The Proteolysis of Human Serum β-Lipoproteins*

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Received August 1, 1961

The effect of trypsin and chymotrypsin on serum β-lipoprotein has been studied. The proteolysis of β-lipoprotein results in definite changes in lipid binding tenacity. Such alterations were demonstrated by empirical partial lipid extractions. Alterations in the β-lipoprotein produced by trypsin or chymotrypsin were also manifested by an increased heat sensitivity. Although not conclusively established, it appears, on the basis of ionographic studies, that the proteolysis of β-lipoprotein occurs with little or no loss of protein fragments from the lipid-protein complex. Partial characterization of proteolytic fragments was accomplished by precipitation with trichloroacetic acid followed by paper chromatography. Studies of the trichloroacetic acid–soluble material of untreated β-lipoprotein indicate that the ultracentrifugal preparations contain detectable quantities of what appears to be peptide material. It is suggested that these materials are weakly held to the surface of the β-lipoprotein particle, since they are not lost during the prolonged dialysis used in the preparation of the β-lipoprotein.

In general, serum lipoproteins are thought to consist of a number of lipids stabilized in their aqueous environment by a specific peptide chain. It has been suggested that the tightness and specificity of the binding of lipids to proteins depends to a large extent on steric arrangement (Gurd, 1960, p. 209). Numerous reports on disruption of the lipoprotein molecule have been reported and were recently reviewed by Gurd (1960, p. 280ff.). In reference to the effect of organic solvents and detergents on the removal of lipid components from the lipoproteins, it is difficult, in these investigations, to decide whether the specific treatment is affecting the protein moiety, the lipid-protein interaction, or both.

Recently Scanu and Hughes (1960) isolated the protein from human α_1 lipoprotein, and found that it exhibits a strong affinity for lipids. Application of similar techniques to the preparation of lipid-free β-lipoprotein were unsuccessful (Scanu and Hughes, 1960).

The purpose of this investigation was to study the effects of proteolysis on human serum β-lipoprotein. In theory, such treatment should drastically alter the steric arrangement of the protein moiety without altering the environment of the lipid-protein interactions. Extraction studies, relying solely on organic solvent treatment, probably affect both the conformation of the protein and the energetics of any hydrophobic lipid-protein bonds. Experiments will be reported which indicate the effects of protein fragmentation on the stability of the lipoprotein complex.

Experimental Procedure

Pooled human serum was obtained from Cook County Hospital, Chicago. It generally represented morning-fasted samples from approximately fifty patients. The α-chymotrypsin (thrice crystallized) and trypsin (twice crystallized) were purchased from Worthington Biochemical Corporation.

The β-lipoproteins were prepared according to the ultracentrifugal procedure described by Havel *et al.* (1955) and modified by McDonald and coworkers (1960). The later procedure eliminates the presence of heavy metal ions in the ultracentrifugal media. Throughout this paper the term "β-lipoproteins" represents the ultracentrifugal fraction in the density range 1.019 to 1.063 g/cc. All ultracentrifugations were carried out in a Spinco Model L ultracentrifuge with the number 40 rotor. Each run was allowed to proceed for about 20 hours at 40,000 rpm (10–12°). Examination of the Lusteroid tube at the termination of the ultracentrifugal run indicated that the β-lipoprotein was packed in the top 2–3 mm of the tube. This was separated from the remainder of the infranatant proteins by about 3 cm of clear salt solution. The tube was sliced just below the floating lipoproteins to minimize loss of the preparation. The ultracentrifugal β-lipoprotein preparation was dialyzed for approximately 90 hours at 2° against phosphate buffer, pH 7.5, 0.05 M, to remove the relatively high concentrations of NaCl and KBr which were present. All solutions were prepared with water redistilled from an all-glass apparatus; this eliminated trace amounts of heavy metal ions.

Protein was determined by the method of Gornall *et al.* (1949). Cholesterol analysis was done according to the procedure of Sperry and Webb (1950). In this investigation the concentration of β-lipoprotein will be reported in terms of both protein and cholesterol concentration. The method of Stewart and Hendry (1935) was used for phospholipid analysis. Proteolysis of the β-lipoprotein was demonstrated by the release of trichloroacetic acid–soluble tyrosine, the tyrosine, in turn, being determined by the method of Folin

* Supported in part by a grant-in-aid from the Chicago Heart Association.

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and Ciocalteu (1927). The activity of chymotrypsin and trypsin was estimated by the spectrophotometric methods of Schwert and Takenaka (1955).

Ionographic separations were carried out on Whatman No. 1 filter paper, at 25°, in conjunction with a Veronal buffer, pH 8.6, 0.05 M, by the horizontal strip technique described by McDonald (1955). Protein material was detected by staining with bromphenol blue or by spraying with ninhydrin as described by Block *et al.* (1958).

To illustrate the effect of proteolysis on the stability of the β-lipoprotein, it was decided to determine the rate of turbidity formation at elevated temperatures. The measurements were made with a Beckman DU Spectrophotometer at 700 mμ. At this wavelength the β-lipoprotein solutions have practically no absorption, so that changes in the optical density reflect the production of turbidity.

Results

The lipoproteins contained in human serum, in view of their variable composition, present a problem to any study of their structure. This is particularly true when the tenacity of lipid binding is demonstrated by empirical extraction procedures. It was hoped that the use of pooled serum (approximately 50 donors) would reduce the variability of the composition. The cholesterol-to-protein ratio of the various preparations which were used in this study (Table I) indicated that some variation did

Table I

Cholesterol-to-Protein Ratio of β-Lipoprotein

No. of Preparations	Cholesterol to Protein Ratio: Highest Value	Lowest Value	Av.	Standard Deviation
21	1.42	0.73	1.08	±0.15

occur. To compensate for this, the experiments to be described were done on several β-lipoprotein preparations.

Evidence for the Proteolysis of β-Lipoprotein.— Preliminary experiments indicated that the ninhydrin reagent could not be used effectively in conjunction with the β-lipoprotein, since precipitates appear when the lipoprotein-ninhydrin solutions are heated. This result was attributed to the large amounts of lipid material present.

Proteolysis by trypsin or chymotrypsin was effectively demonstrated by determination of the release of trichloroacetic acid–soluble tyrosine. The results for one typical experiment are presented in Figure 1. The values were increased as much as eightfold if the β-lipoprotein was frozen (−50°) and thawed before the rate of proteolysis was studied. Freezing of lipoproteins is believed to produce permanent configurational changes in the lipoprotein complex (Gurd, 1960, p. 280ff.). This effect is not observed with ordinary proteins, e.g., albumin. For this reason the increase in proteolysis resulting from the freeze-thaw treatment may be taken as evidence that the release of trichloroacetic acid–soluble material is from the β-lipoprotein.

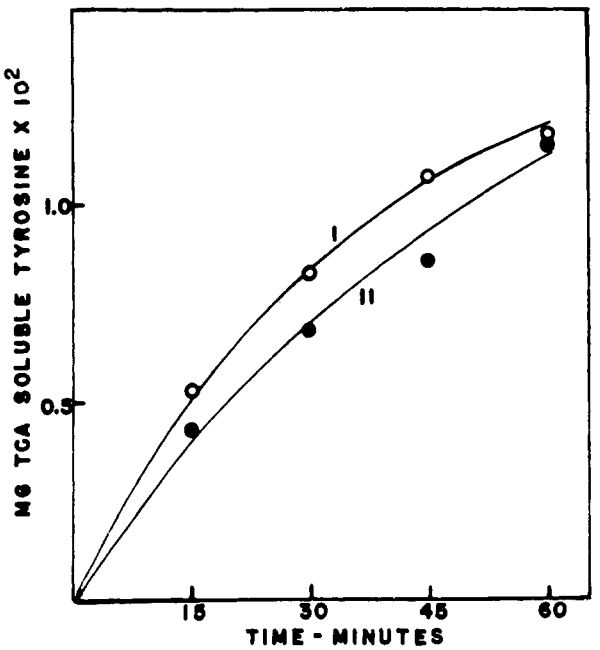

Fig. 1.—Proteolysis of β-lipoprotein. All reactions were carried out in phosphate buffer, pH 7.5, 0.02 M at 30°. I—volume, 2 0 ml; chymotrypsin, 0.05 mg, 610 units; β-lipoprotein, 2.02 mg protein, 2.18 mg cholesterol. II—volume, 1.0 ml.; trypsin, 0.05 mg, 565 units; β-lipoprotein, 0.99 mg protein, 0.77 mg cholesterol.

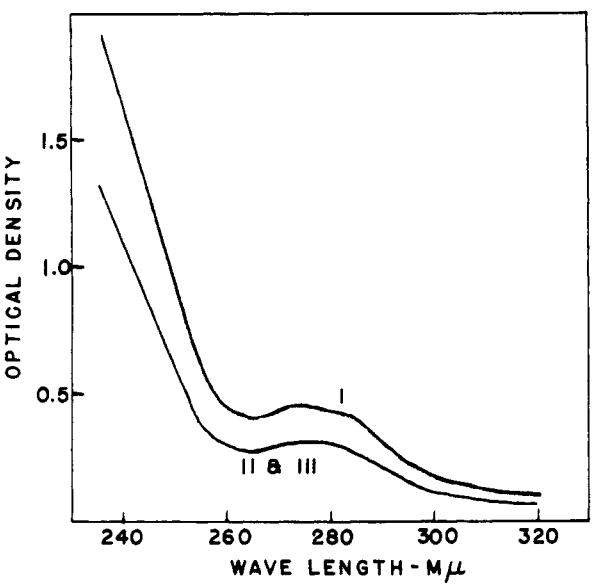

Fig. 2.—Effect of proteolysis on the UV spectrum of β-lipoprotein. I—untreated β-lipoprotein. II and III—treated with chymotrypsin and trypsin respectively. The concentration of β-lipoprotein was 0.186 mg/ml protein and 0.209 mg/ml cholesterol. The concentration of the enzymes was 0.0082 mg/ml. The spectral determinations were carried out in Tris buffer, pH 7.5, 0.05 M.

As illustrated in Figure 2, changes in the ultraviolet absorption spectra of the β-lipoprotein also occur after treatment with trypsin or chymotrypsin. The effects of the two enzymes were so similar that they are represented by one curve in Figure 2.

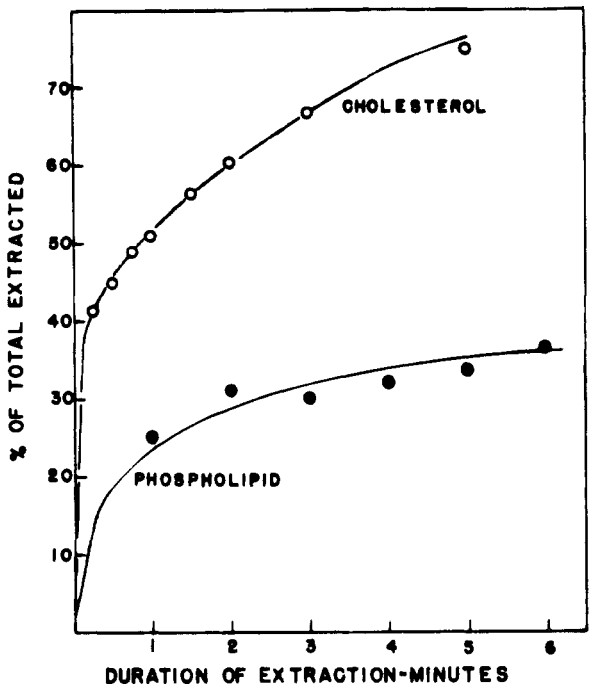

FIG. 3.—Partial extraction of cholesterol and phospholipid from β-lipoprotein. *Cholesterol*—1.0 ml β-lipoprotein (0.930 mg/ml cholesterol, 0.960 mg/ml protein) in phosphate buffer, pH 7.5, 0.02 M extracted with 4.0 ml of ether for the indicated times. *Phospholipid*—1.0 ml β-lipoprotein (1.50 mg/ml cholesterol, 1.06 mg/ml protein, 0.80 mg/ml phospholipid) in Tris buffer, pH 7.5, 0.02 M. Extracted with 4.0 ml of chloroform for the indicated times.

The decrease in UV absorption occurs at a much slower rate than the release of trichloroacetic acid-soluble tyrosine. This fact seems to indicate that the decrease in the UV absorption may not arise solely from proteolysis.

β-Lipoprotein Proteolysis and Lipid "Binding" Capacity.—At present no method is available for determining the tenacity of lipid binding by a lipoprotein. For this reason, an indirect empirical procedure was devised. The removal of lipid from lipoprotein by organic solvent extraction is not an instantaneous process. The rate of removal of the lipid by the organic solvent would seem to be governed by the same factors which are responsible for the stability of the lipoprotein complex in an aqueous environment. Proteolysis of the lipoprotein protein might be expected to increase the rate of removal of lipid components if the maximum stability is provided by the native peptide chain(s).

Figure 3 represents the rate of removal of cholesterol and phospholipid from β-lipoprotein as a function of the duration of solvent extraction. Many factors (composition of lipoprotein, nature of organic solvent, organic solvent-to-lipid ratio, etc.) influence the partial lipid extraction of a lipoprotein. For example, the rate of extraction of cholesterol from β-lipoprotein can be increased considerably by using anhydrous ether instead of ordinary ether. In any series of experiments, however, the variables affecting the partial lipid extractions may be kept constant.

In order to show that proteolysis affects the nature of the lipid binding by the β-lipoprotein, it was decided to make the partial extractions at several time intervals during treatment with the proteolytic enzyme. 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 1-minute partial extraction was chosen. The procedure for the partial extractions was as follows: To the β-lipoprotein (1.0 or 2.0 ml) contained in a 15-ml glass-stoppered centrifuge tube was added 4.0 ml of the organic solvent. The tube was shaken for the indicated time, usually 1 minute. Then the organic solvent was allowed to separate for about 1 minute and an aliquot was withdrawn and analyzed for the respective lipid.

Table II describes the reproducibility of these

TABLE II

PARTIAL ETHER EXTRACTIONS OF β-LIPOPROTEIN

Cholesterol/ Protein Ratio in Preparation of β-Lipoprotein	Number of Determinations	Average % of Total Cholesterol Extracted[a]	Standard Deviation
0.73	6	51.6	±1.4
0.97	4	52.9	±1.3
1.03	2	40.5	±0.9
1.02	2	46.0	±0.2
1.51	2	58.7	±0.2
1.17	3	57.2	±1.7
1.15	3	57.1	±0.2
1.15	3	55.8	±2.1

[a] One minute ether (not anhydrous) extraction.

empirical extraction procedures. For any one β-lipoprotein preparation, the results agree within 1–2%. The error for the partial extraction of phospholipid was of the same magnitude. A somewhat larger variation occurs from one β-lipoprotein preparation to another.

The effect of chymotrypsin on the 1-minute lipid extractions of β-lipoprotein is illustrated in Figure 4. The amount of cholesterol extracted increased about 10% during the chymotryptic proteolysis. A small but definite increase in the extraction of phospholipid also occurred. The results obtained for trypsin treatment were similar. In the two experiments indicated in Figure 5, the amount of cholesterol extracted increased about 30% during incubation with trypsin.

The results in Figures 4 and 5 should be considered in the light of one simple observation. The β-lipoprotein solution after treatment with trypsin or chymotrypsin did not change in appearance. No turbidity or opalescence developed even after storage for periods of 1 week at 2°. This probably means that most of the protein remains with the lipid after proteolysis; the hydrophilic shell is only slightly altered.

Efforts were made to detect β-lipoprotein protein fragments after treatment with trypsin or chymotrypsin. Solutions of the β-lipoprotein were incu-

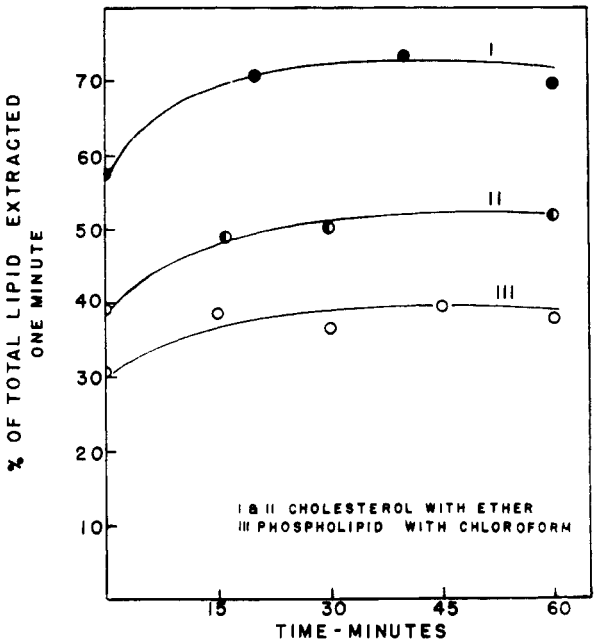

FIG. 4.—Effect of chymotrypsin on partial lipid extractions of β-lipoprotein. I and II in phosphate buffer, pH 7.5, 0.02 M. III in Tris buffer, pH 7.5, 0.02 M. 4.0 ml of the organic solvent was used to extract 2.0 ml of the reaction mixtures containing: I—2.03 mg protein, 2.18 mg cholesterol as β-lipoprotein, 0.10 mg chymotrypsin (850 units). II—1.99 mg protein, 2.06 mg cholesterol as β-lipoprotein, 0.10 mg chymotrypsin (280 units). III—2.15 mg protein, 3.36 mg cholesterol, 2.23 mg phospholipid as β-lipoprotein, 0.10 mg chymotrypsin (930 units).

bated with the enzymes for 18 hours at room temperature. The incubation mixtures were then applied to Whatman 1 paper strips, at 25°, and an electrical potential of 5 volts/cm was applied for 6 hours, with

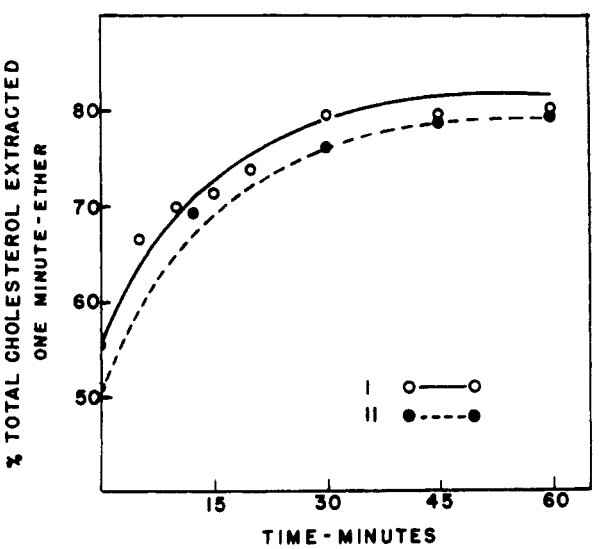

FIG. 5.—Effect of trypsin on partial cholesterol extractions of β-lipoproteins. Carried out in phosphate buffer, pH 7.5, 0.05 M; 4.0 ml of ether was used to extract 1.0 ml of the reaction mixtures containing: I—1.19 mg protein, 1.87 mg cholesterol as β-lipoprotein, 0.05 mg trypsin (412 units). II—0.795 mg protein, 0.875 mg cholesterol as β-lipoprotein, 0.05 mg trypsin (400 units).

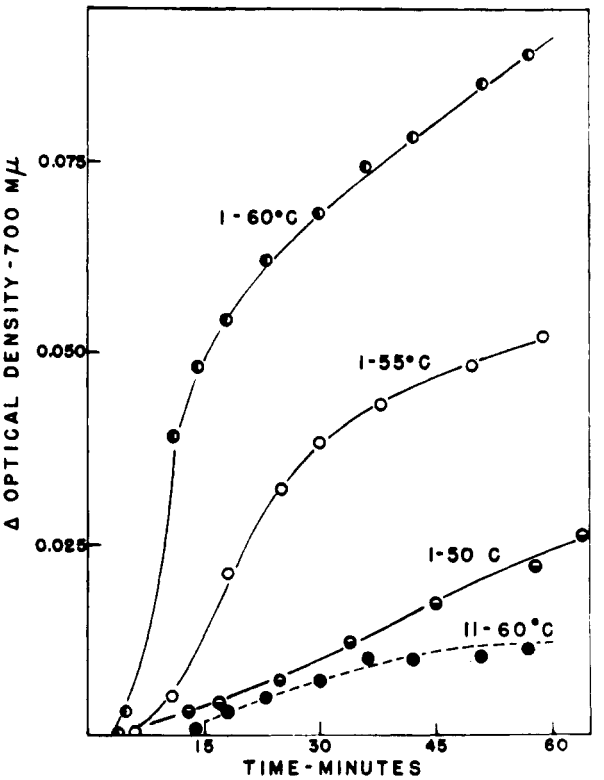

FIG. 6.—Turbidity formation by β-lipoprotein. Incubation mixtures were made up in phosphate buffer, pH 7.5, 0.05 M. The solutions contained 1.31 mg/ml protein and 1.50 mg/ml cholesterol as β-lipoprotein. In addition the curves labeled I contained 0.05 mg/ml chymotrypsin (340 units/ml). The curve labeled II contained 0.05 mg/ml trypsin (330 units/ml).

use of the ionographic technique described by McDonald and co-workers (1955). Duplicate strips were dried and stained with ninhydrin or bromphenol blue. Although this experiment was repeated four times, no ninhydrin-positive material which could be ascribed to the proteolysis of the β-lipoprotein could be detected. Furthermore, there appeared to be no significant differences between the mobilities of the treated and untreated β-lipoprotein.

Since no protein fragments were detected by ionography, and in order to leave little doubt that definite changes had occurred in the β-lipoprotein as a result of proteolysis, a series of studies completely different from the extraction experiments and tyrosine determinations were made. The procedure consisted of incubating the β-lipoprotein with the enzyme for 18 hours and then determining the rate of development of turbidity at temperatures up to 60°. The results are indicated in Figure 6. At the termination of the turbidity determinations, a 2.0-ml aliquot of each of the β-lipoprotein–enzyme solutions was mixed with 2.0 ml of 10% trichloroacetic acid and the optical density of the soluble material was determined at 280 mμ with a Beckman DU spectrophotometer. The results are indicated in Figure 7.

In conjunction with the data presented in Figure

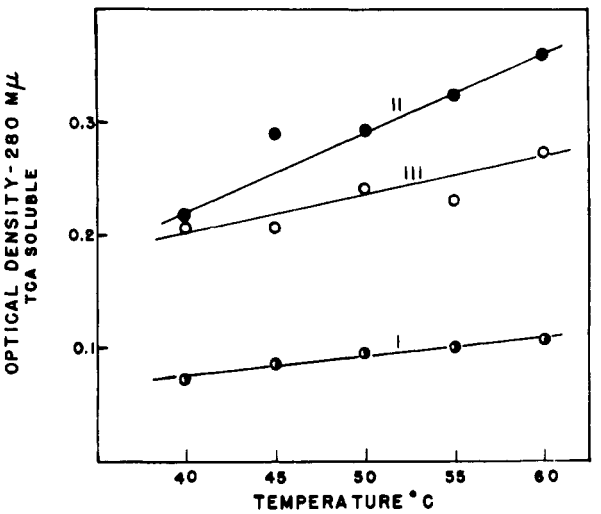

FIG. 7.—Release of trichloroacetic acid-soluble material from β-lipoprotein. I—β-lipoprotein, untreated. II—β-lipoprotein treated with chymotrypsin. III—β-lipoprotein treated with trypsin. (For composition of reaction mixtures see Fig. 6.)

6, it is necessary to point out that the β-lipoprotein blank was stable even after heating at 60° for 1 hour. Turbidity began to appear in the β-lipoprotein treated with trypsin only after the temperature was raised to 60°. Below 45° turbidity formation was absent in even the chymotrypsin-treated β-lipoprotein.

Figure 7 brings out the fact that some trichloroacetic acid-soluble material is found in the untreated preparation of β-lipoprotein. Proteolysis at least doubles the amount of trichloroacetic acid-soluble material which is released; enzyme blanks contained no material soluble in trichloroacetic acid. In all cases, slight increases in the release of trichloroacetic acid-soluble material occurred simultaneously with increasing temperature.

β-Lipoprotein Protein Fragments.—In several experiments the trichloroacetic acid-soluble material from β-lipoprotein before and after proteolysis was lyophilized, redissolved in 0.2 N NaOH, and chromatographed on Whatman No. 1 paper. The chromatograms were developed by the conventional descending technique with butanol-acetic acid-water, 4:1:1, v/v/v. Spraying with ninhydrin produced the results illustrated in Figure 8. Similar results were obtained with two other comparable experiments.

The untreated β-lipoprotein contained three areas of faintly ninhydrin-positive material. The R_F of these substances would seem to indicate that they are of low molecular weight. Since they are not lost during the prolonged dialysis used in the preparation of the lipid-protein complex, it may be presumed that they are weakly bound to the β-lipoprotein molecule and are released during the trichloroacetic acid precipitation. Another possibility is that these "contaminating" fragments are formed during dialysis.

Since estimation of the number of protein frag-

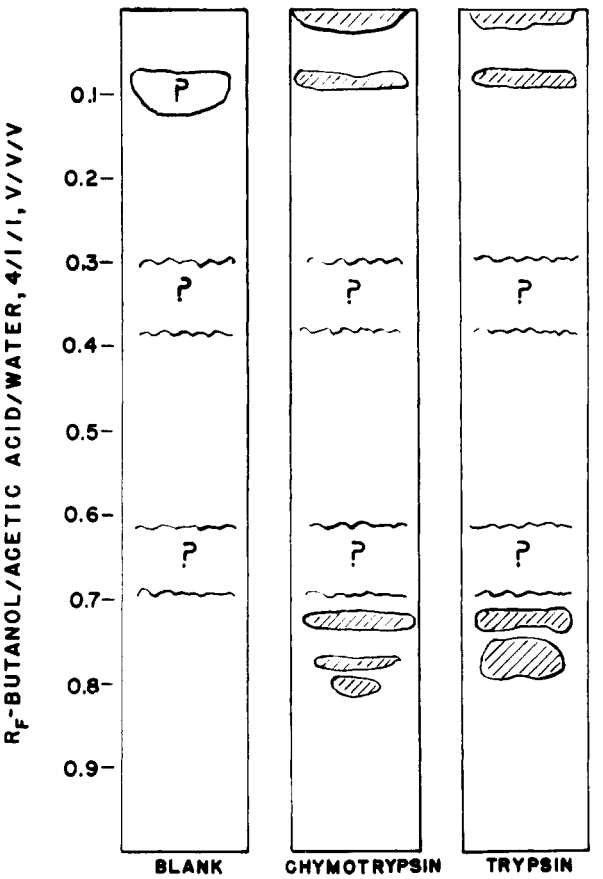

FIG. 8.—Paper chromatography of trichloroacetic acid-soluble material from β-lipoprotein. Shaded areas on the chromatogram are spots resulting from ninhydrin spray. Areas denoted with a question mark are only faintly colored.

ments would be of great help in clarifying the results of this investigation, attempts were made to obtain lipid-free protein before and after proteolysis. To do this the β-lipoprotein was lyophilized prior to organic solvent extraction. The lyophilized β-lipoprotein was extracted four times at 2° with *n*-pentane or ether. When ether was used for the lipid extraction, the final material was lumpy and hard. On the other hand, when *n*-pentane extractions were made, a white flocculent material resulted. If the organic solvent was allowed to evaporate (0°), a brown-black hard mass resulted. It was important, therefore, to redissolve the protein material immediately after the last lipid extraction. The serial *n*-pentane extractions removed all the cholesterol. It was shown that the residual protein from β-lipoprotein was not soluble in the following solutions: (1) water, (2) barbital buffer, pH 8.6, 0.05 M, (3) 0.001 N HCl, (4) 0.2 N 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 protein from β-lipoprotein 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 4 M urea. The protein dissolves instantly in this solution, but a gel begins to float on the surface of the buffer in a few minutes. When the urea was removed by dialysis, the gel persisted. Similar gel formation also occurred in the samples of protein from β-lipoprotein treated beforehand with trypsin or chymotrypsin.

Discussion

The main objective of this study was to observe the effect of chymotrypsin and trypsin on native β-lipoprotein. Considerably different results are obtained if the lipid-free protein is isolated first and then digested as a suspension (Rodbell and Fredrickson, 1959).

Evidence for proteolysis of β-lipoprotein is provided by the release of trichloroacetic acid–soluble peptide material. This evidence is supported by experiments which indicate that changes in the lipid-conjugating capacity occur during incubation with trypsin or chymotrypsin. Such changes were demonstrated with partial lipid-extraction procedures. Alterations in the native β-lipoprotein during proteolysis can also be observed by determining the rate of turbidity formation at elevated temperatures (Fig. 6).

The proteolytic digestion of the native β-lipoprotein is not very extensive. This is evident from the fact that coalescence of lipid components does not occur at room temperature after treatment with trypsin or chymotrypsin. The fact that even after removal of the lipid, isolation of protein fragments was not possible, may be taken as further evidence that only limited proteolysis occurred.

The paper electrophoretic studies of the digestion mixtures would seem to indicate that no peptide fragments are released from the β-lipoprotein after treatment with proteolytic enzymes. However, the possibility also exists that the ninhydrin reagent is not sensitive enough to detect the small amounts of peptide fragments which may formed. It is known that the ninhydrin spray will detect as little as 0.2 to 25 μg of amino acids (Lederer and Lederer, 1957), but the sensitivity decreases with peptides of increasing molecular weight.

Precipitation of β-lipoprotein by trichloroacetic acid does result in the release of peptide fragments. These materials have been partially characterized by paper chromatography (Fig. 8). However, from these data it is still impossible to ascertain how many fragments are present in the ninhydrin-positive material remaining at the origin of the chromatogram.

A suggested mechanism for the effects of trypsin and chymotrypsin is as follows: The first step may be the breaking of a limited number of peptide bonds. Certainly most, if not all, of the protein remains with the β-lipoprotein. In essence what is formed is a "new" lipoprotein. The nature of the new material depends on the specific proteolytic enzyme which has been used. It is suggested that the lipid is now covered with an increased number of peptide chains, and that they have fewer restrictions as to their location on the periphery of the molecule. Upon heating, for example, the peptide chains may relocate on different areas of the surface of the β-lipoprotein. Previously shielded areas of lipid contained in the β-lipoprotein are exposed to the environment and coalescence occurs; for the same reason, lipid extraction is facilitated.

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