

THE ROLE OF LIPIDS AND LIPOPROTEINS IN ATHEROSCLEROSIS

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Atherosclerosis is generally considered to be the major disease of this era. Its consequences in the coronary, cerebral, and peripheral arteries, in the form of occlusive phenomena, are responsible for more death and disability than any other disease. In spite of much study and research there is still no agreement concerning the sequence of pathogenetic events, etiology, or treatment of atherosclerosis. The not-too-rare occurrence of coronary artery occlusions (almost always a consequence of atherosclerosis) in young men from 20-40 years of age testifies to the fallacy of the still prevalent idea that atherosclerosis is a problem of the aged or senile. For the male it is a real threat in the prime of life. The absence of the disease at autopsy in many persons who have survived to be octogenarians is eloquent evidence that atherosclerosis should be regarded as a disease and not as an inevitable consequence of ageing.

For many years it has been known that cholesterol (and its esters), phospholipids, and fatty acids are prominent components of early atheromatous lesions, whereas secondary pathological processes supervening may alter the relative preponderance of certain of these substances in late lesions. Some

* This work was supported (in part) by the Atomic Energy Commission and the United States Public Health Service.

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workers have indicted exogenous (dietary) cholesterol for the production of the disease, while others have denied the significance of this source of cholesterol on the basis that large quantities of cholesterol may be endogenously synthesized from such precursors as water and acetate. The suggestion has also been made that atherosclerosis is the result of the chylomicronemia which follows meals. No agreement has been in sight, largely because no objective means for the evaluation of conflicting ideas have been available.

In any effort to formulate a concept of this disease process one must take cognizance of certain well-established clinical and experimental observations and determine whether a new concept is in harmony with such observations. We shall describe briefly several pertinent features of atherosclerosis and then attempt to show that our experiments and the ideas we have evolved therefrom do provide a reasonable picture of some aspects of this disease.

A. Blood cholesterol levels: Myriad determinations of blood cholesterol levels have been made by workers all over the world in an effort to show whether or not the blood cholesterol (free or esterified) is elevated in those patients who develop atherosclerosis. The result remains highly controversial. Some workers claim a significant elevation in blood cholesterol level for a majority of patients with atherosclerosis, while others debate this finding vigorously. Certainly a tremendous number of people who suffer from the consequences of atherosclerosis show blood cholesterol levels in the accepted normal range. There does exist a group of disease states (including diabetes mellitus, nephrotic nephritis, severe hypothyroidism, and essential familial hypercholesterolemia) in which the blood cholesterol level may be appreciably elevated. Such patients do show, in general, earlier and more severe atherosclerosis than the population at large. Yet no bridge has been established between this relatively small group and the vast population of individuals with normal blood cholesterol levels from which the majority of victims of atherosclerotic disease are drawn.

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B. Experimental Atherosclerosis in the Rabbit: Anitschow (1)

demonstrated that cholesterol feeding in the rabbit results in the production of hypercholesterolemia and of a lesion in the arteries greatly resembling the human atherosclerotic lesions. Controversy has existed in the literature since Anitschow's work, criticism of the significance of his experiments having centered largely around the fact that the rabbit is herbivorous and hence ordinarily ingests essentially no cholesterol. It is our opinion that pertinent clues to the human problem are obtainable from cholesterol-induced atherosclerosis in the rabbit. We shall endeavor to detail the observations and ideas on which this opinion is based below.

C. Incidence of Atherosclerosis in Humans: The common medical knowledge that the incidence of atherosclerosis, its severity, and its complications increase, in general, with age must be accounted for in any overall concept of this disease. This must be done while still reckoning with the observations that severe atherosclerosis may be seen in young individuals, especially males, and that at autopsy many persons of all age groups may be free of atherosclerosis. Another unexplained but striking fact is the occurrence of coronary artery occlusions (secondary to atherosclerosis) in males far more frequently than in females, this differential being most pronounced in the age group below 40 years and decreasing steadily with increasing age above 40 years.

It is reasonably certain that the variations in analytical blood cholesterol levels in the groups just discussed fall far short of affording an adequate explanation of the facts regarding incidence of the disease.

D. Occurrence of Atherosclerosis in Association with Diabetes Mellitus:

As a group diabetic patients are more susceptible to early and severe atherosclerosis than is the population in general. It has been stated by authorities on diabetes that atherosclerosis and its complications represent the major problems

facing diabetics now that insulin is available to prevent fatalities due to the diabetes itself. Hypercholesterolemia alone does not account for the extraordinary susceptibility of diabetics to atherosclerosis.

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Some two years ago the present group of authors undertook a physico-chemical investigation of those giant molecules of serum which may consist of cholesterol, its esters, phospholipids, fatty acids, and protein as building blocks. The basic premise was that it is entirely possible that a defect might exist in certain of these giant molecules, which could be responsible for the development of atherosclerosis, whereas the mere analytical levels of any of the building blocks in serum might be of little or no significance. Thus, in a sense, it would not necessarily be any more logical to study the total level of serum cholesterol if we are interested in a particular molecule containing cholesterol than it would be to study serum alanine or glycine levels if it were serum albumin about which we are concerned. The instrument found by us to be of greatest service in this research has been the preparative and analytical ultracentrifuge (Spinco Model E). Gofman, Lindgren, and Elliott (2) and Lindgren, Elliott, Gofman, and Strisower (3) have explained the ultracentrifugal situation with respect to the lipids and lipoproteins of rabbit and human serum and have shown how the ultracentrifuge may be used to characterize certain physico-chemical properties of these components in the native state. Our subsequent work has revealed that a considerable diversity of components exists in the low-density group heretofore known as the B₁-lipoprotein. Since the research with rabbit atherosclerosis gave us pertinent leads with respect to the human problem, this aspect of the work will be described first.

Serum Lipid and Lipoprotein Changes in the Rabbit Developing Atherosclerosis

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Normal rabbits (3) show a lipoprotein of hydrated density 1.03 gms/cc

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in their serum. This lipoprotein contains approximately 30% cholesterol* by weight. Ultracentrifugally it appears as a single component of flotation rate between 5 and 8 Svedberg units under our specified conditions.**

On feeding rabbits three grams of cholesterol per week a highly interesting sequence of events occurs, which we believe has a direct bearing on the development of atherosclerosis in these animals. The initial increment in serum cholesterol during the feeding is manifested by an increase in the concentration of the previously existing 5-8S_f component. The level of this component may increase as much as four-fold. In other rabbits, however, following the initial increase in level of the 5-8S_f component, a series of new cholesterol-bearing giant molecules appear in the serum, including several detectable components, of S_f class 10-30 (in some cases components of even higher S_f values appear). Meanwhile as these new components develop the level of the 5-8S_f molecule is maintained at an approximately constant value, the serum cholesterol increment thereafter being essentially all in the form of the molecules of higher S_f values. Any particular rabbit may show any number (from none to all) of the new components in spite of the fact that this animal receives the same maintenance ration of cholesterol as do all the other animals. Sera or sodium chloride solutions containing high concentrations of the molecules just described scatter light intensely (are turbid). This is worthy of note since many authors erroneously

* All analytical cholesterol determinations were made by the method of Schoenheimer and Sperry.

** Hereinafter wherever molecules move against a centrifugal field (as they do when they are of lower density than that of the medium in which they are dissolved) we shall refer to flotation, instead of using the more cumbersome term, "negative sedimentation". The Svedberg unit (1S equals 10⁻¹³ cm/sec/dyne/gm.) will be used. Thus a molecule described as having a flotation rate of 20S units under our specified conditions will be referred to as a molecule of the 20S_f class, etc.

All the runs reported in this communication were made at a temperature of 27 ± 2°C in a sodium chloride solution of density 1.0625 (unless otherwise stated), using low-density substances previously isolated by differential preparative ultracentrifugation. Flotation rates have not been converted to a value corresponding to the S_{20,w} value of sedimentation runs, since we feel it may ultimately prove more valuable to retain the data in the form obtained rather than to add much unnecessary calculation and back calculation. No conclusions reported here will be influenced by corrections for concentration, viscosity changes due to slight temperature difference between runs or by the Ogston-Johnston effect, since such corrections are outside the range involved in our consideration.

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attribute the turbidity to the presence of chylomicrons. In fact at no time in the course of cholesterol feeding is a large fraction of the cholesterol in rabbit serum transported in the form of chylomicrons. The literature reports (4),(5) which indicate that chylomicrons contain cholesterol may be in error because the methods of obtaining the chylomicron fraction for analysis would allow considerable contamination with the much lower molecular weight molecules we have described in this section.

The new components that appear in rabbit serum with cholesterol feeding are differentiable by their flotation rates, and in addition by virtue of the fact that their hydrated densities are 1.01 and less, in contrast to the value 1.03 for the molecules of the 5-8S_f class.

The entire group of rabbits was autopsied after fifteen weeks of cholesterol feeding. It was found that those rabbits failing to develop high levels of the components of S_f greater than 5-8 units showed no gross atherosclerosis or showed minimal atherosclerosis, whereas mild to severe atherosclerosis developed in those with high concentrations of the molecules of the S_f10-30 class (See Figures 1 and 2). From the observation that all the rabbits attained comparable levels of the 5-8S_f component but showed widely varying degrees of atherosclerosis one can suggest that probably this component is not a guilty one. On the other hand the correlation between the development of severe atherosclerosis and the presence in blood of high concentrations of components of the S_f10-30 class suggests that at least some of these components either are the molecules which deposit in atheromatous plaques or are a reflection in the blood of the metabolic abnormality which results in cholesterol-induced atherosclerosis. Direct evidence as to the deposition of these molecules in plaques

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is now being obtained by a combination of the ultracentrifugal approach with labeling of the various lipid and lipoprotein components with P^{32} incorporated into the phospholipid and H^3 incorporated into the cholesterol.

Components of S_f greater than 8S do not appear in serum until 30-40 days after the initiation of cholesterol feeding and further, such components do not appear in appreciable concentration until the total serum cholesterol has reached approximately 200-250 mg%. It has been known a long time that experimental rabbit atherosclerosis shows a "latent" period of about 40 days after initiation of feeding and that atherosclerosis is rare or minimal in experiments of this duration in rabbits whose total serum cholesterols never rise above 250 mg%. The correlation between our observations and the older data lend further plausibility to the suggestion that the molecules of the S_f 10-30 class are those intimately involved in the production of atherosclerosis.

One point is worth noting with respect to the size of these molecules. Flotation rates higher than 8 S_f do not infer that the components moving with such rates are of higher molecular weight than the 8 S_f molecules. For two molecules of density 1.00 and 1.03 floating in a medium of density 1.06, there will be approximately a two-fold difference in flotation rates with no difference in molecular weights, assuming identical shape factors. The shape factors and molecular weights are now being determined upon isolated ultracentrifugal components from rabbit serum by the supplementary study of their diffusion and viscometric properties.

Human Atherosclerosis

In parallel with the studies of rabbit hypercholesterolemia and atherosclerosis an investigation of the types of low-density molecules present in human serum has been carried on, including those present in individuals with and without known atherosclerotic disease. It was of course obvious to search for possible similarities in the mechanism of cholesterol transport in the human on ad libitum

feeding and in the rabbit fed cholesterol. The correlation between the findings in the human and the rabbit appear even more extensive than might have been hoped for and enable us to present a somewhat unified concept of certain aspects of the nature of atherosclerosis in both species.

There are present in the isolated low density group of molecules from many human sera components of flotation rate (under our specified conditions) greater than 70S, which may correspond to the class of chylomicrons, and in addition, components of hydrated densities less than 1.00 whose flotation rates are between 40 and 70S units. These components are markedly influenced by the relationship between the time of drawing the blood samples and the time and character of previous meals. For the present discussion of atherosclerosis no further reference will be made to these components. There is also present in every one of some 600 sera studied at least one low-density lipoprotein, of S_f value between 3 and 8 units (the S_f of this component varies from individual to individual), the hydrated density of which is in the range 1.03-1.04 gms/cc. Over short periods (few days) the level of and properties of this component appear uninfluenced by previous meals.

In addition to the components just described there are present in some sera, but not in all sera, low-density lipid and lipoprotein components, containing cholesterol, with flotation rates (under our specified conditions) in the S_f 10-20 class (See Figure 1). These components are easily differentiated from those constituting part of the lipemia of meals. It has been possible to show by runs of flotation versus density of medium that the components of higher S_f values are of lower hydrated densities than the major low-density molecules of the 3-8 S_f class. By differential ultracentrifugation in solutions of graded density we have been able to isolate the individual molecular species in a state of reasonable

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ultracentrifugal homogeneity and to do some studies of chemical composition. Molecules in the $S_f 10-20$ class contain approximately 30% cholesterol by weight, but contain little or no protein in contrast to the major low density group of $3-8S_f$ class which show a protein content of 25% by weight.

Study of several groups of individuals with respect to the presence of molecules of the $S_f 10-20$ class indicates that the presence of these molecules in the serum of humans is related to the development of atherosclerosis. The following groups of individuals have been investigated:

- a. Young women without known disease from 20-40 years of age.
- b. Young men without known disease from 20-40 years of age.
- c. Women without known disease from 40-70 years of age.
- d. Men without known disease from 40-70 years of age.
- e. Diabetic females without additional known disease from 25-70 years of age.
- f. Diabetic males without additional known disease from 35-70 years of age.
- g. Female patients with a proven myocardial infarction. (age group 50-70)
- h. Male patients with a proven myocardial infarction. (age group 30-70)

Since over 95% of myocardial infarctions occur in individuals whose coronary arteries are atherosclerotic, a group of patients who have had an unequivocal episode of this type should represent excellent material for the evaluation of the significance of $S_f 10-20$ molecules with respect to atherosclerosis. To preclude the criticism that metabolic upsets attendant during the period of recovery from an acute infarction might alter the picture, or that any drug therapy might do the same, no cases were studied unless the infarction had occurred at least six weeks before blood was drawn for study. The criteria required for inclusion in this group were (a) a typical clinical history of a myocardial infarction, (b) typical laboratory findings during the episode, and

(c) electrocardiographic changes characteristic of myocardial infarction. Patients in borderline categories with respect to any of the criteria were excluded. Patients with myocardial infarction and co-existent hypertension were excluded in the effort, for the present, to eliminate any obscuring of the data by the hypertensive state. In all, 104 patients with myocardial infarctions, including 87 males and 17 females, were chosen for this study.

The data in Figures 3 and 4 summarize all the findings for all of the groups examined with respect to the presence of and concentration of molecules of the S_{f10-20} class. The following conclusions are drawn from these data:

a. The incidence of occurrence of measurable concentrations of molecules of the S_{f10-20} class is significantly higher in males from 20-40 years of age than in females of the same age group. Assuming that these S_{f10-20} molecules reflect the metabolic disturbance which results in atherosclerosis, these data are in accord with the fact that females of this age group are much less likely to show significant atherosclerosis than are the corresponding males.

b. In the age group over 40 years, normal males and females both show significant increases in the incidence of occurrence of measurable concentrations of molecules of the S_{f10-20} class as compared with the corresponding younger age groups. Further the differential between the sexes in the older age group is lessened compared with that in the younger age group. These observations are both consistent with the clinical observations that atherosclerosis increases in frequency with age in both sexes, and that the differential decreases with increasing age.

c. The data indicate a probably higher incidence of occurrence of measurable concentrations of molecules of the S_{f10-20} class in diabetics than in the normals of the corresponding age groups. However, in certain of the diabetic categories larger numbers of cases will be helpful in establishing significance.

d. One hundred and one out of 104 patients with proven myocardial infarction show the presence of molecules in the S_{f10-20} class in measurable con-

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centration. This would be fully anticipated if the S_{f10-20} class of molecules is directly related to the development of atherosclerosis.

The observation that approximately 50% of presumably normal individuals in the age group over 40 years show appreciable concentrations of S_{f10-20} molecules in their serum is expected from known pathological data (6) which indicate that approximately this percentage of people in this age group are developing atherosclerosis, even though the disease is not clinically evident. The same type of reasoning holds for the other groups studied, including the diabetics.

In the rabbit, components analogous in many respects to the S_{f10-20} class of molecules of humans, appear in serum as the result of dietary overloading with cholesterol. It was of interest, therefore, to know whether the dietary cholesterol intake might alter the level of concentration of the S_{f10-20} molecules in persons whose serum contained them. Our preliminary study of a group of 20 patients whose diet we have restricted in cholesterol and fats has demonstrated that the concentration of the S_{f10-20} class of molecules is definitely reduced or even brought down to a level below resolution ultracentrifugally in 17 of the cases studied within two weeks to one month.

The Relationship between Serum Cholesterol Levels and the Presence of S_{f10-20}

Molecules in Serum:

As mentioned earlier analytical blood cholesterol levels have proven highly unsatisfactory as a measure reflecting the occurrence or progress of atherosclerosis. We believe that our data may indicate the reason for this. A comparison of blood cholesterol levels as determined analytically (Schoenheimer-Sperry method) with the presence or absence of molecules of the S_{f10-20} class reveals that, although there is a general trend toward increased

frequency of occurrence of such molecules in sera with cholesterols over 200 mg%, this is by no means a universal finding. It is quite common, also, to find sera with cholesterol levels well below 200 mg% with appreciable or high concentration of molecules of the S_f 10-20 class. In fact several sera studied with cholesterol levels between 120 and 140 mg% show appreciable concentrations of these molecules. Further, it is common to find sera with cholesterol levels well over 200 mg% cholesterol without showing any measurable concentration of the S_f 10-20 class of molecules. At a particular cholesterol level one person may show 25% of the total serum cholesterol in the form of S_f 10-20 molecules, whereas another person may show essentially none in this form. It should also be noted that the detection of the S_f 10-20 molecules when present in low concentration depends upon the concentration effected in our preliminary ultracentrifugal purification and upon the sensitivity of the optical method of detection subsequently used. We can readily detect 5 mg% of S_f 10-20 molecules by our methods (representing approximately 1 mg% of cholesterol in this fraction). Thus a significant concentration of molecules of the S_f 10-20 class may be present in serum and still represent numerically, but not physiologically, an insignificant fraction of the total serum cholesterol. These facts help explain why it has not been possible for previous workers to reach any definite conclusions concerning atherosclerosis by studying analytical cholesterol values.

The patients with hypercholesterolemia (over 300 mg%), drawn from the individuals without known disease, from the diabetics, and from the patients with myocardial infarctions show no essential differences ultracentrifugally in the nature of the molecules transporting cholesterol, but instead show generally an increase in the quantity of cholesterol of serum bound in the form of the S_f 10-20 class of molecules. However, many normocholesterolemic individuals

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may carry much more cholesterol in this fraction (S_f10-20 molecules) than do the hypercholesteroleemics. Thus since normocholesterolemic individuals and hypercholesterolemic individuals may have appreciable concentrations of S_f10-20 molecules, it is understandable that individuals of both these groups should develop atherosclerosis, assuming our thesis of the relation of S_f10-20 molecules to atherosclerosis to be correct. This would provide, then, a missing link between these groups that had not been available from the study of analytical cholesterol levels.

Summary:

The mechanism of cholesterol transport in the serum of rabbits and humans via giant lipid and lipoprotein molecules of low-density has been characterized. In both species there exist classes of molecules of higher S_f rate and lower density than the major group of cholesterol-bearing lipoproteins. The evidence indicates that the lower density of the molecules of higher S_f values is at least partly due to a lower content of protein per molecule.

Evidence implicating the cholesterol-bearing molecules of the S_f10-30 class in the production of cholesterol-induced atherosclerosis in the rabbit has been presented.

A study of 104 patients with proven myocardial infarctions reveals an almost universal occurrence of cholesterol-bearing molecules of the S_f10-20 class (a class of molecules similar in many respects to the S_f10-30 class in rabbits) at fairly high levels in the blood. All categories of normal humans studied show a lower frequency of occurrence of measurable concentrations of S_f10-20 molecules than do the myocardial infarction patients (a group of patients who almost all have coronary artery atherosclerosis). The findings in the groups other than the myocardial infarction group are also consistent with the expected incidence of atherosclerosis in such groups.

Preliminary evidence indicates that exogenous cholesterol in the human as well as in the rabbit is a factor in influencing the blood level of molecules of the S_p10-20 class.

Studies are now in progress with other categories of patients who develop atherosclerosis to a degree beyond that for supposed normal individuals of corresponding ages. These categories include hypertensive patients, patients with the anginal syndrome but without proven infarctions, nephrotic patients, and hypothyroid patients. In addition long-term studies of the effect of diet, with and without adjunctive drugs as thyroid, lipotropic factors, and possibly sex hormones, on the blood level of molecules of the S_p10-20 class are continuing. All these groups should help further evaluate the role of these giant molecules in the development of atherosclerosis.

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The authors wish to acknowledge with gratitude the generous and invaluable advice and assistance given by Professors Hardin B. Jones and John H. Lawrence.

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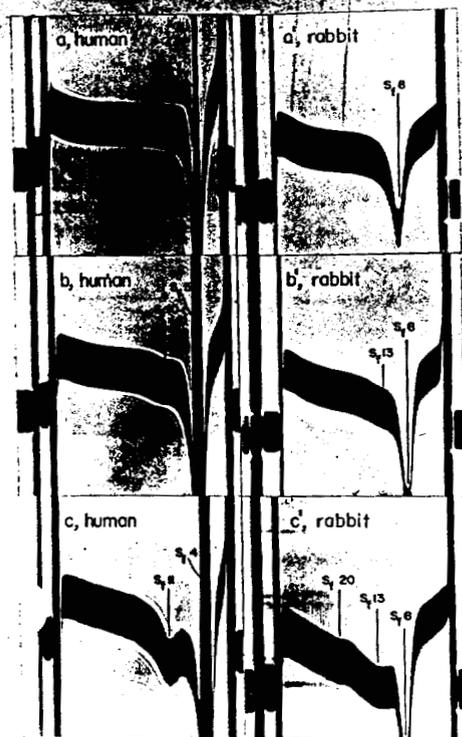
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Figure 1. Ultracentrifugal flotation diagrams of lipids and lipoproteins of the human and rabbit

The normal rabbit (1a') shows only a lipoprotein of the S_f 5-8 class, but during cholesterol induction of atherosclerosis develops additional components of the S_f 10-30 class (1b' and 1c'). The human may show only a single lipoprotein of the 3-8 S_f class (1a), or components of the S_f 10-20 class may be present in variable degree (1b and 1c). The vertical bar through the main inverse peak in (1a, 1b, and 1c) represents a region of refractive index gradient in the cell so great that an entering light beam is thrown out of the optical system. The presence of this bar does not interfere with the resolution of the S_f 10-20 components.

All analytical runs were made at a rotor speed of 52,640 R.P.M. in a cell of 0.8 cc capacity.



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Figure 2. Plot showing the relationship of severity of atherosclerosis (as determined by autopsy) to the concentration of S_F5-8 and S_F10-30 classes of molecules. Each number (plotted horizontally) refers to an individual rabbit.

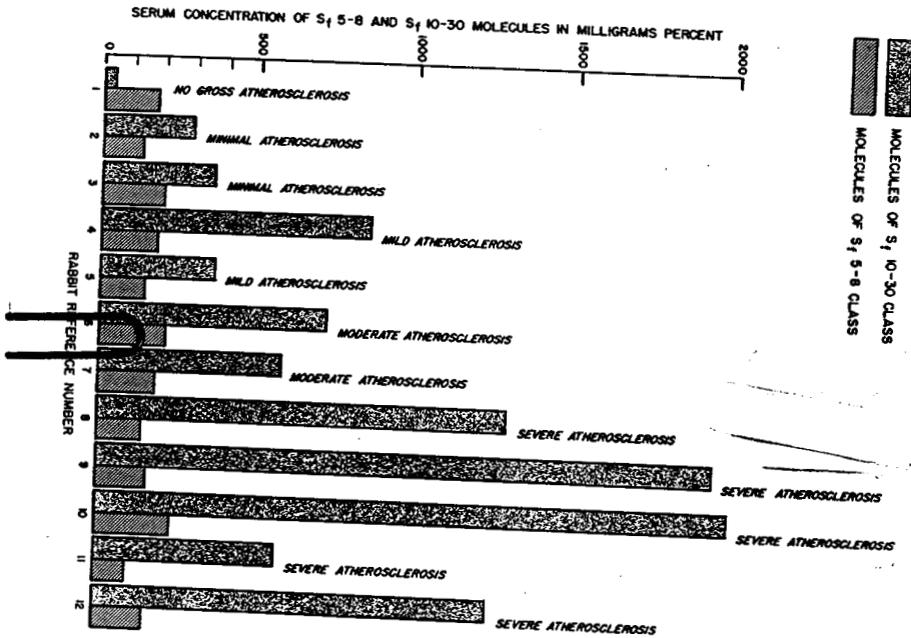
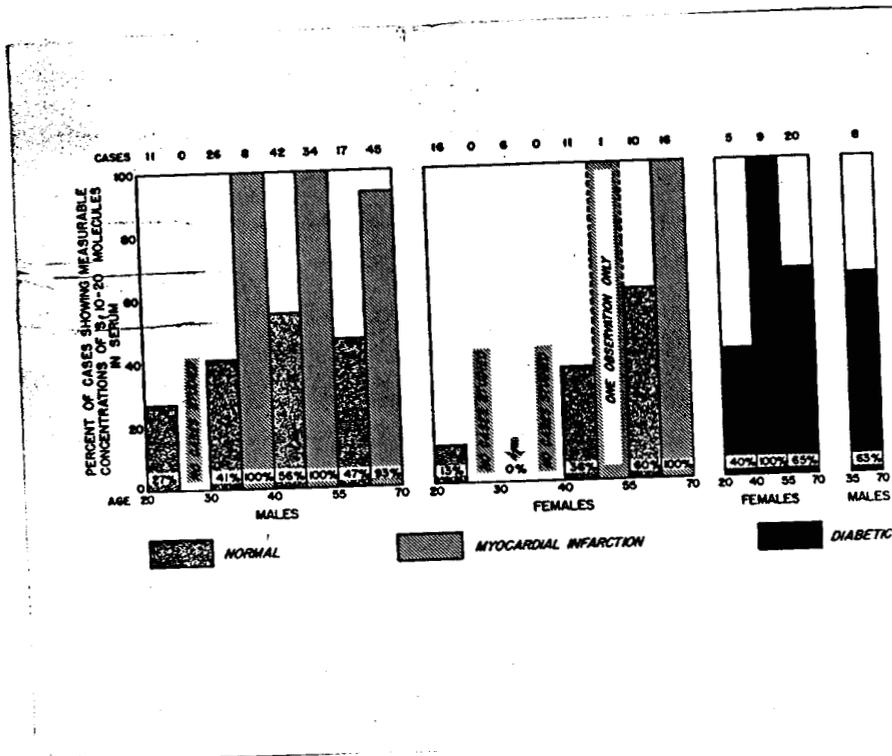


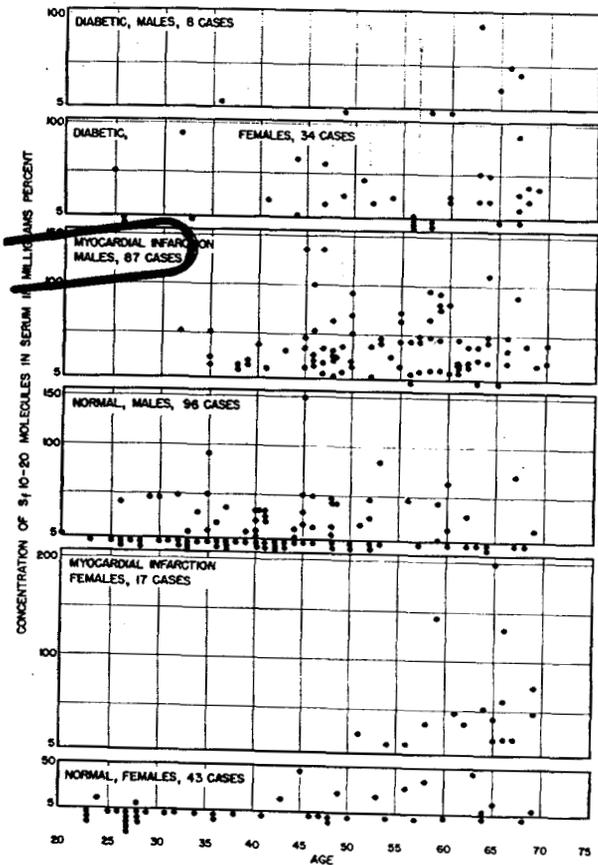
Figure 3. The above histograms indicate the percentages of cases showing measurable concentrations of S₁₀₋₂₀ molecules in serum by age, sex and history of myocardial infarction or diabetes mellitus. Males and females without known disease are compared with proven myocardial infarctions by age increments of 20-29, 30-39, 40-54, and 55 to 70 years inclusive. The frequencies of these molecular types in diabetic group are presented by the ages of 20-39, 40-54, and 55-70 years for females and 35-70 years in the small sample of diabetic males. "No cases studied" is used to denote that no myocardial infarctions in the particular age categories involved were available.



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Figure 4. Presented by scatter diagram are concentrations of S_p10-20 molecules in individual cases grouped by physiological categories. Selection of bloods for analysis have been limited to 20-70 years in order to increase the numbers that could be studied in this age span. The limit of resolution, 5 milligrams percent, has been drawn and those determinations that are essentially "zero" are placed below this limit. No concentrations of these S_p10-20 molecules were found present below the measured concentration of 7.5 milligrams percent.



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