive sulfate may either be administered in vivo or be used with enzyme systems in vitro. The MPS may be isolated and their activity measured in terms of specific activity and/or radioautography.

Radioactive sulfate uptake has been demonstrated by means of radioautography with the aorta of the chicken (106), the adult rat and rabbit (107). Buck (108) has studied the incorporation of radioactive sulfate in the aorta of the normal and the atherosclerotic rabbit. Cholesterol-fed animals and controls were injected with S^{35} labelled sulfate and killed at the end of 48 hours. The uptake of radioactive sulfate was studied by the radioautographic technique, and adjacent sections of arteries were stained metachromatically. A close parallelism was noted between the intensity of metachromatic staining and the distribution of S^{35}. It is of particular interest, that these workers also observed that the intimal lesions of the aorta of the cholesterol-fed rabbits incorporated S^{35} in much higher concentration than did any part of the wall of the normal aorta. However, the media underlying the intimal lesions showed a lower isotopic concentration than that of normal rabbits.

Buck and Heagy (109) further confirmed their former observations of a higher sulfate uptake by arteriosclerotic than by normal aortic tissue of the rabbits. They isolated the MPS material from the aortas and determined the radioactivity and the sulfate content. The results showed that the specific activity from the cholesterol-fed animals was on the

average more than twice that of the control animals. It was further noted that on paper electrophoresis the extracted MPS exhibited a peak of radioactivity corresponding to a bond that stained metachromatically with toluidine blue and was considered to represent chondroitin sulfate.

Dyrbye (110) carried out studies on human aortic tissue. He incubated specimens of aorta (Intimal and medial together) in a medium containing S^{35} labelled sulfate. The acid MPS later were isolated and the radioactivity of the material was determined. A definite decrease in the rate of sulfate incorporation by the aortic tissue with increased age was found.

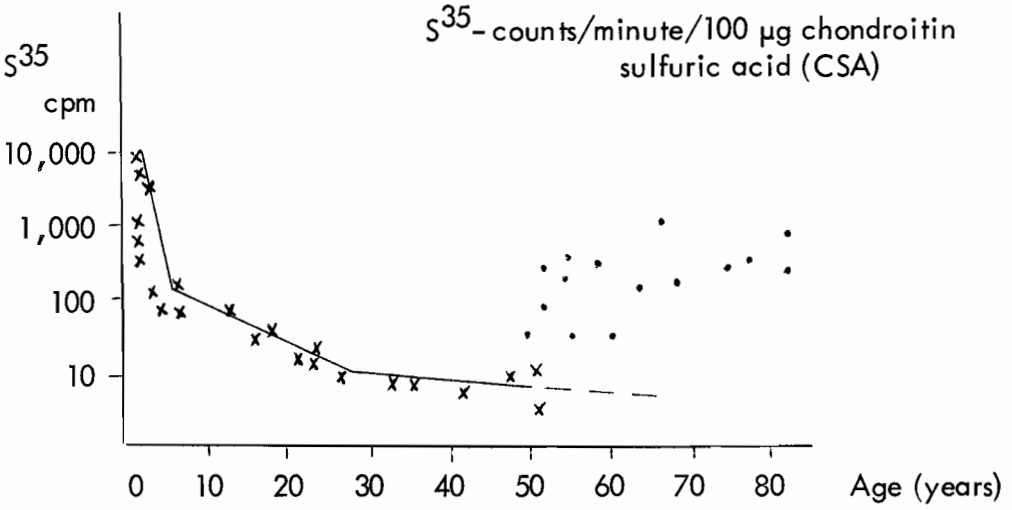
Curran and Crane (74) studied the radioactive sulfate uptake of human autopsy tissue from subjects whose ages ranged from 4 days to 84 years. They found that radioactive sulfate uptake is markedly increased with increasing severity of the atherosclerotic lesions.

Hauss et al. (111) studied the sulfate incorporation in rats with experimental atherosclerosis, as well as in atherosclerotic and normal tissue from human subjects. They found that for both human and animal tissues the specific activity of the isolated chondroitin sulfate tends to decrease with age. With experimental atherosclerosis in the rat there is a marked increase in the radioactive sulfate uptake by the aorta during the early phase of atherosclerosis while in the later phases the uptake tends to fall off. Incubation experiments with human aortas showed that the incorporation of radioactive sulfur into sulfomucopoly-

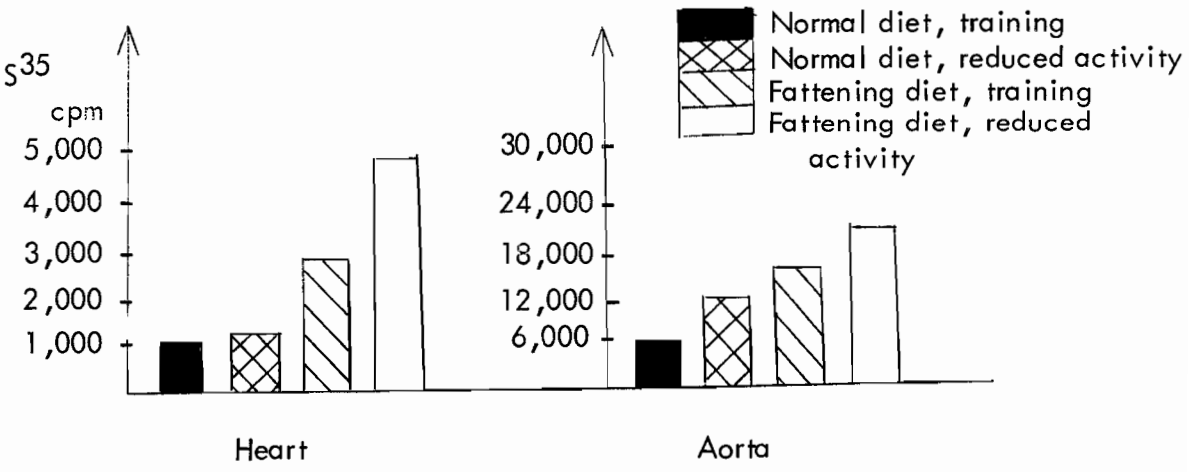
saccharides was several times greater than would be expected from the slope of the age curve (Fig. 4 A). These workers carried out experiments with rats to test the effect of two other factors that are thought to contribute to atherogenesis, namely, lack of physical excercise and excessive fat intake in the diet. They found that the degree of incorporation of S^{35} into the sulfated MPS of the heart and the aorta increased in the experimental group in the following ascending order: the group given normal diet and muscular training, the group on normal diet and reduced physical activity, the group on fattening diet and muscle training followed by the group on fattening diet and reduced physical activity (Fig. 4 B).

Figure 4

A. Incorporation in vitro of radioactive sulfur into chondroitin sulfate of the connective tissue of the normal (x) and atherosclerotic human aorta (.).



B. Influence of physical excercise and excessive fat ingestion on the metabolism of chondroitin sulfuric acids. Incorporation of S^{35} into the CSA of connective tissues of heart and aorta of rats. CSA isolated 24 hours after S^{35} injection.



From Hauss et al., J. Atheroscl. Res. 2, 50 (1962).

4. Comparative Activity of Enzymes in Normal and Atherosclerotic Vascular Tissue.

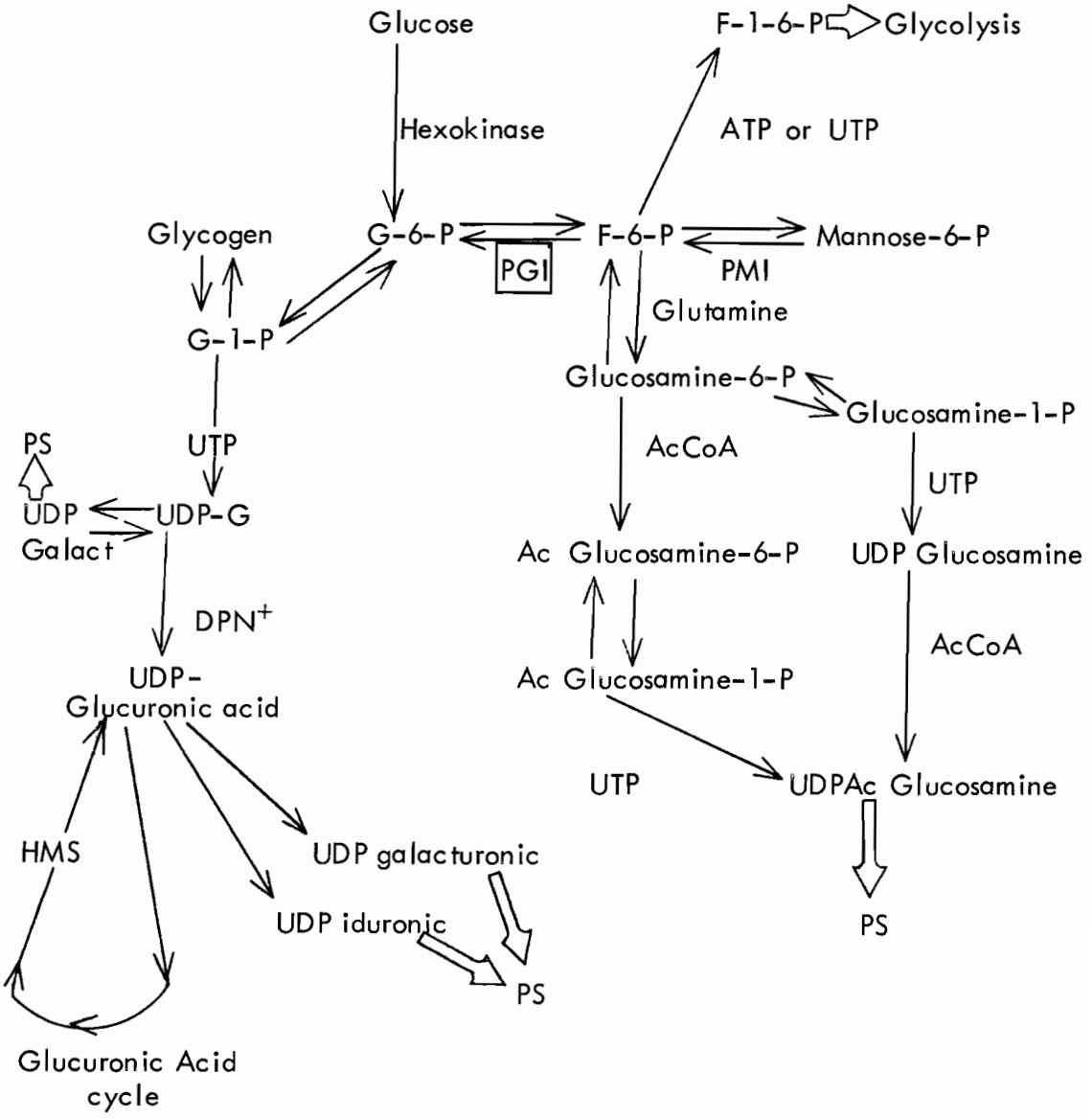
Many studies have been made on the comparative activities of various enzymes in normal and atherosclerotic tissue. This type of comparative study may provide information as to the role of various metabolic pathways involved in the pathological process. For example, if it were shown that the enzymes normally involved in MPS metabolism showed greater activity in the atherosclerotic than in the normal areas, this would afford strong presumptive evidence that the MPS are actively involved in the development of the lesions. However, with the exception of the studies with radioactive sulfate, comparative studies have not been carried out on the MPS metabolic pathways in vascular tissue.

Figure 5 illustrates some of the relationships between the glycolytic and the MPS pathways, as found in other tissues. It is of interest to compare some of the data on the comparative glycolytic activity in arteriosclerotic and normal vascular tissue as presented in Table IV.

It will be noted that the means of the activity of the various enzymes differ greatly. For example, the average value for the hexokinase activity for normal aortic tissue is less than 1 per cent of that of the phosphoglucoisomerase activity. The hexokinase value is remarkably low for a tissue that depends largely on glycolysis for its energy requirement. This observation raises the question whether

Figure 5

Some Metabolic Interelationships between Glycolysis and MPS Precursors.



PS. designates polysaccharides

TABLE IV

Mean Glycolytic Activities of Normal and Atherosclerotic Human Arterial Tissue.

(Intima and Media together).

	Aorta		Coronary Artery
Enzyme	Wet Weight* (g)	Tissue Nitrogen** (%)	Wet Weight* (g)
Hexokinase	0.013	91 (N.S.)	-
Phosphogluco-isomerase	1.85	99 (N.S.)	2.05
Aldolase	0.056	-	0.099
Enolase	0.24	-	0.24
Lactic dehydro-genase	1.06	96 (N.S.)	0.68

*Mean enzyme activities for normal tissue expressed as mM of substrate metabolized/g wet tissue/hour.

**Mean enzyme activities of atherosclerotic aortic tissue expressed in percentage of normal tissue portions. Statistical evaluation in brackets.

Adapted from Kirk, J.E., in "Intermediary Metabolism of Arterial Tissue", (103).

hexokinase is a limiting enzyme system in the glycolytic system in arterial tissue. The very high activity exhibited by phosphoglucoisomerase in the atherosclerotic tissue also is puzzling, particularly in view of the relatively small amounts of glucose-6-phosphate that can be supplied at the low hexokinase activity. It is noteworthy that phosphoglucoisomerase occupies a central position in the metabolism of glucosamine-6-phosphate and of UDP-glucuronic acid, both of which are important intermediates in mucopolysaccharide synthesis.

Activity of phosphoglucoisomerase in the atherosclerotic process.

The presence of phosphoglucoisomerase (PGI) was first demonstrated in extracts of yeast, skeletal muscle, heart, liver, brain and kidney (111). The enzyme is now known to be widely distributed in the tissues of animals, plants and microorganisms (112). It has also been purified from a variety of sources. The optimal pH of the PGI from most sources is between pH 8 and 9. Most authorities report that fructose-6-phosphate (F-6-P) is present at equilibrium to the extent of 30 per cent. The Km for glucose-6-phosphate with serum PGI has been reported as 1.22×10^{-3} M (113). PGI does not act on mannose-6-phosphate nor has it any effect on free hexoses, or on glucose-1-phosphate, glucose-1,6-diphosphate, fructose-1,6-diphosphate (114). No metal or other cofactor is known to be required by PGI.

The PGI activity in the aortic tissue of man was measured by Brandstrup et al. (115) who compared also enzyme activity of normal and atherosclerotic aortic tissue. The mean activity for normal intimal-

medial sections of human aorta was found to be 1.62 mM of F-6-P formed/g wet tissue/hour. A significantly lower value (1.39 mM of F-6-P formed/g wet tissue/hour) was found for atherosclerotic intimal-medial tissue sections. Expressed in terms of tissue nitrogen, the mean activity for normal and atherosclerotic intimal-medial aortic sections was respectively 44 and 43 mM of F-6-P formed/g tissue nitrogen/hour.

The same workers (116) later obtained a higher mean value of 2.05 mM of F-6-P produced/g wet weight/hour for the activity of normal human coronary artery. A significant decrease (23 per cent) in activity with atherosclerotic specimens was obtained. However, the activity was calculated on the basis of the wet weight of the tissue.

Several problems arise with this type of approach. One arises from the circumstance that the intima and media are not involved to the same extent or in the same way in the atherosclerotic process. It will be recalled that the concensus of evidence indicates that atherosclerosis is a disease primarily involving the intima. There is evidence also that the intimal cells undergo changes in ultrastructure during atherosclerosis and give rise to atypical features. It is apparent therefore that significant changes in the enzyme activities of the intima could easily be obscured when the intima and the media are studied as one tissue.

Another difficulty arises from the method of expressing the

enzyme activity. It will be recalled, for example, that studies with radioactive sulfate have indicated that the synthesis of MPS tends to increase during the development of atherosclerosis for any given age group. It would be of particular interest to determine whether the activity of the enzymes involved in MPS synthesis tends to increase, and whether such a change may be due to an increased production of enzyme in the original cells of the intima. This question may not be fully answerable by studies on artificial systems. This would by the case particularly in non-limiting enzyme systems where no change in activity is found. The amount of substrate handled by such an enzyme in vivo could increase several-fold without the synthesis of new enzyme.

Nevertheless it is useful to have a satisfactory index for measuring changes in the enzyme activity related to a tissue constituent. It is preferable to relate activities to a measurement reflecting the number of active cells. In this way changes in enzymatic activities during the pathological process would be more apparent. However, there is no entirely satisfactory method of achieving this; it is evident that with pathological tissues, where there are pronounced alterations in lipid and connective tissue content, expressing enzymatic activities on the basis of wet weight simply indicates total activity changes. No information is provided as to whether enzymatic activity has changed per given unit of cells during the pathological process. The tissue nitrogen content may afford a better basis for expressing activity. However, with aorta

which in atherosclerosis and with ageing is known to undergo changes in collagen (and probably elastin) content (76,101), the nitrogen content is not a satisfactory criteria for the calculation of results. DNA content is considered to be the most reliable method of expressing enzyme activities in tissues that contain relatively functional cells. However, one should be aware that atherosclerotic lesions, especially the more advanced types, contain nonfunctional nuclear material which has escaped from the cells, become insipissated or otherwise degraded. Further, a variety of phagocytic and other inflammatory cells may invade the lesions thus changing the cell population.

As none of these indices by itself is entirely satisfactory, the writer in the experiments presented later in this thesis used several modes of expressions for purposes of comparison.

5. The Postulated Role of Heparin in Atherosclerosis.

A special problem of interest in atherosclerosis is what part heparin plays in its development and whether heparin is released during thrombotic episodes from atherosclerotic or normal vascular tissue.

Of the sulfated MPS present in various connective tissues three possess anticoagulant activity, namely, α-heparin, chondroitin sulfate B (ß-heparin) and heparitin sulfate (84,117). Several authors have reported the observation of anticoagulant and lipid clearing activity with aortic MPS (117,118). These findings raise the question as to whether the MPS with anticoagulant properties may play a role during the occurrence of vascular thrombosis.

Of particular interest is the reported observation by Kudrjashov (119) and Griffith (120), of increased activity of anticoagulant factors in the plasma during thrombosis. Myasnikov et al. (121) also have observed that the concentration of heparin in blood rose during the experimental induction of arterial thrombosis in the rabbit. The latter authors reported also a less pronounced rise in the rabbit when fed a diet containing added cholesterol. These findings suggested to the investigators the possible presence in the blood of a natural anticoagulant system, part of which was considered to be associated with the release of "heparin" during the thrombotic episode. They postulated that the site of release may be the aorta (121). These findings, if confirmed, have important

implications concerning the clinical practice of administering heparin after an arterial thrombosis. It is important to note that Myasnikov et al. estimated the heparin by Pieptea's method (122), which was designed primarily for the estimation of heparin added to blood. As the method involves titration of an acidic substance with a basic dye (toluidine blue) it is relatively non-specific for differentiating between heparin and the MPS normally present in small amounts in blood.

Charles and Scott (123) and Jaques et al. (124) have developed extraction methods by which added heparin in an amount as small as 0.1 mg per 100 ml could be accurately measured. They were unable to demonstrate the presence of heparin in normal blood specimens from the human, the dog or the ox. Gibson et al. (125) also have endeavoured to isolate heparin from normal blood specimens but were unsuccessful. Jaques (126), Gibson (125) and others are doubtful of the existence of free heparin in normal blood. However, Howell (127) obtained fractions with anticoagulant activity from the blood of a dog in peptone shock, and Jaques and Waters (128) isolated the barium salt of pure heparin from the blood of dogs sensitized and made anaphylactic with injections of foreign serum albumin. The writer's study of this problem is described in a later section.

EXPERIMENTAL

Materials and Methods

1. Collection of Human Aortic Samples. Criteria used for Selection of Material.

Human aortic material was obtained at autopsy from the Pathological Institute, McGill University, with the kind co-operation of Professor G.C. McMillan, Chairman of the Department, who also offered valuable suggestions as to the pathological criteria for selection of the material.

In order to work within a homogeneous patient population certain clinical criteria were set up to exclude conditions that are known to accentuate or otherwise complicate atherosclerosis. Similarly, in order to carry out comparable biochemical studies and to minimize variability, certain anatomical and pathological criteria were adopted for selection of the material.

The clinical criteria for selection of the subjects were as follows: patients with the final diagnosis on their chart as "hypertension", "endocrinopathy" (including diabetes mellitus) or "collagen disease" (i.e. polyarteritis nodosa, systemic lupus erythrymatosus, etc.) were excluded. Patients without these charted diagnoses, but whose blood pressure was greater than 110 mm Hg diastolic or 180 systolic recorded on two con-

secutive occasions or with persistent glycosuria or an A.C. blood glucose greater than 120 mg per cent also were excluded. Patients that had received massive steroid therapy for whatever cause likewise were excluded.

The pathological criteria for selection were as follows: all specimens were obtained from the thoracic aorta in the region of $T_3 - T_4$. These samples were generally about two inches long, and a special effort was always made to include a normal area as well as a lesion of the type described below. Samples which did not fulfill these criteria were not used for assay of phosphoglucoisomerase activity or for the analysis of tissue constituents. As soon as removed, the specimens were placed in a special sterile glass container immersed in ice, and were transported in a thermos bottle to the laboratory for analysis. All the material was obtained within 3-18 hours after death, and the phosphoglucoisomerase activity was determined generally within 2-16 hours after the material was obtained.

An effort was made also to select the type of lesion studied. Only discreet, uncomplicated and moderately well developed lesions were studied. Any lesion with ulceration, gross calcification or hemorrhage within the plaque was excluded. No attempt was made to distinguish macroscopically as to whether a lesion was predominantly 'fibrous' or 'fatty'. However, on microscopic examination most of the lesions were found to be of an intermediate type. At the outset adjacent histological sections were taken from each specimen for hematoxylin and eosin staining.

Later on only an occasional lesion was histologically checked.

The degree of overall involvement of the aorta was not used as a criterion, as long as a normal part and a suitable lesion could be obtained in the area $T_3 - T_4$. The aortas, however, were given a grading independently by the resident pathologist and the writer, and the evaluations were recorded. Most of the aortas fell into grades VII-IX according to the standard chart used in the pathology department.

2. Separation of Normal and Atherosclerotic Portions into Intima and Media.

A separation of the intima from the media by dissection is possible by virtue of the circumstance that between these tissues there is a condensation of dense connective tissue, the internal elastic lamina. This lamina forms, at least in normal tissue, a distinct tissue plane.

Having removed the adventitia, the tissue is held on its side with forceps and, with the aid of a pair of five forceps an attempt is made to select the appropriate tissue plane in a normal area. This is not always successful, and a medial smooth muscle plane may have been opened instead. Dissection in another area of the tissue must then be started. It has been found by experience that best results are obtained by dissecting along the thinnest possible layer. Generally speaking, if a smooth muscle plane has been opened a clean separation of sections is impeded by transverse bundles of smooth muscle running across the two parts of the aortic wall. On the other hand, in the correct laminal plane the intima peels off very readily leaving a smooth surface of separation. The foregoing description applies to normal aortic tissue.

In the atherosclerotic regions the internal elastic lamina does not form as distinct a plane. The most satisfactory procedure for separating the tissues in the involved areas is to begin the dissection in an adjacent normal region. The laminal plane is then followed through to the involved area.

The dissection technique is illustrated in Figure 1A and 1B.

Hematoxylin and eosin sections were made to corroborate the macroscopic observations. The separation of intima and media was not perfect, nevertheless a fairly accurate division of the two layers was achieved in most cases.

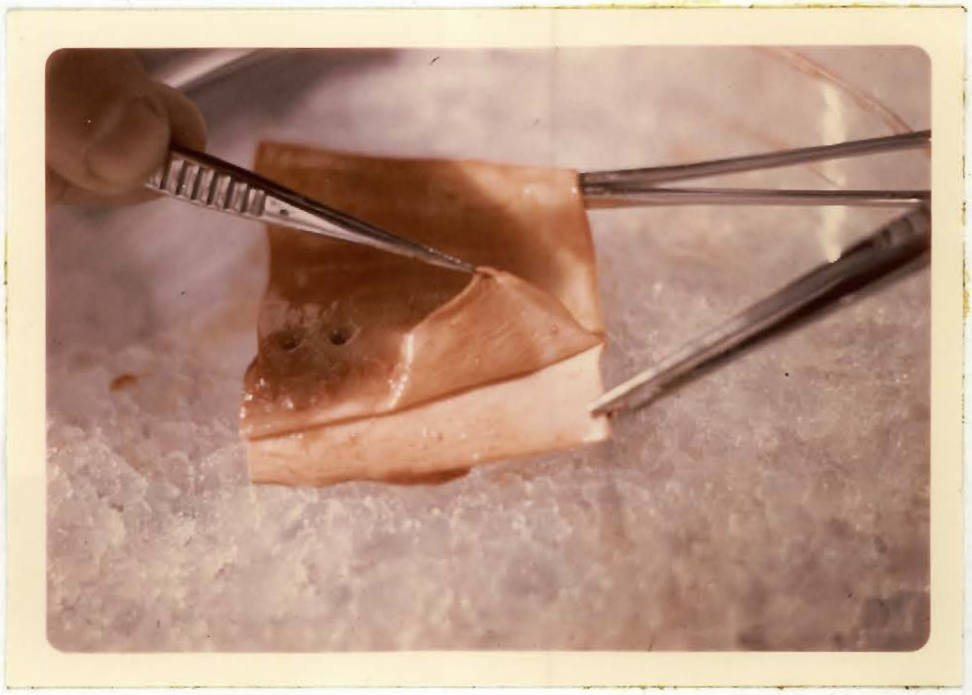
All of the steps described above were carried out in a sterile dissection dish and sterile instruments at 0 °C.

Figure 1

Dissection of the Aorta into Layers.

A. Dissecting the Adventitia

B. Dissecting the Intima from the Media



Technique as described in text.

3. Phosphoglucoisomerase Activity of Homogenates.

The dissected specimens were given the following designations: normal intima (NI), normal media (NM), atherosclerotic intima (AI) and atherosclerotic media (AM). Each specimen was then cut into five pieces with a sterile scissor or scalpel, the pieces thoroughly mixed and then divided into two portions. One portion was weighed on a wet tissue balance, cooled to 0°C, diluted with cold, sterile, distilled water and homogenized in a Potter-Elvehjem glass homogenizer surrounded with ice, to give a final concentration of 5 per cent (w/v).

The other portion was placed in a tared 5 ml beaker and weighed accurately. These specimens were then processed as follows: the four beakers and contents were placed in an air oven and dried at 103°-105°C for four hours. They were allowed to cool in a vacuum dessicator and again weighed accurately. The dried residue was crushed with a glass rod to coarse particle size and stored in individual containers until required for chemical analysis.

(a) Assay of phosphoglucoisomerase (PGI) activity.

The assay of PGI in the aortic homogenates was based on measurement of the amount of fructose-6-phosphate formed from glucose-6-phosphate substrate per unit time in the presence of the enzyme. Fructose-6-phosphate was measured according to the colorimetric method of Roe (129)

which utilizes the reaction between fructose and alcoholic resorcinol in the presence of hydrochloric acid. The optical density is measured at 490 mμ.

The assay procedure was a modification of that described by Brandstrup et al. (115) as follows:

0.3 ml of glucose-6-phosphate (0.1 M, pH 7.9), 0.2 ml of 1.12 M tris buffer (pH 7.9) and 1.7 ml distilled water were placed in a test tube and incubated in a water bath at 38°C for five minutes. 200 μl of homogenate were added with a constriction pipette, the contents mixed and 1 ml transferred immediately to a 15 ml centrifuge tube containing 5.0 ml of 3.3 per cent trichloroacetic acid. This constituted the '0-time' sample. The remainder of the solution was placed immediately in a shaker in a water bath at 38°C and agitated for 10 minutes. At the end of that interval another 1 ml sample was pipetted into a centrifuge tube containing 5.0 ml of 3.3 per cent TCA.

The samples in the centrifuge tubes were then allowed to stand in the cold for 10 minutes, then centrifuged at 2,000xg for ten minutes. The supernatant was then filtered through a No. 40 Whatman filter paper and 2 ml aliquots were used for estimation of fructose-6-phosphate.

Owing to the variability in repeated assays in the pilot experiments, the following procedure was adopted as routine in all the PGI assays. Three nonsimultaneous incubation experiments were carried

out with each set of four homogenates (NI, NM, AI, AM) obtained from a single human aorta. The colorimetric determination of fructose-6-phosphate was then carried out in duplicate on aliquots of the TCA supernatant on each incubation experiment. Thus all the values for PGI activity represent the mean of three nonsimultaneous experiments.

Standards of fructose-6-phosphate (sodium salt) were run simultaneously with all the analyses. It is evident from Figure 2 that fructose-6-phosphate gives an absorbancy-wavelength curve similar to that of pure D-fructose, with a flat peak at 490 mμ under the conditions tested. As indicated in Figure 3 the amount of product formed (indicated by the O.D.) is proportional to time, for both intima and media, for the interval of one hour. Thus the 10 minute incubation period is satisfactory for the assay of activity in terms of the quantity of product formed per hour.

(b) Chromatography of fructose-6-phosphate and other sugar phosphates.

Paper chromatography of fructose-6-phosphate was undertaken to establish that the product measured colorimetrically was indeed fructose-6-phosphate and to exclude the possibility that fructose-6-phosphate was being converted to other products such as mannose-6-phosphate or fructose-1,6-diphosphate.

The procedures used were modifications of the methods of Hanes (130) and Caldwell (131). Descending paper chromatograms

Figure 2

Absorbancy-wavelength Curves for Fructose and Fructose-6-Phosphate.

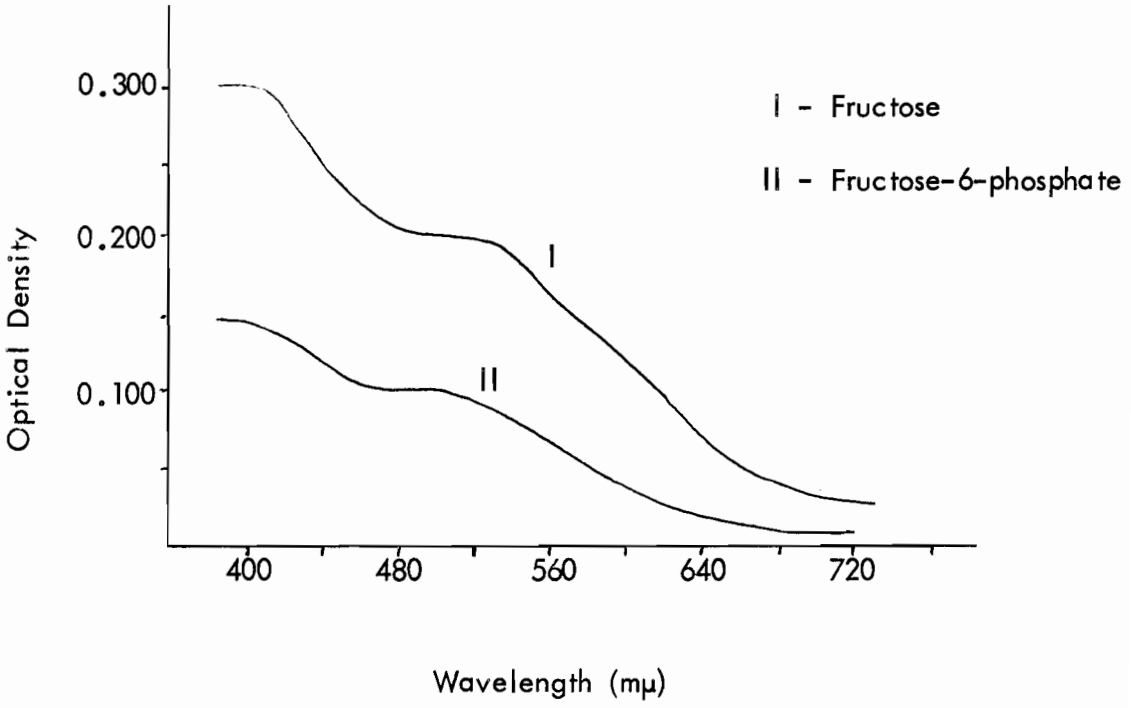
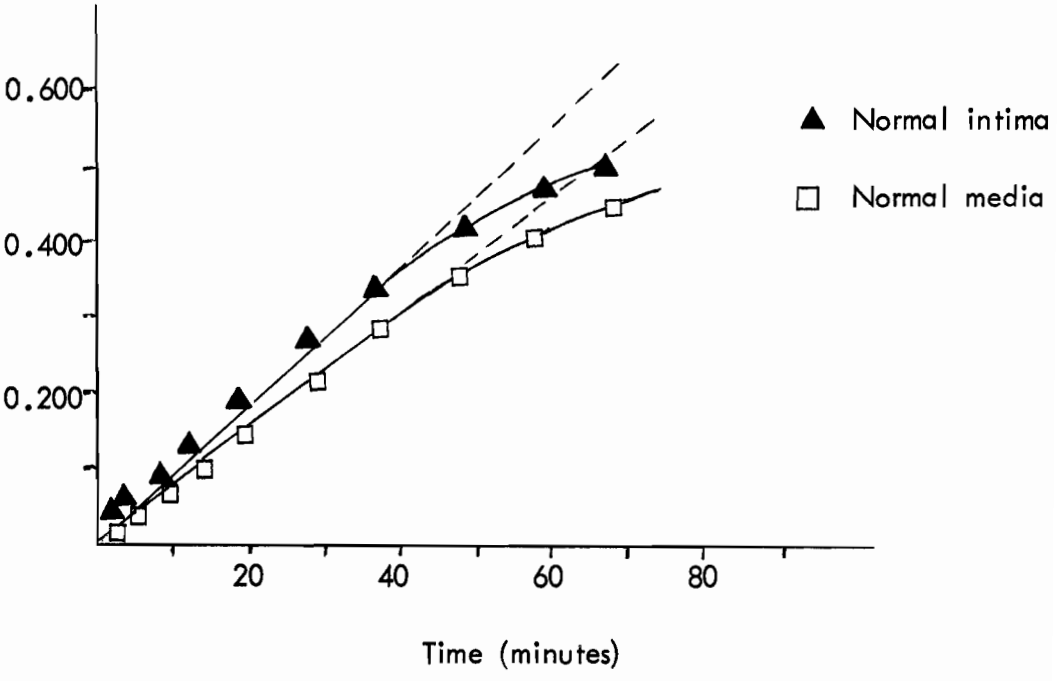


Figure 3

Phosphoglucoisomerase Time-Activity Curves in Aortic Homogenates.



(Whatman No. 4 paper washed with 2 N acetic acid) were run for 24 hours at room temperature with a system of isopropyl ether/90 per cent formic acid [90/60]. The presence of phosphate esters could be indicated by spraying the paper with a solution of 5 ml perchloric acid (60 per cent w/w), 10 ml HCl (IN), 2.5 ml ammonium molybdate (4 per cent w/v) and water to 100 ml. It was found that standards of inorganic phosphate, triose phosphates, fructose-6-phosphate, glucose-6-phosphate, mannose-6-phosphate and fructose-1,6-diphosphate could be differentiated from each other by the respective R_F values and the somewhat different staining properties of fructose-1,6-diphosphate and mannose-6-phosphate from the rest. The colour bands were intensified by suspending the paper for 5 - 10 minutes in a jar containing dilute H_2S gas. The position of the esters was then indicated by the intensely blue spots on a faintly grey background.

Samples taken from the TCA filtrate in the phosphoglucoisomerase assays (where glucose-6-phosphate had been used as the substrate) were run and developed in the aforementioned system. These revealed only spots corresponding to glucose-6-phosphate and fructose-6-phosphate. There was no evidence of fructose-1,6-diphosphate, mannose-6-phosphate or triose phosphate.

In another set of experiments with fructose-6-phosphate as the substrate and the same incubation conditions, chromatography again indicated the presence only of fructose-6-phosphate and glucose-6-phosphate.

In a single experiment with mannose-6-phosphate as the substrate no fructose-6-phosphate could be demonstrated chromatographically.

From the above results it appears that fructose-6-phosphate formed from glucose-6-phosphate is not substantially converted to mannose-6-phosphate or fructose-1,6-diphosphate and/or triose phosphate under the conditions tested. It also appears likely that the substance measured colorimetrically was fructose-6-phosphate.

It will be recalled from Figure 5, page 55, that fructose-6-phosphate may be converted also to glucosamine-6-phosphate. The presence of the latter could not be demonstrated in the TCA filtrate from the phosphoglucoisomerase assays (where glucose-6-phosphate was the substrate) by means of a modification of the Elson-Morgan method (132).

The above evidence thus indicates that the rate of appearance of fructose-6-phosphate from glucose-6-phosphate as substrate, is a satisfactory means of estimating phosphoglucoisomerase activity in human aortic tissue homogenates.

(c) Stability of phosphoglucoisomerase.

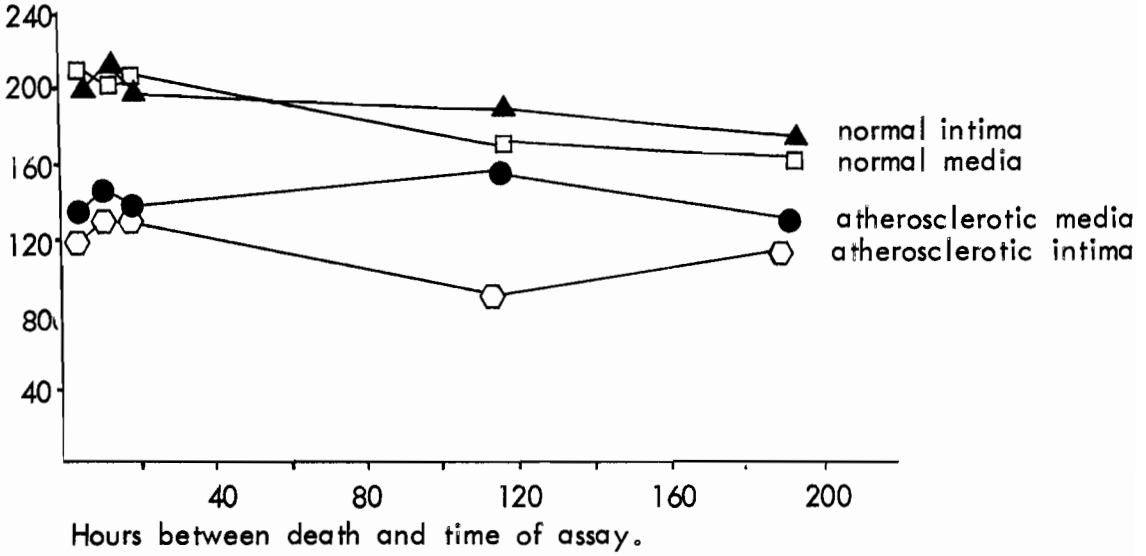
Demonstration of the stability of PGI in the aortic tissue available to us was important in view of the unavoidable delay incurred in obtaining the tissue specimens during autopsy.

Reports in the literature indicate generally that phospho-

glucoisomerase is quite a stable enzyme. For example, the enzyme purified from bovine mammary glands (133) is completely stable for several weeks at 4°C. The activity in the intact mammary glands remains unaltered for several days when kept at 4°C, and for several months at -10°C (133). Wick et al. (134) have made use of the stability of PGI in purifying the enzyme from rat kidney by the use of heat and ammonium sulfate, and Slein (135) in a review of PGI reports that it is quite stable in the cold.

Figure 4

Stability of Phosphoglucoisomerase in Preserved Homogenates. (Homogenates stored at 5°C).



Observations on the phosphoglucoisomerase activity in human aortic tissue as shown in Figure 4 indicate that the activity of the enzyme in a 5 per cent homogenate remained relatively stable for several days. There is no information on the stability of the enzyme in aorta during the six hour period after death of the subject.

4. Determination of Tissue Nitrogen, DNA, Fat, Hexosamine, Hydroxyproline Hexuronic Acid and Sugars in dried Aortic Tissue Samples.

All the determinations were done on dried (103-105°C for 4 hours) samples of tissue of 5 - 30 mg weight, prepared as described. Prior to accurately weighing samples for individual analysis the dry material was removed from the "deep freeze" and placed in the vacuum dessicator for several hours or overnight.

Tissue nitrogen was estimated with the Micro-Kjeldahl method as described in Analytical Chemistry (143).

DNA was determined by a modification of the fluorometric procedure of Kissane and Robins (136) based on the specific reaction of 2,5-diaminobenzoic acid (DABA) with deoxyribose. To get the dry aortic tissue material in a form that can readily react with DABA the following procedure was adopted:

Accurately weighed portions of the aortic material were placed in ground glass stoppered centrifuge tubes to which was added 1 ml $CaCl_2$ (0.1 M, pH 7.9), 1.0 ml Tris buffer (0.154 M, pH 7.9) and a small amount of crystalline trypsin (Sigma Chemical Company, two times crystallized, dialyzed and lyophilized). The tubes were placed in a shaking water bath at 38°C for 6-8 hours and coarse material was occasionally broken down using a stirring rod. 2.0 ml of 20 per cent TCA were then added, the tubes were allowed to stand for

10 minutes at 5°C then centrifuged for 10 minutes at 2,000 r.p.m. The supernatant was then carefully drained off and discarded.

Lipid extraction was carried out according to the procedure of Kissane and Robins (136). Three extractions were carried out with absolute ethanol, the second of which was at 60°C for 15 minutes. The three alcoholic extracts from each sample were combined and saved for the estimation of the lipids. The lipid-free samples were then dried in a vacuum dessicator.

A solution of 2,5-diaminobenzoic acid (Aldrich Co.) was prepared by dissolving 0.3 g of DABA in 1 ml of 4.0 N HCl. The solution was decolourized five times with Norit-A and filtered each time through a millipore filter. 0.2 ml of this solution were then added to the samples. The tubes were tightly closed and kept at 60°C for one half hour with occasional vigorous shaking. 8 ml of perchloric acid, 0.6 N were then added as the tubes were agitated. Particulate matter was centrifuged at 2,000 r.p.m. for 10 minutes and aliquots of the supernatant were appropriately diluted with 0.6 N perchloric acid and the solution was read in a Turner fluorimeter (primary filters 47B, and secondary 2A12).

Standard curves with 20, 40, 60 and 80 µg of a commercial preparation of DNA gave satisfactory proportionality. However, as some variability was noted in the slope of the curve in subsequent trials, the practice was adopted of including standards of 20, 60 and

80 µg with each run of samples. The deviation of the values from the mean among tissue samples run simultaneously was 4 - 8 per cent.

In the tryptic digestion of the samples, controls of the crystalline trypsin did not show any DNA activity, and microscopic examination of the digestion mixture after 6-8 hours confirmed the absence of micro-organisms.

Hexosamine, hexuronic acid, hydroxyproline and sugar determinations were carried out on single dry tissue samples according to the method of P.A. Anastassiadis and R.H. Common (95). In this method sulphonated polystyrene resin (Dowex-50, 200-400 mesh) suspended in 0.07 N hydrochloric acid is used for the hydrolysis of the tissues. The hydrolysis was carried out at 100°C for 20 hours in a specially constructed oven with a rotating system of tube clamps. The sugars and uronic acids were eluted from the resin by draining the resin and washing with water. Hexosamine and hydroxyproline were then eluted with 2 N HCl. Facilities for the hydrolysis and for a number of the steps in this procedure, and helpful advice were kindly provided by Professor P.A. Anastassiadis of the Department of Agricultural Chemistry, Macdonald College, P.Q. Hexuronic acid was estimated according to the method of Dische (137), sugars with the anthrone reaction (138), hexosamine by a modification of the Elson-Morgan method (132, 139), and hydroxyproline according to the method of Neuman and Logan (140).

Total lipid was determined by direct weighing of the vacuum-dried alcohol-extracted fractions from the DNA procedure (p.77). The material was stored at -20°C for further examination or analysis.

5. Possible Release of Heparin during Thrombosis in the Rat.

As mentioned in the introduction a special investigation, distinct from the study on human arterial tissue, was carried out to ascertain whether heparin is released during thrombosis in vivo. Prompted by a report in the literature that less "heparin" is released from atherosclerotic than normal rabbits during thrombosis(121), experiments were designed to study the phenomenon in the rat.

A group of rats was fed the "atherogenic" diet devised by Gresham and Howard (56) and compared with a control group.

The composition of the diet was as follows: arachis oil 400 g, cholesterol 50 g, cholic acid 20 g, thiouracil 3 g, sucrose 169 g, casein 200 g, choline chloride 10 g, salts mixture 40 g, cellophane flaked film 100 mg, MgO 5 g, inositol 2 g, thiamine hydrochloride 16 mg, pyridoxine HCl 16 mg, folic acid 10 mg, calcium pantothenate 40 mg, biotin 0.6 mg, B_{12} 0.05 mg, nicotinamide 0.2 g, and 1000 units vitamin A, 40 International units vitamin D added per rat per week.

The atherogenic diet was kindly provided by Dr. Eugene Donefer of the Department of Animal Science, Faculty of Agriculture, Macdonald College, P.Q. The active ingredients of the diet were: arachis oil, cholesterol, cholic acid and thiouracil.

The U.S.P. sodium heparin standard was obtained from U.S.P. Reference Standards, 46 Park Avenue, New York 16, N.Y.

Technical grade n-octylamine (95 per cent) was redistilled and the fraction boiling between 178-180 °C collected.

Azure A (certified 92 per cent) was obtained from Anachemia Chemicals Limited, Montreal.

(a) Preparation of atherosclerotic animals.

An experimental group of twelve albino male rats (body weight 80-90 g) was fed the atherogenic diet for a period of three months. A corresponding control group was fed Purina Chow. At intervals of 30 days a specimen of blood was taken from the tail vein of each animal.

At the end of three months, experimental arterial thrombosis was produced in the two groups of animals in the following manner: the abdominal aorta was exposed through an anterior approach, and clamped just below the origins of the renal vessels. A quantity (0.2 ml) of saline containing 20 N.I.H. units of thrombin was injected into the aorta at a site distal and close to the clamp. At the end of 20 minutes the clamp was removed. Aortic pulsations usually ceased distal to the site of injection. A small quantity (1.0 ml) of blood was removed from a jugular vein at the end of 30, 60 and 120 minutes, and mixed with isotonic (3.8 per cent) sodium citrate solution. At the conclusion of the experiment the animals were killed with an excess of ether and the aortas removed and prepared for histological examination.

(b) Modification of Jaques' method for extraction of heparin.

A small quantity (0.4 ml) of plasma was extracted with octylamine and brucine according to a modification of the procedure of Jaques (124), adapted for measurement of very small quantities (1-40 µg/ml).

In this procedure the quantity of octylamine added to the plasma is critical for obtaining optimal recovery of heparin (126). Table I indicates the recovery of added heparin from 0.4 ml of plasma with various amounts of octylamine.

TABLE I

Heparin Recovered from Plasma with Octylamine

Heparin added (µg/0.4 ml plasma)	Octylamine added (ml)	Recovery (%)
20	0.05	51
20	0.10	92
20	0.20	69
20	0.40	65

For optimal recovery in our procedure, therefore, 0.1 ml of octylamine was added per 0.4 ml of plasma in a graduated centrifuge tube. The mixture was allowed to stand for 2 minutes and centrifuged

at 3000xg for 10 minutes. The supernatant was discarded. To the precipitate was added 0.5 ml of 0.1 N NaOH, and the tube placed in a water bath at 70°C for 15 minutes. The solution was then extracted with 0.8 ml of ether and the ether layer removed with a pipette. The residual ether was removed by placing the tubes in the water bath at 37°C for 10 minutes. The specimen was cooled and to it 0.8 ml brucine phosphate was added, mixed, and the sample allowed to stand for five minutes. It was then centrifuged at 3000xg for 10 minutes, the supernatant carefully decanted and the remaining liquid allowed to drain. The precipitate was washed successively with 1.2 ml of 95 per cent ethanol and 1.2 ml ether, centrifuged and the supernatant decanted after each washing. The precipitate was placed in a dessicator for one hour after which 0.5 ml of 0.05 N NaOH was added and the specimen placed in a water bath for 15 minutes. It was then cooled to room temperature and neutralized stepwise with 0.5 N HCl (usually about 0.4 ml HCl was required). Phosphate buffer 0.15 M, pH 7.3, was then added to bring the volume to 1.0 ml. Finally, the solution was assayed by the metachromatic or antithrombin assay.

(c) Modification of the metachromatic assay procedure of Jaques et al. (126).

The metachromatic procedure also was modified for micro-estimation as follows: to 0.5 ml of heparin standard, or to the 'unknown'

plasma extract, was added 0.3 ml of phosphate buffer 0.15 M, pH 7.3. A stock solution (0.01 per cent) of Azure A was prepared and diluted ten times with water immediately before use. A quantity (0.5 ml) of this solution was added to the heparin-buffer solution and mixed thoroughly. Immediately after the mixing the optical density was read at 500 mμ (Beckman DU spectrophotometer with 1 ml cuvettes).

A coagulation test system based on the antithrombin activity of heparin (126) also was set up. The thrombin in this system was standardized against the U.S.P. sodium heparin standard.

The minimum amount of heparin that could be determined by the assay procedures was 0.2 μg with the metachromatic assay and 0.1 U.S.P. unit with the antithrombin assay.

Experimental Results

1. Phosphoglucoisomerase in Normal Aortic Tissue.

Investigations were carried out to determine some of the characteristics of PGI in aortic tissue and to compare the findings with PGI from other sources, reported by other workers.

(a) Influence of hydrogen ion concentration.

Figure 5 indicates the influence of the hydrogen ion concentration on the activity of PGI in the aorta. The pH optimum of the enzyme is 7.9. This hydrogen ion concentration was maintained in all the PGI assays.

(b) Influence of substrate concentration.

Influence of substrate concentration on reaction velocity is indicated in Figure 6A. The Lineweaver-Burk plot is indicated in Figure 6B.

A value for Km of 1.1×10^{-3}M has been calculated from this plot. This value is close to that (1.22×10^{-3}M) reported by Bedansky (103) for the PGI of the plasma. Bruns et al. (140), on the other hand, reported a value of 4×10^{-3}M for plasma, and Ramasarma (142), a value of 3.45×10^{-3}M for the PGI of the bean. It is noteworthy that

Figure 5

The Effect of Hydrogen Ion Concentration on Phosphoglucoisomerase Activity.

Conditions: Glucose-6-phosphate, 0.1M, 0.3 ml, adjusted to the appropriate pH.

Tris buffer, 1.12 M, 0.2 ml at appropriate pH.

Distilled water, 1.7 ml.

Aortic homogenate from normal intima, 0.2 ml.

Incubation and subsequent steps as per page 70.

Figure 5

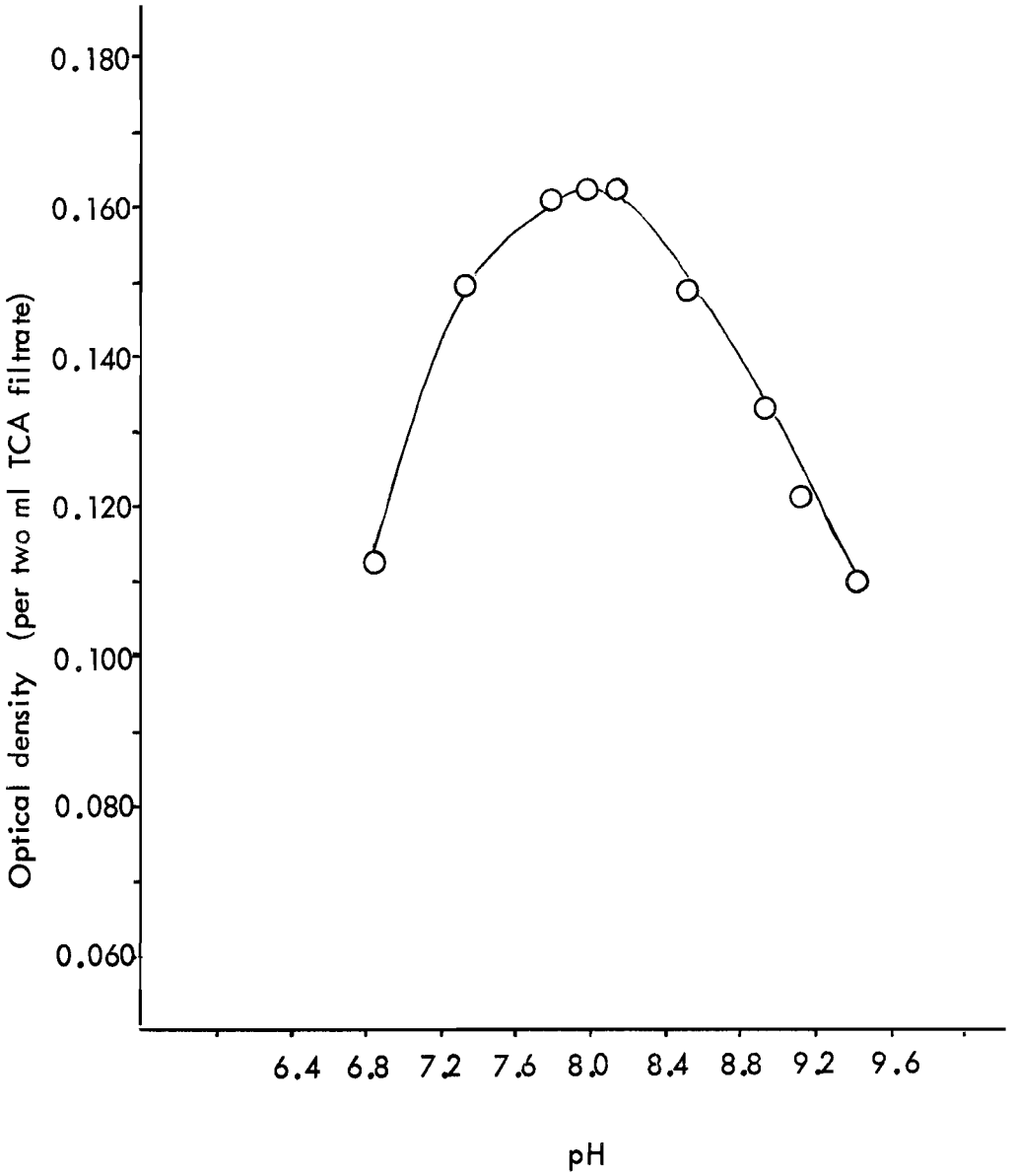

0.180
0.160
0.140
0.120
0.100
0.080
0.060
Optical density (per two ml TCA filtrate)
6.4
6.8
7.2
7.6
8.0
8.4
8.8
9.2
9.6
pH

Figure 6

The Effect of Substrate Concentration on Velocity.

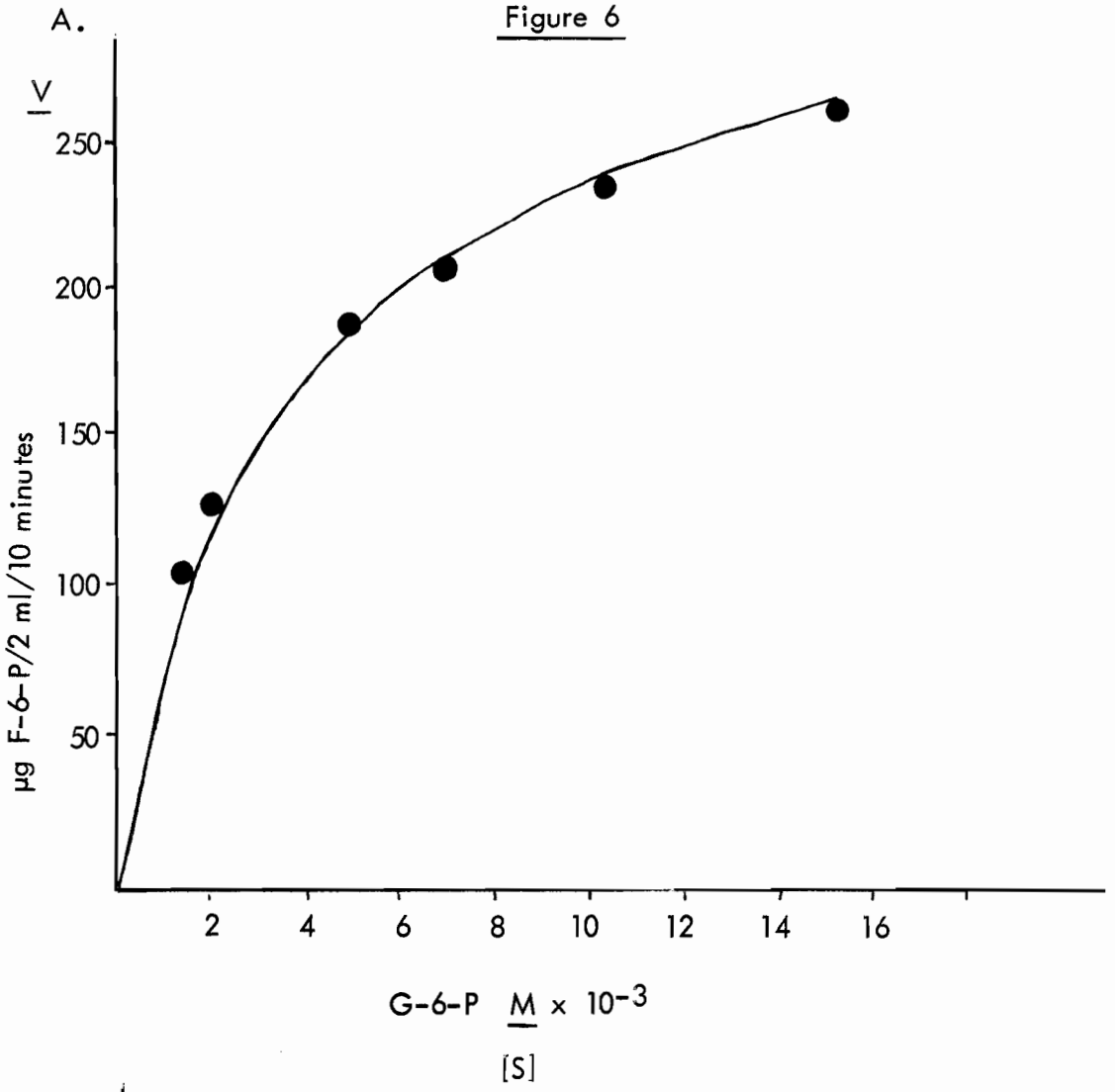
A. Plot of substrate concentration (glucose-6-phosphate x 10^{-3} M) against velocity of reaction (fructose-6-phosphate formed, µg in 2 ml TCA filtrate per 10 minutes incubation).

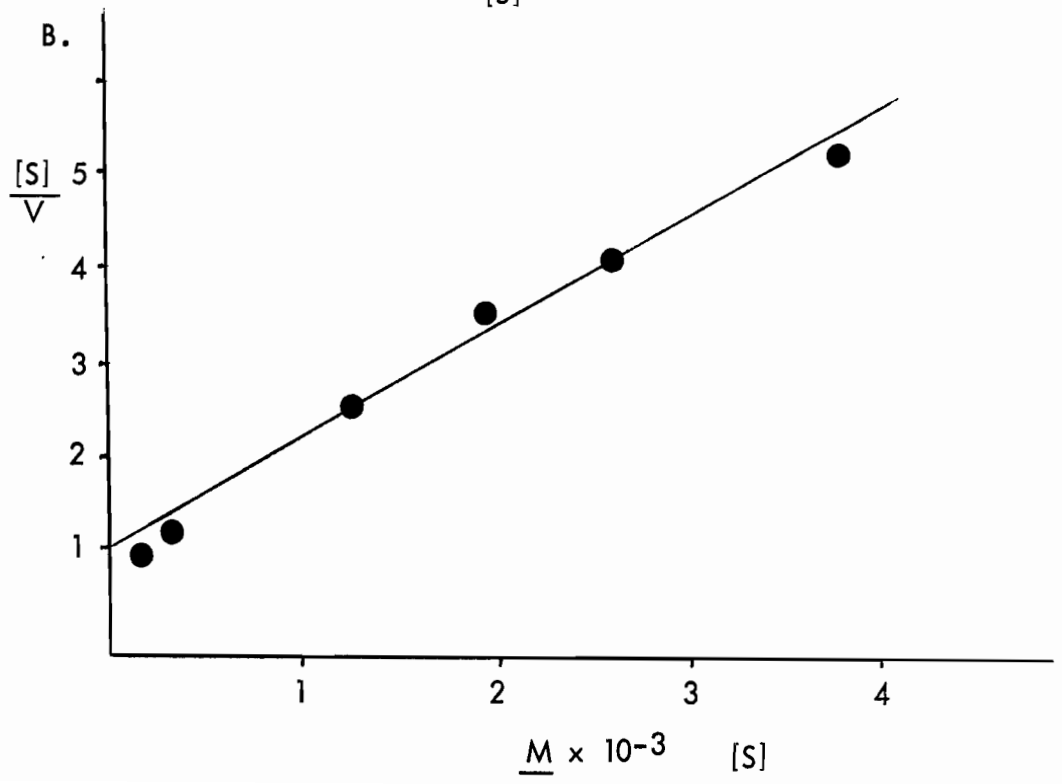
B. Plot of substrate concentration over velocity against substrate concentration.

Slope of the line = $\frac{1}{V}$ = 1.1

Intercept at ordinate = $\frac{Km}{V}$ = 1.0

Figure 6


A.
V
250
200
150
100
50
µg F-6-P/2 ml/10 minutes
2
4
6
8
10
12
14
16
G-6-P M x 10-3
[S]


B.
[S]
V
5
4
3
2
1
1
2
3
4
M x 10-3
[S]

our value of 1.1×10^{-3}M was obtained with simple aortic homogenates.

(c) Influence of enzyme concentration.

Some experiments were carried out to establish the relationship between the enzyme concentration and the velocity of reaction.

Figure 7 shows that a linear relationship persisted with concentration up to 0.3 ml of enzyme per incubation tube. With higher concentrations, the curve showed a tendency to level off. This tendency may be attributable to relative enzyme excess for the substrate concentration used.

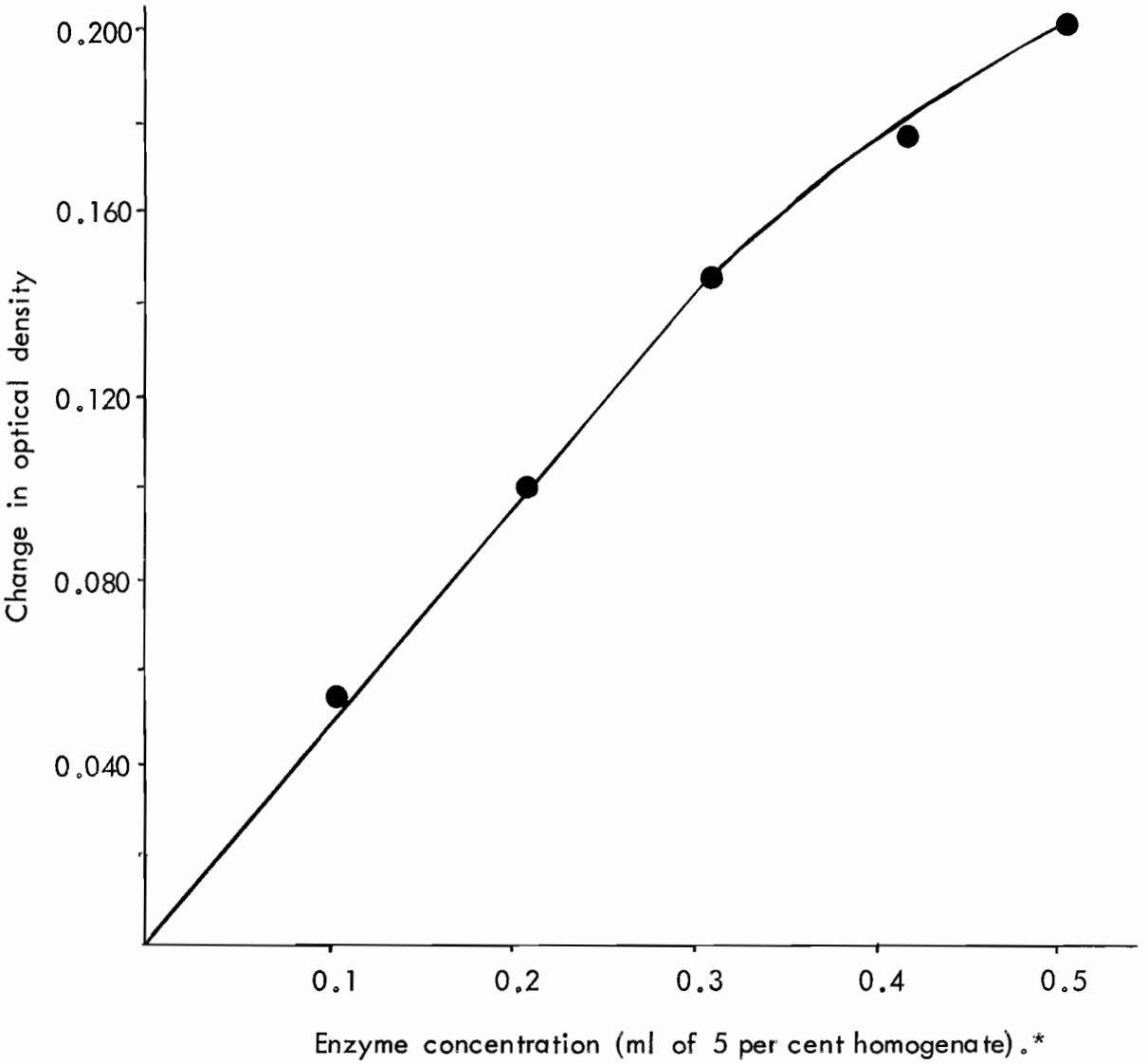
2. Phosphoglucoisomerase Activity Expressed on the Basis of Dry, Wet and Fat-Free Weights of the Tissue for the NI, NM, AI and AM.

As already observed from the literature, most of the values for the activity of PGI in aortic tissue have been expressed in terms of dry or wet weights (102, 115). For comparison purposes, the PGI activity was measured in aortas from ten individuals and expressed on the basis of the dry and wet weight. However, in our study the important innovation was made of measuring the activity of the NI, NM, AI and the AM separately. The results are presented in Tables II and III.

Expressed on the basis of wet weight the ratios of activities are NI:AI = 1:0.8, and for NM:AM = 1:1.0. Closely similar ratios

Figure 7

Effect of Enzyme Concentration on Activity.



*Experiments carried out in standard incubation system described on p. 70.

TABLE II

Phosphoglucoisomerase Activity of the Intima and the Media of Normal and Atherosclerotic Aortic Tissue.

Specimen from case No.	Tissues: NI	NM	AI	AM
	(mM F-6-P produced/g wet tissue/hour)*			
6	1.54	1.88	1.32	2.03
7	1.44	1.69	0.72	2.11
8	1.95	2.31	1.75	2.38
9	2.10	1.71	1.64	1.62
10	1.78	2.18	1.72	1.57
11	1.60	2.30	1.74	1.56
12	0.96	1.29	0.87	1.41
13	1.13	1.64	1.06	2.10
14	0.67	1.14	0.82	1.35
15	1.07	1.23	0.99	1.39
Mean	1.42	1.74	1.26	1.75

*The group of values for any case represents the mean of three non-simultaneous experiments. The TCA filtrate from each experiment was analyzed in duplicate.

NI:AI = 1:0.8

NM:AM = 1:1.0

TABLE III

Phosphoglucoisomerase Activity of the Intima and the Media of Normal and Atherosclerotic Aortic Tissue.

Specimen from case No.	Tissues: NI	NM	AI	AM
	(mM F-6-P produced/g dry tissue/hour)*			
6	4.84	7.25	3.17	5.60
7	6.12	6.65	2.42	7.25
8	7.62	9.83	5.94	10.03
9	10.07	6.92	6.57	5.75
10	6.55	8.77	8.47	6.24
11	7.10	10.04	6.95	5.67
12	4.98	6.01	3.78	5.17
13	2.72	4.85	2.57	2.42
14	0.78	1.41	2.30	2.42
15	4.39	5.37	4.68	7.00
Mean	5.52	5.81	4.69	5.76

*Dry weight was determined individually for each tissue type.

NI:AI = 1:0.9

NM:AM = 1:1.0

were obtained with the values based on the dry weight of the tissue; NI:AI = 1:0.9 and NM:AM = 1:1.0.

The differences in the values of NI and AI were, however, not found to be statistically significant. If the NI and the NM be considered together and likewise the AI and the AM, the difference between the activity of the normal and the pathological tissues becomes obscured. Thus, the mean activity for normal tissue (NI + NM) = 1.58 and for atherosclerotic tissue (AI + AM) = 1.51. This gives a ratio between the combined activity values of the intima and media section of the atherosclerotic and those of the normal tissues of 1:1.0. These values correspond closely to those reported by Brandstrup et al. (115) for sections of the undissected intima - media.

The total lipid was determined in some of the samples (see also section 3). The mean PGI activity expressed in terms of fat-free weight was respectively 6.03, 6.24, 6.35 and 6.95 mM of F-6-P produced per gram dry defatted tissue per hour for the NI, NM, AI and the AM.

3. Moisture, Nitrogen, DNA and Total Lipid Content of Aortic Tissue.

Few systematic studies appear to have been done to demonstrate the differences in the chemical composition between the intima and media separately in the normal and atherosclerotic tissues.

Such a study has been carried out by the author and the results are presented in Tables IV, V and VI.

It is apparent from Table IV that the moisture content of the normal intima is significantly greater than that of the atherosclerotic intima ($p < 0.05$). The difference in the moisture content between normal and atherosclerotic media is much less pronounced.

Table V indicates the total nitrogen content in the components of the aorta. It is of interest that the mean nitrogen content of AI was 3.4 per cent lower than the corresponding value for NI. The difference is statistically significant to $p=0.05$($t = 2.397$) for ten degrees of freedom. A 2.4 per cent difference exists between the nitrogen content of NM and the AM, but it is not statistically significant. These observations indicate that the AI, and to a lesser extent the AM, contain a greater proportion of nonprotein material, such as fat or mucopolysaccharide, than the NI and the NM.

Table VI gives tha values for the DNA content of the two component tissues of the aorta. The NI contains approximately twice as much DNA as the AI. This difference is statistically significant to $p = 0.025$ ($t = 2.808$) for ten degrees of freedom. On the other hand, there is little difference in the DNA content between the NM and the AM, thus indicating that the number of cells in the media was likely not affected by the atherosclerotic process.

It is of interest to compare the ratios of the means of the

TABLE IV

Moisture Content of the Component Tissues of the Aorta.

Specimen from case No.	Moisture Content of Tissues: NI	NM	AI	AM
	(Per cent)			
5	83.9	77.0	79.5	75.5
6	67.8	74.3	58.7	64.0
7	76.2	74.7	50.3	72.7
8	80.7	75.2	75.2	73.0
9	77.6	76.4	71.0	77.8
10	79.6	76.5	71.5	75.5
11	77.7	75.2	70.9	72.6
12	80.7	75.7	75.5	78.9
13	75.7	65.8	58.3	63.5
14	77.4	74.9	69.8	73.3
15	75.2	77.0	73.9	79.5
Mean	77.51	74.78	69.58	73.30

TABLE V

Nitrogen Content of Normal and Atherosclerotic Intima and Media.

Specimen from case No.	Nitrogen content of component tissues of aorta: NI	NM	AI	AM
	(per cent nitrogen in dry tissue)			
5	10.82	10.98	8.37	10.03
6	10.15	10.25	6.16	11.40
7	11.60	12.70	9.10	11.10
8	11.15	12.80	5.90	11.40
9	13.00	13.60	8.60	11.40
10	13.50	13.80	13.10	13.10
11	13.50	13.40	5.40	10.00
12	12.90	15.60	11.60	11.80
13	10.70	12.60	6.80	11.60
14	10.20	10.40	7.10	7.90
15	9.80	12.30	7.00	11.20
Mean	11.52	12.57	8.10	10.09

TABLE VI

DNA Content of Component Tissues of Aorta.

Specimen from case No.	DNA of Tissues: NI	NM	AI	AM
	(µg DNA per mg dry tissue)			
5	3.4	4.7	3.9	5.4
6	5.3	5.9	2.7	7.1
7	7.3	6.1	2.5	3.7
8	4.1	4.1	2.7	4.7
9	5.0	3.9	2.5	3.4
10	4.4	3.7	2.4	4.7
11	8.9	3.0	3.2	2.7
12	6.0	4.8	4.1	2.2
13	3.0	3.0	2.8	3.3
14	2.9	2.7	2.6	3.5
15	5.1	3.3	3.4	5.9
Mean	5.04	4.11	2.73	4.21

Ratio of means of DNA content:

NI:AI = 1:0.6
NM:AM = 1:1.0
NI:NM = 1:0.8
AI:AM = 1:0.5

DNA content NI:NM and AI:AM as given at the foot of Table VI. It it apparent that the DNA content of the intima of the normal aorta is greater than that of the media. This presumably is attributable to the greater cellularity of the normal tissue. During atherosclerosis this relation is reversed. The reversal apparently is attributable simply to a decrease in the DNA content in the intima, as the content of the media does not undergo a significant change.

The total lipid content was determined gravimetrically. However, as some of the samples were too small to be weighed individually with accuracy the means of the values were: NI, 8.4; NM, 7.0; AI, 26.0 and AM, 16.7 per cent of the dry weight of the tissue.

4. The PGI Activity Expressed on the Basis of Tissue Nitrogen, DNA and Noncollagen Nitrogen Content of the Tissues.

As previously noted it is difficult to make comparisons between the enzyme activity of normal and pathological tissue, since the latter may have undergone extensive changes in composition. The merits or demerits of various commonly used modes of expression of enzyme activity have been reviewed in a preceeding section (p. 59-60). The writer has chosen to express activity in several ways.

Tables VII, VIII and IX present the activity of phospho-

TABLE VII

Phosphoglucoisomerase Activity of the Intima and the Media in Normal and Arteriosclerotic Aortic Tissue.

Specimen from case No.	Tissues: NI	NM	AI	AM
	(mM F-6-P produced/g tissue nitrogen/hour)			
6	47.6	70.7	51.5	49.1
7	52.7	52.5	26.6	65.2
8	68.7	76.9	100.1	88.0
9	81.5	51.8	76.5	50.5
10	48.2	63.5	64.3	47.3
11	52.0	74.4	105.0	76.0
12	38.2	38.6	32.4	43.8
13	25.4	38.6	37.8	12.2
14	7.7	13.6	32.4	30.6
15	44.7	43.7	69.5	62.5
Mean	46.7	52.4	59.6	52.5

Ratios:

NI:AI = 1:1.3
NM:AM = 1:1.0

TABLE VIII

Phosphoglucoisomerase Activity of Intima and Media in Normal and Arteriosclerotic Aortic Tissue.

Specimen from case No.	Tissues: NI	NM	AI	AM
	(mM F-6-P produced/μg DNA/hour)			
6	1.22	1.25	1.16	0.79
7	0.84	1.09	0.97	1.96
8	1.86	2.40	2.20	2.13
9	2.07	1.77	2.63	1.69
10	1.48	2.37	3.54	1.33
11	0.80	3.34	2.13	2.11
12	0.83	1.25	0.92	2.35
13	0.91	1.62	0.92	0.74
14	0.27	0.52	0.88	0.69
15	0.86	1.63	1.37	1.18
Mean	1.11	1.72	1.67	1.50

Ratios:

NI:AI = 1:1.5
NM:AM = 1:0.9

TABLE IX

Phosphoglucoisomerase Activity of Intima and Media in Normal and Arteriosclerotic Aortic Tissue.

Specimen from case No.	Tissues: NI	NM	AI	AM
	(mM F-6-P produced/µg noncollagen nitrogen/hour)			
6	8.17	9.97	22.70	6.92
7	10.04	6.47	4.00	8.85
8	11.18	10.00		12.50
9	11.06	6.71	12.27	6.85
10	8.17	8.90	13.82	6.28
11	8.30	10.18	40.00	10.08
12	4.67	4.70	6.15	5.62
13	4.05	5.76	7.55	2.90
14	1.42	1.90	6.07	4.30
15	10.90	5.62	18.20	8.26
Mean	8.80	6.36	14.97	7.25

Ratios:

NI:AI = 1:1.7
NM:AM = 1:1.2
AI:AM = 1:0.5
NI:NM = 1:0.7

glucoisomerase on the basis of nitrogen, DNA and the noncollagen nitrogen content respectively.

It is evident from Table VII that the PGI activity for the AI is greater than that of the NI and the difference is statistically significant to 0.025 ($t = 3.009$ for 9 degrees of freedom). No difference was observed between the NM and the AM. The mean values given in Table VII on the basis of the nitrogen content are of the same order of magnitude as those reported by Brandstrup et al. (115) for the undissected intima-media sections - 44 and 43 mM of F-6-P formed per gram tissue nitrogen per hour (i.e. a ratio of undissected normal intima-media to arteriosclerotic intima-media of 1:1).

Comparison of our values for the dissected aorta with those cited above gives a ratio NI:AI of 1:1.3, but for (NI+NM):(AI+AM) of 1:1.1. It is evident that any difference in PGI activity between the intima and the media tends to be obscured when the two are analyzed together as one tissue.

It may be significant furthermore that Zemplenyi (105), with undissected rabbit aorta failed to demonstrate any difference in activity between the normal and atherosclerotic tissue (Zemplenyi expressed his results as arbitrary units per mg nitrogen of aortic extract).

Table VIII shows PGI activity expressed on the basis of the DNA content of the tissue. It is apparent that the activity is greater for the AI than for the NI. The ratio of NI:AI was 1:1.5.

This difference between the activity of these components is statistically significant to 0.05. The activity of the NM is somewhat greater than that of AM, however, the difference is not statistically significant.

In Table IX the PGI activity is expressed on the basis of the noncollagen nitrogen content of the tissues as calculated from the hydroxyproline analysis referred to on page 76. For this calculation the conversion factor of 7.46, recommended by Neuman and Logan (140), has been used to estimate the collagen equivalent. The nitrogen content of collagen is then calculated using the generally accepted value of 17.86 per cent. The noncollagen nitrogen is obtained by subtracting the nitrogen content of collagen per gram dry tissue from the total nitrogen per gram dry tissue as estimated by the micro-Kjeldhal method. The noncollagen nitrogen is considered to afford a satisfactory index of the amount of cellular material in the tissue. It is evident from the data in Table IX that the PGI activity in the AI was considerably greater than that of the NI and this difference is statistically significant ($p < 0.01$). The ratio of NI:AI was 1:1.7. The difference between the value for the NM and the AM (ratio NM:AM = 1:1.2) is not significant. It is noteworthy that the difference in activity was pronounced between AI and AM (ratio AI:AM = 1:0.5), and less conspicuous between the NI and the NM (ratio NI:NM = 1:0.70).

5. Changes in Connective Tissue in Atherosclerosis.

(a) Hexosamine, hexuronic acid and sugar content of NI, NM, AI and AM.

The results for analysis of hexosamine are presented in Table X.

It is evident that the normal intima tends to have a higher content of hexosamine than the atherosclerotic intima, but the difference proved not to be statistically significant. Likewise, there was no significant difference in the glucosamine content between the NM and the AM, the NI and the NM, and the AI and the AM respectively.

The mean values of the analytical results calculated on the basis of tissue nitrogen and DNA, and the corresponding ratios of NI:AI, NM:AM, NI:NM and AI:AM are given in Table XI. It is readily apparent, when expressed on these bases, that the NI contains more hexosamine than the AI and the differences between the values for the normal and the arteriosclerotic tissues are statistically significant. On the other hand, the difference between the values for the normal media and intima in no instance was statistically significant. The NM contains significantly more hexosamine than AM and the AI more than the AM when the values are expressed on the basis of DNA and nitrogen.

Table XII shows the analytical values for hexuronic acid expressed on the basis of dry weight of the tissues. The NI contains more

TABLE X

Hexosamine Content of the Normal and Atherosclerotic Aorta.

Specimen from case No.	Tissues: NI	NM	AI	AM
	(µgper mg dry tissue)			
5	10.4	12.9	7.7	8.8
6	9.2	11.4	11.9	10.7
7	11.2	14.7	10.8	9.7
8	17.6	14.8	15.0	12.2
9	13.6	11.2	13.2	11.7
10	11.9	13.3	11.6	11.1
11	15.7	12.7	9.3	9.5
12	9.2	11.1	7.7	7.2
13	11.5	12.5	9.7	13.5
14	11.9	10.3	10.5	10.3
15	14.3	11.7	12.1	12.0
Mean	12.4	12.5	10.9	10.6

TABLE XI

Hexosamine Content of Normal and Atherosclerotic Tissue Expressed on the Basis of Tissue Nitrogen and DNA.

Expressed on basis of	Tissues: NI	NM	AI	AM
	Mean values for specimens from 10 cases.			
N*	108.2	100.2	145.7	97.7
DNA**	2.7	3.2	3.8	2.7
	Ratios of values NI:AI	NM:AM	NI:NM	AI:AM
N	1 : 1.3	1 : 1.0	1 : 0.9	1 : 0.7
DNA	1 : 1.4	1 : 0.8	1 : 1.2	1 : 0.7
	Statistical analysis (t-test) NI vs AI	NM vs AM	NI vs NM	AI vs AM
N	p 0.01	N.S.	N.S.	p 0.01
DNA	p 0.05	p 0.05	N.S.	N.S.

N* Micrograms of hexosamine per mg per cent dry tissue nitrogen.
DNA** Micrograms of hexosamine per microgram DNA per mg dry tissue.
N.S. - Not significant.

TABLE XII

Hexuronic Acid Content of Aortic Tissues.

Specimen from case No.	Tissues: NI	NM	AI	AM
	(μg per mg dry tissue)			
5	5.0	3.1	2.9	2.4
6	8.6	5.1	5.7	4.6
7	7.0	5.9	3.1	4.0
8	1.0	12.2	2.5	5.7
9	7.1	3.5	3.0	3.4
10	3.8	5.1	2.9	3.0
11	5.1	5.1	2.4	3.9
12	3.7	4.3	2.6	2.8
13	3.4	5.2	2.8	4.4
14	3.2	4.1	2.7	2.3
15	7.5	5.1	4.4	4.2
Mean	5.0	5.3	3.2	3.7

hexuronic acid than the AI, and the NM more than the AM (ratios: NI:AI of 1:0.6 and NM:AM of 1:0.7). The difference in both cases is statistically significant to $p < 0.01$. On the other hand, there was no significant difference between the hexuronic acid content of the NI and that of the NM, or of the AI and the AM.

When hexuronic acid is calculated on the basis of the tissue nitrogen or the DNA, as given in Table XIII and Tables XXVI and XXVII in the appendix (p.156-7) the picture changes. Thus, the differences between the values for the NI and the AI, and also between those of the NM and the AM now become nonsignificant. Likewise the differences between the content of the NI and that of the NM, and between the AI and the AM become nonsignificant.

The values for the sugar content for the 10 cases expressed on the basis of dry weight are given in Table XIV. The respective differences in the values between NI and AI, NM and AM, NI and NM and AI and AM are statistically not significant.

However, when expressed in terms of nitrogen or DNA as in Table XV (Tables XXVIII and XXIX in the appendix), a striking parallelism is evident in the pattern of the sugars and the hexosamine (Table XI) distribution, in the various parts of the aorta. As with the hexosamines, conspicuous change is the increase of sugar content of the atherosclerotic intima compared to that of the normal intima. The increase

TABLE XIII

Hexuronic Acid Content of Normal and Atherosclerotic Tissue Expressed on the Basis of Tissue Nitrogen and DNA.

Expressed on basis of	Tissues: NI	NM	AI	AM
	Mean values for specimens from 10 cases			
N*	44.4	42.7	42.8	32.9
DNA*	1.0	1.37	1.1	0.94
	Ratios of values NI:AI	NM:AM	NI:NM	AI:AM
N	1:1.0	1:0.7	1:1.0	1:0.8
DNA	1:1.1	1:0.7	1:1.4	1:0.9
	Statistical analysis (t-test) NI vs AI	NM vs AM	NI vs NM	AI vs AM
N	N.S.	N.S.	N.S.	N.S.
DNA	N.S.	N.S.	N.S.	N.S.

*Values expressed as in Table XI.

TABLE XIV

Sugar Content in Normal and Atherosclerotic Aorta.

Specimen from case No.	Tissues: NI	NM	AI	AM
		(μg per mg dry tissue)*		
5	-	9.6	7.9	7.4
6	10.2	9.5	10.0	8.3
7	12.5	9.7	7.4	8.5
8	17.8	9.6	16.4	8.9
9	9.6	7.8	10.4	8.4
10	10.7	11.9	13.5	7.2
11	10.8	9.6	8.8	8.8
12	7.7	8.0	8.7	8.1
13	9.5	11.1	8.8	10.5
14	9.5	11.3	8.1	8.2
15	15.6	17.5	12.5	18.9
Mean	11.4	10.7	10.1	9.8

*Estimated by means of the anthrone reaction (p. 78).

TABLE XV

Sugar Content of Normal and Atherosclerotic Tissue Expressed on the Basis of Tissue Nitrogen and DNA.

Expressed on basis of	Tissues: NI	NM	AI	AM
	Mean values for specimens from 10 cases			
N*	101.6	84.9	136.4	86.3
DNA*	2.43	2.96	3.57	2.39
	Ratios of values			
	NI:AI	NM:AM	NI:NM	AI:AM
N	1:1.3	1:1.0	1:0.8	1:0.6
DNA	1:1.5	1:0.8	1:1.2	1:0.7
	Statistical analysis (t-test)			
	NI vs AI	NM vs AM	NI vs NM	AI vs AM
N	p 0.005	N.S.	N.S.	p 0.05
DNA	p 0.005	N.S.	N.S.	N.S.

*Values expressed as in Table XI.

is statistically highly significant, whether expressed on the basis of tissue nitrogen or DNA. The difference between the sugar content of the NM and the AM, or the NI and the NM, on the other hand, is not significant. The AM tends to contain less sugar than the AI, expressed either on the basis of nitrogen or DNA but the difference is statistically significant only on the basis of the tissue nitrogen.

A survey of all these findings reveals the following:

First, the part of the aorta that undergoes the most change during the development of atherosclerosis is the intima. This is most apparent from the analytical results when the values are expressed on the basis of the nitrogen or DNA content of the tissues. The hexosamine and the sugar content of the intima undergo a parallel increase. The hexuronic acid content, on the contrary, remains relatively unchanged.

Second, the changes in the media are much less pronounced. In some specimens there was a small decrease in the hexosamine and sugar content of the media during atherosclerosis but this was apparent only when results are expressed on the basis of the DNA content of the tissue. The hexuronic acid analyses showed a decrease comparable in magnitude with that of hexosamine, but the differences are not statistically significant.

Third, the intima and media of the normal aortas showed no statistically significant changes in composition with respect to any of the constituents when expressed on the basis of nitrogen or DNA.

It is noteworthy, however, that normal media tends to have a higher content of hexuronic acid than normal intima (when the results are expressed on the basis of DNA), but the differences observed are not statistically significant.

Finally, as may be anticipated from the foregoing, the atherosclerotic intima contains more hexosamine and sugar than the atherosclerotic media, while the hexuronic acid remains relatively unchanged.

(b) Hydroxyproline content of NI, NM, AI and AM.

The hydroxyproline content of tissues affords an index of the collagen content.

It is apparent from Table XVI that there is practically no difference in the hydroxyproline content of the NI as compared to the AI, or of the NM as compared to the AM, when the results are expressed on the basis of dry weight. However, the intima contained more hydroxyproline than the media both in the normal (NI:NM = 1:0.7), and atherosclerotic (AI:AM = 1:0.7) tissues. The difference in both cases is statistically significant [$p = 0.005$ and $p = 0.025$ respectively].

Table XVII indicates that when the results are calculated on the basis of the tissue nitrogen or the DNA (see also appendix Tables XXX and XXXI), the content of hydroxyproline in the AI is significantly higher than in the NI. There is no considerable difference

TABLE XVI

Hydroxyproline Content of Normal and Atherosclerotic Aorta.

Specimen from case No.	Tissues: NI	NM	AI	AM
	(per mg dry weight)			
5	33.5	20.8	29.8	19.4
6	31.9	22.5	35.8	24.8
7	42.9	18.3	22.8	21.8
8	32.5	22.2	46.0	23.8
9	28.0	24.8	24.8	23.0
10	41.3	29.7	52.4	24.1
11	37.2	25.4	25.2	32.7
12	16.8	20.6	33.5	18.9
13	30.0	31.5	26.3	24.6
14	35.4	22.4	24.8	17.2
15	43.3	20.7	33.1	20.4
Average	33.9	23.5	32.2	22.8

TABLE VXII

Hydroxyproline Content of Normal and Atherosclerotic Tissue Expressed on the Basis of Tissue Nitrogen and DNA.

Expressed on basis of	Tissues: NI	NM	AI	AM
	Mean values for specimens from 10 cases			
N*	297.90	189.29	420.03	196.23
DNA*	7.57	6.26	11.03	6.04
	Ratios of values			
	NI:AI	NM:AM	NI:NM	AI:AM
N	1:1.4	1:1.0	1:0.7	1:0.5
DNA	1:1.5	1:1.0	1:0.8	1:0.5
	Statistical analysis (t-test)			
	NI vs AI	NM vs AM	NI vs NM	AI vs AM
N	p 0.025	N.S.	N.S.	p 0.005
DNA	p 0.05	N.S.	N.S.	p 0.025

*Values expressed as in Table XI.

between the hydroxyproline content of NM and that of the AM. The NM tended to contain less hydroxyproline than the NI, but the difference is not statistically significant. The AI, on the other hand, contained twice as much hydroxyproline as the AM and when calculated either on the basis of the tissue nitrogen or the DNA, the difference is significant.

These findings indicate that the collagen content of the intima increases significantly during the development of atherosclerosis. The media, on the other hand, undergoes no such change in composition.

6. Possible Release of Heparin During Thrombosis in the Rat.

As indicated on page 80, the question whether heparin is released during thrombosis in the normal or atherosclerotic rat, was investigated and a group of rats was fed an atherogenic diet.

During the three months feeding period, the animals on the atherogenic diet showed the following changes: loss of weight as indicated in Figure 8, the passing of abnormal fatty stool, and signs of severe hypothyroidism such as underactivity and changes in the skin and hair.

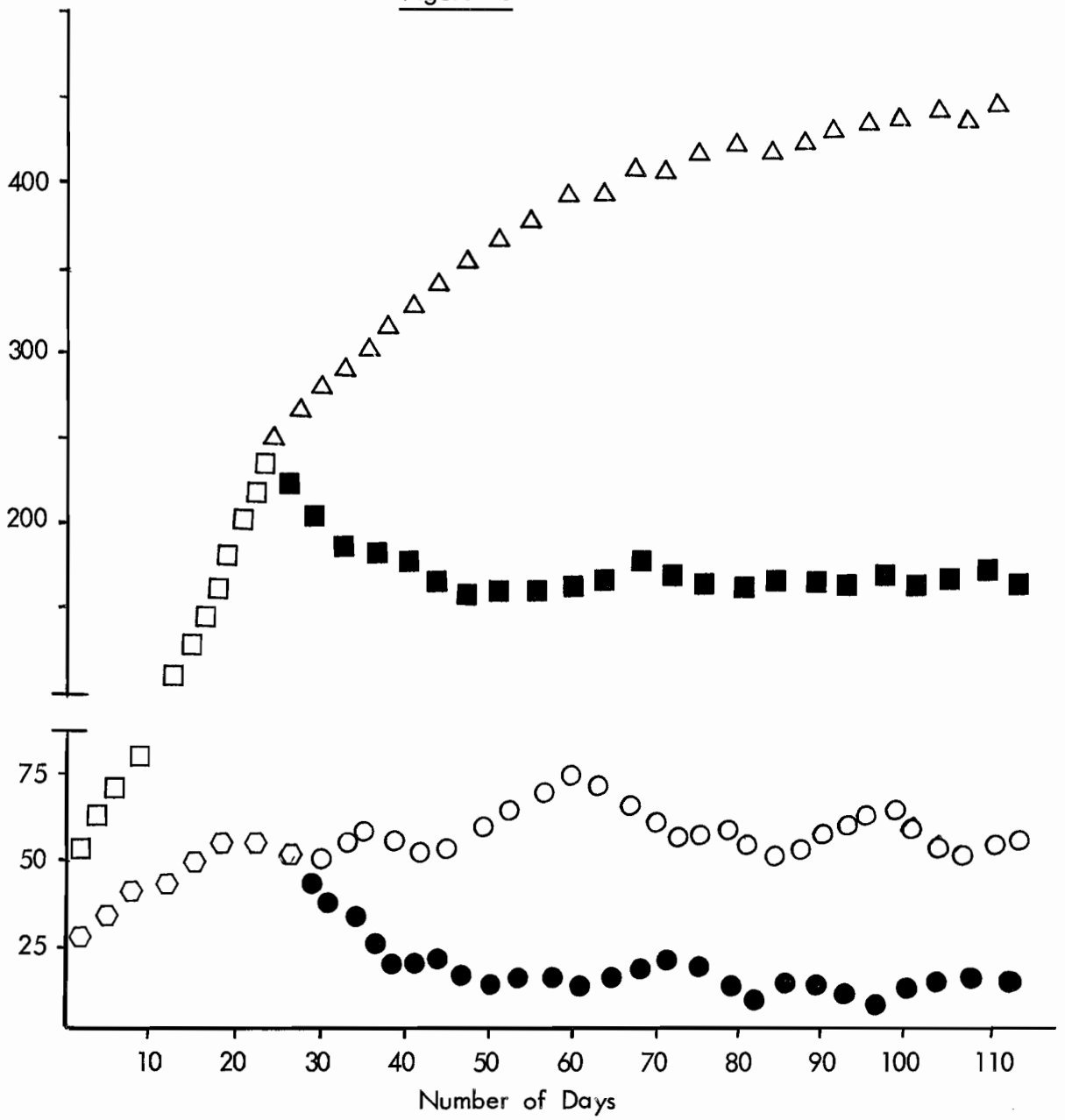
Histological examination of the aortas of the animals on the atherogenic diet showed early changes characterized by intimal proliferation and accumulation of intercellular material (Figure 9 A,B). Fragmentation of the internal elastic lamina was observed in some cases.

Figure 8

The Effect of the "Atherogenic" Diet on Body Weight and Food Consumption of Rats.

The control and experimental groups consist of 12 rats each. Weights of food consumed or body weights during the control period represent the mean of 24 measurements. All other weights represent the mean of 12 measurements.

Figure 8



⬡ Average weights of "Purina" consumed during control period.

● Average weights of "atherogenic" diet consumed by experimental group.

○ Average weight of "Purina" consumed by control group.

□ Average body weight during control period.

■ Average body weight of group on "atherogenic" diet.

△ Average body weight of group on "Purina" diet.

Figure 9

Changes in Aortas of Rats Fed the "Atherogenic" Diet.

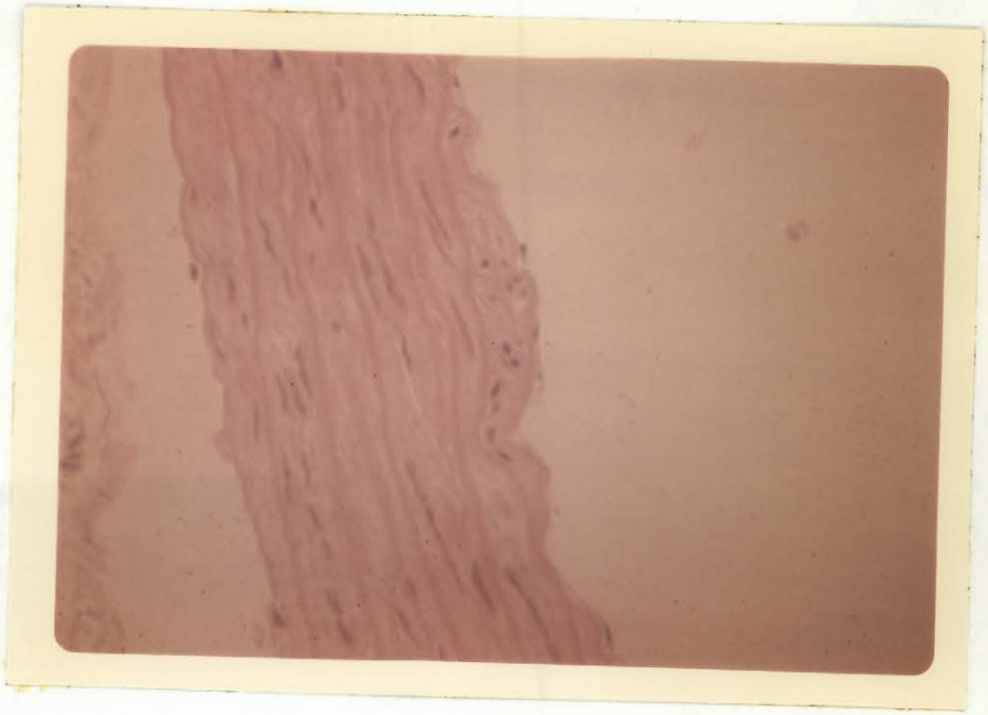
A. Hematoxylin and eosin.

Photomicrograph of rat aorta demonstrating a typical lesion. The intima is to the right, and the proliferation of the intimal cells may be noted. A more normal intimal area is seen directly below the lesion.

B. Higher magnification of the same lesion as in A. In addition to the intimal proliferation, accumulation of intercellular material is evident.

Figure 9

A.



B.

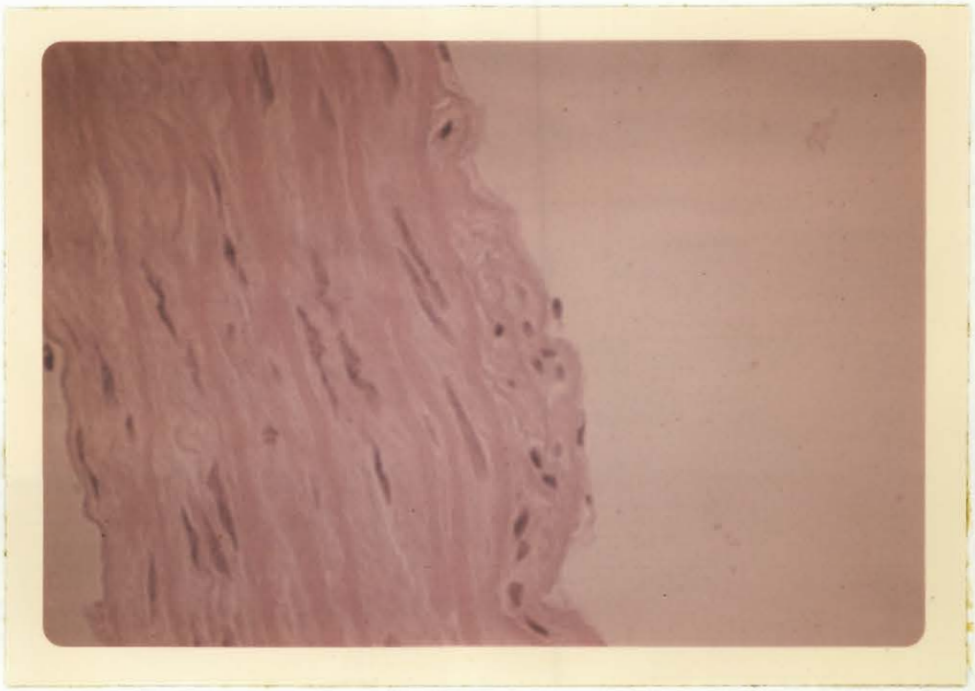
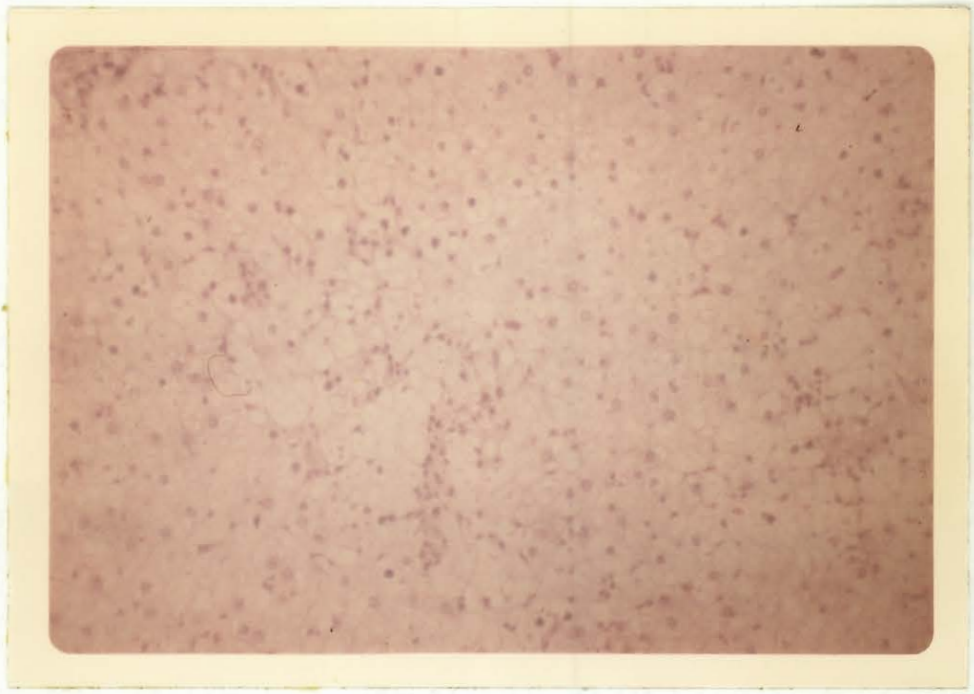


Figure 9C

Changes in the Livers of Rats Fed the "Atherogenic" Diet.



Hematoxylin and eosin staining.

The pronounced fatty infiltration is evident. Nearly all of the cytoplasm of the parenchymal cells has been replaced by fat. The nuclei and cell outlines are clearly visible.

Advanced lesions were not evident. The abdominal aorta in the region of the thrombin injection showed fresh thrombus formation. It is noteworthy that the liver in all the animals on the atherogenic diet showed pronounced fatty infiltration (Figure 9C), while the heart and kidney in some of the animals showed fatty changes.

A summary of the results of octylamine-brucine extraction of rat plasma and the metachromatic and anticoagulant assay of the extracts is given in the following Tables.

Table XVIII indicates the recovery of heparin obtained with the two methods of assay. It is evident that minute amounts of heparin added to serum were satisfactorily estimated.

Table XIX gives the results obtained with the extraction and assay procedures applied to plasma specimens from the control and the atherosclerotic groups before and after the production of thrombosis. It is apparent from Table XIX that the presence of a significant amount of heparin could not be demonstrated in the blood serum of either group of animals.

With these extraction and assay procedures it was not possible to demonstrate the presence of free heparin in arterial blood of the dog or in the venous blood from the ox and the human. As in preceding experiments, however, heparin added in the concentration of 20 µg per ml of plasma could be accounted for satisfactorily by both assay methods.

TABLE XVIII

Recovery of Added Heparin from Rat Plasma.

Heparin added to 1 ml of plasma	No. of Expts.	Assay			
		Metachromatic Method Assay Recovery		Antithrombin Method Assay Recovery	
(μg)		(μg/ml plasma)	(%)	U.S.P. units /ml plasma	(%)**
0	4	0.4 (±0.2)*	-	nil	-
1	4	0.9 (±0.3)	90	nil	-
5	4	5.0 (±0.3)	100	0.4 (±0.1)	64
10	4	8.8 (±0.4)	88	0.8 (±0.2)	64
20	4	18.1 (±0.8)	90	1.9 (±0.2)	76

*Standard deviation

**Calculated on basis of Na heparin added, which assayed at 125 U.S.P. units per mg.

TABLE XIX

Metachromatic and Anticoagulant Activity of Rat Plasma.

Group	Assay	
	Metachromatic Method	Antithrombin Method
	(μg/ml plasma)	(U.S.P. units)
Control group		
(1) Before thrombosis	0.6 (±0.3)*	nil
(2) After thrombosis	0.8 (±0.4)	nil
Atherosclerotic group		
(1) Before thrombosis	0.5 (±0.4)	nil
(2) After thrombosis	0.6 (±0.6)	nil

*Standard deviation

12 animals in each group.

DISCUSSION

The results described have affirmed our anticipation at the outset of the study that the enzymological approach might help to elucidate the metabolic and the chemical changes that underlie the pathogenesis of atherosclerosis. The writer considers that he has established the following premises which of necessity must be affirmed as a basis for valid interpretation of the experimental findings:

First, by studying the enzyme activity of the intima and the media individually, of the normal and atherosclerotic aorta it has been possible to show that the enzyme activity of the two main components of this vessel, particularly that of the intima is altered in atherosclerosis. This is in conformity with, and may afford evidence for, the accepted view that atherosclerosis is primarily a disease of the intima. Furthermore, segregation of the two components permits comparison of the enzyme activity between normal intima and normal media, which may reflect differences in physiological activity, and between atherosclerotic intima and media. Such differences become obscured when the two components are treated together as one tissue.

Second, variability was minimized by setting up rigid clinical and pathological criteria for the selection of cases and the location and type of lesion.

Third, we have demonstrated the advantage of expressing

the enzyme activity on the basis of several, rather than a single criterion. Expressed on the basis of tissue nitrogen, DNA and non-collagen nitrogen, changes per unit mass of within the cells during atherosclerosis would be more apparent than when expressed on the basis of dry weight alone.

Fourth, the enzyme phosphoglucoisomerase in homogenates from human aortas obtained at autopsy was shown to be stable for several days at 4°C. We have also obtained indirect evidence indicating that no significant alteration in PGI activity occurred from the time of death of the patient to the time of assay.

Fifth, the study has shown that the PGI in human aortic tissue is similar in behaviour with that reported by various workers with other tissues.

Sixth, we have demonstrated the desirability of applying the first three points mentioned (i.e. separation of intima and media, clinical and pathological criteria and expression of results in several parameters) to chemical analysis of the connective tissue constituents. Thus, determinations of hexosamine, hexuronic acid, sugars and hydroxyproline in the various parts of the normal and atherosclerotic aorta have been expressed on the basis of dry weight as well as tissue nitrogen and DNA. In this manner, noteworthy changes in connective tissue composition during atherosclerosis were demonstrated particularly in the intima.

Finally, in the special study concerning the possible release of heparin from the normal and atherosclerotic rat during thrombosis, we demonstrated the feasibility of producing atherosclerosis in the rat and we were able to extract and assay minute quantities of added heparin from the rat plasma.

1. Phosphoglucoisomerase Activity in Homogenates of Aortic Tissue.

In a comparative study of the enzyme activity of normal and pathological tissue, the advantage of using the simple tissue homogenate, rather than a purified enzyme preparation from the same tissue, is evident. The point to be established is whether changes in enzyme activity per unit of cell mass had occurred during the development of the pathological process. It is essential therefore to take into consideration, at least initially, all the factors that may influence the enzyme activity. This can be done with the simple homogenate, whereas fractionation and purification may result in variable loss of enzyme and other factors.

One of the first points we established with the homogenate system was whether PGI from human aorta behaved similarly to PGI from other sources. The obtained pH optimum of 7.9 is within the range reported by other workers from a variety of tissues (135). The Km value, 1.1×10^{-3} M, with glucose-6-phosphate as the substrate, is also within the range reported for the enzyme from other sources (103, 141, 142).

Furthermore, with the aid of chromatography, it was possible to demonstrate the production of fructose-6-phosphate with glucose-6-phosphate as the substrate. It is noteworthy that no conversion of fructose-6-phosphate to mannose-6-phosphate, glucosamine-6-phosphate, fructose-1,6-diphosphate or to triose phosphate was demonstrable in the absence of ATP and under the conditions tested. It is reasonable to suppose, therefore, that the rate of production of fructose-6-phosphate from glucose-6-phosphate, in the aortic homogenate system used may be comparable to that which might be obtained with a purer system. This conjecture is reasonable in view of the close agreement between the Km values obtained and those reported for purified PGI from other sources (103, 104, 142).

Another point that we sought to establish, at the outset of the study, was the stability of PGI in the aortic homogenate system. As already indicated, various workers (133,134,135) report that PGI from a variety of sources is quite stable in the cold. Our findings confirmed this for the human aorta. Nevertheless, we had no direct data for the minimum time lag from the time of death of the patient to the time of assay, for obvious reasons. However, several indirect lines of evidence suggest that PGI activity probably underwent no significant alterations during that time. First, there were no major differences in activity for samples obtained at different times, during the interval of 6 - 18 hours after death.

Second, there was no correlation between the time after death that a sample was obtained and its PGI activity over the above

mentioned time interval. Third, there was little variation between the ratios of the activities of the various homogenates (i.e. NI, NM, AI and AM) of the same aortic specimen on storing at 4°C. The last point is particularly relevant with respect to comparative activity studies.

It has been indicated that special attention has been paid to the problem of expressing enzyme activities in pathological vascular tissue, and that several manners of expressing results have been chosen which better reflect the concept of "unit cell mass", than does the tissue weight basis alone. Nevertheless, expressing enzyme activities on the basis of chemical tissue constituents is not the only way the problem may be approached. For example, the suggestion was made to the author that activity may be also expressed in terms of unit area of tissue. However, such an approach would suffer from difficulties of standardization, for it is a common observation that pathological aortas show variable degrees of dilatation of their surfaces. Thus, a unit area of a normal part may not be equivalent to a unit area of a pathological part, and such differences would become even more variable when attempting to evaluate results from different aortas. We therefore felt that expressing the results on the basis of a number of chemical parameters afforded the most satisfactory approach to the problem.

(a) Comparison of PGI activity of the NI with the AI and of the NM with the AM.

The phosphoglucoisomerase activity of the aortic homogenates

of the NI, on the average, was found to be lower than that for the AI expressed on the basis of wet or dry tissue weights (Table XX). This small difference proved not to be statistically significant.

TABLE XX

PGI Activities of Normal and Atherosclerotic Intima Expressed on the Basis of various Parameters.

	Mean values of activity calculated on the following bases:				
Tissue	Wet(1) weight	Dry(2) weight	Tissue(3) nitrogen	DNA(4)	Noncollagen(5) nitrogen
NI	1.42	5.52	46.6	1.11	8.80
AI	1.26	4.69	59.6	1.67	14.97
Ratio of activities (NI : AI)	1 : 0.8	1:0.9	1:1.3	1:1.5	1:1.7
Statistical analysis (NI vs AI)	N.S.	N.S.	p 0.025	p 0.05	p 0.01

(1) - (5): Units as in Tables II, III, VII, VIII and IX respectively.

Expressed on the basis of the tissue content of nitrogen, DNA or noncollagen nitrogen, the AI demonstrates significantly greater PGI activity than the NI. This is readily evident from Table XX by the values of the ratios of NI : AI.

On the other hand, as seen from Table XXI, the media does not show pronounced alterations in PGI activity during atherosclerosis. There is no statistically significant variation between the NM and the AM, no matter on what basis the activity is expressed.

TABLE XXI

PGI Activities of Normal and Atherosclerotic Media Expressed on the Basis of Various Parameters.

	Mean values of activity calculated on the following bases:				
Tissue	Wet(1) weight	Dry (2) weight	Tissue(3) nitrogen	DNA(4)	Noncollagen(5) nitrogen
NM	1.74	5.81	52.4	1.72	6.36
AM	1.75	5.76	52.5	1.49	7.25
Ratio of activities (NM:AM)	1:1.0	1:1.0	1:1.0	1:0.9	1:1.2
Statistical analysis (NM vs AM)	N.S.	N.S.	N.S.	N.S.	N.S.

(1) - (5):Units as in Tables II, III, VII, VIII and IX respectively.

The observed increase in PGI activity of the intima during atherosclerosis, while the media shows little change, is in consonance with the prevailing view that atherosclerosis is primarily a disease of the intima. The writer appreciates, however, that caution is necessary in

assessing the functional significance of this finding, in view of the high phosphoglucoisomerase activity in aortic tissue. It will be recalled, for example, that the hexokinase activity in aortic tissue is only a small fraction of that of PGI and consequently PGI would not appear to be a limiting system, at least with reference to the enzymes of the glycolytic pathway which are reported in the literature. The central position of PGI in the metabolism of the mucopolysaccharide precursors (glucosamine-6-phosphate and glucuronic acid) is noteworthy in this connection, but no definite conclusions may be drawn, as activities of individual enzymes in these pathways have not been reported for aortic tissue. However, it will also be recalled that S^{35} uptake experiments indicate that the metabolic activity of the mucopolysaccharide pathways undergoes a pronounced increase during atherosclerosis (74, 108, 109, 111). Thus, the very high activity of PGI in aortic tissue as well as the demonstrated increase in activity of the intima during atherosclerosis, may be explained by making the conjecture that both of these findings are associated with a very active mucopolysaccharide metabolism in the normal and atherosclerotic aorta.

(b) Comparison of PGI activity of the intima and media in normal parts of the aorta.

From Table XXII it is apparent that in the macroscopically normal portions of the aortas, the media shows a greater PGI activity than the intima when the values are expressed in terms of wet or dry

tissue weight, tissue nitrogen or DNA. However, expressed on the basis of noncollagen nitrogen, the PGI activity is less in the media than in the intima of the normal tissue. Of these, only the difference expressed on the basis of DNA is statistically significant ($p = 0.01$).

TABLE XXII

PGI Activity for Normal Intima and Media Expressed on the Basis of Various Parameters.

	Mean values of activity calculated on the following bases:				
Tissue	Wet(1) weight	Dry (2) weight	Tissue(3) nitrogen	DNA(4)	Noncollagen(5) nitrogen
NI	1.42	5.52	46.6	1.11	8.80
NM	1.74	5.81	52.4	1.72	6.36
Ratio of activities (NI:NM)	1:1.2	1:1.1	1:1.1	1:1.5	1:0.7
Statistical analysis (NI vs AI)	N.S.	N.S.	N.S.	p 0.01	N.S.

(1) - (5): Units as in Tables II, III, VII, VIII and IX respectively.

The reason for the discrepancy of PGI activity between that expressed on the basis of DNA, and that expressed on the basis of collagen, is readily apparent when one compares the values obtained

for the DNA and noncollagen nitrogen content of the normal intima and media (Tables IV and XXXII). Thus, the ratio of noncollagen nitrogen to DNA content is $1:1.4 \times 10^{-2}$ for the NI but prominently increases to $1:2.3 \times 10^{-2}$ for the NM. In other words, assuming that noncollagen nitrogen represents a reliable index of "protoplasmic" protein material and that DNA is a reliable index of the number of cells, then the increase in the ratio indicates that the cells in the NM are larger than the NI. This point is elaborated on later (p. 138).

In view of the foregoing, caution must be excercised in interpreting the physiological role of the significantly greater PGI activity of the NM, as compared to the NI, when the results were expressed on the basis of DNA content. It appears likely that this difference may not indicate a potentially higher metabolic activity by the cells in the normal media, but may simply reflect the larger size of cells in this layer of the aorta.

(c) Comparison of PGI activity of the Intima and Media in atherosclerosis.

It can be seen from Table XXIII that the AM shows a higher PGI activity than AI when the results are expressed in terms of dry or wet weights. As has been pointed out (p. 58) , not too much emphasis should be placed on activities expressed on these bases. The activities expressed in terms of nitrogen, DNA or noncollagen nitrogen

TABLE XXIII

PGI Activities for Atherosclerotic Intima and Media Expressed on the Basis of Various Parameters.

Tissue	Mean values of activity calculated on the following bases: Wet(1) weight	Dry (2) weight	Tissue(3) nitrogen	DNA(4)	Noncollagen(5) nitrogen
AI	1.26	4.96	59.6	1.67	14.97
AM	1.75	5.76	52.5	1.49	7.25
Ratio of activities (AI:AM)	1:1.4	1:1.2	1:0.9	1:0.9	1:0.5
Statistical analysis (NI vs AI)	N.S.	N.S.	N.S.	N.S.	p 0.01

(1) - (5): Units as in Tables II, III, VII, VIII and IX respectively.

are all higher for AI than AM, although the difference is significant only in the case of noncollagen nitrogen. It will also be noted that the difference of the activity expressed in terms of DNA is quite small. This is as might be expected from the previous results (Tables XX - XXII), as the approximate ratio of activities (expressed on the basis of DNA) for NI : AI : NM is 1 : 1.5 : 1.5 and the activity for the NM is roughly the same as for the AM.

Again, the apparent discrepancy in activity, expressed

on DNA basis as compared to noncollagen nitrogen basis, is readily accounted for by the different noncollagen nitrogen to DNA ratios for the two aortic layers (AI = $1:1.2 \times 10^{-2}$, AM = $1:2.0 \times 10^{-2}$). This difference re-emphasizes the importance of measuring activities separately in the intima and the media, and the care that must be taken in interpreting comparisons of enzyme activities of similar tissues which, however, may have varying cell types.

2. Changes in the Aortic Tissues During the Development of Atherosclerosis.

(a) Changes in moisture, nitrogen, lipid, DNA and noncollagen nitrogen content of the normal and atherosclerotic aorta.

The greater moisture content of the NI as compared to the AI (Table V) may be anticipated from the greater proportion of hydrophobic lipid in the AI. There is no statistically significant difference in the moisture content of the NI as compared to the NM (although the latter tends to be somewhat lower), nor between the AI or AM.

The finding that AI has significantly lower nitrogen content than NI tissues generally indicates replacement of protein material by lipid and mucopolysaccharide during the atherosclerotic process. Indications exist that parallel changes occur in the media.

The nitrogen content of the NM was slightly higher than the NI, but the difference was not found statistically significant.

The higer DNA content of the NI as compared to the AI is probably a reflection of the smaller number of cells in the AI. No difference in the DNA content of the normal an atherosclerotic media was observed, indicating that the cell population of this layer was not greatly affected by the atherosclerotic process. NI had a somewhat higher DNA content than NM, indicating a larger number of cells per gram dry weight. This emphasized the diversity in the cell populations of the two layers.

Some interesting relationships were derived by comparing the amounts of DNA to noncollagen nitrogen (Appendix, Table XXXII). Thus, the noncollagen nitrogen to DNA ratio was found to be 1.4×10^{-2} for NI and 1.3×10^{-2} for AI, indicating little relative change in cell size in the intima during atherosclerosis. As already discussed, there is a prominent difference in the cell size between the normal intima and media (ratio of noncollagen nitrogen to DNA for NI = $1:1.4 \times 10^{-2}$ and NM = $1:2.3 \times 10^{-2}$), and a distinct but smaller difference between the cell sizes of the atherosclerotic and normal media (ratio of noncollagen nitrogen to DNA for AI = $1:1.3 \times 10^{-2}$ and AM = $1:1.9 \times 10^{-2}$).

(b) Hexosamine, hexuronic acid and sugars.

The reader will recall that the changes in mucopolysaccharide constituents during atherosclerosis are evident when the values are calculated on the basis of the nitrogen or the DNA content of the tissue, and that they were most pronounced in the intima. Further, the hexosamine and sugars in the tissues follow similar patterns of change, both tending to be increased in the atherosclerotic intima, while hexuronic acid content remained relatively unchanged. Thus, the hexosamine : sugar : hexuronic acid ratio was 1:0.9:0.4 for the NI and was altered to 1.3:1.3:0.4 in the AI, calculated on the basis of tissue nitrogen (the ratios calculated by taking the value of hexosamine content in the NI as unity). Similar relationships were evident when the results were expressed in terms of the DNA content of the tissue. The theoretical ratio of hexosamine to hexuronic acid in the acid mucopolysaccharides is approximately 1:1 and should not be altered by an increase in the acid fraction. On the other hand, neutral mucopolysaccharides contain hexosamine and neutral sugars but are free from uronic acid (76, 82, 84). Since in the AI there was an increase in the hexosamine and the sugar content while the hexuronic acid remained constant, one may infer that in the intima during arteriosclerosis the neutral mucopolysaccharide fraction was increased while the acid fraction has remained unchanged.

It is of interest to compare this finding with the results

of other workers who have determined hexosamine directly in the aortic tissue or isolated MPS from individuals of various ages. It may be recalled that Bertelsen (93, 94) found increases in both the neutral and the acid MPS with ageing. However, the increases were small and the number of cases few. A number of other workers (90, 92, 96, 98) studied the acid MPS fraction and some reported a decrease in hyaluronic acid with ageing. Hexosamine determinations performed directly on the tissue showed an increase with ageing (91, 94) when results are expressed in terms of fat-free tissue. As pointed out (p.46) the main difficulty with interpreting these studies has been the failure to distinguish between ageing and the simultaneous increase in degree of atherosclerosis. Our study may have overcome this difficulty by virtue of the fact that all our cases were from subjects in the sixth and seventh decade of life. Thus, by controlling the age variable, valid conclusions may be drawn about the effect of atherosclerosis per se. Our findings (with respect to the intima) are in general agreement with both the reported (91,94) increase in hexosamine and neutral MPS (93,94) with increasing age, if one makes the reasonable assumption of a parallel increase in degree of atherosclerosis with ageing. However, our results do not indicate an increase in the acid MPS fraction in the AI as compared to the NI (hexuronic acid content of NI:AI equals 1:1.0 on the basis of nitrogen and 1:1.1 on the basis of DNA). This is in contradiction to the increase in the acid fraction

with ageing reported by Bertelsen (93, 94). One explanation for this difference may be that the increase in the acid fraction is more typically on effect of ageing, while the increase in the neutral fraction is more characteristic of the development of the atherosclerotic lesions. Most, although not all, of the histochemical evidence would tend to support this conjecture. The increase in metachromatic substance (thought to represent acid MPS) up to the fourth decade, in the virtual absence of PAS-positive material (thought to represent neutral MPS) (76), is part of this evidence. This view was also supported by the demonstration of the pronounced PAS-positive staining of fibrils in atheromatous plaques (75) in a background of only faint metachromatic reaction. On the other hand, it may be recalled that Curran and Crane (74), using a colloidal iron-PAS combination felt they demonstrated increases in acid MPS during atherosclerosis. Our own results, with particular reference to hexuronic acid, should be interpreted with caution, as the resin hydrolysis method although reducing the destruction of hexuronic acid (95), did not entirely eliminate it. It may be that no definite answer can be given to this problem, until the MPS of the various parts of the normal and atherosclerotic aorta are isolated quantitatively and characterized in a study that has been age controlled.

From the foregoing it is evident that our results indicate that the most striking changes in MPS content during atherosclerosis have taken place in the intima. Changes in the media have been less

impressive. Thus, hexuronic acid showed no statistically significant changes in any of the relationships given in Table XIII, although there is a tendency to decrease in the AM as compared to NM. Similarly, hexosamine and sugars showed only small changes when the NM was compared to the AM or the NI was compared to the NM, but, as may be anticipated, both the hexosamine and sugar content of the AI was greater than that of the AM (the differences were statistically significant when the values were expressed on the basis of tissue nitrogen - Tables XI and XV). These changes again suggest an increase in neutral MPS content of the AI as compared to the AM.

(c) Hydroxyproline

As noted from Table XIX the hydroxyproline content of the AI significantly increased with respect to the NI. This agrees with the findings of Bertelsen (76) who reported a pronounced increase in hydroxyproline in sclerotic aortic tissues. Our findings also agree with the same author's work (76) on the higher hydroxyproline content of the NI as compared to NM. There is practically no change in the hydroxyproline content of the normal and atherosclerotic media, indicating that also with respect to collagen content, the atherosclerotic process involves mostly the intima. The significantly higher hydroxyproline content of AI as compared to the AM also supports this point.

In the foregoing the assumption has been made that the

hydroxyproline content of the tissue accurately reflects the collagen content. This is generally valid (140), although it must be kept in mind that elastin also contains hydroxyproline to the extent of about one per cent (144). Thus, in tissues such as the media, in which a high content of elastin has been demonstrated by histochemical methods, the value of collagen content calculated on the basis of hydroxyproline should probably be regarded as being somewhat higher than the true value.

3. The Postulated Release of Heparin During Thrombosis in the Rat.

The efficacy of the common clinical practice of administering heparin to patients with certain types of thrombosis is now held in question (145, 146). The current trend of scepticism among clinicians has arisen from the large volume of conflicting morbidity and mortality statistics. Of particular relevance to this question is the postulate that heparin may be released in the circulation during thrombosis, as a neutral anticoagulant response (119,121) to counteract the thrombotic episode. Free heparin is well known to inhibit coagulation *in vitro* and *in vivo* and thrombosis *in vivo* (147, 148). Engelberg (149) has suggested that heparin may be present in the blood in a dissociable combination with protein and, as such, may function as an anticoagulant, *in vivo*. This postulate, however,

remains to be established.

With our octylamine and brucine extraction procedure for free heparin we have demonstrated the presence in blood of traces of metachromatic material but not anticoagulant activity. Extracts of plasma from atherosclerotic and normal rats after thrombosis, likewise, showed no indication of anticoagulant activity. We conclude therefore that under the conditions tested free heparin is not released during arterial thrombosis.

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APPENDIX

TABLE XXIV

Hexosamine Calculated on the Basis of Tissue Nitrogen.

Case No.	NI	NM	AI	AM
	(μg hexosamine/mg per cent tissue nitrogen)			
5	96.2	117.3	91.7	88.0
6	90.2	110.7	192.7	93.9
7	96.6	115.7	118.7	87.3
8	157.1	115.6	254.2	107.0
9	104.6	83.9	153.5	100.9
10	88.1	96.4	88.5	84.7
11	116.3	94.8	172.2	97.7
12	71.3	71.2	66.4	61.0
13	107.5	102.4	142.6	116.4
14	116.7	99.0	147.9	130.4
15	145.9	95.1	172.9	107.1
Average	108.2	100.2	145.7	97.7

TABLE XXV

Hexosamine Calculated on the Basis of DNA.

Case No.	NI	NM	AI	AM
	(μg hexosamine per μg DNA)			
5	3.1	2.7	2.0	1.6
6	1.7	1.9	3.8	1.5
7	1.5	2.4	4.3	2.6
8	4.3	3.6	5.6	2.6
9	2.7	2.9	5.3	3.4
10	2.7	3.6	4.8	2.5
11	1.8	4.2	2.9	3.5
12	1.5	2.3	1.9	3.3
13	3.8	4.3	3.5	4.1
14	4.1	3.8	4.0	2.9
15	2.8	3.5	3.6	2.0
Ave.	2.7	3.2	3.8	2.7

TABLE XXVI

Hexuronic Acid Calculated on the Basis of Tissue Nitrogen.

Case No.	NI	NM	AI	AM
	(μg hexuronic acid/mg per cent tissue nitrogen)			
5	46.48	28.42	35.00	24.00
6	84.73	49.66	92.58	40.44
7	60.34	46.22	33.85	35.86
8	9.15	95.31	42.50	50.00
9	54.46	25.74	34.90	29.60
10	27.78	37.24	22.10	22.40
11	38.00	37.98	44.90	30.58
12	28.84	27.37	22.76	23.47
13	31.96	40.44	41.03	37.76
14	30.98	39.13	37.89	29.62
15	76.02	41.63	63.14	37.59
Ave.	44.43	42.65	42.79	32.85

TABLE XXVII

Hexuronic Acid Calculated on the Basis of DNA.

Case No.	NI	NM	AI	AM
	(μg hexuronic acid/μg DNA)			
5	1.00	0.66	0.75	0.45
6	1.62	0.86	2.09	0.65
7	0.96	0.96	1.23	1.08
8	0.25	2.98	0.93	1.21
9	1.42	0.90	1.20	0.99
10	0.85	1.39	1.21	0.63
11	0.58	1.70	0.76	1.33
12	0.62	0.89	0.64	1.26
13	1.14	1.72	0.98	1.33
14	1.09	1.51	1.03	0.67
15	1.46	1.55	1.30	0.72
Ave.	1.00	1.37	1.10	0.94

TABLE XXVIII

Sugar Content Calculated on the Basis of Tissue Nitrogen.

Case No.	NI	NM	AI	AM
	(μg/sugar/mg per cent tissue nitrogen)			
5	101.8	87.0	93.8	74.5
6	100.3	92.7	161.5	73.5
7	107.1	76.6	81.4	76.5
8	169.1	74.2	282.3	78.0
9	73.0	52.1	120.5	73.9
10	79.2	85.9	102.3	54.3
11	80.2	71.7	162.8	87.5
12	59.4	51.3	74.5	67.8
13	98.4	91.4	129.2	90.5
14	93.0	108.7	114.5	103.7
15	156.0	142.0	177.9	168.8
Ave.	101.8	84.9	136.4	86.3

TABLE XXIX

Sugar Content Calculated on the Basis of DNA.

Case No.	NI	NM	AI	AM
		(μg sugar per μg DNA)		
5	2.43	2.02	1.99	1.37
6	1.93	1.61	3.69	1.16
7	1.71	1.59	2.97	2.29
8	4.34	2.36	6.07	1.89
9	1.91	1.99	4.15	2.48
10	2.43	4.98	5.63	1.49
11	1.22	3.21	2.74	3.24
12	1.28	1.67	2.12	3.65
13	3.16	3.68	3.11	3.18
14	3.28	4.17	3.13	2.34
15	3.06	5.30	3.66	3.20
Ave.	2.43	2.96	3.57	2.39

TABLE XXX

Hydroxyproline Calculated on the Basis of Tissue Nitrogen.

Case No.	NI	NM	AI	AM
	(μg hydroxyproline/mg per cent tissue nitrogen)			
5	309.6	189.4	356.0	193.4
6	309.6	219.5	581.2	217.5
7	369.8	144.1	250.5	184.7
8	291.5	173.4	779.7	208.8
9	215.4	182.4	288.4	201.8
10	305.9	215.2	400.0	184.0
11	275.6	192.4	466.7	196.2
12	130.2	132.1	288.8	160.2
13	280.4	250.0	386.8	212.1
14	347.1	215.4	349.3	217.7
15	441.8	168.3	472.9	182.1
Ave.	297.9	189.3	420.0	196.2

TABLE XXXI

Hydroxyproline Calculated on the Basis of DNA.

Case No.	NI	NM	AI	AM
	(μg hydroxyproline/μg DNA)			
5	9.85	4.43	7.64	3.59
6	6.02	3.81	11.03	3.49
7	5.88	3.00	9.12	5.89
8	7.93	5.41	17.04	5.06
9	5.60	6.36	9.92	6.76
10	9.39	8.03	21.83	5.13
11	5.18	8.47	7.88	12.11
12	2.80	4.29	8.17	8.59
13	10.00	10.50	9.39	7.45
14	12.21	8.30	9.54	4.91
15	8.49	6.27	9.74	3.46
Ave.	7.58	6.26	11.03	6.04

TABLE XXXII

Noncollagen Nitrogen (Total N-Collagen N) µg/mg Tissue Dry Weight.

	Case No.	NI	NM	AI	AM
Total N	5	108.20	109.80	80.70	100.30
Collagen N(µg)		44.55	27.66	39.63	25.80
		63.7	82.1	41.1	74.5
Total N	6	101.50	102.50	61.60	114.00
Collagen N(µg)		42.42	29.92	47.61	32.98
		59.1	72.6	14.0	81.0
Total N	7	116.00	127.00	91.00	111.00
Collagen N(µg)		57.05	24.34	30.32	28.99
		59.0	102.7	60.7	82.0
Total N	8	111.50	128.00	59.00	114.00
Collagen N(µg)		43.23	29.53	61.18	31.65
		68.3	98.5		82.4
Total N	9	130.00	136.00	86.00	114.00
CollagenN(µg)		37.24	32.98	32.98	30.59
		92.8	103.0	53.0	83.4
Total N	10	135.00	138.00	131.00	131.00
Collagen N(µg)		54.93	39.50	69.69	32.05
		80.1	98.5	61.3	99.0
Total N	11	135.00	134.00	54.00	100.00
Collagen N(µg)		49.50	33.80	36.60	43.60
		85.5	100.2	17.4	56.4
Total N	12	129.0	156.0	116.0	118.0
Collagen N(µg)		22.4	27.4	44.6	25.2
		106.6	128.6	61.4	92.8
Total N	13	107.0	126.0	68.0	116.0
Collagen N(µg)		40.0	42.0	34.0	32.8
		67.0	84.0	34.0	83.2
Total N	14	102.0	104.0	71.0	79.0
Collagen N(µg)		47.2	29.8	33.1	22.9
		54.8	74.2	37.9	56.1
Total N	15	98.0	123.0	70.0	112.0
Collagen N(µg)		57.7	27.6	44.1	27.2
		40.3	95.4	25.9	84.8
Average		70.7	94.5	37.0	78.8