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The Proteolysis of Human Serum Beta Lipoproteins

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THE PROTEOLYSIS OF HUMAN SERUM BETA LIPOPROTEINS

BY

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LIFE

Leonard Jerome Banaszak was born in Milwaukee, Wisconsin, on February 1, 1933. He graduated from Custer High School in Milwaukee in June, 1951. From September, 1951, to June, 1955, he attended the University of Wisconsin, and received the Bachelor of Science degree in chemistry in June, 1955.

He was employed by the Rahr Malting Company, Manitowoc, Wisconsin, from June, 1955, till February, 1956, where he held the position of Junior Research Chemist in the Biochemistry Research section.

In March, 1956, he was drafted into the U.S. Navy for two years, and served for twelve months in the Bio-Med Division of the U.S. Naval Radiological Defense Laboratory, San Francisco, California.

In January, 1958, he began his studies at Loyola University and held the position of Teaching Assistant in the Department of Biochemistry, Stritch School of Medicine, from January 1958, to June, 1958. From July 1, 1958, until June 30, 1959, he held the New Horizons Fellowship for fundamental studies of separation processes, supported by Labline, Inc., Chicago. He received his M.S. in biochemistry from Loyola University in January, 1960. He was awarded a National Science Foundation Cooperative

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He is co-author of the following publications: "Centrifugal Force in Paper Chromatography and Electrophoresis", Anal. Chem. 31, 825 (1959); "Paper Chromatographic Separation of Human Serum Lipoproteins", Clin. Chem. 5, 270 (1959); "The Fading of Sudan Black B Bound to Lipoproteins", Fed. Proc. 19, 231 (1960); "The Separation of Human Serum Lipoproteins by Conventional Descending and Centrifugally Accelerated Paper Chromatography", Anal. Biochem. 1, 44-59 (1960); "The Fading of Sudan Black B Bound to Human Serum Lipoproteins", Clin. Chim. Acta 6, 25-30 (1961); "The Proteolysis of Human Serum Beta Lipoprotein", Fed. Proc. 20, 267 (1961).

PREFACE

A knowledge of various types of lipoproteins is becoming increasingly important to an understanding of certain processes in biochemistry. Some of these substances have, however, defied isolation and purification, and consequently very little is known about them. To cite an example in the plant world, lipoproteins are thought to be of structural significance in the chloroplasts of certain plant cells. This laminated regular array of chlorophyll molecules, stabilized by lipoprotein, appears to be an absolute requirement for photosynthesis to occur.

Similarly in the animal kingdom, lipoproteins are of great importance but again very little is known about most of them. The problem here also is chiefly one of isolation. For example, one might consider the so-called structural lipoproteins which are believed to form an integral part of most membranes, including both plant and animal cells. The cellular membrane serves to maintain the structural integrity of the cell. Moreover the lipide-protein-carbohydrate components of the membrane by nature of their spatial arrangement probably provide this membrane with its characteristic permeability. The latter needs no further elucidation regarding its importance.

In mammals, these exists a series of lipoproteins which serve to transport lipide to and from various tissues. Studies

of these serum lipoproteins have been in progress for over three decades and much preliminary information regarding their isolation composition and characterization has been accumulated.

It is the purpose of this dissertation to study and discuss some properties of one particular lipoprotein -- human serum beta lipoprotein. Occasional reference is made to various other lipide-protein interactions, a knowledge of which has added much to the present day picture of the serum lipoproteins.

I wish to extend my sincere appreciation to Dr. Hugh J. McDonald for his guidance and encouragement throughout this investigation.

I offer gratitude to my wife, Joyce, for her patience and understanding throughout my studies.

And lastly, I thank my parents, Mr. and Mrs. Leonard Banaszak, Sr., whose indirect guidance and aid were beyond measure.

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CHAPTER I

INTRODUCTION TO THE BIOCHEMISTRY OF LIPOPROTEINS

While the meaning of the term "lipoprotein" may appear self-evident, any specific discussion of the subject requires a more detailed definition. One may consider biological lipide-protein complexes as belonging to one of two categories. Proteolipides are the lipide-protein systems which for the most part retain the solubility characteristics of the lipide components. The other category, lipoproteins, are lipide-protein complexes which have solubility characteristics common to most proteins. They are soluble in aqueous solutions. It becomes apparent that these two types of lipide-protein combinations, by nature of their solubility characteristics alone, require different approaches as regards isolation and study.

Limited knowledge of structural lipoproteins, which are thought to be constituents of most cells, prevents classification of them in either category. The structural lipoproteins are important components of cellular and sub-cellular membranes. The lipide-protein compounds contained in blood serum are lipoproteins according to the previously stated definitions.

Several thorough and relatively recent reviews on the investigation of serum lipoproteins (5, 6) and on lipoproteins in general (28) have been utilized in preparing this dissertation.

Some older review articles have also been used (18,54). An attempt has been made in the following discussion to emphasize work published in the past two years and to limit the material which has already been reviewed.

HISTORY

The first serum lipoprotein of a homogeneous nature was isolated by Macheboeuf in 1929 (44). This was done by adjusting the pH of horse serum to 3.9 and precipitating the lipoprotein with ammonium sulfate. Although not termed lipoprotein, vaguely similar material was known to exist in post-prandial blood serum for centuries before Macheboeuf's work. This is in reference to the microscopic fat particles called chylomicra. Chylomicra are 0.5 to 1.0 microns in diameter and they consist mainly of triglycerides and derivatives which are stabilized by small amounts of protein.

Rapid advances in the isolation and characterization of lipoproteins were brought about with the adaptation of the ultracentrifuge to the analysis of serum. McFarlane in 1935 (53) had noticed a new substance which sedimented in the ultracentrifuge at about the same rate as serum albumin. About ten years later, Pedersen (65) isolated a low density substance from serum which he called "X-protein", This material floated upon ultracentrifugation if the density of the medium was raised above 1.04 g/ml. Because of this unusually low density, Pedersen postulated that the

"X-protein" consisted of a complex of albumin, globulin, and plasma lipide.

CHEMICAL METHODS OF ISOLATION AND DETERMINATION

In 1946, during the course of fractionating plasma proteins, Cohn and his colleagues (16) were able to isolate and characterize two distinct lipoproteins. The method involved fractional precipitation of the serum proteins by altering ionic strength, pH and ethanol concentration. The same group (17) in 1950 developed an improved isolation procedure for serum protein fractionation. The new method took advantage of the reduced solubility of the protein salts formed with bivalent cations. This permitted a reduction in the ethanol concentration needed for precipitation. The pH range used during the isolation procedure was also reduced from 4.4 - 7.4 to 5.5 - 7.4.

Bernfeld (8) in 1958 developed a chemical method for the determination of serum lipoproteins. He found that beta lipoproteins are the only proteins of human serum to interact with polyanions at pH 8.6. The polyanions are the sulfate esters of various polysaccharides. They form a precipitate with low density lipoproteins. The quantity of the precipitate is determined nephelometrically; the beta lipoprotein concentration is proportional to the resulting turbidity. Bernfeld and co-workers (9) studied a large number of polyanions and their reactions with the low density lipoproteins. It was evident from this investigation that an

increase in the molecular weight of the polyanion decreases the solubility of the resulting lipoprotein complex. It was also found that carboxyl groups on the polyanion make the complex more soluble. The presence of hexosamine residues, either N-acetylated or N-sulfated, seems to reduce markedly the affinity between the low density lipoproteins and the polyanion. Substances such as polyvinyl sulfate, polystyrene sulfonate and cellulose sulfate behave in a similar manner toward the low density lipoproteins. Thus the chemical nature of the polyanion appears to be unimportant. The interaction and the characteristics of the resulting complex depends mainly on the groups attached to the main body of the polymer, viz. sulfate, N-acetyl, N-sulfatyl, and carboxyl.

ULTRACENTRIFUGAL ISOLATION TECHNIQUES

In spite of the availability of chemical techniques for isolation and determination, most of the serum lipoprotein studies have been carried out on ultracentrifugally prepared fractions. The theory and application of the ultracentrifuge has been described in detail by Svedberg and Pedersen (84). The principle which is the operational basis for the ultracentrifuge is relatively simple. Measurements of the sedimentation velocity of colloidal particles in the earth's gravitational field to determine particle weight have been used for about sixty years. Subliminal colloidal particles such as proteins would have an infinitely small sedimentation rate under gravitational forces and, in some cases, sedi-

mentation is obliterated by diffusion forces. In 1923, Svedberg showed that particle weights of small colloids could be obtained by determining the sedimentation velocity in centrifugal fields. Calculations at that time indicated that such measurements on particles of the size of proteins would require centrifugal fields of the order of 100,000 times gravity. In 1935, the Svedberg group had developed an ultracentrifuge which would develop forces up to 750,000 times gravity.

The application of the ultracentrifuge to the study of serum lipoproteins is somewhat different from its application to proteins. It was found that upon ultracentrifugation, the lipoproteins floated rather than sedimented. The densities of serum lipoproteins was considerably less than that of ordinary protein; this was due to the presence of lipides. Table I presents the densities of some of the components of lipoproteins (63).

TABLE I

APPROXIMATE DENSITIES OF LIPOPROTEIN COMPONENTS

Component	density	Component	density
Peptide	1.35	Cholesterol esters	0.99
Triglyceride	0.92	Phospholipide	0.97
Cholesterol	1.06		

Because of this tendency toward flotation rather than sedimentation, it was necessary to set up a different system of units. The

original system expressed the rate of sedimentation in water. The flotation unit (S_f) is defined as the rate of flotation in aqueous sodium chloride solution of density 1.063 (25°C) under a unit centrifugal field. The application of the ultracentrifuge to the study of lipoproteins has been reviewed by several investigators (63, 21).

Information regarding the structure and size of lipoproteins can be obtained from analytical ultracentrifugation. Analytical ultracentrifugation refers to the methods by which the rate of sedimentation or flotation is determined. The preparation and purification of serum lipoproteins can also be accomplished by ultracentrifugation. Rather than measuring the rate of flotation, in this method each particular lipoprotein is floated to the top of the tube singularly and removed. This is done by adjusting the density of the solution gradually, and each time ultracentrifuging for an empirical time interval. The duration of ultracentrifugation is a function of the S_f and is determined by the interval required to get the desired component reasonably concentrated at the top of the tube. One such preparative technique has been described by Lindgren and his associates (44). A slightly different but acceptable method was introduced by Havel et al (33).

IONOGRAPHY AND CHROMATOGRAPHY

Techniques much less elaborate than those described

above have been devised for the determination of serum lipoproteins. The use of ionography, i.e. electromigration in paper-stabilized media, for the estimation of serum lipoproteins has been described and reviewed by McDonald (51) and by Block et al (11). A careful comparison of ultracentrifugal fractions with those obtained by ionography has been made by Pezold, deLalla and Cofman (70). A similar comparison between serum lipoprotein fractions obtained by ultracentrifugation, ionography and a relatively new paper chromatographic procedure has been made by McDonald, Banaszak and Kissane (53).

Recently Carlson (14) has described a column chromatographic procedure for the separation of serum lipoproteins. The chromatography is performed on glass powder columns. Application of serum at pH 8.8 to these columns results in the absorption of the lipoproteins. All other serum proteins are washed through. Increasing the pH to 9.6, then to 9.8 and finally adding 0.4 M potassium carbonate resulted in the elution of three lipoprotein fractions. They are eluted in the following order: alpha-1, alpha-2 and beta lipoprotein. Unfortunately, ultracentrifugal fractions were not chromatographed and the exact nature of these fractions are unknown.

COMPOSITION

Taking advantage of mainly the ultracentrifugal procedure for fractionating lipoproteins, the composition of these com-

TABLE II

COMPOSITION OF LIPOPROTEINS

Form	Avg. Conc. mg %	Density	S_f	Lipoprotein composition: Protein %	Lipoprotein composition: Lipide %	Lipide composition as % total lipide: TG	PL	Chol ester	Chol	NEFA
Chylomicrons	0-50	Less than 0.96	10^4-10^5	1	99	88	8	3	1	-
Low density (beta) lipoproteins										
LDF 1	150	0.96-1.006	20-400	7	93	56	20	15	8	1
LDF 2	50	1.006-1.019	12-20	11	89	29	26	34	9	1
LDF 3	350	1.019-1.063	0-12	21	79	13	28	46	10	1
High density (alpha) lipoproteins										
HDF 1	50	1.063-1.125		33	67	16	43	31	10	-
HDF 2	300	1.125-1.210		57	43	13	46	29	6	6
Albumin-NEFA	4000			99	1	0	0	0	0	100

Non-esterified fatty acids-NEFA, triglycerides-TG, phospholipide-PL.

See reference 61.

pounds has been studied by numerous investigators. Table II is a summary of six such reports (61). It demonstrates the main vehicles for fat transport in man. Table II also indicates inherent differences in the lipoproteins. This is in light of their different compositions. It must be emphasized that the results are average postabsorptive values for a healthy and well-nourished 40-year-old man. Not only do wide differences exist in the concentration of the various fractions in serum but, the lipide composition of a given fraction varies considerably with the individual's previous history. For example, the density class 1.019 to 1.063, referred to hereafter simply as beta lipoprotein, may have a cholesterol to protein ratio ranging from nine-tenths to three.

Because of the use of different techniques for isolation and characterization of the serum lipoproteins, there is a lack of uniformity in their nomenclature. The terms alpha and beta lipoproteins have been derived from electrophoretic studies of serum (13). It is also acceptable to name a serum lipoprotein by either its S_f value or the hydrated density range. The terms high density and low density lipoproteins as well as the names alpha and beta refer to classes or groups of lipoproteins. There is one exception to this system. Many investigators have chosen to call the S_f 0-12 class, beta lipoprotein. In this discussion, the author has followed the latter practice.

The fatty acid composition of the lipides of lipopro-

teins has recently been reported by Green and Oncley (31). This included the fatty acid composition of the phosphatides, sterol esters and triglycerides. The phosphatides had a very high content of the highly unsaturated fatty acids. Earlier Swell et al (85) demonstrated that about seventy per cent of the total cholesterol ester fatty acids were polyunsaturated.

The types of phospholipide contained in the various serum lipoprotein fractions has been studied by Phillips (72). Both low density and high density lipoproteins contained similar amounts of cephalin and lecithin. The high density lipoproteins contained about three-fourths of the total serum lysolecithin; sphingomyelin was present mainly in the low density fractions. About five per cent of the total serum hexosamine is associated with the various serum lipoproteins (23, 1).

The purification and characterization of a number of lipoproteins from egg yolk has recently been performed by Bernardi and Cook (7). The composition of egg yolk lipoproteins has been studied by Abraham et al (1). The molecular weights of these lipoproteins are relatively low; they are of the order of 400,000. Sub-units of about one-half the size are obtained in alkaline and urea solutions.

A lipoprotein with a density greater than 1.210 has been isolated from serum (71). It contains essentially no cholesterol but has varying amounts of phospholipides. The latter is mainly in the form of lysolecithin.

Only rudimentary information is available regarding the amino acid composition of the protein moiety from serum lipoproteins. This is presented in table III (64). There does not appear to be anything strikingly unusual about the amino acid profile of the lipoprotein protein. A relatively large proportion of leucine is present. It has been suggested that the aliphatic side chain of leucine may be of importance for non-polar interactions with lipides. The data in table III for low density lipoproteins represents at least two fractions. Two fractions are also present in the high density lipoproteins. Analysis of the amino acids contained in the proteins of the individual high density fractions has recently been reported by Shore (80). Both fractions contained similar amounts of all the amino acids and the results were about the same as for the combined fractions presented in table III.

METABOLISM

Many investigators have felt that the lipoproteins of serum are metabolically interrelated. The differences in the size and density was thought to arise from variations in lipide composition. The confusion regarding the interrelationships of serum lipoproteins was reduced mainly through the determination of the N- and C-terminal amino acids of the protein portion from the lipoproteins along with the previously described amino acid composition.

TABLE III

AMINO ACID COMPOSITION OF THE PLASMA LIPOPROTEINS AND ALBUMIN

	Amino acid residues per 100,000 g of peptide moiety		
	Low density lipoproteins[a]	High density lipoproteins	Albumin
glycine	33.4	36	21.4
alanine	49.3	66	
valine	49.5	55	66.0
leucine	131.7	135	84
isoleucine	ca 0	ca 0	13
phenylalanine	45.0	38	47.3
proline	28.6	(28)	44.4
tryptophan	?	?	1.0
serine	51.1	55	35.3
threonine	49.5	45	42
half-cystine	41.1	26	52.2
methionine	ca 0	ca 0	8.7
aspartic acid and asparagine	76.0	62	78.2
glutamic acid and glutamine	73.5	131	118.6
tyrosine		19	26.0
lysine		57	84.3
histidine	ca 6.5	17	22.6
arginine	20.0	42	35.7

[a] Excluding S_f 12-20 lipoprotein

See reference 64.

Shore (81) has reported that the two alpha lipoprotein fractions of density 1.09 and 1.12 have similar amounts of both N- and C-terminal amino acids. They are N-terminal aspartic acid and C-terminal threonine. The beta lipoproteins, density 1.019 to 1.063, contained C-terminal serine and N-terminal glutamic acid (2). This data would seem to contradict the theory that low density lipoproteins are converted to high density lipoproteins simply by loss of lipide. Such a conversion had been suggested to occur *in vivo* by Graham (30) and *in vitro* by Boyle (12).

Oncley (64) and co-workers using iodinated lipoprotein protein have found evidence that the lipoprotein of density 0.98 is converted to that of density 1.02. This was not reversible. The experiments consisted of injecting tracer amounts of iodinated (I^{131}) lipoproteins into human subjects. At various time intervals, serum samples are drawn and the lipoprotein fractions isolated. The iodine was shown to remain quantitatively in the peptide moiety. Oncley also showed that little or none of the protein from alpha lipoproteins is converted to low density lipoprotein or chylomicrons. This seems to conflict with the work of Rodbell (76) who has shown that part of the protein associated with the chylomicrons is very similar to that obtained from alpha lipoproteins. This evidence was obtained by removing the lipide from the lipoproteins and then treating the remaining protein with proteolytic enzymes. Peptides or proteins are compared by running two-dimensional chromatograms of the digest, a procedure

which is referred to as "finger printing". At present no unifying hypothesis of the interrelationships of serum lipoproteins consonant with all the facts can be offered.

Lastly, the synthesis of lipoproteins by rat liver slices and perfused rat liver has been demonstrated by two independent groups (50, 73). The synthesis includes both the incorporation of labeled amino acids into the protein portion and labeled acetate into the lipide moieties. The removal and metabolism of chylomicrons by adipose tissue *in vitro* has been demonstrated by Rodbell (75).

CLINICAL ASPECTS OF LIPOPROTEINS

It is now fairly well established that the cholesterol concentration in blood serum is a primary factor in the formation of atherosclerotic patches in blood vessels. Very recently several drugs have been found which interfere with the biosynthesis of cholesterol. Dietary control of cholesterol intake is also becoming prominent. The general objective now seems to be the reduction of blood serum cholesterol. The final objective is, of course, the prevention of cholesterol or other lipide deposition on/in the arterial wall and the resulting atherosclerosis.

Such an approach, while it may be effective in controlling fat deposition in arteries, will not offer much information as to the mechanism or the actual processes involved. Since it is recognized now that practically all serum lipide material is associated with protein as biochemical entities, principally lipo-

proteins, interest then shifts from serum cholesterol to lipoproteins. From a fundamental viewpoint, it appears that the important question to be answered is how and why lipide leaves the lipoproteins of serum and deposits on the arterial wall.

There seems to be little doubt that the low density lipoproteins of serum are the agents of atherogenesis (61). However, atherosclerosis is undoubtedly a disease of multiple causation. Thus the agent, the low density lipoproteins, must be an essential though not necessarily a sufficient cause. Olson (61) has suggested as a working hypothesis, that the rate of atherogenesis may be a product of three main factors: a high concentration of low density lipoproteins, elevated mean arterial blood pressure and the susceptibility of the artery to atheromatous change.

The author suggests one other possibility. Since the lipide to protein ratio of a lipoprotein is variable, it is conceivable that a given lipoprotein molecule may become "overloaded" with lipide. This extra lipide is perhaps only loosely held. It may therefore leave the lipoprotein and become bound to the artery wall. With this in mind, it will be necessary to gain further information as to the structure of the lipoproteins. It will also be necessary to obtain data regarding the energy or spectra of energies involved in the lipide-protein and lipide-lipide interactions.

CHAPTER II

THE NATURE AND STRUCTURE OF LIPOPROTEINS

It has been suggested that in order to determine the structure of a protein the following requirements must be fulfilled (19): 1. Isolation and characterization of a single chemical individual, including the accumulation of adequate proof that such an objective has been reached. 2. Determination of molecular size. 3. Determination of functional groups. 4. Degradation to smaller fragments with identification of these fragments. 5. Derivation of the manner in which the known fragments are connected. 6. Synthesis.

The determination of the structure of serum lipoproteins is in the earliest stages. Isolation, characterization and the determination of some of the functional groups has been discussed in the previous chapter. The case for lipoproteins is complicated because a given species of molecules is undoubtedly non-homogeneous in respect to lipide composition. This fact necessitates a rather loose interpretation of the term "single chemical individual". However, all evidence to date would indicate that the protein moiety of the lipoprotein is well defined. It is the structure of this protein which probably defines the final structure of the lipoprotein. Because of the ease with which lipoproteins undergo non-polar interactions with lipides, one might expect many

other materials to be weakly bound to them. Non-polar interactions are briefly defined as those not involving the coulombic forces between oppositely charged particles. Definite proof of "contamination" is not easy to obtain, but some evidence will be provided in the experiments comprising this investigation.

The following discussion is designed to present in part the physical and chemical properties of lipoproteins. Precedence is naturally given to serum lipoproteins. Some theoretical aspects of lipide-protein interactions is also presented.

SIZE AND SHAPE

Practically all of the information regarding the molecular weight and size of the lipoproteins has been derived at least in part from analytical ultracentrifugal data. A summary of this information is presented in figure 1 (45). The significance of the S_f values has been described earlier. The hydrated densities in grams per milliliter are the densities of the molecules with their bound water. There is an approximately linear relationship between the hydrated densities and the lipoprotein protein content (80, 67). The protein content becomes greater with increased hydrated density. In order to exemplify the relative sizes of the molecules in figure 1, they were assumed to be spherical in shape. The ultracentrifugal pattern in figure 1 is indicative of the number of lipoprotein species present in human serum.

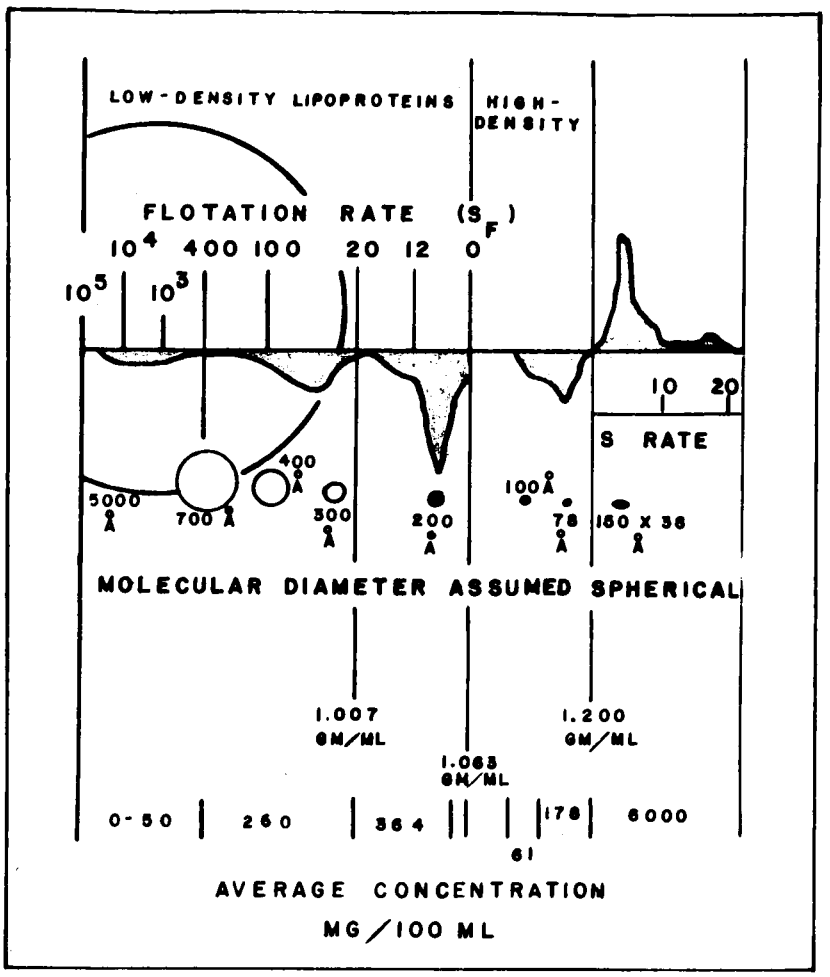


Figure 1

THE ULTRACENTRIFUGAL COMPOSITION OF HUMAN SERUM

See reference (45).

The molecular weights of the various serum lipoproteins are given in table IV. These values were obtained by analytical ultracentrifugation. A recent molecular weight estimation of the lipoproteins of density 1.03 has also been done by electron microscopy (35). The values obtained were about 6 x 10^6. This is approximately twice the value indicated in table IV and it has been suggested that dimerization may occur during fixation of the material.

TABLE IV

MOLECULAR WEIGHTS OF HUMAN SERUM LIPOPROTEINS

Hydrated density[a]	Molecular Weight
0.98	5 to 25 x 10^7 (ref. 64)
1.03[b]	1,300,000 (ref. 66)
1.03	3,200,000 (ref. 10)
1.09	365,000 to 435,000 (ref. 64)
1.14	165,000 to 195,000 (ref. 64)
1.14[c]	200,000 (ref. 66)

[a]For simplicity, all names which have been applied to various fractions are not listed. If any confusion arises, it is suggested that the reader examine table II or figure 1.

[b]Calculated for the anhydrous protein.

[c]Isolated by chemical techniques.

The beta lipoprotein particles visualized in an elec-

tron microscope (34) appeared to have the shape of an oblate ellipsoid. They had a diameter of 350 Å and a height of about 175 Å. An oblate ellipsoid is flattened or depressed at the poles. Earlier work had assigned the shape of a prolate ellipsoid with dimensions of 350 Å x 160 Å (10). The latter type is elongated in the direction of a line joining the poles.

Unesterified cholesterol has molecular dimensions of 7.2 x 4.5 x 20 Å (18). Since unesterified cholesterol comprises about eight per cent of the beta lipoprotein, whose molecular weight is 3×10^6, there are about 600 molecules of cholesterol in every molecule of lipoprotein. There are roughly three times as many molecules of cholesterol esters as of unesterified cholesterol. From these rough calculations and the foremontioned size of the lipoproteins, one may probably eliminate any linear array of these lipides with respect to the protein. Another way of stating this would be to say that it is unlikely that each molecule of lipide, cholesterol for example, interacts with a portion of the protein. Oncley (65) made slightly different considerations. He pointed out that if the protein of the lipoprotein is considered to occupy the periphery of the molecule, about one-half the surface of the particle could be covered by peptide. Some of the lipide molecules can only interact with other lipides. A heterogeneity of binding energies is therefore to be expected.

CHEMICAL STUDIES

For organizational purposes, the chemistry of the lipoproteins may be divided into three categories: 1. Reactions of the whole molecule. 2. Reactions involving only the lipide portion. 3. Reactions of the protein moiety.

Lipides for the most part are insoluble in an aqueous (polar) environment. One of the most interesting properties of serum lipoproteins is their ability to take up more lipide and still retain their original solubility characteristics. This can be demonstrated by a number of experiments. One of the simplest but perhaps least obvious is the pre-staining technique introduced by McDonald and co-workers (54,56). They used Sudan Black B, a material which is insoluble in water. When this material is dissolved in a non-polar solvent and added to serum or a solution of lipoproteins, the Sudan Black B interacts with the lipide moiety. The result is a lipoprotein which is stained a deep blue-black color. One is lead to assume that this interaction is limited. In no case is enough of the stain taken up so that flocculation or precipitation of the lipoprotein occurs. Furthermore, the rate of fading of Sudan Black B catalyzed by hydrogen peroxide is different for high and low density lipoproteins (52). It has been suggested that this result follows from the relationship of the stain to the environment. In the case of the high density lipoproteins, the lipide is believed to be completely shielded by a shell of protein. In the low density lipo-

proteins, portions of the lipide and therefore probably the stain are exposed to the environment. Consequently, the Sudan Black B is more rapidly attacked by the hydrogen peroxide.

Cholesterol and other steroids may also be incorporated into serum lipoproteins (4). To do this a rather simple procedure has been devised. It consists of incubating serum or a solution of the lipoprotein with Celite (Johns-Manville Co.) coated with the respective lipide. A more recent method consisted of incubation of the lipoprotein with a cholesterol dispersion (87). The latter is prepared by mixing cholesterol with a solution of Tween 20 in methanol. The methanol may then be evaporated and the material re-dissolved in saline (0.9% NaCl). After incubation with the dispersion, the lipoproteins may be isolated by ultracentrifugation. When serum is treated in this manner, practically all of the cholesterol is taken up by the low density lipoproteins. Most of the cholesterol taken up by the low density lipoproteins is found in the beta lipoprotein.

The distribution of fatty acids between the serum proteins is different from that obtained with sterols. Goodman and Shafrir (27) have studied the binding of oleic, palmitic and linoleic acids to plasma proteins. These fatty acids represent eighty to eighty-five per cent of all the fatty acids in the blood serum. Goodman's results indicated that only three-tenths per cent are bound to the low density lipoproteins. The high density lipoproteins bind even less of the fatty acids. Serum albumin

is responsible for the transport of practically all of the unesterified fatty acids. Similar results were obtained by two other independent groups (6,29).

The work of Goodman and Shafrir (27) also indicated that two classes of binding sites for the fatty acids existed on the beta lipoprotein. The large difference between the numbers of these two binding sites permitted speculation on the mechanism of the fatty acid interaction. Their reasoning was that in one class, the large number of available sites indicated relatively little specificity. The main factor may be that of the "solubility" of the fatty acid hydrocarbon chain in the lipide portion of the lipoprotein. In a small number of instances, the carboxylate end of the fatty acid may interact with one or more charged side groups of the protein portion.

Certain other non-polar materials have been found to be associated with the beta lipoprotein fraction (62). This includes estriol, vitamin A and vitamin E, and some of their metabolic homologues.

Some idea of the factors influencing the lipide binding by the lipoprotein may be obtained by observing the factors which influence the removal of lipide by organic solvents. Macheboeuf and co-workers (49) studied the extraction of lipides from a chemically prepared serum lipoprotein. It was shown that low concentrations of ethanol (6-12%) and a number of homologous alcohols promoted the extraction of lipides. The mechanism for this effect

was believed to be the partial removal of the water hydration shell from the lipoprotein. This facilitated attack by ether with the consequent removal of lipide. It is the loss of this bound water which is probably the source of the instability of lipoproteins to lyophilization (46).

Avigan (3) has also described some extraction studies with ultracentrifugally prepared serum lipoproteins. Partial extraction of lipides from the beta lipoprotein resulted in no change in the electrophoretic mobility. This seems to show that the number of charges on the molecule is not changed appreciably by the lipide extraction. Avigan (3) also showed that the resistance of the beta lipoprotein to denaturation by urea is abolished after ether extraction. He suggested that the sterols may serve an important function in maintaining the protein configuration.

Only limited information is available regarding the reactivity of the functional groups of lipides bound to lipoproteins. One such lipide reaction which has been studied in great detail is the oxidation of carotenoids (6,32). During this reaction, there is a decrease in the absorption spectra of the lipoprotein above 410 mu. The decrease is attributed to the change in the carotenes contained in the lipoproteins. This reaction along with other lipide oxidations may account for other slow changes occuring in the lipoproteins during isolation and aging. Such changes have been demonstrated by a flattening of the ultracentrifugal

peak and an increase in the ionographic mobility (74,6). The elimination of cupric ion from solutions of the lipoproteins prevents the oxidations; cupric ion is a catalyst for these reactions. Preparing and storing serum lipoprotein solutions under nitrogen also prevents oxidative alterations. Curd (32) has pointed out that some of the changes which occur in the absorption spectra of serum lipoproteins might also result from cholesterol and unsaturated fatty acid oxidation.

Specific reactions which would test the reactivity of some of the functional groups in lipoprotein lipide might be of great help in obtaining a better picture of lipoprotein structure. Some of these groups which must eventually be studied are: 1) the phenolic hydroxyl of cholesterol and other sterols. 2) the phosphate groups of phospholipides. 3) the aliphatic hydroxyl of serine. 4) the amino groups of serine and ethanolamine. Functional groups of lipides which are "buried" in the lipoprotein complex would be expected to be unreactive.

There is very little to be said about lipoprotein protein. For some time now, investigators have been trying to prepare lipide-free but undenatured lipoprotein. The word "undenatured" here refers to the recombining capacity of the protein toward lipide. Studies of such a protein would offer valuable information regarding the protein structure(s) which are conducive to lipide conjugation. In 1960, Scanu and Hughes were able to isolate protein from serum alpha lipoprotein which fulfilled

these requirements (77,78). The removal of lipides was accomplished by ethanol-ether extractions at -20°C. This was followed by prolonged ether extraction at the same temperature. After the extraction, the protein was labeled with I^{131}. This protein rapidly took up lipide from serum to form a lipoprotein which was indistinquishable from the original lipoprotein. The characterization of the re-formed lipoprotein was done by ultracentrifugation and starch gel electrophoresis. Scanu (77) could not prepare a similar lipide-free undenatured protein preparation from serum beta lipoprotein.

EXCHANGE OF LIPIDES BETWEEN LIPOPROTEINS

It has already been pointed out that phospholipides, triglycerides, cholesterol and cholesterol esters are very insoluble in water. Because of this fact, the rapid exchange of lipides between lipoprotein fractions probably does not occur via the existence of monomolecular lipide in the aqueous environment. A rapid exchange of lipide between lipoprotein fractions and between lipoproteins and tissue has been demonstrated by a number of investigators (77, 24, 47).

Before describing the character of lipide-protein association and exchange, it is necessary to define the words reversible and irreversible as they will be used in this brief discussion. A reversible association colloid is in equilibrium with its component monomolecular species (40). For example, an aqueous

solution of sodium laurate would contain sodium ions, laurate ions and micelles of the fatty acid. At very low concentrations, only the monomolecular species would be present. In some colloid systems however, no individual molecules of the lipide are present in the solution. Such systems are frequently referred to as irreversible. An oil emulsion would be an example of an irreversible system. The lipide material is stabilized in an aqueous environment by absorbed layers of ions and a water hydration shell. The lipide-protein interaction of serum lipoproteins are believed to be irreversible. One exception is the fatty acid-lipoprotein interaction which has been discussed earlier. The idea of irreversibility as defined here stems mainly from the fact that lipide material in blood serum is always associated with lipoprotein or protein.

If no unimolecular lipide is present in serum, how then does exchange of these substances occur between lipoproteins? Gurd (32) has recently proposed a theory of lipide exchange. He suggests that exchanges or transfers are brought about by actual collision between the lipoprotein molecules. Exchange occurs in the resulting collision complex; the complex then decomposes into molecules very closely resembling the original lipoproteins. The transfer of a lipide molecule would be a diffusion process by which the molecule moves out of the original lipoprotein and into the other lipoprotein without passing completely into the aqueous environment.

Because of the irreversible character or apparent irreversible character of lipide-protein interactions in lipoproteins, no method is yet available for quantitatively measuring the affinity of lipoproteins for their major lipide constituents. It is impossible, therefore, to define the conditions which lead to a net uptake or release of lipoprotein lipide.

THE LIPIDE-PROTEIN BOND

The affinity of lipoproteins for lipide material is determined by the type of chemical bond(s) which compose the interaction. Pauling (68) defined a chemical bond as a bond between two atoms or groups of atoms when the forces acting between them are such as to lead to the formation of an aggregate with sufficient stability to make it convenient for the chemist to consider it as an independent molecular species. In general, the weak Van der Waals' forces between molecules are not considered to lead to chemical bond formation, but in exceptional cases, according to Pauling, such as that of the O_4 molecule, it may happen that these forces are strong enough to make it convenient to describe the corresponding intermolecular interaction as bond formation.

Although it is believed that many of the interactions involving the lipoprotein are the result of Van der Waals' forces, serum lipoproteins are accepted as being independent molecular species. These lipide-protein combinations may rightly be considered as being joined by chemical bonds and a comparison of Van der

Waals' forces with other bonds may be made. Despite this fact, the lipoproteins are in many respects unique in biochemistry. These compounds are composed of a heterogeneous group of lipides of somewhat variable composition stabilized in an aqueous environment by what must be considered to be a specific peptide chain. The contradiction indicated by the statements "independent molecular species"....."of variable composition" is only one of semantics.

Because of the great complexity of the lipoproteins, the chemical interactions which serve to link the components can only be discussed in a general sense. The possible types of bonds may be divided into four categories: 1) Covalent 2) Electrostatic 3) Van der Waals and 4) Hydrogen bond.

The possibilities for formation of covalent bonds between lipide and protein are minimal. Such covalent links would only seem possible between the phosphate of phospholipide and a hydroxyl group of serine or threonine or one belonging to another phospholipide.

Similarly, electrostatic bonds between lipide and protein are probably of little importance. Most lipides do not contain groups which are likely to have a net electrostatic charge. One exception is again the phospholipides which via negative charges on the phosphate portion could be bound to very basic groups in the protein residue. The possibility of electrostatic interaction could also exist between positively charged amino groups

(i.e. choline) in the phospholipids and acidic groups in the protein. Goodman (27) had suggested that a part of the fatty acid anion binding to low density lipoproteins could be accounted for by electrostatic interactions.

Because of the lack of charged groups in lipides, it seems likely that most of the interactions between lipides and between lipide and protein involve Van der Waals' forces. This term is frequently misused in biochemistry, particularly when used synonymously with the term non-polar interaction. Classically Van der Waals' forces are divided into three types, two of which involve polar or coulombic forces (37). Keesom suggested one type of Van der Waals' forces. This is the electrostatic interaction between two permanent dipoles. Debye proposed another type of polar interaction. This is the attraction between a permanent dipole and the moments induced by this dipole in a neighboring molecule.

Finally there are the London forces. These are the type of Van der Waals' forces which are involved in the so-called non-polar interactions. Ketelaar (37) described the forces as follows: "If we could take instantaneous photographs at certain instants of a molecule or atom, we should usually find an arrangement of nuclei and electrons such that the whole exhibits an electric dipole moment. This holds also for an atom with a spherically symmetrical charge distribution as in the inert gases and in the hydrogen atom in the ground state." Averaged over any period

of time, there is, of course, no moment. However this rapidly varying dipole gives rise to an electric field and this polarizes the other atom in which the induced dipole is in phase with the first; this interacts with the instantaneous dipole. The London interaction between two large molecules is a sum of the interaction between atom components. Thus maximum interaction will obtain when the contours of the interacting groups allow the apposition of large fractions of their surfaces.

It has recently been suggested that protein-protein interactions may also result from fluctuation in surface charge density (26). This could occur by the migration of protons between basic sites on the protein surface. In a sense this effect may be analogous to the London forces but involving fluctuations of protons rather than electrons.

Under certain conditions, an atom of hydrogen is attracted by rather strong forces to two atoms instead of one, so that it may be considered to be acting as a bond between them. This is called the hydrogen bond. It is largely ionic in character, and it is formed only between the most electronegative atoms --nitrogen, oxygen and fluorine. The presence of oxygen and nitrogen in both protein and lipides permit their linkage by hydrogen bridges. At present there is no way to evaluate their importance in lipoproteins.

GLOBULAR PROTEINS

Serum lipoproteins are occasionally referred to as lipo-

globulins. This resulted from the fact that their electrophoretic mobility and solubility properties were very similar to those of ordinary serum globulin. Globular proteins differ greatly from the somewhat linear alpha helical structure of fibrillar proteins (26). Some investigators feel that the globular proteins retain an alpha helical basic structure but this is folded and cross-linked in a very complicated and, at present, unknown manner. Varied procedures which denature, that is, alter the native structure, act by interfering with or destroying the forces which maintain this complicated folding.

In the case of lipoglobulins, the native structure of the protein core may be maintained in part by interlinking lipide molecules. Thus removal of the lipide may in part denature the protein. It is not at all unreasonable to speculate that the native folding of lipoprotein provides the molecule with its lipide conjugating property. Furthermore, one must also consider the protein to be the main factor in stabilizing the lipide in the aqueous environment. In some manner the protein structure must also serve to limit the amount of lipide uptake. The protein moiety of lipoprotein may be imagined to serve both as a shield to prevent lipide coalescence and to provide the basic structure for lipide addition.

RATIONALE OF PROPOSED INVESTIGATION

It has been pointed out that the protein moiety of lipo-

proteins is an important factor in maintaining the stability of a large amount of lipide in an aqueous environment. Substances which alter the native structure of the protein core also reduce the stability of the lipide-protein complex. However, most substances which denature protein may also reduce the energy of the lipide-protein interaction. This would be the case for alcohols, ether and other organic solvents. These compounds "pull" the lipide from the protein and probably simultaneously denature the protein moiety. Thus it is not possible to say whether the agent is effecting the protein structure or the lipide-protein interaction. If it were possible to obtain lipide-free protein having an affinity for lipide, the significance of protein structure to lipide conjugation could easily be evaluated. Isolation of "undenatured" lipide-free protein has only recently been accomplished for serum alpha lipoprotein (77). Application of similar techniques to the serum beta lipoproteins was without success. Another approach to the problem would be to disrupt the protein structure with proteolytic enzymes. It would seem extremely unlikely that these enzymes could affect the lipide-protein interaction in any way other than that which would be mediated through changes in the protein moiety.

The purpose of this investigation is to see if proteolytic enzymes affect the native lipoproteins. In the cases where proteolytic activity is established, the effect on lipide binding will be evaluated. Investigation of the materials following pro-

teolysis of serum beta lipoproteins will be initiated.

The irreversible character of lipide binding of lipoproteins was described earlier in this chapter. This hypothesis has never been demonstrated. An experimental approach to substantiate the irreversible character of the lipoprotein-cholesterol interaction will also be presented in this dissertation.

CHAPTER III

MATERIALS AND METHODS

The materials and methods used in this investigation are described in the following section. In some instances, special techniques or procedures will be described along with the experimental results in the succeeding chapter. This has been done in cases where an understanding of the procedure is required to interpret the results.

CHEMICALS

Unless otherwise indicated, all chemicals used in this study are analytical reagent grade. Chemicals which are obtained from special suppliers or are of unusual importance to the investigation are listed in table VIII, Appendix I.

The preparation of buffers used for dialysis, incubations and ionography are described in table IX, Appendix I.

FRACTIONATION OF HUMAN SERUM LIPOPROTEINS

Pooled human serum was obtained from Cook County Hospital. It represented samples from approximately fifty unselected patients. In general, the type of blood specimen was that required for determination of fasting blood sugar. Ultracentrifugal fractionation of the serum was begun on the same day that the blood was drawn.

Small amounts of cells were invariably present in the

blood serum. The cells were removed by centrifuging for ten minutes at full speed in a clinical centrifuge. The density of the serum was then adjusted by the addition of stock salt solutions of known density. A detailed description of the preparation of the salt solutions and of other techniques used in this preparation are presented in table X, Appendix I.

Ultracentrifugation was carried out for eighteen hours at 40,000 RPM in the number 40 rotor of a Spinco Model L Ultracentrifuge. Because of the age of the rotor, the last few preparations were obtained by centrifuging for twenty hours at 38,000 RPM. Temperature in the head chamber was maintained throughout the run at 10-12° C.

Beta lipoproteins, density 1.019 to 1.063, were obtained by first adjusting the density of the serum to 1.019 and centrifuging as described above. The lipoproteins which floated to the top of the tube were removed and discarded. The contents of the bottom portion of the tube (infranatant) were adjusted to density 1.063 and centrifuged for twenty hours. The beta lipoproteins floated as a distinct layer at the top of the tube and were collected. If a preparation of alpha lipoproteins, density 1.063 to 1.21, was desired, the infranatant was collected and adjusted to density 1.21. After centrifuging again for twenty hours, the alpha lipoproteins floated to the top of the tube and were collected.

When enzymic studies were to be made, it was necessary to remove the relatively high concentration of sodium chloride and

potassium bromide which is present in the lipoprotein solutions. To remove these salts, the lipoproteins were placed in Visking cellophane sacks which, prior to use, were washed for twenty-four hours against about five changes of glass distilled water. Dialysis of the lipoprotein was carried out for approximately ninety hours at 2°C. During this time the external buffer was changed at least six times. This amounted to a total buffer volume of about 1200 ml. In the case of the beta lipoproteins, the resulting solution was a clear yellow color.

ANALYTICAL PROCEDURES

A detailed description of analytical procedures, while being important to any chemical investigation, is still rather cumbersome reading. For this reason, only a brief mention of the analytical methods employed in this investigation will be presented here. For a detailed discussion of these procedures, the reader is referred to Appendix II. The latter also contains the standard curves from which the unknown values were derived.

Protein was determined by the biuret method of Gornall et al (28). This method was chosen over any method involving Kjeldahl digestion because the lipoproteins contain relatively large amounts of phospholipide. The nitrogen contained in phospholipides would result in significant amounts of ammonia following Kjeldahl digestion. This would give fallaciously high protein values.

Cholesterol analysis was done by the method of Sperry and Webb (82). Before this procedure was adopted, two other methods were tried. One technique depended on the color produced when cholesterol was mixed with ferric chloride (43). The other method consisted of the ultra-violet spectrophotometric estimation of cholesterol (86). Both methods (43, 86) proved less reproducible and more cumbersome than the procedure of Sperry and Webb. The values appearing in later sections of this dissertation are for total cholesterol; no attempt was made to distinquish between the free and esterified forms.

In the experiments in which it was necessary to perform phospholipide analysis, these were done by the method of Stewart and Hendry (83). The procedure involved the extraction of phospholipide with hot ethanol and acetone. The phospholipide was then digested with concentrated sulfuric acid and the inorganic phosphate was determined by standard techniques.

Proteolysis of the beta lipoproteins was demonstrated by the release of trichloroacetic acid soluble tyrosine. Tyrosine was determined by the method of Folin and Ciocalteu (25). The use of the ninhydrin technique (60) for determining the production of alpha amino groups during proteolysis was difficult. Production of ninhydrin color depends on heating the solutions to 100°C for ten minutes. Large amounts of a precipitate appeared upon heating the lipoprotein with the ninhydrin reagent. The precipitate was attributed to the presence of lipide material in the

lipoproteins.

The activity of chymotrypsin and trypsin was initially measured by determining the rate of release of trichloroacetic acid soluble material from a hemoglobin solution. Later, artificial substrates of the proteolytic enzymes were obtained and the assay procedures were then done by the much simpler ultra-violet spectrophotometric technique of Schwert and Takenaka (79). Trypsin activity was determined by measuring the rate of hydrolysis of N-benzoyl-L-arginine ethyl ester; the substrate used to determine chymotryptic activity was N-acetyl-L-tyrosine ethyl ester.

IONOGRAPHY AND CHROMATOGRAPHY

Ionographic separations were done with a Precision Scientific Company Ionograph. A description of the principal features of this apparatus has been published (55). The specific conditions used in different experiments often varied and for this reason they will be reported in the sections devoted to the results. The paper strips were placed in the ionograph during the night preceding a run, in order to equilibrate the buffer through the strips. The electrical potential was applied for one hour preceding the application of the migrant. Upon completion of the run, the strips were removed and dried for about thirty minutes at 110°C. Protein material was detected by staining with bromphenol blue or by spraying with ninhydrin. Cholesterol was detected with phosphotungstic acid spray. The preparation and use of these detecting solutions is described in Appendix II.

Paper chromatographic separations were carried out in a large chromatography jar, sixty centimeters in height and thirty centimeters in diameter. In the bottom of the jar was placed a large dish which contained a sheet of Whatman 3MM paper. The bottom of this paper was immersed in the developing solution and extended above the liquid for about three or four inches. It was felt that this paper facilitated equilibration of the atmosphere within the jar. All the paper chromatographic experiments were carried out at room temperature, approximately 25°C. Separation of trichloroacetic acid soluble material from the lipoproteins following proteolysis was done as follows: The migrant is applied to Whatman #1 paper and development of the chromatogram is carried out in a conventional descending manner with butanol:acetic acid:water, 4:1:1; v:v:v. The chromatogram is dried and peptide material was detected with ninhydrin spray.

TURBIDOMETRIC STUDIES

It was felt that changes in the stability of a lipoprotein complex would be manifested by gradual flocculation of the material. To illustrate the effect of proteolysis on the beta lipoprotein complex, it was decided to determine the rate of turbidity formation at elevated temperatures. The measurements were made in a Beckman DU Spectrophotometer equipped with thermospacers. The thermospacers in conjunction with a Bronwill thermoregulator and water bath can be used to control the temperature of the spectrophotometer's cell compartment. Turbidity formation at

various temperatures was determined by measuring the optical density of the lipoproteins at 700 millimicrons. At this wave length, the lipoprotein solutions have practically no absorption. Therefore, changes in the optical density at 700 millimicrons reflect the production of turbidity.

CHAPTER IV

THE PROTEOLYSIS OF HUMAN SERUM BETA LIPOPROTEINS
RESULTS AND DISCUSSION

At the onset of this investigation several limitations had to be imposed. Some were necessary from a practical standpoint; others were necessary because of the quality of materials which were available. First of all, the enzymes to be used in this study had to be chosen. Chymotrypsin and trypsin were selected because they are active in a pH range where the lipoproteins are stable and are available in very pure form. Furthermore, solutions of these enzymes are stable over relatively long periods of time and their activities are easily determined.

The next consideration was the selection of the particular serum lipoprotein fraction to be studied. The fractionation of serum lipoproteins by ultracentrifugation is a relatively long process and only about twenty milligrams of a given lipoprotein are obtained from each series of ultracentrifugal runs. It was decided to use serum beta lipoproteins, in the density range of 1.019-1.063, mainly because of the homogeneity of this fraction. The lipoprotein fraction of density less than 1.019 contains more than one type of lipoprotein and is contaminated by chylomicra. The alpha lipoproteins, in the density range 1.063 to 1.210, are thought to consist of two fractions.

Another problem arose during the course of the investigation. The concentration of the beta lipoproteins varied from

different ultracentrifugal preparations. Furthermore, the concentration of protein was relatively low, about two milligrams per milliliter. It would have been possible to dilute each preparation to the same concentration. However, this procedure was discarded because the values obtained in a variety of experiments would have approached the lower limit of the analytical techniques. No satisfactory method is presently available for further concentrating the lipoproteins.

Another difficulty related to the small amout of material obtained from each preparation was that it was often impossible to run corresponding experiments with both trypsin and chymotrypsin. Early in this investigation it became apparent that the effect of chymotrypsin appeared to be more reproducible. When only enough lipoprotein for one experiment was available, chymotryptic studies were made.

EVIDENCE FOR THE PROTEOLYSIS OF LIPOPROTEINS

One of the first experiments which was done was to survey the effect of chymotrypsin on the various serum lipoprotein fractions. This was accomplished by determining the rate of release of trichloroacetic acid soluble tyrosine. The results are illustrated in figure 2 and the data tabulated in table XVI, Appendix III. At this point the materials necessary for assaying the chymotrypsin were not yet available. To compensate for this, a solution of hemoglobin was also used in conjunction with chymo-

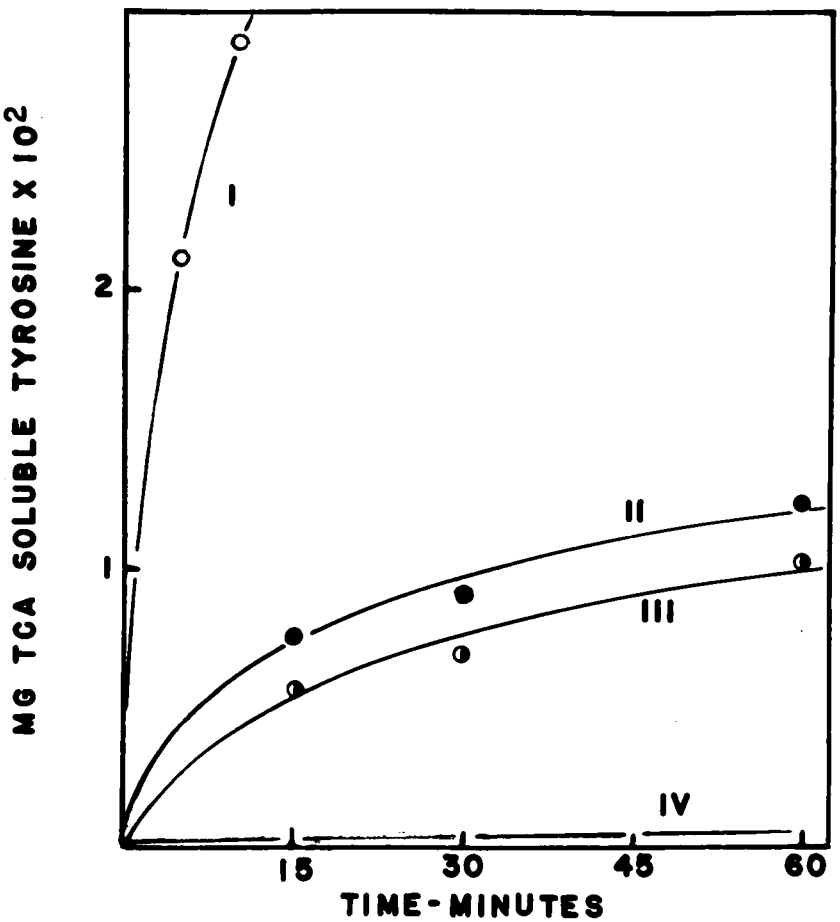


Figure 2

EFFECT OF CHYMOTRYPSIN ON SERUM LIPOPROTEINS

The reaction was carried out in 2.0 ml of phosphate buffer, pH 7.5, 0.02 M, at 30°C. Each reaction mixture contained 0.10 mg of chymotrypsin. I-Hemoglobin, approximately 5 mg. II-Beta lipoprotein, 2.23 mg of protein. III-Lipoproteins of density less than 1.019, 1.80 mg of protein. IV-Alpha lipoproteins, density 1.063 to 1.210, 8.82 mg of protein.

trypsin. This served not only to establish the activity of the enzyme but also to show that proteolysis of ultracentrifugally prepared lipoproteins is very slight. A satisfactory explanation as to why proteolysis did not occur with alpha lipoproteins cannot be offered at the present time.

It was at this point that attention was centered on the beta lipoprotein fraction. In order to demonstrate the fact that proteolysis was occuring with both trypsin and chymotrypsin, further measurements of the release of trichloroacetic acid soluble peptides (as tyrosine) were made. The conditions used in the experiments appear along with the data. All the studies described here and those that follow were done in a water bath at 30 ± 0.5°C. The solutions used in preparation of the enzyme-lipoprotein incubation mixtures were equilibrated beforehand to 30°C. Usually only 0.10 mg of the chymotrypsin or trypsin was used. No trichloroacetic acid soluble tyrosine could be detected from the enzymes alone.

Typical rate curves for the proteolysis appear in figure 3. The data for this experiment along with confirmatory experiments are recorded in table XVII, Appendix III. Throughout this discussion the concentration of the lipoprotein is expressed by both the protein and cholesterol concentration. The results for the series of experiments were reasonably similar; only a comparison of the results obtained with one enzyme should be made. The curves presented in figure 3 would seem to indicate that the proteolytic reaction is almost complete after one hour. It is

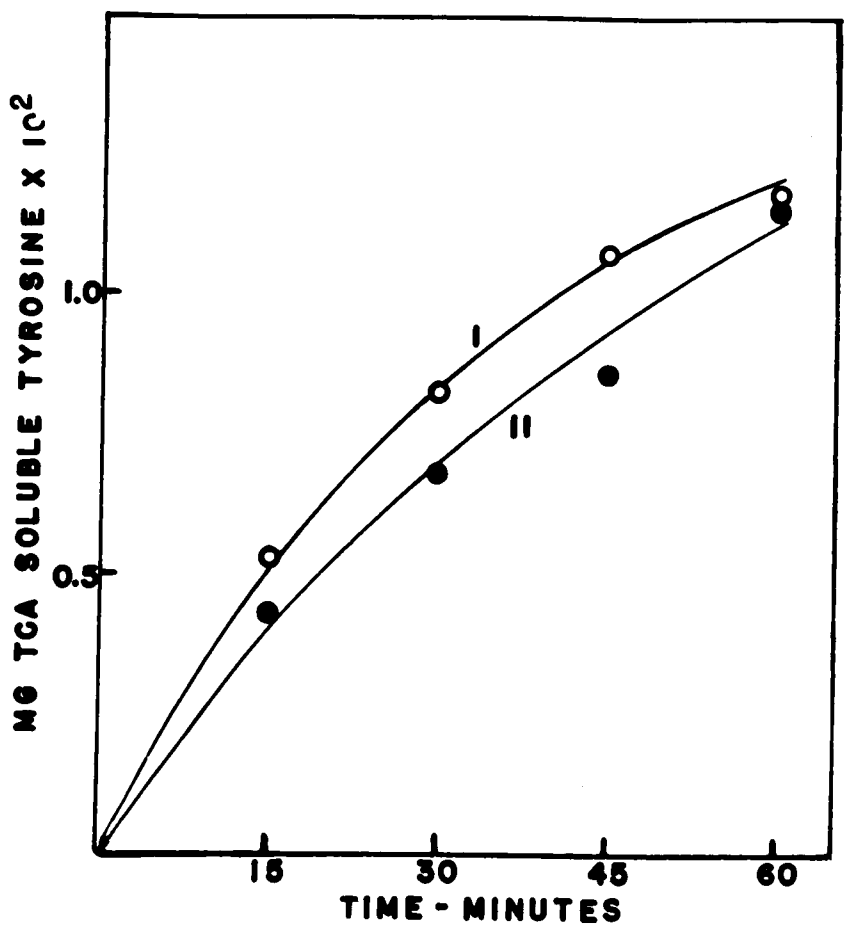


Figure 3

PROTEOLYSIS OF BETA LIPOPROTEIN

All reactions were carried out in phosphate buffer, pH 7.5, 0.02 M at 30° C. I--Volume-2.0 ml; chymotrypsin-0.05 mg, 610 units; beta lipoprotein, 2.02 mg protein, 2.18 mg cholesterol. II--Volume-1.0 ml; trypsin-0.05 mg, 565 units; beta lipoprotein, 0.99 mg protein, 0.77 mg cholesterol.

unfortunate that no estimate of the number of peptide bonds which were broken could be made from this data. Such calculations can be made when the rate of proteolysis is determined by changes in the ninhydrin color. As was pointed out in Chapter III, the use of the ninhydrin reagent was not possible. There was no detectable cholesterol in the trichloroacetic acid soluble fraction.

An interesting sidelight of these studies is the observed effect of chymotrypsin on beta lipoproteins that are briefly frozen and then thawed. Freezing is thought to damage the native structure of the lipoprotein. To test the effect of freezing on the chymotryptic proteolysis, a preparation of beta lipoprotein was frozen in a dry ice-isopropyl alcohol bath (approx. -50° C). The frozen lipoproteins were immediately thawed and a small amount of turbidity removed by centrifugation. The rate of proteolysis by chymotrypsin was then determined in the usual manner. The results are illustrated in figure 4 and the data is presented in table XVIII, Appendix III. The striking difference between the frozen-thawed and the untreated beta lipoprotein indicated that definite changes have occurred in the protein moiety. This is in agreement with the suggestion of Gurd (32). He pointed out that in the absence of water, the lipoprotein complex may rearrange to allow some form of internal compensation, and assume a configuration which does not lead back uniquely to the original structure when water is readmitted to the system. This is not the case with simple proteins.

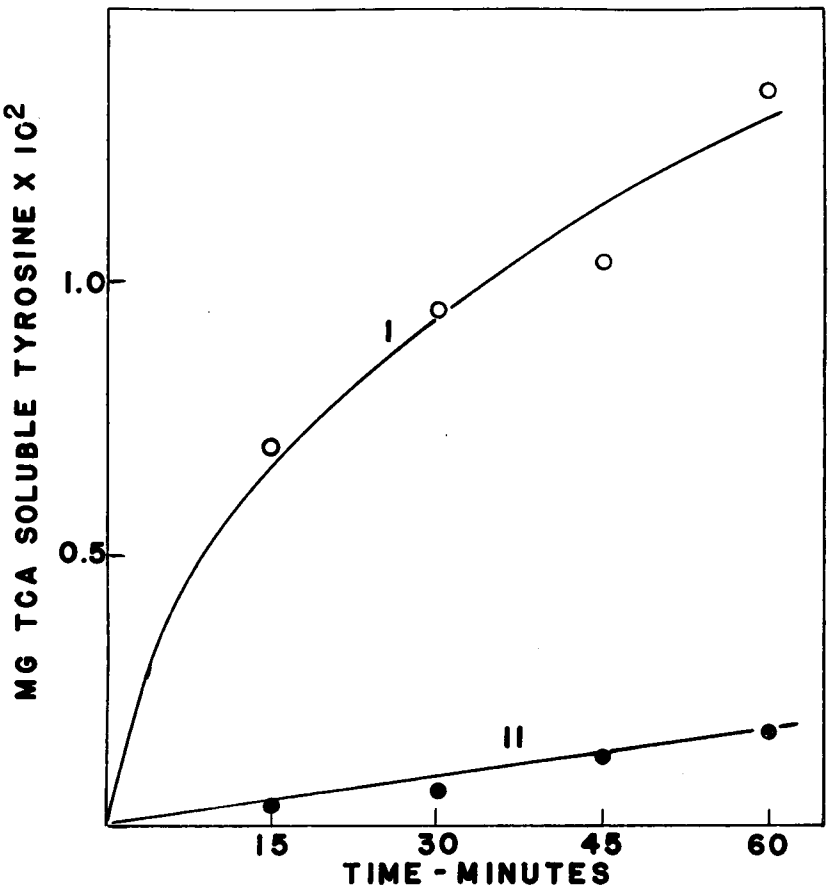


Figure 4

EFFECT OF FREEZING ON THE PROTEOLYSIS OF BETA LIPOPROTEIN

Carried out in 2.0 ml of phosphate buffer, pH 7.5, 0.02 M, at 30°C. Chymotrypsin-0.10 mg, 1500 units; Beta lipoproteins-1.85 mg protein, 1.35 mg cholesterol. I--Frozen-thawed. II--Untreated.

Early in this study attempts were made to find a simple procedure for determining the rate of proteolysis. Kinetic estimation of the factors influencing the proteolysis of beta lipoprotein could then be made. The main objective was to find a method which would require less of the lipoprotein than is needed when tyrosine determinations are made. Viscosimetric and dilatometric studies of the lipoprotein undergoing proteolysis indicated no change. The significance of these findings will be discussed in a later section.

Slight but detectable changes in the ultra-violet absorption spectra of the beta lipoprotein after treatment with trysin and chymotrypsin were found. The results are shown in figure 5 and the data presented in table XIX, Appendix III. There was no need to correct for the enzyme protein; the concentrations of chymotrypsin and trypsin were below detectable limits. The change resulting from either trypsin or chymotrypsin was so similar that only one curve is drawn in figure 5.

The decrease in ultra-violet absorption occurs very frequently during the proteolysis of undenatured proteins (41). Attempts to use this change to determine the rate of proteolysis were unsuccessful. The decrease in ultra-violet absorption represents at most, 25% of the net absorption of the beta lipoprotein. Furthermore, it is not at all unlikely that some of the decrease in the ultra-violet absorption spectra occurs after proteolysis has stopped. Gradual rearrangement of the molecule may occur

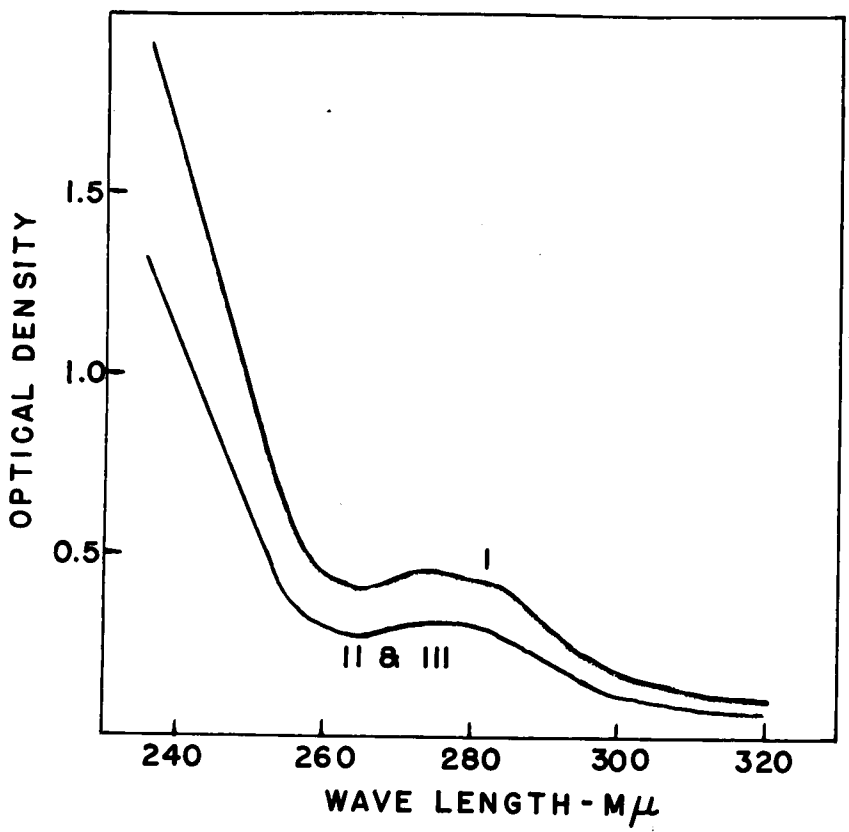


Figure 5

EFFECT OF PROTEOLYSIS ON THE ULTRA-VIOLET SPECTRUM OF BETA LIPOPROTEIN

I--untreated beta lipoprotein. II & III treated with chymotrypsin and trypsin. The concentration of beta lipoprotein was 0.186 mg/ml protein and 0.209 mg/ml cholesterol. The concentration of the enzymes was 0.0082 mg/ml. The spectral determinations were carried out in a Tris buffer, pH 7.5, 0.05 M.

following peptide bond breakage. This would account for the fact that two different proteolytic enzymes produced essentially the same change. At any rate, this spectral change may be taken as added evidence that proteolysis is occurring.

The next step was to determine if the protein alterations were affecting the binding of lipids by the lipoprotein. This is a difficult problem because there are no direct methods for measuring the extent or capacity for lipide binding by a protein in an aqueous media.

LIPOPROTEIN PROTEOLYSIS AND LIPIDE "BINDING" CAPACITY

Since no direct procedure is presently available for determining the energy of the lipoprotein-lipide interaction, an indirect empirical technique was devised. The removal of lipide from lipoproteins by organic solvent extraction is not an instantaneous process. The rate of removal of the lipide by the organic solvent would seem to be governed by the same factors which maintain the stability of the lipoprotein complex in an aqueous environment. Proteolysis of the lipoprotein might be expected to increase the rate of removal of lipide if the maximum stability is provided by the native peptide chain(s). However, it is important to realize that the ease of lipide extraction from lipoproteins is a function of many variables. A few of them are 1) organic solvent 2) the ratio of organic solvent to lipoprotein 3) factors which directly affect the lipoprotein--e.g. pH, denaturing agents, salt concentration, etc. 4) temperature. For the most part these

variables do not present any difficulty because they may be kept constant during a proteolytic rate study. The main problem was to find the system which would result in only partial extraction of the lipide and which could be done fairly rapidly.

Preliminary experiments indicated that partial extraction of cholesterol from beta lipoprotein could be obtained with ether. The removal of cholesterol and phospholipide from beta lipoprotein as a function of the duration of extraction is illustrated in figure 6. The data for this experiment is presented in table XX of Appendix III. The extraction procedure was briefly as follows: To the lipoprotein solution contained in a 15 ml glass stoppered centrifuge tube was quickly added 4.0 ml of ether (A.R., not anhydrous). The tube was shaken by hand and the duration of shaking was timed with a stop watch. The ether phase was then allowed to separate for about one minute and an aliquot removed with a pipette and placed in a colorimeter tube. The ether was evaporated and cholesterol was determined as previously described.

With a few exceptions, the procedure for the phospholipide extraction was the same as that for cholesterol. Preliminary work had shown that the extraction of phospholipide from the beta lipoprotein by ether is very slow. For this reason chloroform was used in place of ether. The use of chloroform presented a difficulty. A precipitate which did not separate from the chloroform phase occurred after the phospholipide

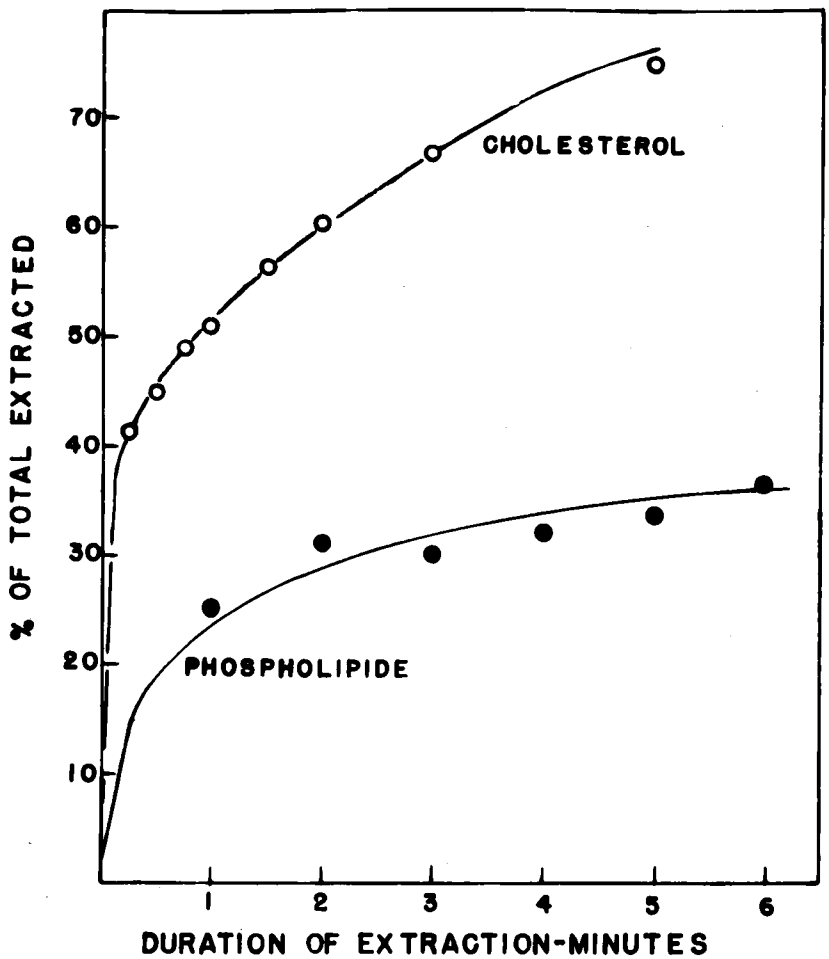


Figure 6

PARTIAL EXTRACTION OF CHOLESTEROL AND PHOSPHOLIPIDE FROM BETA LIPOPROTEIN

Cholesterol--1.0 ml beta lipoprotein (0.930 mg/ml cholesterol, 0.960 mg/ml protein) in phosphate buffer, pH 7.5, 0.02 M extracted with 4.0 ml ether for the indicated times.
Phospholipide--1.0 ml beta lipoprotein (1.50 mg/ml cholesterol, 1.06 mg/ml protein, 0.80 mg/ml phospholipide) in Tris buffer, pH 7.5, 0.02 M. Extracted with 4.0 ml of chloroform for the indicated times.

extraction. To eliminate the precipitate, the aqueous phase was removed immediately after the extraction and the chloroform extract was filtered through Whatman #1 paper. An aliquot of the filtrate was analyzed for phospholipide. The removal of the aqueous layer and the filtration step only took about two minutes so that evaporation of the chloroform was minimized. Tris buffer was used in place of the phosphate buffer during these phospholipide studies. This eliminated the possible contamination of the chloroform extract with small amounts of aqueous phosphate.

The partial extraction curves presented in figure 6 indicate that the initial removal of cholesterol or phospholipide is fairly rapid. In the cholesterol curve, the amount extracted tapers off and the removal of the last 50% occurs at a much slower rate. In order to show that proteolysis affects the nature of the lipide binding, it was decided to make the partial extractions at several time intervals during proteolysis. This presented a problem. It is doubtful that the organic solvent extraction immediately stops the proteolytic reaction. Therefore in order to negate the time error which would result from proteolysis continuing during the extraction, a short extraction period was necessary. For this reason the one minute partial extraction was chosen.

Before discussing the reproducibility of these one minute extractions, one other point needs clarification. As was pointed out earlier, the rate of extraction of lipide from lipoproteins depends on several variables, two of which could not be

kept constant from one experiment to another. One was the "loading" of the lipoprotein with cholesterol. It is to be expected that if a greater amount of cholesterol is bound to each lipoprotein molecule, a larger percentage will be extracted in one minute. The "loading" of the beta lipoprotein would be reflected in the cholesterol to protein ratio. Variations in this ratio are apparent from the data appearing in table V. The values were obtained by protein and cholesterol analysis of the beta lipoprotein prepared by ultracentrifugation. Each set of values represents a different preparation.

The variations in the concentration of the beta lipoprotein also presents some complications. The differences in concentration alter the lipoprotein to ether ratio since the amount of ether used in the one minute partial extraction was kept constant, 4.0 ml. For the previously mentioned reasons, the per cent of the total cholesterol which is extracted in one minute will vary with the particular lipoprotein preparation.

Evidence for the reproducibility of the one minute partial extractions of cholesterol is described in table VI. The results of each part were obtained with a different preparation. This accounts for the variation between the different sections. Only one value is reported in cases where the remainder of the beta lipoprotein preparation was needed for another experiment. The first column in table VI described the concentration and volume of lipoprotein present.

TABLE V

COMPOSITION OF HUMAN SERUM BETA LIPOPROTEIN

Protein mg/ml	Cholesterol mg/ml	Cholesterol/Protein
2.23	2.16	0.97
2.58	2.78	1.08
2.02	2.18	1.08
1.85	1.35	0.73
2.44	2.32	0.95
1.98	1.55	0.78
1.92	1.86	0.97
1.99	2.06	1.03
2.11	3.00	1.42
2.07	2.32	1.12
2.23	2.51	1.12
2.53	2.90	1.15
2.37	2.71	1.14
2.63	2.96	1.10
2.38	2.73	1.15
3.00	3.41	1.14
3.18	3.49	1.10
2.46	2.84	1.15
2.30	2.77	1.20
2.36	2.91	1.23
2.61	3.00	1.15

TABLE VI

ONE MINUTE PARTIAL ETHER EXTRACTIONS OF BETA LIPOPROTEIN

Solution of lipoprotein	% of total cholesterol One minute ether extraction	Average*
A. 2.0 ml-2.02 mg protein, 2.18 mg cholesterol	57.8	
B. 2.5 ml-0.925 mg protein, 0.675 mg cholesterol	51.9	
	50.6	
	52.7	
	51.2	51.6 ± 2.0
	49.8	
	53.6	
C. 2.0 ml-2.44 mg protein, 2.31 mg cholesterol	53.9	
D. 2.0 ml-0.96 mg protein, 0.77 mg cholesterol	55.5	
E. 2.0 ml-0.96 mg protein, 0.93 mg cholesterol	50.7	
	54.1	
	52.9	52.9 ± 2.2
	53.8	
F. 2.0 ml-1.99 mg protein, 2.06 mg cholesterol	39.8	
	41.2	40.5 ± 0.7
G. 0.50 ml-0.95 mg protein, 1.06 mg cholesterol	45.8	
	46.1	46.0 ± 0.2

TABLE VI (CONT'D)

Solution of lipoprotein	% of total cholesterol One minute ether extraction	Average*
H. 2.0 ml-1.31 mg protein, 1.98 mg cholesterol	58.2	58.7 ± 0.5
	59.1	
I. 1.0 ml-1.27 mg protein, 1.48 mg cholesterol	59.0	
	57.0	57.2 ± 1.8
	55.6	
J. 2.0 ml-2.38 mg protein, 2.73 mg cholesterol	56.8	
	57.8	57.1 ± 0.3
	56.8	
K. 1.0 ml-1.19 mg protein, 1.37 mg cholesterol	54.9	
	54.3	55.8 ± 2.4
	58.2	

*Data reported is the average value ± the maximum deviation from this average.

Two anomalous experiments were omitted from the table. In one preparation duplicate one minute partial extractions resulted in an average value of 31.1 ± 0.5%; in another instance, a beta lipoprotein preparation released over 70% of the total cholesterol present. No reasonable explanation can be offered for these discrepancies. Nonetheless, the values for the one minute ether extraction agreed within about 2%; in many cases the variation was even less.

The next step was to find out what effect limited proteolysis of the beta lipoprotein had on the amount of cholesterol which was removed by the partial extractions. Before presenting the results of the extraction studies, it is necessary to describe one simple observation made throughout this study. Usually upon completion of an experiment, whatever was left of the beta lipoprotein solution was stored in the cold (2°C.). One would expect that if much damage to the lipoprotein occurred by proteolysis, precipitation of the material would occur very rapidly. This was not the case. Beta lipoproteins containing either trypsin or chymotrypsin were as stable as the untreated substances. It would appear therefore that the alteration in the native structure is very slight. It is even possible that no peptide material is lost from the lipoprotein. This point will be discussed in a later section.

Definite changes in the structure of the beta lipoprotein do occur during proteolysis. This can be demonstrated by

removing aliquots of the beta lipoprotein-proteolytic enzyme system at various time intervals and measuring the amount of cholesterol released during the one minute ether extraction. Similar effects are noted on the release of phospholipide by one minute chloroform extractions. Typical results for the action of chymotrypsin on beta lipoprotein are illustrated in figure 7 and the data recorded in table XXI of Appendix III. Experiments similar to those illustrated in curves I and II, figure 7 were repeated several times. The results were nearly the same in all the experiments. Proteolysis of the beta lipoprotein by chymotrypsin resulted in a 10 to 20% increase in the amount of cholesterol removed by the one minute ether extraction. The change in the lipoprotein which produces this effect occurs fairly early in the proteolytic reaction.

The results obtained from trypsin treatment were similar to those resulting from chymotrypsin. Some typical rate curves for the tryptic proteolysis of the beta lipoprotein are illustrated in figure 8 and the data presented in table XXII of Appendix III.

The data contained in figures 7 and 8 offers good evidence that alterations in the lipide "binding" capacity of the lipoproteins result from proteolysis. The cause of these changes is obscure but two of the most obvious possibilities are: (1) Proteolysis results in loss of some of the protein which "shields" the lipide from the environment. (2) Proteolysis causes a rear-

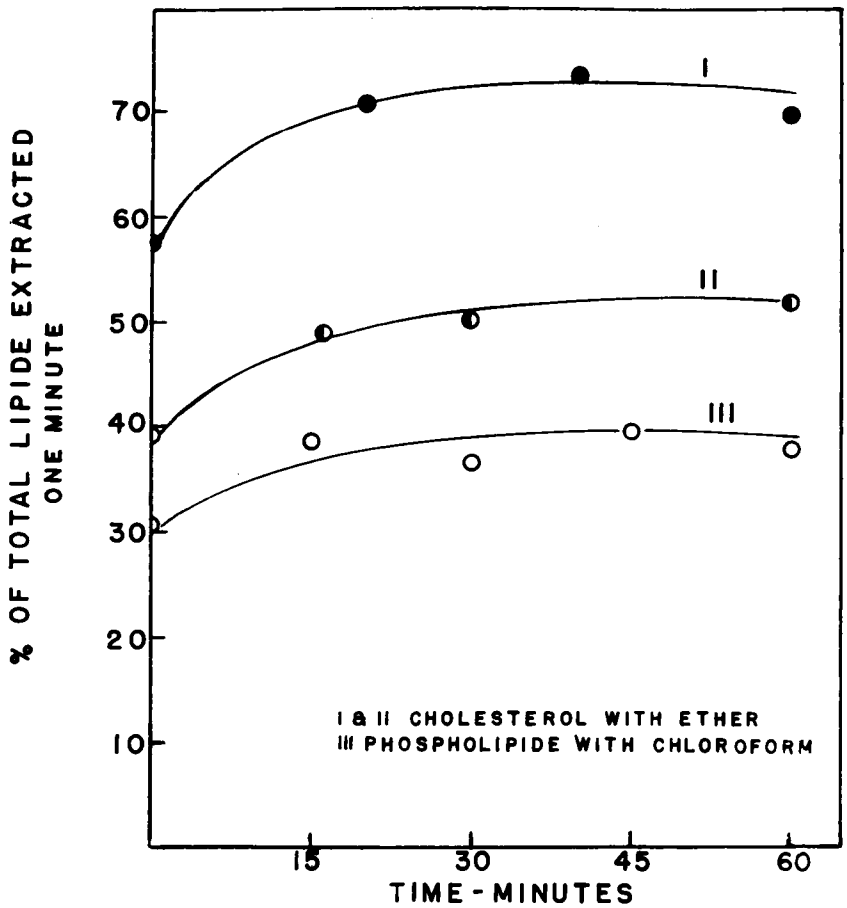


Figure 7

EFFECT OF CHYMOTRYPSIN ON PARTIAL LIPIDE EXTRACTIONS OF BETA LIPOPROTEIN

I & II in phosphate buffer, pH 7.5, 0.02 M. III in Tris buffer pH 7.5, 0.02 M Tris. 4.0 ml of the organic solvent was used to extract 2.0 ml of the reaction mixtures containing: I--2.03 mg protein, 2.18 mg cholesterol as beta lipoprotein, 0.10 mg chymotrypsin (850 units). II--1.99 mg protein, 2.06 mg cholesterol as beta lipoprotein, 0.10 mg chymotrypsin (280 units). III--2.15 mg protein, 3.36 mg cholesterol, 2.23 mg phospholipide as beta lipoprotein, 0.10 mg chymotrypsin (930 units).

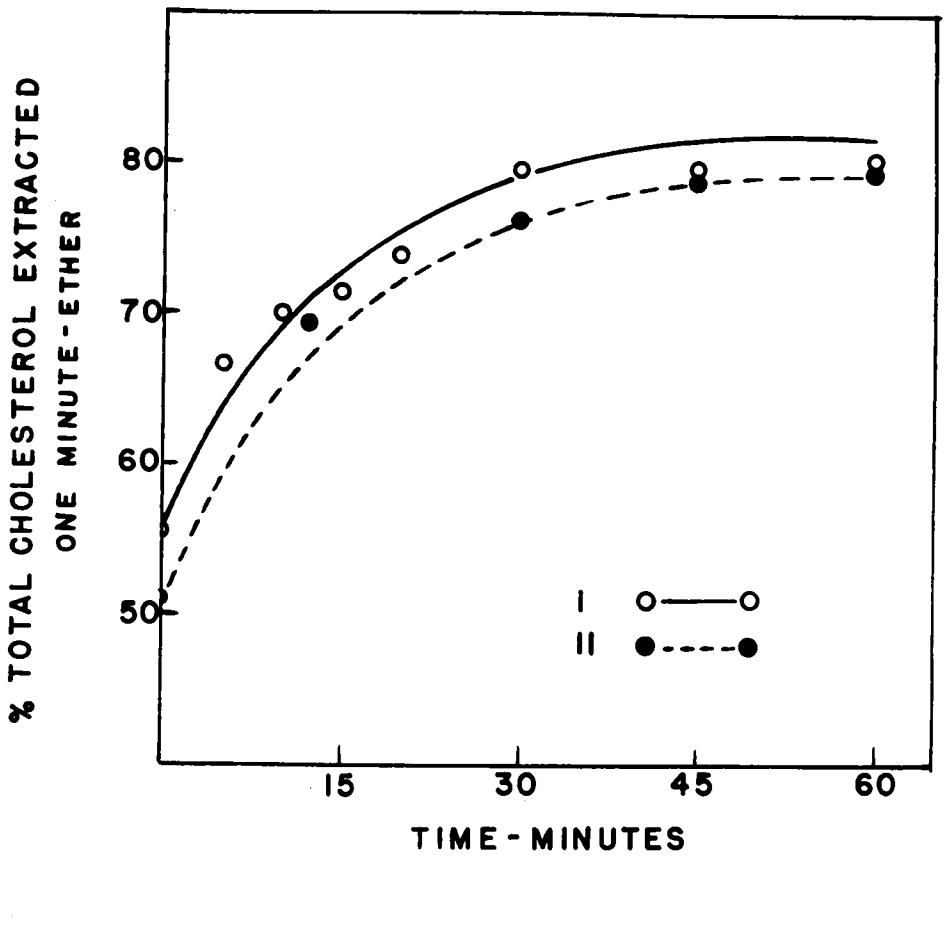


Figure 8

EFFECT OF TRYPSIN ON PARTIAL CHOLESTEROL EXTRACTIONS OF BETA LIPOPROTEIN

Carried out in phosphate buffer, pH 7.5, 0.05 M. 4.0 ml of ether was used to extract 1.0 ml of the reaction mixtures containing: I--1.19 mg protein, 1.87 mg cholesterol as beta lipoprotein, 0.05 mg trypsin (412 units). II--0.795 mg protein, 0.875 mg cholesterol as beta lipoprotein, 0.05 mg trypsin (400 units).

rangement of the lipoprotein molecule and as a result its lipide conjugating ability is reduced. This may or may not be accompanied by loss of peptide material from the lipoprotein.

TURBIDOMETRIC STUDIES

In order to leave little doubt that definite changes have occurred in the beta lipoprotein as a result of proteolysis, an approach completely different from the extraction studies and tyrosine determinations would be valuable. It has already been pointed out that no change in the viscosity of the beta lipoprotein could be detected during proteolysis at 30°C. Similarly no volume changes were detected following treatment with chymotrypsin or trypsin. There was no way of determining whether the instruments used for the viscosimetric and dilatometric studies were sensitive enough to detect small changes. For this reason, a different approach was taken. It seemed likely that the new lipoprotein formed by proteolysis would not be as stable as the native material. But it has already been pointed out that no precipitation of the beta lipoprotein occurred upon standing at 2°C. The most obvious experiment was then to determine if the stability of the treated lipoprotein was the same as the untreated at elevated temperatures. A procedure for the determination of turbidity formation at elevated temperatures was devised; it has been described in Chapter III.

The procedure for the experiment was as follows: The beta lipoprotein-enzyme solutions were set up the day preceding

the turbidity formation studies. The total volume of each mixture was 10.0 ml and contained 13.1 mg protein and 15.0 mg of cholesterol as beta lipoprotein. One tube contained 0.5 mg of chymotrypsin (3400 u) and another mixture contained 0.5 mg of trypsin (3300 u). The third tube contained no enzyme and served as a blank. The proteolysis was allowed to continue for 18 hours at room temperature. At the end of this time, three milliliters of each mixture were placed in Corex Beckman spectrophotometer cells and the rate of turbidity formation determined. At the end of each determination, 2.0 ml of the lipoprotein-enzyme solutions and of the lipoprotein blank were pipetted into 2.0 ml of 10% trichloroacetic acid. The precipitate which formed was removed by centrifugation and the optical density of the trichloroacetic acid soluble material was determined at 280 mu.

The rate of turbidity formation for the treated lipoproteins is presented in figure 9 and the data contained in table XXIII, Appendix III. The initial lag period which appears on the curves probably results from the time required to bring the lipoprotein solution from room temperature to that of the spectrophotometer cell compartment. The untreated beta lipoprotein was completely stable even after heating at 60°C for one hour. Turbidity started appearing in the beta lipoprotein treated with trypsin only after the temperature was raised to 60°C. Below 45°C turbidity formation was absent in even the chymotrypsin treated lipoproteins.

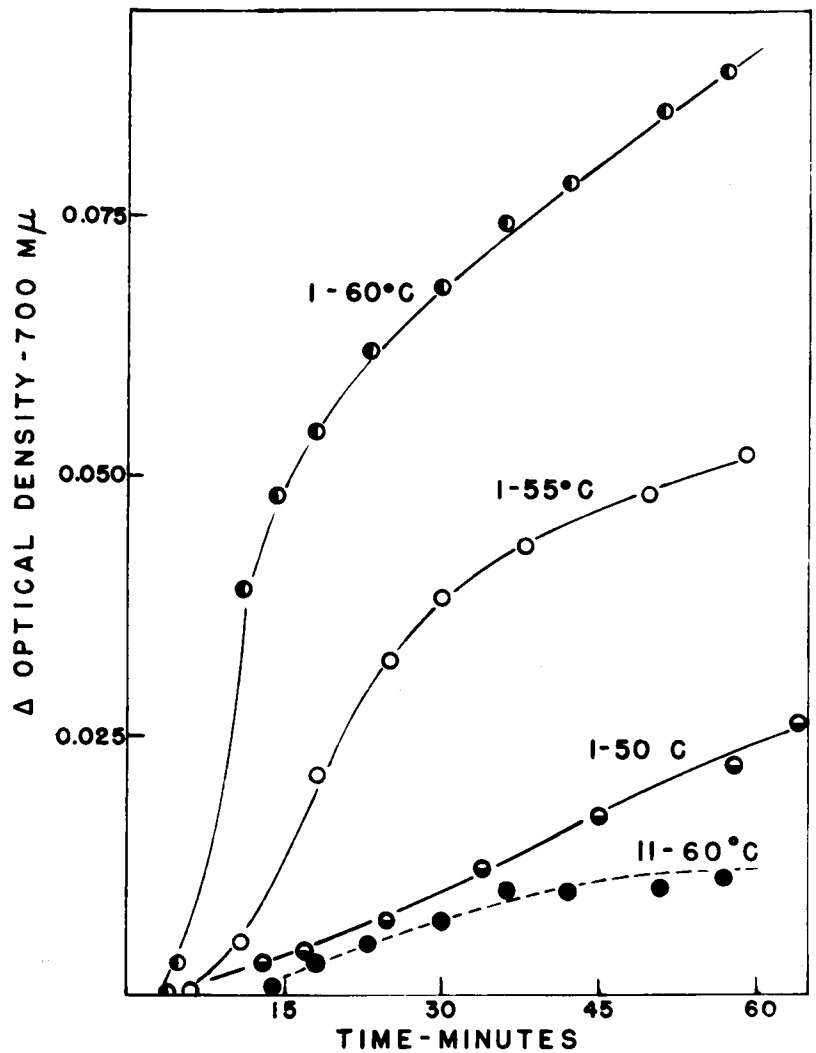


Figure 9

TURBIDITY FORMATION BY BETA LIPOPROTEIN

Incubation mixtures were made up in phosphate buffer, pH 7.5, 0.05 M. The solutions contained 1.31 mg/ml protein and 1.50 mg/ml cholesterol as beta lipoprotein. In addition, the curves labeled I contained 0.05 mg/ml chymotrypsin (340 units/ml). The curve labeled II contained 0.05 mg/ml trypsin (330 units/ml).

Figure 10 represents the results obtained from the determination of the trichloroacetic acid soluble material after the turbidity determinations (see also table XXIV, appendix III). Raising the temperature increases only slightly the amount of material released from the blank. The results for trypsin parallel those of the blank. However the large differences establishes the fact that tryptic proteolysis occurred. Similarly the results for chymotrypsin in figure 10 are definite evidence that proteolysis occurred.

The development of turbidity following proteolysis may be taken as evidence that the stability of this complex molecule has been altered. It is interesting that although only small differences exist between the amount of proteolysis which occurred for trypsin and chymotrypsin (see figure 10), the altered lipoproteins which resulted were very different. The material which resulted from chymotrypsin was very sensitive to heat coalescence. This would seem to indicate that the amount of damage to the lipoprotein by the enzymes depends on the specific bonds broken. It also emphasizes the specific nature of the beta lipoprotein protein. If the protein was of a relatively unspecific nature, it would seem that very little differences should be found between the lipoproteins formed from breaking two different types of peptide linkages. The possibility also must be considered that the large differences in heat stability (chymotrypsin vs trypsin treatment) is a result of the amount of proteolysis which has oc-

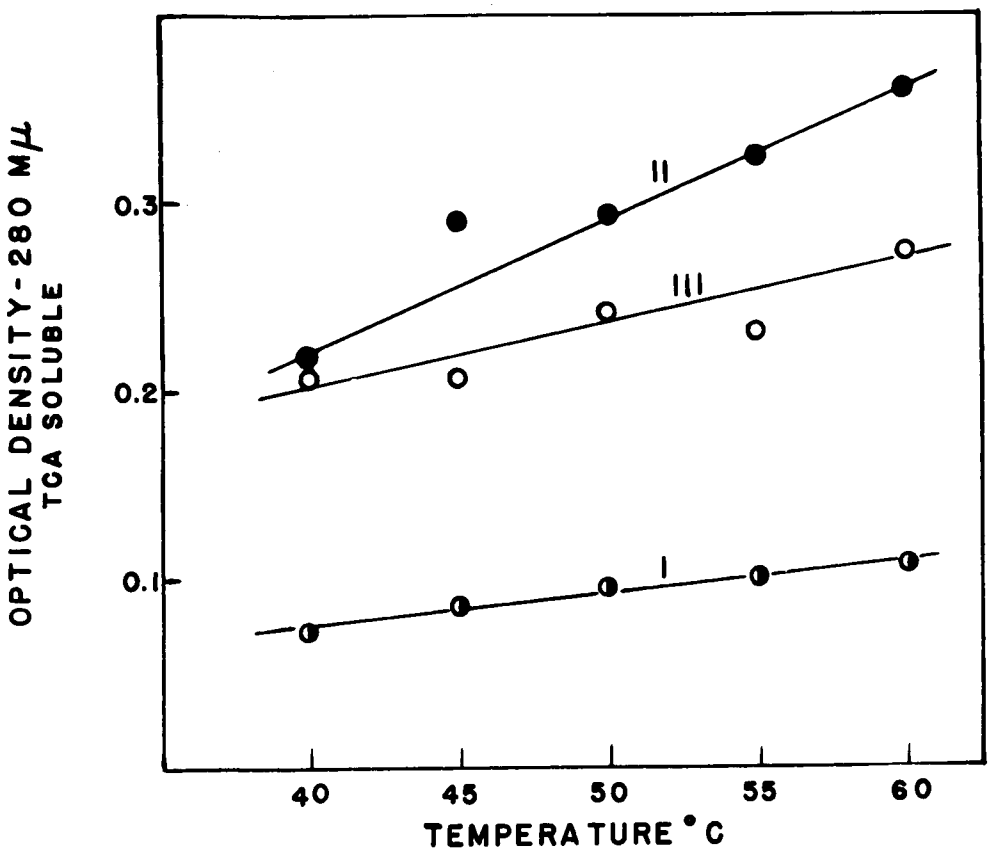


Figure 10

RELEASE OF TCA SOLUBLE MATERIAL FROM BETA LIPOPROTEIN

I. Beta lipoprotein, untreated. II. Beta lipoprotein treated with chymotrypsin. III. Beta lipoprotein treated with trypsin (see tables XXIII and XXIV, Appendix III).

curred.

IONOGRAPHY OF BETA LIPOPROTEIN FOLLOWING PROTEOLYSIS

It has been mentioned earlier that the proteolysis of beta lipoprotein may occur without the loss of any peptide fragments from the molecule. In other words, peptide linkages may be broken but the fragments which are formed may remain attached to the lipoprotein via the bonds which join the lipide to the protein. Certainly, the beta lipoprotein molecule after treatment with trypsin or chymotrypsin must retain most of the protein moiety. It would be extremely unlikely for cholesterol to exist in aqueous solution at a concentration level of 2-3 mg/ml in the absence of some of the protein.

Evidence for the fact that beta lipoprotein may remain as one molecular unit following proteolysis was obtained by ionography. The procedure was to incubate the beta lipoprotein with the proteolytic enzymes overnight. After this incubation period, duplicate samples of treated beta lipoproteins and untreated blanks were applied to Whatman #1 paper strips. The ionograms were allowed to develop under an electrical potential of about 0.1 volts/cm for about 6 hours. A veronal buffer system, pH 8.6, ionic strength 0.05 was used for the ionography. At the completion of the run, one strip was stained with bromphenol blue and a duplicate was sprayed with ninhydrin reagent. The amount of either chymotrypsin or trypsin was below the detectable limits

of bromphenol blue stain or the ninhydrin spray.

On the ionograms, the beta lipoproteins appear as a rather diffuse band with a low ionographic mobility. In no case, and this experiment was done at least four times, was it possible to detect any ninhydrin positive material which could be ascribed to the proteolysis of the beta lipoprotein. Furthermore, there appeared to be no significant difference between the mobilities of the treated and untreated beta lipoprotein.

Besides the hypothesis that no peptide material is actually broken off, one other possibility exists. It may well be that the color developing reagents, ninhydrin and bromphenol blue are simply not sensitive enough to detect small amounts of the peptide fragments. The ninhydrin spray will detect as little as 0.2 to 25 micrograms of amino acids (42). In the case of peptide, the minimal amounts detectable increases with the molecular weight of the peptide.

TRICHLOROACETIC ACID SOLUBLE MATERIAL FROM BETA LIPOPROTEIN

Although no peptide-like material could be detected in the beta lipoprotein-enzyme mixture by ionography, precipitation by trichloroacetic acid (TCA) causes the release of protein fragments. This point has already been demonstrated by using the Folin-Ciocalteu reagent for tyrosine. If the TCA soluble material is chromatographed on paper, the separation of ninhydrin positive material is possible.

Preliminary experiments had shown that the concentration

of peptide material in the TCA soluble portion of the beta lipoproteins was very low. It was decided, therefore, to concentrate the material prior to paper chromatography. Concentration was accomplished by lyophilization.

A typical procedure for the chromatographic analysis of the TCA soluble material from beta lipoproteins was as follows: Three incubation mixtures, each having a volume of 3.0 ml, were prepared. All of them contained 7.38 mg protein and 8.53 mg of cholesterol as beta lipoprotein. In addition one contained 0.1 mg of chymotrypsin (1630 u) and another contained 0.1 mg of trypsin (2050 u). These mixtures were allowed to stand at room temperature overnight. At the end of the incubation, 2.0 ml of 10% TCA was added and the precipitate which formed was removed by centrifugation. The TCA soluble material had a volume of approximately four milliliters. This was lyophilized to dryness and the solid material re-dissolved in 1.0 ml of 0.2 N NaOH. One-tenth of a milliliter of the re-dissolved material was slowly applied to the paper chromatogram. Whatman #1 paper was used. The chromatogram was developed by the conventional descending technique with butanol:acetic acid:water, 4:1:1, v:v:v. The development of a 40 cm long chromatogram took 14½ hours. At the completion of the development, the chromatogram was dried in a current of warm air and peptide material located with ninhydrin reagent as described in Chapter III.

The results of this experiment are presented in

in figure 11. Similar results were obtained with two other comparable experiments. Areas on the chromatogram which gave barely detectable ninhydrin colors are denoted with a question mark. The untreated beta lipoprotein contained three areas of faintly ninhydrin positive material. This is in agreement with the data presented in figure 10. The TCA soluble material from beta lipoprotein contains some material which has a low absorption at 280 mu. The R_f of these substances would indicate that they are of low molecular weight. Since they are not lost during the prolonged dialysis used in the preparation of the beta lipoprotein, it may be presumed that they are weakly bound to the beta lipoprotein molecule and are released during the TCA precipitation. The material which appears at the origin of the chromatograms corresponding to treatment with chymotrypsin or trypsin, are probably high molecular weight peptides formed during the proteolytic reaction. Other ninhydrin positive substances resulting from proteolysis appear in the R_f range 0.7 to 0.8.

LIPIDE-FREE BETA LIPOPROTEIN

The studies of the trichloroacetic acid soluble material from beta lipoprotein following proteolysis are interesting but limited. A better understanding of the proteolytic reaction would be possible if all of the protein or protein fragments could be obtained from the lipoprotein after treatment with trypsin or chymotrypsin. Studies of the amino acid sequence and structure

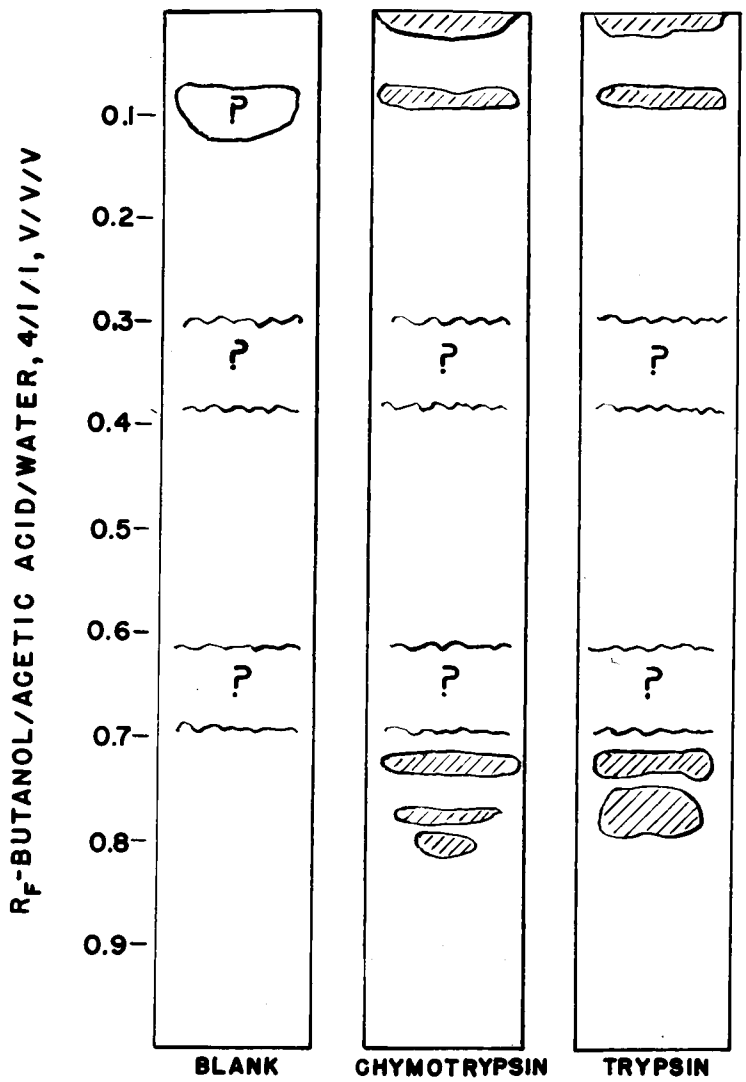


Figure 11

PAPER CHROMATOGRAPHY OF TCA SOLUBLE MATERIAL FROM BETA LIPOPROTEINS

Shaded areas on the chromatogram are spots resulting from ninhydrin spray. Areas denoted with a question mark are only faintly colored.

of the protein moiety from beta lipoprotein would also be facilitated if the corresponding undenatured lipide-free protein could be isolated. In this discussion, the term, undenatured, is used operationally to mean that the protein material would be soluble in dilute aqueous solutions and would demonstrate recombining capacity toward lipide. Attempts were made to obtain lipide-free protein before and after proteolysis of the beta lipoprotein.

Scanu (77) was able to remove the lipide from alpha lipoproteins by ether extraction. This has been discussed in Chapter II. He was unable to apply the same techniques to beta lipoprotein, and for this reason a different approach was used in the experiments to be described below.

With little or no background information to start with, the procedures were chosen on a trial and error basis. As will be seen, only very limited success was achieved. Despite this fact, the experiments which will be described provide a few guide-posts for further investigation and for this reason are reported. All the experiments were done on three different samples. One was untreated beta lipoprotein; the other two were incubated beforehand with either trypsin or chymotrypsin.

It was decided to attempt the preparation of beta lipoprotein protein by extracting the lipide from the solid rather than a solution of the preparation. To do this the beta lipoprotein was lyophilized prior to organic solvent extraction. The material which resulted had the typical "fluffy" appearance of

ordinary lyophilized proteins.

Extraction of the lyophilized beta lipoprotein samples were carried out in 15 ml glass stoppered centrifuge tubes. In the first series of experiments both pentane and ether were used to remove the lipide. The procedure was as follows: about ten milligrams of the beta lipoprotein preparation was shaken with five milliliters of the organic solvent for one minute. The suspension was allowed to stand in an ice bath for ten minutes. The organic solvent was separated from the protein by centrifuging for one minute at full speed in a clinical centrifuge. The organic solvent was poured off and the extraction procedure repeated four times. The final material was lumpy and hard when ether was used for the lipide extraction. On the other hand, if the extractions were made with pentane a white flocculent material resulted. If now the organic solvent was allowed to evaporate, even at 0°C, a brown-black hard mass resulted. It is important, therefore, to re-dissolve the protein material immediately after the last lipide extraction. Because of the better appearance of the protein material after pentane extraction, as shown in the preliminary experiments, the use of ether was dropped. It was shown that the serial pentane extractions removed all the cholesterol. No determination of other lipide material in the protein residue were made.

The problem of re-dissolving the lipoprotein protein was complicated by the fact that whenever the protein could not

be dissolved in an aqueous solution, the experiment had to be repeated starting with the ultracentrifugal preparation. It was shown that the residual protein from beta lipoprotein was not soluble in the following solutions: 1) water 2) veronal buffer, pH 8.6, 0.05 M 3) 0.001 N HCl 4) 0.2N H_2SO_4 5) 50% ethanol in water 6) acetate buffer, pH 4.0, 0.05 M 7) carbonate buffer, pH 10.2, 0.05 M. This includes the lipoprotein protein which had been treated beforehand with trypsin or chymotrypsin. Solution of the protein moiety could be accomplished in carbonate buffer, pH 10.2, 0.05 M if it contained urea (final concentration-4 molar). The protein dissolves instantly in this solution, but after standing a few minutes, a clear gel begins to float on the surface of the buffer. Gel formation occurs frequently upon treating globular proteins with urea. This is thought to occur as a result of increased protein-protein interaction made possible by the unfolding of the structure during the treatment with urea.

When the urea was removed by dialysis, the gel persisted. It could be temporarily broken up by vigorous mixing. This was done and ionography of the resulting solutions were performed in the usual manner (Veronal buffer, pH 8.6, u 0.05). Color development of the finished ionograms with bromphenol blue or ninhydrin indicated that the material streaked outward from the origin to the positive pole. No definite bands were detected. However, the samples treated with chymotrypsin or trypsin had a mobility of roughly three times that of the untreated beta lipo-

protein protein. The measurements of the migrating distance were made to the leading edge of the diffuse protein spots.

The failure of this particular aspect of the study, and for that matter, the failure of other investigators (77) to isolate the protein from beta lipoprotein provides little data for discussion. It is necessary to keep in mind that isolation of lipide-free beta lipoprotein may not be possible. Loss of cholesterol and other lipides from the beta lipoprotein have been shown to increase the sensitivity of the molecule to urea denaturation. It is possible therefore that the protein backbone of the beta lipoprotein is unstable in the absence of lipide material. Perhaps the mild proteolysis effected with trypsin or chymotrypsin does little to alter this situation.

CHAPTER V

THE CHARACTER OF THE LIPOPROTEIN-CHOLESTEROL INTERACTION

In the previous chapter, it has been suggested that even following mild proteolysis, dissociation of the protein-lipide complex may not occur. At present there are no methods available for quantitatively determining the affinity of lipoproteins for their major lipide constituents. However, using procedures to be described presently, it was possible to demonstrate indirectly the irreversible character of the lipoprotein-cholesterol interaction in a partially aqueous environment.

The rationale behind this study was the idea that if some free (unimolecular) cholesterol was in equilibrium with the lipoproteins, this "free cholesterol" would then pass through a dialysis bag and could be determined. Klotz (39) has described the use of the equilibrium dialysis technique for studying protein-ion interactions. The equilibrium dialysis technique involves dialysis of the protein solution contained in a cellophane sack, against aqueous buffers containing the ion to be studied. Obviously this method cannot be strictly applied to the binding of cholesterol by lipoproteins. First of all, cholesterol is essentially insoluble in aqueous solutions. Secondly, the permeability of cholesterol through cellophane membranes is questionable. Klevens and Carr (38) have shown that equilibrium of detergent molecules through a cellophane membrane requires periods

of dialysis ranging from 250 to 2500 hours. Such long periods of dialysis could not be used in conjunction with the lipoproteins. Serum lipoproteins undergo many forms of slow changes upon standing for long periods of time (32).

It was necessary therefore, to construct a membrane which would be readily permeable to cholesterol. Attention was directed to collodion membranes since they are relatively easy to cast. In 1939, Holmes (36) described the preparation of an artificial membrane which he claimed resembled an animal cell membrane. It contained lecithin in collodion but was used only in conjunction with toluene and other very non-polar solvents. More recently Carr et al (15) has described the preparation of reproducible collodion membranes. It was decided to attempt to construct a lipide permeable membrane by incorporating non-polar material into a simple collodion dialysis membrane.

After a few preliminary experiments the possible use of lecithin was eliminated. The only preparation of lecithin available was shown to contain relatively large amounts of cholesterol. Attempts to remove the contaminating cholesterol were largely unsuccessful. It was decided therefore to look for a non-polar material which was not of biological origin. Paraffin wax was not soluble in the collodion solution. However paraffin oil dissolved readily in a solution containing the collodion. Paraffin oil is a mixture of saturated hydrocarbons of lower molecular weight than that composing paraffin wax.

The technique of casting collodion membranes is fairly simple. Twenty grams of collodion (Parlodion, Mallinckrodt, AR) is dissolved in 500 ml of ether:95% ethanol, 50:50, v:v. The casting tubes (mandels) consist of test tubes which are 2 cm in diameter and 15 cm in length. A small hole is made in the bottom of the test tube. The hole is plugged with molten caramelized sugar which hardens on cooling. The choice of test tube (mandel) size depends on the desired size of the final membrane. The mandel is attached to a stirring motor by means of a rubber stopper and can then be rotated while in a horizontal position. The apparatus for casting collodion membranes is illustrated in figure 12. The collodion solution is poured on the rotating mandel moving toward the bottom of the tube with a continuous motion. This is done three times allowing three minutes between each pour. The mandel should be rotating at about 20 RPM. If it is going much slower than 20 RPM, the collodion solution will tend to drip off the mandel. At higher rotational speeds "rippling" of the membrane occurs. Ten minutes should elapse between the last pour and the termination of rotation. The membrane is dried for one hour at room temperature and then soaked overnight in distilled water. A small crystal of thymol may be used in the water soak as a preservative. The sugar dissolves and the membrane may be pulled off the casting tube. If not, soaking for one hour in 70% ethanol permits easy removal of the membrane from the mandel. The drying and soaking procedures should be kept as constant as possible (15). The mineral oil-

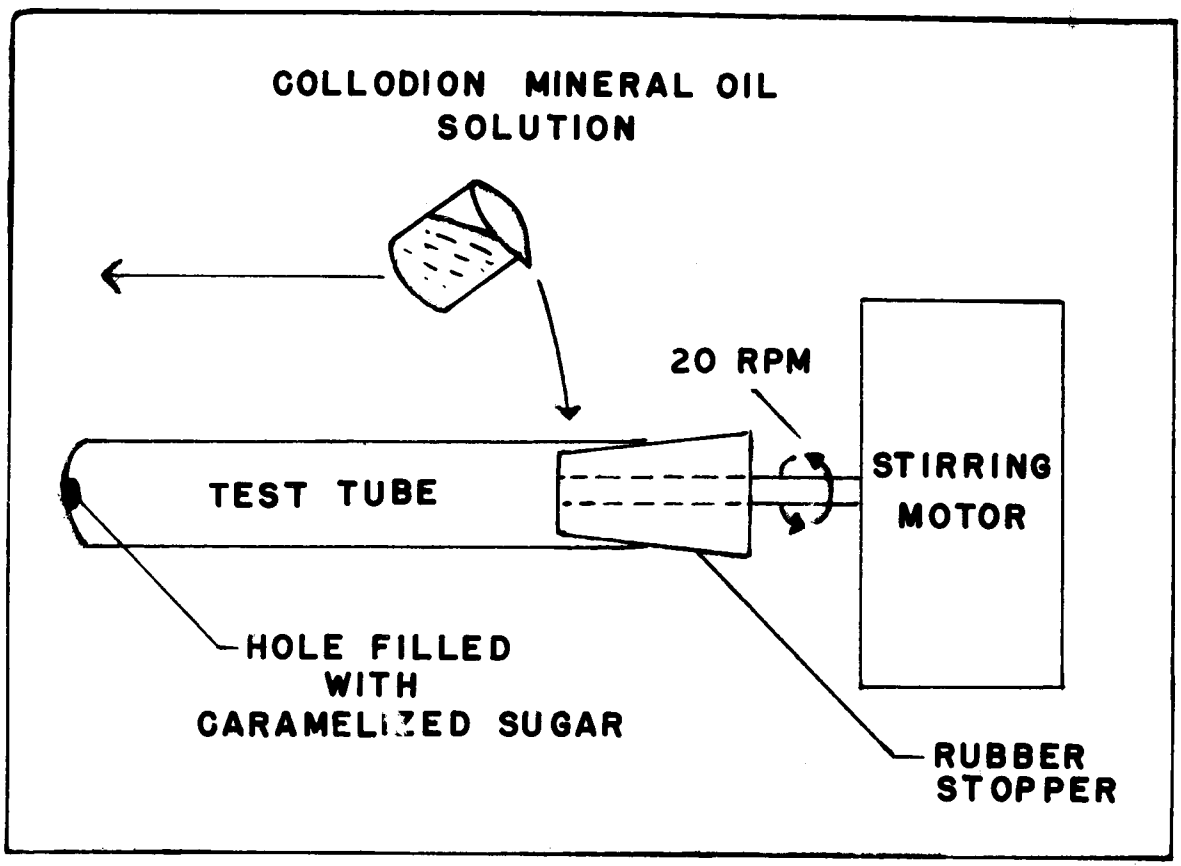


Figure 12

APPARATUS FOR CASTING COLLODION MEMBRANES

See text.

collodion membranes are prepared in the same manner except the collodion solution is made to contain 1 g% of mineral oil.

Larger amounts of mineral oil may also be incorporated into the membrane; concentrations up to 5 g% have been used. However, when the concentration is above 3 g%, some of the mineral oil appears in the soaking baths or later in the dialysis solutions.

Differences in the permeability of the membrane to non-polar material are striking. In one experiment, 10.0 ml of a saturated cholesterol solution in 60% ethanol was dialyzed for 45 hours, 25° C., against 10.0 ml of 60% ethanol. After dialysis, the solutions and bags were analyzed for cholesterol. The results are indicated in figure 13. It is necessary to point out that these membranes completely retain bovine serum albumin.

The somewhat low recoveries indicated on figure 13 probably result from the technique used to determine the membrane binding capacity. This was done simply by cutting the bag in half longitudinally and putting the pieces in a 15 ml centrifuge tube along with 4.0 ml of chloroform. The tube was shaken for one hour as described in Chapter III and cholesterol was determined in the usual manner. In no case was cholesterol found external to the ordinary collodion membrane.

Since the lipoproteins would be precipitated by the 60% ethanol used in the forementioned experiments, other dialysis systems had to be used. In one group of experiments the lipoproteins

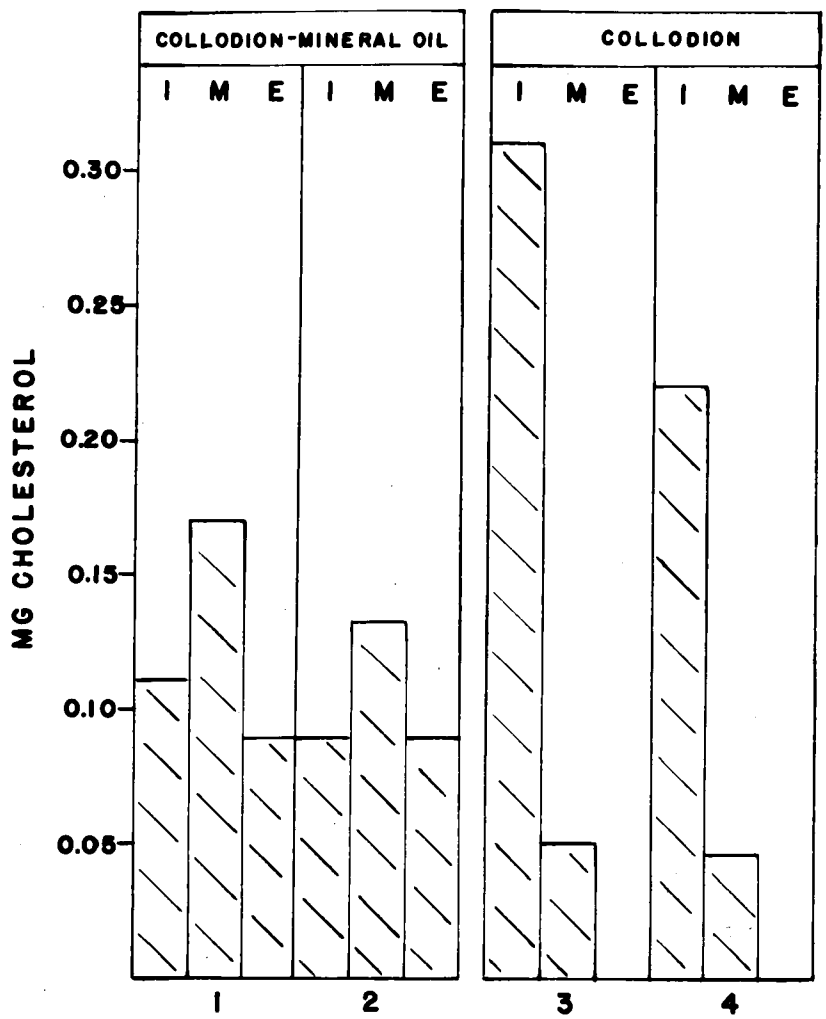


Figure 13

LIPIDE PERMEABILITY OF COLLODION MEMBRANES

I--internal solution, M--membrane bag, E--external solution. The recoveries for cholesterol were 1) 82% 2) 69% 3) 80% 4) 59%. Dialysis was carried out against 60% ethanol for 45 hours at 25°C.

were dialyzed against phosphate buffer, pH 7.5, 0.02 M. Another dialysis system was the same phosphate buffer containing 10% ethanol. This gives a final ethanol concentration of 5% when dialyzed against an equal volume of lipoprotein solution. In the last group of experiments, the lipoproteins were dialyzed against a solution composed of 10 mg% sodium oleate in phosphate buffer, pH 7.5, 0.02 M. This system is capable of forming stable micelles with cholesterol (86). The rationale behind the use of sodium oleate was that it would serve to stabilize any cholesterol passing through the membrane; the oleate would act in a manner similar to the ethanol used in the second system. The concentration of oleate was calculated to be well in excess of that which could be bound to the lipoproteins.

The initial experiments indicated that no cholesterol was present in the solutions external to the membrane. However these determinations were done by the cholesterol procedure which has been discussed earlier (see Chapter III). This method will not detect quantities much less than 100 micrograms of cholesterol or cholesterol esters.

In order to increase the sensitivity of the cholesterol test, a paper chromatographic procedure was chosen (59). Although it was not possible to determine the actual amount of cholesterol present, the procedure would detect quantities as low as 20 micrograms. The procedure was as follows: Whatman 3MM paper is first dipped into a 10% mineral oil in ether solution

(v:v). The ether is allowed to evaporate and the unknown samples are applied. The chromatograms are developed in a conventional descending manner with a system of acetic acid:chloroform: mineral oil, 130:70:20, V:V:V. Cholesterol containing compounds are detected with phosphotungstic acid (see Chapter III). Cholesterol has an R_f of about 0.63 and cholesterol esters have an R_f around 0.10.

The dialysis experiments were carried out as follows: 1) The mineral oil collodion membranes were prepared. 2) 1.0 ml of the lipoproteins was dialyzed against 1.0 ml of the respective system. 3) After dialysis solutions external to the membranes and the membranes were extracted with 4.0 ml of chloroform for one hour. The chloroform extract was collected, taken to dryness and re-dissolved in 0.20 ml of chloroform. 4) The concentrated chloroform extract was chromatographed as described earlier.

The results of one series of experiments are presented in table VII. No cholesterol could be detected in the solutions external to the cholesterol permeable membrane. The lipoprotein fractions contained about 2 mg of cholesterol compounds so that if only 1% dissociation took place it would have been detected. Some cholesterol was found associated with the membranes when the sodium oleate-phosphate buffer system was used. Since this cholesterol did not pass through the dialysis sack, it can probably be attributed to adsorption by the membrane of intact lipo-

TABLE VII

DIALYSIS OF LIPOPROTEINS CONTAINED IN CHOLESTEROL PERMEABLE MEMBRANES

Lipoprotein	Dialysis system	CHOLESTEROL & CHOLESTEROL-ESTERS* Membrane bag**	External solution
1) Beta lipoprotein	Phosphate buffer, pH 7.5, 0.02 M, 90 hours	none detectable	none detectable
2) Alpha lipoprotein	As Part 1.	none detectable	none detectable
3) Beta lipoprotein	Phosphate buffer, pH 7.5, 0.02 M, containing 10% ethanol, 90 hours	none detectable	none detectable
4) Beta lipoprotein	Phosphate buffer, pH 7.5, 0.02 M, containing 10 mg% sodium oleate, 115 hours	present	none detectable
5) Alpha lipoprotein	As part 4.	present	none detectable

*As determined by the paper chromatographic procedure described in the text.

** Cholesterol permeable mineral-oil--collodion membranes.

protein molecules. The surface of the collodion-mineral oil membrane may have been altered by the relatively high concentration of sodium oleate present.

One explanation for the absence of cholesterol in the extra-dialyzate is that the lipoprotein-cholesterol interaction is irreversible. Such interactions are thought to occur in various artificial colloid systems (40). Exchange of lipids between lipoproteins most probably occurs by some sort of interaction between the lipoproteins. The collision complex theory of Gurd (32) has already been discussed (See Chapter II). It is necessary to keep in mind that the term "irreversible" is not used in a strict thermodynamic sense. The lipoprotein-lipide interaction may be destroyed by extraction with organic solvents. An example of this was the one minute ether extractions described in the previous chapter. Data obtained by organic solvent extractions cannot be used to calculate binding energies for two reasons. First of all, the use of non-polar solvents in conjunction with aqueous lipoprotein solutions represents a two phase system. This obscures any equilibrium processes which may exist. Secondly, the organic solvent must alter the native structure of the lipoprotein. If then equilibrium data could be obtained, it would not be relevant to the native lipoprotein.

CHAPTER VI

CONCLUSIONS

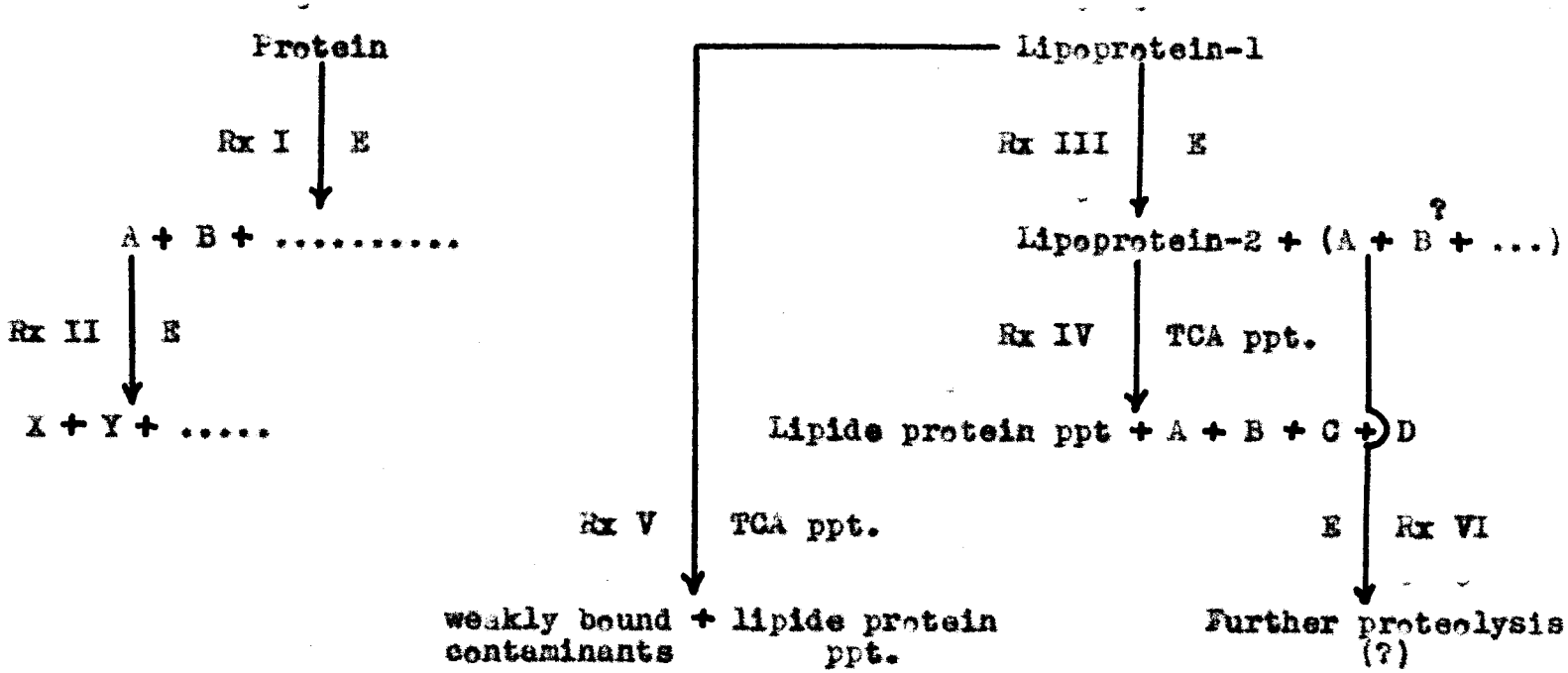
The fragmentation of proteins by proteolytic enzymes followed by careful examination of the resulting peptides has lead to the elucidation of the structure of ribonuclease. Other workers are using similar techniques to study a variety of proteins. The study presented in this dissertation shows that the reaction of proteolytic enzymes with beta lipoprotein is somewhat different from that with ordinary proteins. Since practically nothing is known about the nature of the interaction between protein and lipide in serum lipoproteins, the effects of proteolysis offer some interesting insights on the structure of the beta lipoprotein.

The course of the proteolytic reaction of beta lipoproteins as evidenced by this study is presented in figure 14. For contrast, a simplified diagram of the proteolysis of an ordinary protein is presented. In figure 14, the proteolytic enzymes are indicated by the symbol E. It is apparent that the products of the reaction will in each case depend on the specific enzyme which is used. This results from the specificity of the proteolytic enzyme for a particular series of peptide bonds.

During the proteolysis of a protein, the number of peptide fragments formed depends mainly on the number of peptide

Figure 14

PROTEOLYSIS OF LIPOPROTEINS



E-proteolytic enzyme

A, B, C, etc.-protein fragments

bonds in the protein which may be specifically hydrolyzed by the enzyme chosen (Rx I, figure 14). However the number of peptide bonds which are broken also depends on the exposure of the enzyme sensitive peptide linkage to the enzyme. This is why "denatured" proteins are more sensitive to proteolysis than "undenatured" proteins. For this reason also the proteolytic reaction may proceed via a series of reactions (See Rx II & VI, figure 14). Breaking of one peptide bond in a protein may then expose in one of the fragments an enzyme sensitive peptide bond. Proteolysis may therefore continue on the initial fragments which are formed. For single chain protein molecules, the number of protein fragments formed during proteolysis will be one greater than the number of peptide bonds that have been broken. Necessary exceptions must be made when cyclic areas of the protein are involved. This refers to portions of a protein which are cross-linked with, for example, disulfide bridges.

It would appear from this study that the proteolysis of lipoproteins differs significantly from that of ordinary proteins. Ionographical studies of the reaction of beta lipoprotein with proteolytic enzymes seem to indicate that no protein fragments are released during proteolysis (Rx III, figure 14). Because of this fact, much effort was directed to establish the point that proteolysis was occurring. That definite changes in the lipoprotein occur during treatment with trypsin or chymotrypsin was evident from the data presented in Chapter IV. It can be

summarized as follows: 1) Increases in the amount of TCA soluble peptide material (TCA soluble tyrosine) occurred during incubation with the proteolytic enzyme. 2) Partial extraction of lipide components of the lipoprotein was facilitated by the enzyme treatment. 3) The altered lipoprotein formed during proteolysis was much more sensitive to heat than the native material. This was detected by the coalescence which occurred in the turbidometric studies at elevated temperatures.

Protein fragments formed during proteolysis are released following TCA precipitation. This is not difficult to rationalize. During the TCA precipitation, the native structure of the lipoprotein is drastically altered. Any weakly bound fragments of sufficiently low molecular weight would be released. Although not conclusively shown, it seems that even the untreated beta lipoprotein contained small amounts of low molecular weight peptide material which was weakly bound to the native lipoprotein. Evidence for this suggestion has already been discussed.

The partial lipide extraction studies, while serving to establish the fact that proteolysis was occurrring, were designed with another purpose in mind. One objective was to determine what effect the structural configuration of the protein moiety has on the lipide binding capacity of the lipoprotein. A limited number of investigations on the factors which influence the lipide-protein interaction in lipoproteins have already been discussed (See Chapters I and II). The organic solvents employed

these studies may have twofold effects. The non-polar solvent in one instance serves to "pull" the lipide from the protein. Probably occurring simultaneously with the lipide removal are the configurational changes in the protein moiety resulting from the presence of the organic solvent. However the effect which have been demonstrated to occur during proteolysis could only be mediated by changes in the protein moiety.

The partial lipide extraction studies point to the fact that the lipoprotein protein serves to shield the lipide from the aqueous environment in a rather specific manner. Since little or no peptide material is lost following proteolysis, the amount of protein around the lipide "core" remains essentially constant. On the other hand, it has been shown that is is much easier to extract the lipide from the lipoprotein following proteolysis. Furthermore, the new lipoprotein is considerably more sensitive to heat coalescence.

What follows is a suggested explanation of the changes which occur during the proteolysis of beta lipoprotein. The first step is the breaking of a limited number of peptide bonds. Certainly most, if not all, of the protein material remains with the altered lipoprotein. The nature of the new material depends on the specific proteolytic enzyme which has been used. However, this new lipoprotein unless subjected to unusual conditions (heat, TCA precipitation, etc.) is almost as stable as its native precursor; no precipitation or coalescence of the lipide occurs.

It is suggested then that the lipide is now covered with an increased number of protein or peptide chains. The peptide chains have less restrictions as to their location on the periphery of the molecule. Upon heating, for example, the peptide chains may relocate on the surface of the lipoprotein. Perhaps some of these peptide fragments during their relocation may interact with other peptide chains preventing further movement. In any case, it would seem that areas of lipide contained in the lipoprotein become more exposed to the environment. Thus when the lipoprotein is heated following proteolysis, coalescence occurs; similarly lipide extraction is facilitated. The fact that the lipoproteins formed by the action of chymotrypsin is much more sensitive to heat than that formed by trypsin would seem to indicate that the extent of relocation of protein material depends on the number or location of peptide bonds broken. It was shown that the amount of peptide material liberated by TCA precipitation and after heating was slightly greater in the case of chymotrypsin as compared to tryptic proteolysis.

The difficulties encountered in the attempts to isolate lipide-free lipoprotein are not surprising. In the case of the untreated beta lipoproteins this serves to emphasize the stabilizing influence of the lipide on the protein structure. Failure to isolate definite protein fragments after treatment with proteolytic enzymes is a little more disturbing. However

it bears out the limited extent of proteolysis which has occurred on the native beta lipoprotein.

SUMMARY

Proteolysis of simple proteins as compared to beta lipoproteins has been discussed. The effect of chymotrypsin and trypsin on serum beta lipoprotein has been studied. The results indicate that only a limited amount of proteolysis occurred. This was illustrated by the determination of the release of trichloroacetic acid soluble peptides. Ionographic studies of the beta lipoprotein-proteolytic enzyme mixtures seemed to show that no protein fragments are released simply by proteolysis.

In order to demonstrate the importance of the intact protein moiety to the stability of the lipoprotein, two new techniques were introduced. Partial lipide extraction studies, particularly in reference to cholesterol, were done. Although variations in the one minute extractions did occur, this technique was very reproducible with any one beta lipoprotein preparation. Factors influencing the partial extractions have been discussed. Employing this technique significant changes in the tenacity of lipide binding by the lipoproteins were shown to occur during treatment with trypsin or chymotrypsin. Another technique was to determine the rate of coalescence of the beta lipoprotein upon heating. Again, significant alterations in the lipoprotein following proteolysis were found. A mechanism for the effects noted has been proposed.

Attempts to isolate lipide-free beta lipoprotein have

been made. Although these attempts were largely unsuccessful some information regarding possible approaches to the isolation of lipoprotein protein was obtained.

A slightly different approach to the study of the lipide-protein interaction in lipoproteins was also made. In this work, a dialysis membrane which is readily permeable to lipide was constructed. Studies of serum lipoproteins in conjunction with the lipide permeable membrane seem to bear out the irreversible character of the lipide-protein interaction even in partially non-polar environments.

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APPENDIXES

APPENDIX I

MATERIALS AND METHODS

TABLE VIII

LIST OF CHEMICALS

1) Albumin, crystalline bovine serum, lot # 684, 99% pure, mobility -7.0 (cm/sec/v/cm), Armour & Co., Chicago, Illinois.
2) Benzoyl-L-arginine ethyl ester HCl, lot # c2215, chromatographically pure, Mann Research Lab., New York 6, New York.
3) Bromophenol blue, lot # 341273, Matheson Co., East Rutherford, New Jersey.
4) Cholesterol, lot # 5177, Matheson Co.
5) Chymotrypsin, alpha, 3X crystallized, lot # 700-706, Worthington Biochemical Corp., Freehold, New Jersey.
6) N-acetyl-L-tyrosine ethyl ester, lot # c1338, M.P. 78-79°C, Mann Research Lab.
7) Ninhydrin (1,2,3-Indantrione monohydrate), lot 363202, Matheson Co.
8) Pentane, purified by permanganate, B.P. 35-36°C, Matheson Co.
9) Tris (tris hydroxymethyl amino methane) lot # 87-84, Primary standard, Sigma Chemical Co., St. Louis, Missouri.
10) Trypsin, 2X crystallized, salt free, lot # TRSF 771, Worthington Biochemical Corp.

TABLE IX

PREPARATION OF BUFFERS

1) Phosphate buffer: To obtain a pH of 7.5, dissolve 1.840 g of $NaH_2PO_4 \cdot H_2O$ and 9.11 g of Na_2HPO_4 in four liters of water*. This gives a molarity of 0.02. For a 0.05 M buffer, multiply the indicated quantities of phosphate compounds by 2.5 and dissolve in four liters of water.

2) Tris buffer: To obtain a pH of 7.5, dissolve 9.68 g of Tris (see table VIII) in three liters of water. Add about 60 ml of 1 N HCl, to pH 7.5. Dilute to four liters. This buffer is 0.02 M in respect to Tris. To obtain a 0.05 M buffer, multiply the amount of Tris by 2.5 and dissolve it in four liters of water.

3) Barbiturate buffer: To obtain a pH of 8.6, 2.797 g of veronal and 20.6 g of sodium veronal are dissolved in one liter of water. This gives an ionic strength of 0.1.

*The water used in the preparation of buffers was re-distilled from an all-glass apparatus.

TABLE X

ULTRACENTRIFUGAL TECHNIQUES

1) Stock salt solutions: The solutions of various densities were prepared as follows: A stock salt solution of density 1.346 was prepared by dissolving 153.0 g of sodium chloride and 354.0 g of potassium bromide in one liter of water. This solution was extracted with a saturated solution of dithizone (diphenylthiocarbazone) in carbon tetrachloride in order to remove heavy metal ions. The stock salt solution was then extracted five times with a total of 500 ml of carbon tetrachloride. Traces of the carbon tetrachloride were removed by boiling the salt solution for thirty minutes. Solutions of various densities were prepared by diluting the stock salt solution with doubly distilled water. Densities were determined with a Westphal Balance. Checks were made by using either a five or ten milliliter specific gravity bottle.
2) Preparation of alpha lipoproteins: To the Lusteroid ultracentrifuge tubes were added 2.0 ml of the salt solution of density 1.346 and 10.0 ml of serum, a procedure which results in a solution having a final density of 1.063 (33). The tubes were then filled with a solution of density 1.063. After centrifugation for about eighteen hours at 40,000 RPM, the combined chylomicrons and beta lipoproteins fractions float to the top. The alpha lipoproteins and the other serum proteins remain in the bottom portion of the tube. The infranatant was adjusted to a density of 1.210 by adding 1.12 g of potassium bromide and 0.15 ml

of glass distilled water for every 4.50 ml of infranatant. Ultracentrifugation was repeated for eighteen hours at 40,000 RPM and the alpha lipoprotein fraction which floated to the top was collected.

3) Preparation of beta lipoproteins: To 10.0 ml of serum in the Lusteroid ultracentrifuge tubes was added 2.0 ml of 1.085 density salt solution. This gives a final density of 1.019. After the tubes were capped and filled with a salt solution of density 1.019, they were centrifuged for eighteen hours at 40,000 RPM. The lipoproteins of density less than 1.019 which floated to the top were removed. To 9.0 ml of the infranatant was added 3.0 ml of a salt solution of density 1.196. This results in a final density of 1.063. The tubes were capped and filled with a salt solution of density 1.063. After again centrifuging for about eighteen hours at 40,000 RPM, the beta lipoproteins floated to the top of the tubes and were removed.

4) Collection of lipoprotein fractions: After ultracentrifugation, the tubes were carefully removed from the ultracentrifuge rotor. The lipoproteins appeared as a distinct layer on the top of the salt solutions. The tube was sliced directly below the supernatant lipoprotein concentrate by means of a tube slicer (Beckman Instruments Inc., Spinco Division, Belmont, Calif.). The tube slicer consists of a series of plastic and rubber rings, which hold the Lusteroid tube firmly in place, and a horizontal knife

blade. The blade is forced rapidly through the Lusteroid tube directly below the supernatant lipoproteins. The blade remains in place until the floated lipoproteins are removed by aspiration with a syringe.

APPENDIX II

ANALYTICAL PROCEDURES

Protein analysis

The biuret reagent was prepared as follows: 0.640 g of anhydrous $CuSO_4$ and 3.0 g of $KNaC_4H_4O_6 \cdot 4H_2O$ is dissolved in about 500 ml of water. To this was added 300 ml of 10% NaOH and the solution was diluted to one liter. When this solution is added to protein solutions, a violet color results.

One (1.0) ml of the color reagent was added to the unknown or standard contained in 9.0 ml of phosphate buffer, pH 7.5 After mixing, the color was allowed to develop for thirty minutes and then determined in a Klett colorimeter using a # 54 filter. Crystalline bovine serum albumin (see table VIII) was used to prepare the standard curve illustrated in figure 15. The albumin standard was prepared fresh every two weeks. Standards were always run simultaneously with the unknown lipoproteins. The color reagent was prepared fresh every two months although it appears to be stable indefinitely. The data used to prepare figure 15 is presented in table XI.

Cholesterol analysis

The reagent used to produce the color was prepared by mixing 20 ml of acetic anhydride with 1.5 ml of concentrated sulfuric acid. All glassware used in preparing this solution should

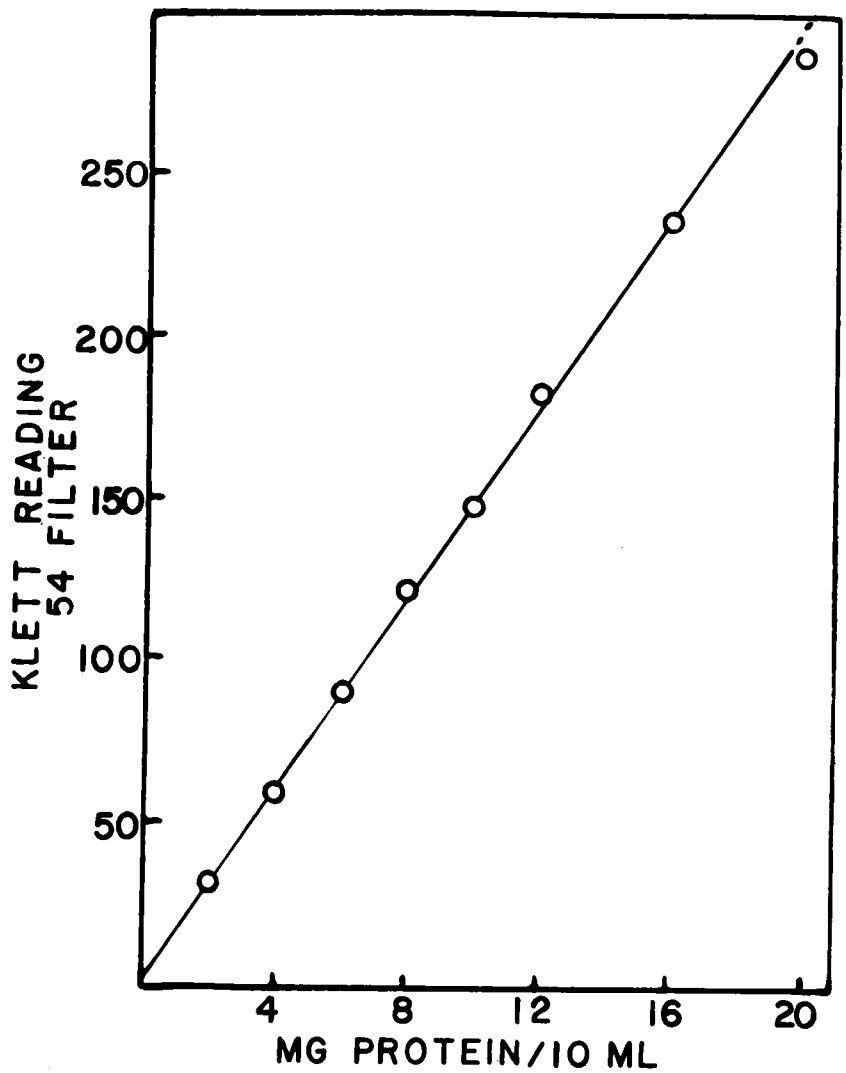

Figure 15

STANDARD CURVE FOR PROTEIN ANALYSIS

TABLE XI

PROTEIN BIURET REACTION

mg albumin	Klett reading #54 filter	mg albumin	Klett reading #54 filter
0	40	8	161
2	71	10	187
4	99	12	222
6	130	16	273
		20	327

TABLE XII

SPERRY AND WEBB CHOLESTEROL DETERMINATION

mg cholesterol	Klett reading #62 filter	mg cholesterol	Klett reading #62 filter
0.000	35	0.375	120
0.125	65	0.500	145
0.125	65	0.500	148
0.250	95	0.625	170
0.250	92	0.625	168
0.375	121		

be free of even traces of water. The acetic anhydride should be obtained from a freshly opened bottle. The color reagent can be used for about twenty-four hours, but the blank increases slowly during this time.

A standard curve for the determination of cholesterol is presented in figure 16 and the data recorded in table XII. Since the color reaction is very sensitive to changes in the acetic anhydride reagent, standards were run with every analysis.

The solutions used to prepare the standard curve were made by diluting a stock solution of cholesterol with chloroform. Prior to preparation of the standard stock solution, the cholesterol was purified as follows: 10 g of cholesterol was dissolved in 115 ml of boiling 95% ethanol. The solution was then filtered and cooled slowly to about 5°C. The white crystals of cholesterol which formed were removed by filtration through Whatman # 42 filter paper. The re-crystallized cholesterol was dried in a vacuum desicator. The standard solution was prepared fresh every 2-3 weeks. Although it was very stable, gradual loss of chloroform alters the concentration; for this reason, the cholesterol standard was stored in the refrigerator.

The procedure for analysis of the unknowns is as follows: 0.20 ml of the lipoprotein solution was first mixed with 0.10 ml of 95% ethanol in a 15 ml glass stoppered centrifuge tube. Four milliliters (4.0) of 10% H_2SO_4 is added, followed by 4.0 ml of chloroform. The tubes are then shaken for one hour in a Precision

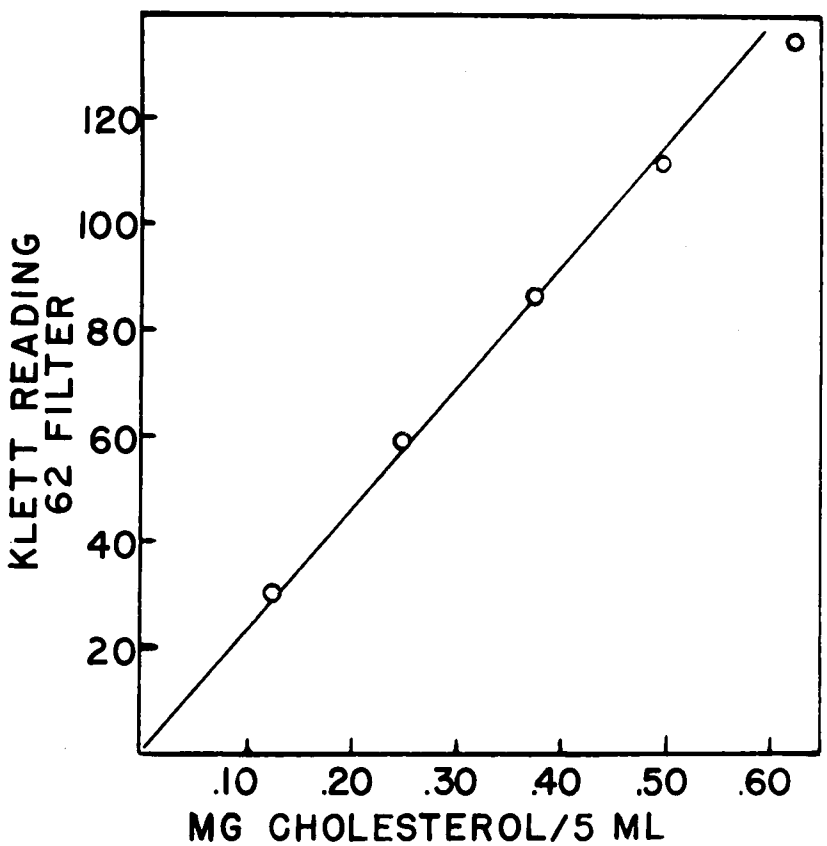

Figure 16

STANDARD CURVE FOR CHOLESTEROL ANALYSIS

Scientific Co. Shaker. The mixture was centrifuged for about five minutes and the aqueous layer and protein button at the interface were removed by aspiration. Three (3.0) ml of the chloroform extract was pipetted into a Klett colorimeter tube and 1.0 ml of chloroform and 1.0 ml of the color reagent were added. The solutions were carefully mixed and the color was determined after exactly thirty minutes with a Klett colorimeter using a #62 filter.

Phospholipide analysis

Phospholipide analysis may be divided into three steps: 1) The extraction of phospholipide. 2) Digestion of organic phosphorus to inorganic phosphate. 3) Determination of inorganic phosphate. A standard curve for the analysis of phosphate is illustrated in figure 17 and the corresponding data is presented in table XIII.

TABLE XIII

INORGANIC PHOSPHATE DETERMINATION

micromoles PO_4	Klett reading #66 filter	micromoles PO_4	Klett reading #66 filter
0.00	3	0.70	160
0.10	24	0.80	183
0.20	47	0.90	202
0.30	72	1.00	226
0.40	94	1.10	242
0.50	113	1.20	260
0.60	140	1.30	293

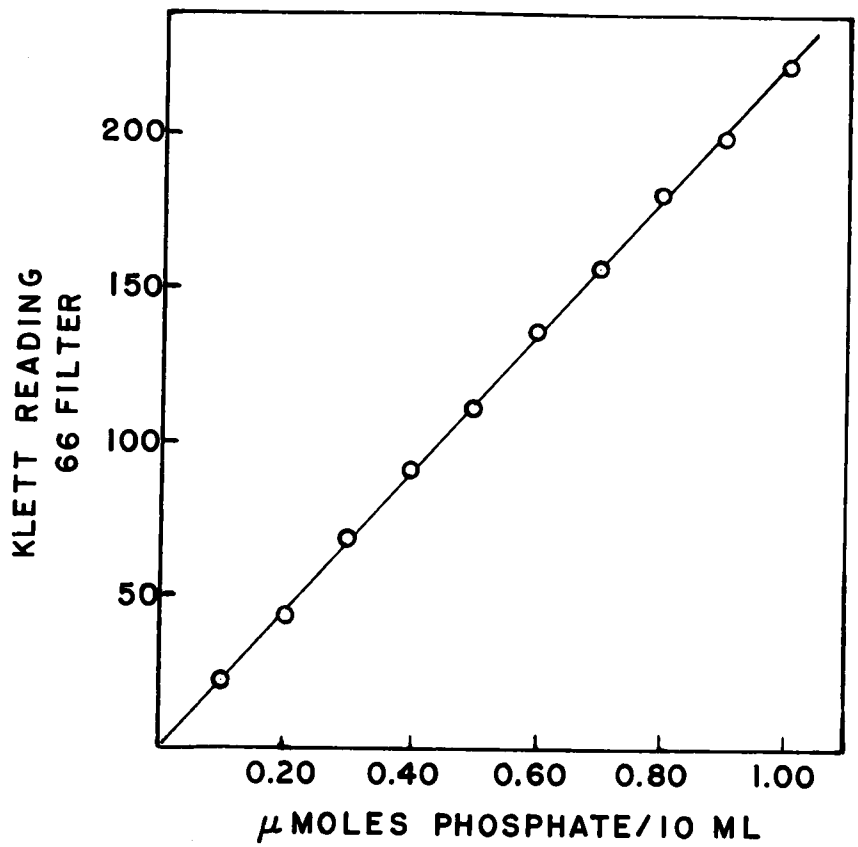

Figure 17

STANDARD CURVE FOR PHOSPHATE ANALYSIS

Milligrams of phospholipides are obtained when milligrams of phosphorus are multiplied by 25.

The procedure for analysis of the unknowns is as follows: 0.05 ml of the lipoprotein solution was placed in a 10 ml volumetric flask along with about 8 ml of Bloor's reagent (ethanol:acetone, 3:1, v:v). This mixture was heated to boiling, cooled and then diluted to 10 ml with the ethanol:acetone solution. The solution was filtered through Whatman #1 filter paper and a 4.0 ml aliquot of the filtrate was removed and placed in a six inch test tube. The ethanol and acetone were evaporated, and 1.0 ml of 5 N H_2SO_4 was added. The tube was heated over a flame until charring occurs and one drop of 2 N HNO_3 was added to clear the solution. Excess nitric acid was removed by heating until white fumes appeared. If upon cooling charring reappears, the treatment with nitric acid is repeated. When digestion was complete, 1.0 ml of water was added and the solution was heated in a boiling water bath for ten minutes.

The phosphate analysis was done as follows: The standard or unknown was placed in a Klett colorimeter tube. One milliliter of 5 N sulfuric acid and one milliliter of a 25% ammonium molybdate solution was added. This was followed by 0.10 ml of the reducing solution. The solution was mixed, diluted to 10 ml and the color was determined in a Klett colorimeter using a #66 filter.

The reducing reagent was first prepared in a powdered form by mixing thoroughly 0.2 g of 1-amino-2-naphthol-4-sulfonic

acid, 1.2 g of sodium bisulfite and 1.2 g of sodium sulfite. For phosphate analysis, 0.25 g of the powdered mix was measured with a small spoon and dissolved in 10 ml of water. Solutions of the reducing mixture were stable for about one week.

Tyrosine determination

The standard curve for tyrosine determinations is illustrated in figure 18 and the data is presented in table XIV. The standard solution was prepared by dissolving 12.5 mg of tyrosine in 250 ml of 1.5 M Na_2CO_3. Aliquots of the standard tyrosine solution were diluted in a colorimeter tube to 3.0 ml with 1.5 M Na_2CO_3. One (1.0) ml of the Folin Ciocalteu reagent was then added and the resulting color was determined after twenty minutes in a Klett colorimeter equipped with a #60 filter

TABLE XIV

TYROSINE DETERMINATION

mg tyrosine	Klett reading #60 filter	mg tyrosine	Klett reading #60 filter
0.000	17	0.040	368
0.010	85	0.045	414
0.015	133	0.050	459
0.020	179	0.060	559
0.025	228	0.070	665
0.030	266	0.080	790
0.035	316		

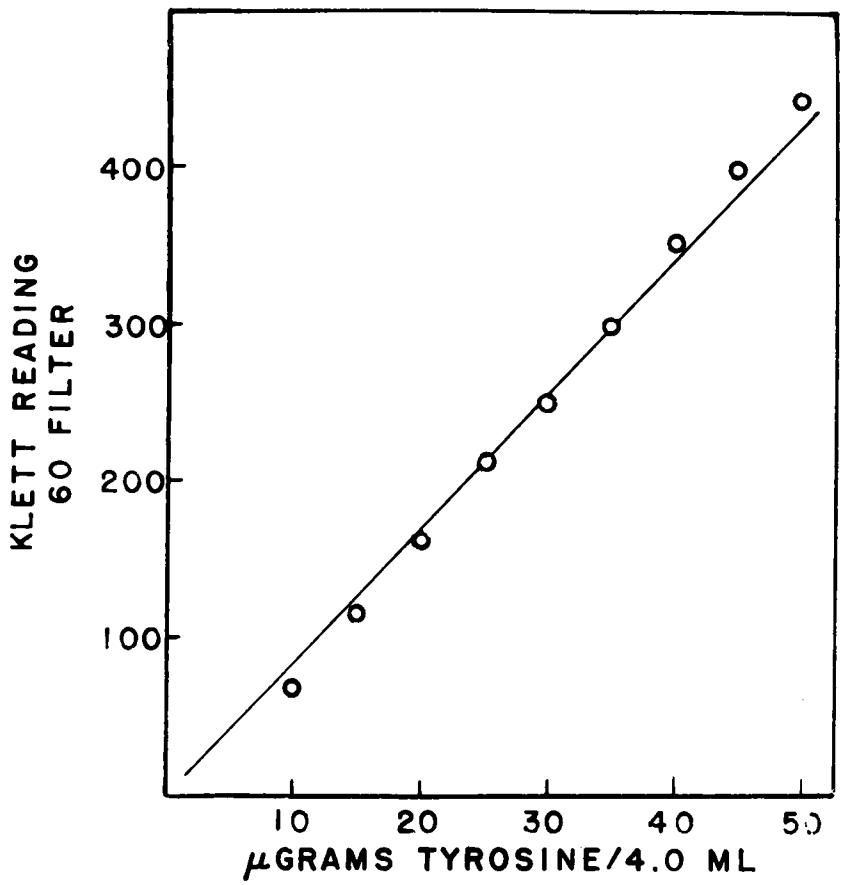

Figure 18

STANDARD CURVE FOR TYROSINE DETERMINATION

The trichloroacetic acid soluble tyrosine from lipoproteins was determined as follows: To 2.0 ml of an enzyme-lipoprotein incubation mixture in a 15 ml centrifuge tube was added 1.0 ml of 10% trichloroacetic acid. The tube was centrifuged for ten minutes at full speed in a clinical centrifuge. This procedure usually removes the precipitate. When small particles of the lipoprotein did not sediment to the bottom of the tube, the supernatant was poured into a clean tube and re-centrifuged for ten minutes. One(1.0) ml of the clear supernatant (TCA soluble) was removed and placed in a Klett colorimeter tube along with 2.0 ml of 1.5 M Na_2CO_3. Finally, 1.0 ml of the Folin Ciocalteu reagent was added and the resulting color was determined after twenty minutes in a Klett colorimeter equipped with a #60 filter.

The Folin Ciocalteu reagent was prepared as follows: 250 g of sodium tungstate, 63 g of sodium molybdate, 1750 ml of water, 105 ml of phosphoric acid (85%) and 250 ml of concentrated hydrochloric acid was placed into a three liter flask fitted with a heating mantle and a standard tapered reflux condenser. The solution was refluxed for 10 hours and then cooled. 375 g of lithium sulfate and 1.3 ml of bromine was added and the solution was boiled until no more bromine was liberated (about 20 minutes). It was then cooled and diluted to 2.5 liters with water.

Assay of chymotrypsin and trypsin

The activity of chymotrypsin solutions was determined as follows: 3.0 ml of 0.00025 M N-acetyl-L-tyrosine ethyl ester

(ATEE) was pipetted into each of two Beckman silica cells. This was allowed to equilibrate to 30°C in the Beckman DU Spectrophotometer. To one cell, which is to be the blank, was added 20 lambdas of 0.001 N HCl. The optical density scale was adjusted with the blank to read 0.200 at 237 millimicrons. Then 20 lambdas of the enzyme, dissolved in 0.001 N HCl and previously equilibrated to 30°C, was added to the other cell. The optical density was determined every 30 seconds for about six minutes. A unit of activity is that which causes a decrease in optical density of 0.001 units per minute under the specified conditions.

The procedure for trypsin assay was the same as that for chymotrypsin with a few exceptions. Instead of ATEE, N-benzoyl-L-arginine ethyl ester (BAEE) is used (0.00025 M). The spectrophotometer set at 253 millimicrons was adjusted to read zero on the optical density scale with the blank. After addition of trypsin to the BAEE, the increase in optical density was determined at regular intervals for about six minutes. A trypsin unit was that activity causing a rise in optical density of 0.001 per minute under the specified conditions. The buffer used in the assay was the same as that which was to be used for lipoprotein proteolysis.

The results of a typical assay are illustrated in figure 19 and the data is presented in table XV. Activities are calculated from the slope of the linear portions of the curve. In the examples presented in figure 19, the calculated activities were:

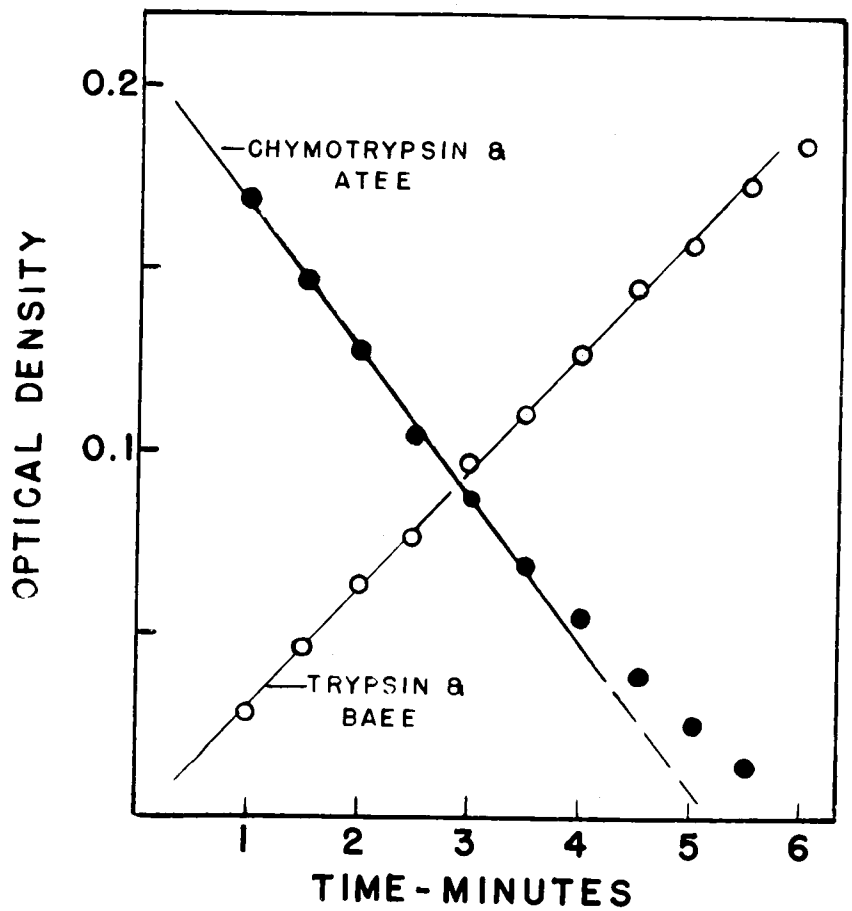

Figure 19

ASSAY OF CHYMOTRYPSIN AND TRYPSIN

TABLE XV

ASSAY OF CHYMOTRYPSIN AND TRYPSIN*

Time-minutes	Chymotrypsin Optical density-237 mu	Trypsin Optical density-253 mu
1.0	0.169	0.028
1.5	0.146	0.046
2.0	0.127	0.063
2.5	0.104	0.076
3.0	0.087	0.096
3.5	0.068	0.110
4.0	0.054	0.126
4.5	0.038	0.144
5.0	0.025	0.157
5.5	0.014	0.173
6.0		0.184

*In phosphate buffer, 0.05 M, pH 7.5

trypsin-1630 units/ml and chymotrypsin-2050 units/ml.

Color development of ionograms and chromatograms

The ninhydrin spray for detection of amino acids and proteins was prepared by dissolving 0.20 g of ninhydrin in 95% butanol containing 5% 2 N acetic acid. The dried paper strip, following chromatography or ionography, was sprayed with this reagent and placed in an oven at 110°C. Color develops after 10-20 minutes. Peptide material appears as spots of various shades of violet.

Protein was detected by staining with bromphenol blue. The staining solution contains 0.1 g of bromphenol blue and 10 g of $HgCl_2$ dissolved in 100 ml of 95% ethanol. The dry paper strips were immersed in the stain for five minutes. Background color was removed by washing in running tap water for about eight minutes. Protein material appears as blue to blue-green spots on a white background.

Because lipoproteins contain large amounts of cholesterol, they may be detected by cholesterol sensitive sprays. This was accomplished by spraying the paper strips with a 10% solution of phosphotungstic acid in ethanol. The strips were then placed in an oven at 110°C for 1-2 minutes. Cholesterol spots appeared pink on a white background. If the chromatogram was heated for longer than two minutes, the whole paper becomes a black-brown color and interpretation was impossible.

APPENDIX III

EXPERIMENTAL RESULTS

TABLE XVI

EFFECT OF CHYMOTRYPSIN ON SERUM LIPOPROTEINS

Incubation time minutes	mg TCA soluble tyrosine X 10	Composition
Part A (See figure 2, II)		
0	0.00	Carried out in 2.0 ml phosphate buffer, pH 7.5, 0.02 M. Temperature was 30°C. 0.10 mg chymotrypsin, 2.23 mg beta lipoprotein protein.
15	0.75	
30	0.90	
60	1.23	
Part B (See figure 2, III)		
0	0.00	As above. Density fraction less than 1.019. 1.80 mg of lipoprotein protein.
15	0.57	
30	0.69	
60	1.02	
Part C (See figure 2, IV)		
0	0.00	As Part A. 8.82 mg alpha lipoprotein protein.
15	0.00	
30	0.00	
60	0.00	

TABLE XVI (CONT'D)

Incubation time minutes	mg TCA soluble tyrosine X 10^2	Composition
Part D (See figure 2, I)		
0	0.00	As Part A. Approximately 5 mg of hemoglobin was present.
5	2.10	
10	2.88	
15	3.30	
21	3.60	
30	3.90	

TABLE XVII

PROTEOLYSIS OF BETA LIPOPROTEIN

Incubation time minutes	mg TCA soluble tyrosine X 10^2	Composition
Part A (See figure 3, I)		
0	0.00	Volume-2.0 ml. Phosphate buffer, pH 7.5, 0.02 M. Temperature-30°C. 0.05 mg chymotrypsin, 610 units. Beta lipoprotein-2.02 mg protein, 2.18 mg cholesterol.
15	0.53	
30	0.83	
45	1.07	
60	1.18	
Part B		
0	0.00	As in Part A except chymotrypsin-0.05 mg, 850 units.
20	0.47	
40	0.83	
60	1.22	
Part C		
0	0.00	As in Part A except chymotrypsin-0.10 mg, 1330 units. Beta lipoprotein-2.44 mg protein, 2.32 mg cholesterol.
15	0.45	
30	0.77	
45	1.21	
60	1.26	
90	1.89	

TABLE XVII (CONT'D)

Incubation time minutes	mg TCA soluble tyrosine X 10^2	Composition
Part D		
0	0.00	Volume-1.0 ml. Phosphate buffer, pH 7.5, 0.02 M. Trypsin-0.10 mg, 1130 units. Beta lipoprotein-1.22 mg protein, 0.93 mg cholesterol.
15	0.20	
30	0.30	
45	0.38	
60	0.48	
Part E (See figure 3, II)		
0	0.00	As Part D except beta lipoprotein-0.99 mg protein, 0.77 mg cholesterol.
15	0.43	
30	0.68	
45	0.86	
60	1.15	
90	1.60	

TABLE XVIII

EFFECT OF FREEZING ON THE PROTEOLYSIS OF BETA LIPOPROTEINS

Incubation time minutes	mg TCA soluble tyrosine X 10^2	Composition
Part A-Untreated (See figure 4, II)		
0	0.00	Volume-2.0 ml. Phosphate buffer, pH 7.5, 0.02 M. Temperature-30°C. Chymotrypsin-0.10 mg, 1500 units. Beta lipoprotein-1.85 mg protein, 1.35 mg cholesterol.
15	0.07	
30	0.12	
45	0.25	
60	0.34	
Part B-Frozen-thawed (SEE figure 4, I)		
0	0.00	As above.
15	0.70	
30	0.95	
45	1.03	
60	1.35	

TABLE XIX

ULTRA-VIOLET SPECTRA OF BETA LIPOPROTEINS

Wave length millimicrons	OPTICAL DENSITY Untreated	Chymotrypsin	Trypsin
320	0.102	0.071	0.069
318	0.104	0.072	0.071
316	0.107	0.074	0.073
314	0.113	0.077	0.076
312	0.117	0.081	0.079
310	0.124	0.085	0.083
308	0.131	0.089	0.087
306	0.136	0.094	0.092
304	0.142	0.101	0.098
302	0.152	0.108	0.105
300	0.164	0.118	0.114
298	0.179	0.129	0.126
296	0.205	0.145	0.141
294	0.236	0.167	0.162
292	0.276	0.194	0.191
290	0.314	0.219	0.214
288	0.351	0.244	0.239
286	0.389	0.270	0.264
284	0.406	0.286	0.281
282	0.420	0.296	0.291
280	0.429	0.304	0.296

TABLE XIX (CONT'D)

Wave length millimicrons	OPTICAL DENSITY Untreated	Chymotrypsin	Trypsin
278	0.437	0.309	0.301
276	0.449	0.314	0.307
274	0.458	0.318	0.309
272	0.453	0.314	0.306
270	0.443	0.306	0.298
268	0.429	0.298	0.291
266	0.419	0.295	0.286
264	0.411	0.294	0.286
262	0.425	0.296	0.287
260	0.441	0.307	0.298
258	0.472	0.327	0.316
256	0.530	0.363	0.351
254	0.630	0.429	0.412
252	0.764	0.512	0.496
250	0.925	0.612	0.594
248	1.093	0.710	0.692
246	1.238	0.799	0.780
244	1.366	0.881	0.859
242	1.488	0.975	0.950
240	1.640	1.086	1.064
238	1.780	1.202	1.181
236	1.925	1.327	1.313

TABLE XIX (CONT'D)

Conditions: The enzymes were incubated with the beta lipoprotein for twenty hours at room temperature. The concentration of beta lipoprotein was 0.186 mg/ml protein and 0.209 mg/ml cholesterol. The concentration of the enzymes was 0.0082 mg/ml for both trypsin (121 units) and chymotrypsin (111 units). Incubations and spectral determinations were carried out in a Tris buffer, pH 7.5, 0.05 M. The Beckman DU Spectrophotometer was adjusted to zero on the optical density scale with the Tris buffer.

TABLE XX

PARTIAL EXTRACTION OF CHOLESTEROL AND PHOSPHOLIPIDE FROM BETA LIPOPROTEIN

Duration of extraction minutes	% of total extracted	Conditions
Cholesterol-ether		
0.25	41.1	1.0 ml beta lipoprotein (0.93 mg/ml cholesterol, 0.96 mg/ml protein) in phosphate buffer, pH 7.5, 0.02 M, extracted with 4.0 ml of ether for the indicated times.
0.50	44.9	
0.75	48.8	
1.0	50.7	
1.0	54.1	
1.0	52.9	
1.0	53.8	
2.0	60.2	
3.0	66.5	
5.0	74.8	
Phospholipide-chloroform		
1.0	25.1*	1.0 ml beta lipoprotein (1.50 mg/ml cholesterol, 1.06 mg/ml protein, 0.80 mg/ml phospholipide) in Tris buffer, pH 7.5, 0.02 M. Extracted with 4.0 ml of chloroform for the indicated time.
2.0	31.0*	
3.0	29.9*	
4.0	31.8*	
5.0	33.1*	
6.0	36.4	

*Average of two determinations. The average experimental error was 1.8%

TABLE XXI

EFFECT OF CHYMOTRYPSIN ON PARTIAL LIPIDE EXTRACTIONS OF BETA LIPOPROTEINS

Incubation time-minutes		% total cholesterol extracted by ether in one minute	Conditions
I.	0	57.8	See figure 7, #I, carried out in phosphate buffer, pH 7.5, 0.02 M. 2.03 mg protein, 2.18 mg cholesterol (as beta lipoprotein), 0.10 mg chymotrypsin (850 units) all in 2.0 ml. This was extracted with 4.0 ml of ether at the indicated times.
	20	70.6	
	40	73.4	
	60	69.6	
II.	0	39.8	See figure 7, #II, as above except: 1.99 mg protein, 2.06 mg cholesterol (as beta lipoprotein), 0.10 mg chymotrypsin (280 units) all in 2.0 ml.
	16	48.9	
	30	50.0	
	45	50.5	
	60	51.9	

Incubation time-minutes		% total phospholipide extracted by $CHCl_3$ in one minute	Conditions
III.	0	30.8	See figure 7, #III, carried out in Tris buffer, pH 7.5, 0.02 M. 2.15 mg protein, 3.36 mg cholesterol, 2.23 mg phospholipide (as beta lipoprotein) 0.10 mg chymotrypsin (930 unit) all in 2.0 ml. This was extracted with 4.0 ml $CHCl_3$ at the indicated times.
	15	38.7	
	30	36.5	
	45	39.7	
	60	37.7	

TABLE XXII

EFFECT OF TRYPSIN ON PARTIAL CHOLESTEROL EXTRACTION OF BETA LIPOPROTEIN

	Incubation time-minutes	% total cholesterol extracted by ether in one minute	Composition
I.	0	55.8	See figure 8, #I, carried out in phosphate buffer, pH 7.5, 0.05 M. 1.19 mg protein, 1.87 mg cholesterol (as beta lipoprotein), 0.05 mg trypsin (412 units) all in 1.0 ml. This was extracted with 4.0 ml of ether at the indicated times.
	5	66.6	
	10	69.9	
	15	71.3	
	20	73.9	
	30	79.6	
	45	79.6	
	60	80.0	
II.	0	50.9	See figure 8, #II, as part I except: 0.795 mg protein, 0.875 mg cholesterol (as beta lipoprotein), 0.05 mg trypsin (400 units) all in 1.0 ml.
	15	69.5	
	30	76.1	
	45	79.1	
	60	79.6	

TABLE XXIII

TURBIDITY FORMATION BY BETA LIPOPROTEINS
TREATED WITH TRYPSIN AND CHYMOTRYPSIN

Time minutes	BETA LIPOPROTEINS: Δ OPTICAL DENSITY-700 mu Untreated	Chymotrypsin	Trypsin
Part A: 40°C			
1-62	0.0	0.0	0.0
Part B: 45°C			
2	0.0	0.0	0.0
8	0.0	0.0	0.0
17	0.0	0.004	0.0
32	0.0	0.007	0.0
53	0.0	0.010	0.0
62	0.0	0.011	0.0
Part C: 50°C			
1	0.0	0.0	0.0
6	0.0	0.0	0.0
13	0.0	0.003	0.0
17	0.0	0.004	0.0
25	0.0	0.007	0.0
34	0.0	0.012	0.0
45	0.0	0.017	0.0
58	0.0	0.022	0.0
64	0.0	0.026	0.0

TABLE XXIII (CONT'D)

Time minutes	BETA LIPOPROTEINS: Δ OPTICAL DENSITY-700mm Untreated	Chymotrypsin	Trypsin
Part D: 55°C			
1	0.0	0.0	0.0
5	0.0	0.003	0.0
11	0.0	0.005	0.0
18	0.0	0.021	0.0
25	0.0	0.032	0.002
30	0.0	0.038	0.005
38	0.0	0.043	0.005
50	0.0	0.048	0.006
59	0.0	0.052	0.007
Part E: 60°C			
1	0.0	0.0	0.0
4	0.0	0.0	0.0
11	0.0	0.039	0.0
14	0.0	0.048	0.001
18	0.0	0.054	0.003
23	0.0	0.062	0.005
30	0.0	0.068	0.007
36	0.0	0.074	0.010
42	0.0	0.078	0.010
51	0.0	0.085	0.010
57	0.0	0.089	0.011

TABLE XXIII (CONT'D)

Solutions: 1) All the incubation mixtures were made up in phosphate buffer, pH 7.5, 0.05 M.

2) All the solutions contained 1.31 mg/ml protein and 1.50 mg/ml cholesterol as beta lipoprotein.

3) In addition, one incubation mixture contained 0.05 mg/ml (340 units/ml) chymotrypsin and another contained 0.05 mg/ml (330 units/ml) of trypsin.

4) At the end of each turbidity determination, 2.0 ml of the incubation mixes were added to 2.0 ml of 10% trichloroacetic acid. The precipitate was removed by centrifugation and the TCA soluble material estimated by determining the optical density at 280 mu. See table XXIV.

TABLE XXIV

TCA SOLUBLE MATERIAL FOLLOWING TURBIDITY DETERMINATIONS

Incubation temperature	OPTICAL DENSITY-280 mu* Untreated	Chymotrypsin	Trypsin
40°C	0.074	0.219	0.207
45°C	0.083	0.290	0.207
50°C	0.094	0.286	0.341
55°C	0.101	0.323	0.230
60°C	0.107	0.360	0.275

*Corrected for TCA present.

APPROVAL SHEET

The dissertation submitted by Leonard J. Banaszak has been read and approved by five members of the faculty of the Stritch School of Medicine, Loyola University.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

May 22, 1961
Date

Hugh A. McDonald
Signature of Adviser