# Soluble and Immobilized Trypsin as Structural Probes of Human Plasma High-Density Lipoproteins: Enzyme Properties and Kinetics of Proteolysis†

Robert E. Byrne* and Angelo M. Scanu

ABSTRACT: The proteolytic action of Sepharose-bound and soluble L-1-(tosylamido)-2-phenylethyl chloromethyl ketone treated bovine trypsin on native human high-density lipoprotein (HDL) of density 1.125–1.21 g/mL ($HDL_3$) and on its lipid-free apoprotein (apo $HDL_3$) was evaluated. For $HDL_3$, initial rate studies of proteolysis by Sepharose-bound trypsin (SphB-T) and soluble trypsin (SOL-T) gave comparable $K_m$ values: 7.1 ± 1.0 and 7.5 ± 1.0 mg of protein/mL, respectively. On the other hand, the $V_{max}$ by SOL-T [52.9 ± 5.0 μmol of $H^+$ produced $min^{-1}$ (mg of active trypsin)$^{-1}$] was significantly higher than that by SphB-T [3.5 ± 0.5 μmol of $H^+$ produced $min^{-1}$ (mg of active trypsin)$^{-1}$]. For apo $HDL_3$, the $K_m$ values obtained from SphB-T (1.6 ± 0.3 mg of protein/mL) and SOL-T (0.9 ± 0.1 mg of protein/mL) were both lower than the corresponding ones with native $HDL_3$. Furthermore, the $V_{max}$ of apo $HDL_3$ hydrolysis by SphB-T [17.6 ± 2.5 μmol of $H^+$ produced $min^{-1}$ (mg of active trypsin)$^{-1}$] was considerably higher than when this enzyme was tested against $HDL_3$. In contrast, similar $V_{max}$ values were obtained when either $HDL_3$ or apo $HDL_3$ was hydrolyzed by SOL-T [52.9 ± 5.0 or 60.4 ± 6.0 μmol of $H^+$ produced $min^{-1}$ (mg of active trypsin)$^{-1}$, respectively]. Prolonged digestions of $HDL_3$ by SOL-T required multiple additions of the enzyme or the presence of $Ca^{2+}$ to maintain a constant activity. In contrast, under comparable conditions and in the presence or absence of $Ca^{2+}$, the activity of SphB-T remained essentially constant for at least 6 h. Under constant enzyme activity, prolonged digestions of $HDL_3$ (1.5 mg of protein/mL) by either SphB-T or SOL-T (0.15 mg of active trypsin/mL) followed pseudo-first-order kinetics with respect to $HDL_3$ for the majority of the reaction; the experimental first-order rate constant of proteolysis by SOL-T ($k_{exp}$ = 1.25 × $10^{-2}$ $min^{-1}$) was approximately 2.5-fold higher than that by SphB-T ($k_{exp}$ = 4.92 × $10^{-3}$ $min^{-1}$). In spite of the different kinetics of proteolysis by SOL-T or SphB-T, the sodium dodecyl sulfate–polyacrylamide gel electrophoresis patterns of $HDL_3$ samples removed at timed intervals were similar although differences in product yields were observed. Both apo A-I and apo A-II were hydrolyzed by either SphB-T or SOL-T; however, in both cases, the apo A-II band disappeared faster than that corresponding to apo A-I. With apo $HDL_3$ as a substrate, the electrophoretic patterns of hydrolysis by SOL-T and SphB-T were similar although they were significantly different from those obtained with $HDL_3$. From the kinetics of both initial and prolonged proteolysis and from the electrophoretic analysis of the products, we conclude that SOL-T hydrolyzes $HDL_3$ more rapidly than SphB-T but the latter form of the enzyme is comparatively more stable and does not require Ca ions to maintain activity. Both SOL-T and SphB-T can be used as structural probes for studying the apoprotein organization in HDL provided that the differences in enzyme activity and kinetics are taken into account.

The physical and chemical evidence available for the structure of high-density lipoproteins (HDL)[1] is consistent with a model of spherical or quasi-spherical particles comprising a highly packed core of cholesteryl esters and triglycerides surrounded by a monolayer of phospholipids, unesterified cholesterol, and apoprotein (Verdery & Nichols, 1975; Shen et al., 1977; Edelstein et al., 1979). Although the apoproteins are located near or at the lipoprotein surface, details regarding the structural organization of individual apoproteins are limited.

Some supporting evidence for the surface location of the apoproteins is suggested by the finding that the amino groups of lysine residues of the HDL apoproteins can be extensively succinylated (ca. 90%) without a major alteration in lipoprotein structure (Scanu et al., 1968). This finding suggests an exterior orientation of the lysine residues. Immunological studies suggest, however, that more than 90% of the antigenic sites of apolipoprotein A-I (apo A-I) in intact HDL are inaccessible to antibodies against this apolipoprotein and become exposed only after removal of lipids or heating (Schonfeld & Pfleger, 1974; Schonfeld et al., 1975; Karlin et al., 1976). In contrast, nearly 100% of the antigenic sites of apo A-II, apo C-II, and apo C-III of intact HDL react with their respective antibody preparations (Schonfeld et al., 1977, 1979). Recently, Jeng et al. (1980) combined Pronase digestion and immunological techniques to differentiate between exposed and buried regions of the apoproteins in HDL. Following digestion of HDL with Pronase, all apo A-II, apo C-II, and apo C-III lost their specific antibody reactivity; in the case of apo A-I, 80% was retained. Since in the intact HDL only approximately 10% of the apo A-I reacted with anti apo A-I antisera, it was suggested that Pronase causes the hydrolysis of only the exposed regions of the HDL apoproteins.

Studies on the proteolysis of human HDL by soluble (Camejo, 1969) and immobilized trypsin (Shore et al., 1979) have been conducted previously. Results with soluble trypsin suggested that the extent of proteolysis and the products formed were similar in both HDL and apo HDL. In contrast, under conditions which ensured complete digestion of apo HDL, proteolysis of human $HDL_3$ by immobilized trypsin was incomplete. In addition, the peptides released from $HDL_3$

† From the Departments of Medicine and Biochemistry, The University of Chicago, Pritzker School of Medicine, Chicago, Illinois 60637. *Received December 8, 1982.* This work was supported by Program Project Grant PHS HL-18577 and Cardiovascular Training Grant PHS 5 T32 HL-7237.

[1] Abbreviations: HDL, high-density lipoprotein(s); $HDL_3$, HDL with a density of 1.125–1.21 g/mL; apo $HDL_3$, delipidated $HDL_3$; apo A-I, apolipoprotein A-I; apo A-II, apolipoprotein A-II; SOL-T, soluble trypsin; SphB-T, Sepharose-bound trypsin; BAEE, $N^{\alpha}$-benzoyl-L-arginine ethyl ester; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane; DFP, diisopropyl phosphorofluoridate; $NaDodSO_4$, sodium dodecyl sulfate; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; ME, β-mercaptoethanol; $V_0$, initial rate; S, substrate; $V_{max}$, maximal attainable rate; $K_m$, concentration of S which causes $V_0$ to be 50% of $V_{max}$.

0006-2960/83/0422-2894$01.50/0 

differed from those released from apo HDL. The differences in proteolysis by soluble and immobilized trypsin were explained primarily on the basis of differences in trypsin penetration into the HDL particle; no consideration was given on possible differences in kinetic properties between soluble and immobilized enzyme. Moreover, the studies using soluble trypsin utilized $Ca^{2+}$ for the purpose of maintaining enzymatic activity. However, the direct effect of this cation on the reactants was not evaluated, and $Ca^{2+}$ ions were not included in the studies with immobilized trypsin. In fact, the studies were carried out for many hours without an independent assessment of enzyme activity.

Additional evidence for the preferential masking of apo A-I in HDL has been reported from our laboratory which indicates that exposure of human $HDL_3$ to mixed human leukocytes (Ritter & Scanu, 1980), or more recently to isolated granulocytes (Polacek & Scanu, 1982), results in a complete hydrolysis of apo A-II whereas apo A-I is only modestly affected. However, when apo HDL instead of $HDL_3$ was incubated with the cells, both apo A-I and apo A-II were extensively hydrolyzed (Ritter & Scanu, 1980).

In order to further understand the differences in peptide bond cleavage accessibility between apoproteins in the intact lipoprotein and those in a lipid-free state, we have investigated initial-rate and prolonged kinetics of human $HDL_3$ and apo $HDL_3$ proteolysis by SOL-T and SphB-T. We have emphasized the importance of critically defining the conditions of incubation and assessing enzyme activity for the purpose of deriving structural information by using proteolytic enzyme probes. We have also compared the soluble and immobilized trypsins as potential proteolytic probes for the study of the surface structure of plasma lipoproteins and, in particular, $HDL_3$.

## Experimental Procedures

### *Materials*

*Chemicals.* Diisopropyl phosphorofluoridate (DFP) was a product of Aldrich Chemical Co. (Milwaukee, WI). $N^{\alpha}$-Benzoyl-L-arginine ethyl ester (BAEE) was purchased from Sigma Chemical Co. (St. Louis, MO), and *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride (NPGB) was from Vega Biochemicals (Tucson, AZ). Sepharose 4B was a product of Pharmacia Fine Chemicals, Inc. (Piscataway, NJ). Cyanogen bromide was from Pierce Chemical Co. (Rockford, IL). Enzyme-grade acrylamide, bis(acrylamide), β-mercaptoethanol (ME), and Coomassie brilliant blue R-250 were purchased from Eastman (Rochester, NY). $Na^{125}I$ (carrier free) was purchased from Amersham Corp. (Arlington Heights, IL). Urea (J. T. Baker Chemical Co., Phillipsburg, NJ) was recrystallized from 95% ethanol, and aqueous solutions were deionized through a mixed-bed resin (Bio-Rad A6-501-X8; Richmond, CA). Guanidine hydrochloride (ultrapure) was purchased from Heico, Inc. (Delaware Water Gap, PA). All other chemicals used were reagent grade.

*Proteins.* Bovine pancreatic trypsin treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone (TPCK) was obtained from Worthington Biochemical Corp. (Freehold, NJ). Crystallized bovine serum albumin was from Miles Laboratories (Elkhart, IN). Horse heart cytochrome *c* and lactoperoxidase were from Sigma Chemical Co. Purified human insulin was a gift from Dr. Donald Steiner, The University of Chicago.

### *Methods*

*Isolation of Human $HDL_3$ and Apo $HDL_3$.* Venous blood from selected healthy normolipidemic male human donors was collected in 250-mL plastic bottles containing 2.5 mL of 0.31 M ethylenediaminetetraacetic acid (EDTA), pH 7.4. The plasma was separated by centrifugation at 4 °C to remove blood cells. The $HDL_3$ ($d$ = 1.125–1.210 g/mL) was isolated from plasma by sequential ultracentrifugal flotation as previously described (Granda & Scanu, 1966; Scanu, 1966). The $HDL_3$ preparation contained apo A-I and apo A-II (about 95% of the total protein mass) as the major apoprotein constituents by $NaDodSO_4$–polyacrylamide gel electrophoretic criteria. The $HDL_3$ preparations gave a single band with α mobility by agarose electrophoresis (Noble, 1968). After flotation, the $HDL_3$ was stored in the presence of DFP ($10^{-3}$ M) under argon gas at 4 °C. Before use, stock $HDL_3$ was dialyzed extensively against 0.15 M NaCl adjusted to pH 7.4 with 0.1 M NaOH. Human apo $HDL_3$ was obtained by delipidation of $HDL_3$ with ethanol–ether at −20 °C as previously described (Scanu & Edelstein, 1971). In some experiments, apo HDL was prepared from HDL ($d$ = 1.063–1.21 g/mL). In these instances, single-spin ultracentrifugal analysis (Foreman et al., 1977) of plasma samples from each of these donors indicated that essentially only $HDL_3$ was present in $d$ = 1.063–1.210 g/mL fraction of the lipoprotein profile. Before use, the dry apoprotein was dissolved in 0.05 M Tris–0.15 M NaCl, pH 7.4, containing 6 M guanidine hydrochloride followed by extensive dialysis at 4 °C against 0.15 M NaCl adjusted to pH 7.4 with 0.1 M NaOH. Analysis of the apo HDL or apo $HDL_3$ by $NaDodSO_4$–polyacrylamide gel electrophoretic criteria indicated that apo A-I and apo A-II comprised the majority of the apolipoprotein of these preparations, with little or no apo E or apo C present as judged by Coomassie blue staining. The relative ratio of apo A-I to apo A-II calculated by gel scanning [approximately (1.5–2.0):1 A-I:A-II] and the lack of significant quantities of apo E and apo C were consistent with the protein composition of apo $HDL_3$ and not apo $HDL_2$.

*Preparation of Immobilized Trypsin.* Sepharose 4B was activated with CNBr (0.2 g of CNBr/mL of settled gel) by the method of Cuatrecasas (1970). Trypsin was then coupled to CNBr-activated Sepharose 4B according to the method of Knights & Light (1974). The coupling mixture contained 50 mg of trypsin in 40 mL of a 50% (v/v) suspension[2] of CNBr-activated Sepharose 4B in 0.025 M borate buffer at pH 10.2 containing 0.02 M $CaCl_2$. The coupling reaction was maintained at 4 °C for 16 h and the mixture suspended by continuous inversion with the aid of a mechanical rotator. After completion of the coupling reaction, the SphB-T was washed extensively with a series of alternating pH 8.0 and pH 4.0 buffers as described by Knights & Light (1974). The immobilized trypsin was stored at 4 °C in 0.01 M acetate–0.1 M NaCl (pH 4.0) containing 0.1% sodium azide. Before use, the suspension was washed repeatedly with 0.05 M sodium barbital containing 0.15 M NaCl and 0.02 M $CaCl_2$ (pH 8.3) for activity measurements. No loss of activity was noted for at least 2 months when the enzyme was stored at 4 °C in the pH 4.0 buffer.

*Assay of Concentration of Trypsin Active Sites.* The concentration of active sites of SOL-T and SphB-T was determined by titration (Chase & Shaw, 1967) with NPGB as described by Knights & Light (1974) employing the molar extinction coefficient of 16 595 at 410 nm for *p*-nitrophenolate at pH 8.3 (Chase & Shaw, 1967). In order to calculate the concentration of active trypsin on a weight basis, we converted the molar concentration of trypsin in active sites to weight

[2] A 50% suspension contained equal volumes of swollen gel and supernatant fluid when settled.

amounts by using a molecular weight of 23 800 (Cunningham, 1954).

*Potentiometric Assay of $HDL_3$ and Apo $HDL_3$ Proteolysis by SphB-T or SOL-T.* The initial concentration of $HDL_3$ or apo $HDL_3$ varied in concentration between 0.4 and 15.0 mg of protein/mL. The rate of proteolysis in either system was determined by pH-stat titration in a 0.15 M NaCl solution maintained at pH 7.4 by the automatic addition of 0.01 M NaOH. The temperature was maintained at 37 °C, with the aid of a thermostat-equipped vessel holder, by stirring the mixture within the reaction vessel continuously. The time course of the hydrolysis was followed in a TT2 automatic titrator by using an ABU 12 autoburet, a 0.25-mL buret, a REC 51 recorder, and a GK 2322C combination electrode (all from Radiometer, Copenhagen, Denmark).

In a typical experiment, 0.12 mL of human $HDL_3$ (1.8 mg of protein) in 0.15 M NaCl was added to the reaction vessel containing 1.035 mL of 0.15 M NaCl. The pH was adjusted to 7.4 and the base line maintained steady for several minutes before the addition of the enzyme. At this time, 0.045 mL of a 50% suspension of SphB-T (0.40 mg of active trypsin/mL of stock in 0.15 M NaCl, pH 7.4) was added to the reaction vessel containing $HDL_3$. Control experiments were routinely performed by using $HDL_3$ but no enzyme in order to assess the amount of base needed to compensate for the aliquot of SphB-T added to the system. For this control, the amount of base added was quite small provided the stock SphB-T suspension was pretitrated to pH 7.4 in a solvent containing 0.15 M NaCl in the absence of buffer. The method for examining the reactions catalyzed by SOL-T was analogous to that described for SphB-T except that the stock SOL-T was dissolved in $1 \times 10^{-3}$ M HCl in order to prevent loss of enzyme activity which in the absence of $Ca^{2+}$ readily occurs at pH 7.4. Furthermore, to minimize the amount of base required to maintain pH 7.4, we routinely prepared relatively concentrated stock solutions of SOL-T (approximately 3 mg of protein/mL).

*Assessment of SOL-T or SphB-T Enzymatic Activity during Incubation in the Presence and Absence of $HDL_3$.* Aliquots (0.12 mL) of human $HDL_3$ (1.8 mg of protein) in 0.15 M NaCl or a control NaCl solution without $HDL_3$ were mixed by continuous stirring at 37 °C with 0.03 mL of 1.0 M Tris-HCl (pH 7.4) followed by the addition of 0.18 mg of active SOL-T or SphB-T in a final volume of 1.2 mL made up with 0.15 M NaCl. When $Ca^{2+}$ was included in the system, the required volume of 0.1 M $CaCl_2$ in 0.15 M NaCl was added to the incubation mixture prior to the addition of SOL-T or SphB-T. At timed intervals, aliquots were assayed against BAEE by the pH-stat method. The assay was initiated by immediately adding 0.02–0.05-mL aliquots to 1.5 mL of 0.015 M BAEE substrate in 0.15 M NaCl containing 0.02 M $CaCl_2$. Under these conditions, the BAEE hydrolysis rate at pH 8.0 and 37 °C was linear for several minutes and directly proportional to the amount of trypsin activity added. Assays of SOL-T and SphB-T activity by the pH-stat method were unaffected by the presence of $HDL_3$ under these conditions. This was due to the relatively high BAEE/$HDL_3$ weight ratio present in the assay system, as well as the lower $K_m$ and higher $V_{max}$ values of BAEE by trypsin compared to those corresponding to $HDL_3$ as a substrate. Control experiments were conducted to assess the BAEE hydrolysis rates of aliquots from incubation mixtures which did not contain SOL-T or SphB-T, and these values were subtracted as blanks.

*Kinetics of Prolonged $HDL_3$ Proteolysis by SOL-T or SphB-T.* The rate of $HDL_3$ proteolysis by SOL-T or SphB-T during prolonged incubations was monitored by the potentiometric assay as described above. The amount of base added with time, $[base]_{(t)}$, during the reaction was a measure of the product formed. The total amount of base added, $[base]_{(T)}$, at the end of the reaction was determined by the method of Kezdy et al. (1958). The total amount differed by less than 6% from the amount of base added experimentally after five successive half-times of reaction. The course of the reaction, $\{[[base]_{(T)} - [base]_{(t)}]/[base]_{(T)}\} \times 100$ (where T and $t$ represent total and time point base added), was plotted on a semilog scale against time (minutes) in order to determine the reaction order and relative rates of proteolysis by either SOL-T or SphB-T (Segal, 1975).

*Initial Rate of Kinetics of $HDL_3$ and Apo $HDL_3$ Proteolysis by SOL-T or SphB-T.* By the potentiometric assay described above, the initial rate ($V_0$) of base added per minute was determined from the slope of the tangent to the titration curve which intersected at the origin. At low substrate concentrations where the initial rate was linear for only a short time, the tangent was determined with the aid of a French curve superimposed on the titration curve. The $V_0$ values of proteolysis as a function of substrate concentration were expressed as micromoles of $H^+$ produced per minute per milligram of active trypsin. A correction factor ($\alpha$) was necessary since only the dissociated protons were detected at pH 7.4. The $\alpha$ value was dependent on the $pK_a$ values of the $\alpha$-$NH_3^+$ groups of the product peptides. By assuming a value of 7.5 for the $pK_a$ of the $\alpha$-$NH_3^+$ groups (Tanford, 1962), we calculated an $\alpha$ value $[10^{pH-pK_a}/(1 + 10^{pH-pK_a})]$ of 0.44 at pH 7.4 according to the method of Jacobsen et al. (1957). Thus, the number of micromoles of $H^+$ produced was equal to $0.44^{-1}$ $\mu$mol of base added. The maximal attainable rate ($V_{max}$) and the concentration of substrate ([S]) which causes $V_0$ to be 50% of $V_{max}$ ($K_m$) were determined from Lineweaver–Burk plots.

*Iodination of Apo A-I and Apo A-II with $Na^{125}I$.* The apoproteins were iodinated with carrier-free $Na^{125}I$ by a lactoperoxidase method as described by Edelstein et al. (1982). Approximately 0.8–1.0 atom of iodine was incorporated per molecule of apoprotein.

*Preparation of $^{125}I$-Apo A-I ($HDL_3$) or $^{125}I$-Apo A-II ($HDL_3$).* $HDL_3$ (3 mg of protein) in 0.15 M NaCl, 0.02% azide, $1.5 \times 10^{-3}$ M EDTA, pH 7.4, was incubated with 0.5 $\mu$Ci (approximately 100 ng) of either $^{125}I$-apo A-I or $^{125}I$-apo A-II at 37 °C for 1 h in the presence of $1 \times 10^{-3}$ M DFP in a total volume of 1.5 mL. Both $^{125}I$-apo A-I ($HDL_3$) and $^{125}I$-apo A-II ($HDL_3$) preparations had a high specific activity (200–300 dpm/$\mu$g) under conditions where very little $^{125}I$-apoprotein mass was actually added to the $HDL_3$ substrate. Thus, essentially no change in apoprotein composition in $HDL_3$ occurred. Reisolation of $^{125}I$-apo A-I ($HDL_3$) or $^{125}I$-apo A-II ($HDL_3$) and removal of the free iodine were accomplished by isopycnic density gradient centrifugation in an SW-40 rotor at 14 °C as previously described (Foreman et al., 1977), and the effluents were analyzed at 280 nm and collected in an ISCO Model 640 fractionator (Lincoln, NE). Individual fractions were counted for $^{125}I$ in a Tracor Analytic (Elk Grove, IL) Model 1190 $\gamma$ counter with an efficiency of 70%. Only $HDL_3$ fractions having essentially the same relative specific activity (cpm/$A_{280nm}$) were pooled. The pooled fractions were dialyzed extensively against 0.15 M NaCl, pH 7.4 (4 °C), before use in proteolysis studies.

*NaDodSO$_4$–Polyacrylamide Gel Electrophoresis.* Analyses in NaDodSO$_4$–polyacrylamide gel electrophoresis were performed by using 10% acrylamide (w/v) as described by Weber & Osborn (1969). Gels were stained with 0.25% (w/v) Coomassie brilliant blue R in 45% methanol–9% acetic acid

and destained in 18% methanol–10% acetic acid. Gel patterns were scanned at 565 nm in a Varian Model DMS 90 spectrophotometer (Varian Instruments, Sunnyvale, CA) equipped with a gel scanning accessory. Gels containing $^{125}I$-labeled apoproteins were either stained with Coomassie blue or sliced into 3-mm sections immediately following electrophoresis and counted for radioactivity. Molecular weight estimations on gels were performed according to Weber & Osborn (1969).

*Protein Determination.* Protein was measured by a modified procedure of Lowry et al. (1951) in the presence of $NaDodSO_4$ as described by Markwell et al. (1978) with bovine serum albumin as a standard.

## Results

*Analysis of the Activity of SOL-T and SphB-T against Human Plasma HDL$_3$.* SOL-T (0.15 mg/mL active trypsin) was incubated with $HDL_3$ (1.5 mg of protein/mL) in 0.15 M NaCl, pH 7.4 at 37 °C, and the liberation of protons during the course of hydrolysis was measured by pH-stat titration. Under these conditions, the initial phase of proteolysis was rapid and linear; thereafter, the rate exhibited a continuous decrease (Figure 1A, open squares). This result suggested that the enzyme was undergoing inactivation, and/or the rate of hydrolysis was dependent on $HDL_3$ concentration. For assessment of the former possibility, the activity of trypsin was independently determined by assaying aliquots of the incubation mixture, at timed intervals, against the synthetic substrate BAEE. Under the same conditions as described in Figure 1A and in the presence of $HDL_3$ (see Figure 1B), only 60% of the initial SOL-T esterase activity remained after 1 h of incubation. In the absence of $HDL_3$, only 20% of the initial SOL-T activity was retained after 1 h. The addition of 5 mM $Ca^{2+}$ to the incubation mixture containing $HDL_3$ resulted in no detectable loss of SOL-T hydrolytic activity for at least 1 h.

Since the preliminary experiments showed that the SOL-T activity was essentially unchanged during incubation with $HDL_3$ in the presence of 5 mM $Ca^{2+}$, the effect of this cation on the proteolysis of $HDL_3$ was investigated (Figure 1B, inset). $HDL_3$ (1.5 mg of protein/mL) was incubated with SOL-T (0.014 mg/mL) in 0.15 M NaCl, pH 7.4 and 37 °C, in the presence or absence of 5 mM $Ca^{2+}$ and the proteolysis monitored by pH-stat titration. As seen in Figure 1B (inset), the rate of proteolysis of $HDL_3$ was somewhat slower in the presence than in the absence of $Ca^{2+}$. At 5 mM, this cation had a stabilizing effect on SOL-T activity but did not enhance the $V_0$ of $HDL_3$ proteolysis and in fact slightly inhibited the $V_0$ of the reaction.

When SphB-T replaced SOL-T under identical conditions of initial $HDL_3$ and active enzyme concentrations (Figure 1A, closed circles), the rate of proteolysis by SphB-T was slower than that with SOL-T. In contrast to the results with SOL-T, 97% of the initial SphB-T activity was unchanged during incubation at 37 °C for at least 6 h in the absence of $Ca^{2+}$ and in the presence or absence of $HDL_3$ (Figure 1B). Thus, no addition of fresh enzyme was needed in the system containing SphB-T.

For investigation of the kinetics of prolonged $HDL_3$ hydrolysis by SOL-T in the absence of $Ca^{2+}$, an attempt was made to maintain SOL-T activity in the $HDL_3$ reaction mixture by the addition of fresh enzyme to approximate the activity of SOL-T lost during incubation. The addition of the corresponding amount of SOL-T lost after 120 min of incubation (indicated by the arrow in Figure 2, open squares) resulted in an increase of the rate of protons released ($HDL_3$, 1.5 mg of protein/mL; SOL-T, 0.15 mg/mL). Since under

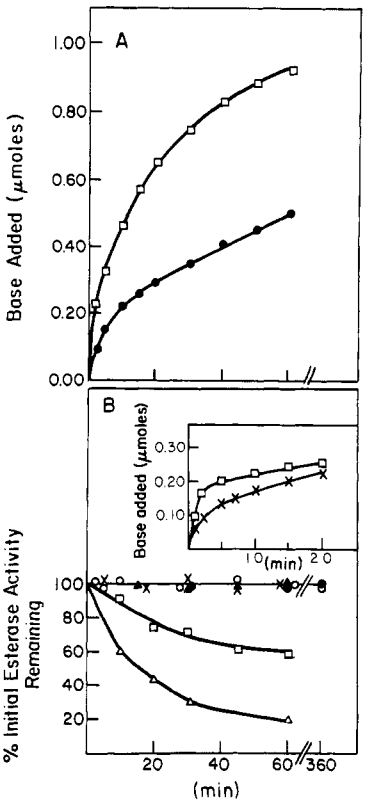


FIGURE 1: Potentiometric analyses of human $HDL_3$ proteolysis by SOL-T and SphB-T and the effect of $Ca^{2+}$ on the stability of enzyme activity during incubation. (A) Human $HDL_3$ proteolysis with SOL-T (□) and SphB-T (●) at 37 °C in 0.15 M NaCl. The pH was maintained at 7.4, and the proteolysis was monitored by pH-stat titration as described under Experimental Procedures. The initial concentration of $HDL_3$ was 1.5 mg of protein/mL, and the initial concentration of SOL-T or SphB-T was 0.15 mg of active trypsin/mL as determined by active-site titration (see Experimental Procedures). (B) Effect of $Ca^{2+}$ on the esterase activity of SOL-T and SphB-T during incubation with human $HDL_3$. At timed intervals, aliquots of the incubation mixtures were removed and assayed for BAEE esterase activity as described under Experimental Procedures. The initial concentrations of $HDL_3$ and SOL-T or SphB-T were the same as described in (A) unless noted otherwise: (△) with SOL-T minus $HDL_3$ and minus $Ca^{2+}$; (□) with SOL-T and $HDL_3$ minus $Ca^{2+}$; (×) with SOL-T, $HDL_3$, and 5 mM $Ca^{2+}$; (▲) with SOL-T and 5 mM $Ca^{2+}$ minus $HDL_3$; (●) with SphB-T and $HDL_3$ minus $Ca^{2+}$; (○) with SphB-T minus $HDL_3$ and minus $Ca^{2+}$. (Inset) Effect of $Ca^{2+}$ on the proteolysis of $HDL_3$ with SOL-T. Incubation conditions and the initial $HDL_3$ concentration were the same as described in (A) except that the active SOL-T concentration was 0.014 mg/mL and the amount of $Ca^{2+}$ added was (×) 5 or (□) 0 mM.

the initial reaction conditions approximately 25% of the initial SOL-T activity was lost after 25 min of incubation, in separate experiments equivalent amounts of lost enzyme activity were added at 25-min intervals (Figure 2, closed squares). As shown in Figure 2 (inset) for the SOL-T–$HDL_3$ system, the plot of log $\{\{[[\text{base}]_{(T)} - [\text{base}]_{(t)}]/[\text{base}]_{(T)}\} \times 100\}$ against time (minutes) was linear after the initial rapid phase of the proteolysis (0–5 min) was reached. The pseudo-first-order rate constant for this reaction was $k_{exp} = 1.25 \times 10^{-2}$ min$^{-1}$. The data obtained in the very early stages of proteolysis were not included in the calculation of the first-order rate constant since the reaction order was intermediate between zero and first order. As compared to SOL-T, the initial rate of $HDL_3$ proteolysis by SphB-T at an equivalent enzyme concentration was significantly lower (Figure 2, closed circles). After the initial rapid phase of proteolysis (0–5 min), the reaction followed pseudo-first-order kinetics with a $k_{exp} = 4.92 \times 10^{-3}$ min$^{-1}$ (Figure 2, inset).

Table 1: Dependence of the Initial Rate of Human $HDL_3$ Proteolysis Catalyzed by Sepharose-Bound or Soluble TPCK-Treated Bovine Trypsin on the Initial Concentration of Human $HDL_3$

| trypsin | initial [$HDL_3$] (mg of protein/mL) | [trypsin] (mg of protein/mL)[a] | [$HDL_3$]/[trypsin] (wt ratio) | initial rate of proteolysis[b,c] [μmol of $H^+$ produced $min^{-1}$ (mg of active trypsin)$^{-1}$] |
|---|---|---|---|---|
| Sepharose bound | 1.5 | 0.015 | 100 | 0.63 ± 0.02 |
| | 5.0 | 0.015 | 333 | 1.26 ± 0.10 |
| | 8.0 | 0.015 | 533 | 2.05 ± 0.10 |
| | 8.0 | 0.150 | 53 | 1.93 ± 0.15 |
| | 12.0 | 0.030 | 400 | 2.93 ± 0.20 |
| soluble | 0.8 | 0.015 | 53 | 5.04 ± 0.60 |
| | 1.5 | 0.015 | 100 | 7.97 ± 0.20 |
| | 3.0 | 0.015 | 200 | 14.52 ± 1.30 |
| | 8.0 | 0.015 | 533 | 23.56 ± 1.60 |
| | 15.0 | 0.015 | 1000 | 37.12 ± 3.00 |

[a] The molar concentration of trypsin in active sites was converted to weight amounts by using a molecular weight of 23 800 (Cunningham, 1954). [b] The proteolysis was conducted at 37 °C in a final reaction volume of 1.5 mL containing 0.15 M NaCl maintained at pH 7.4 by pH-stat titration with 0.01 M NaOH. [c] The initial rate was calculated on the basis of a correction for proton yield ($\alpha$ = 0.44) according to the method of Jacobsen et al. (1957) by assuming a $pK_a$ value of 7.5 for the $\alpha$-amino groups of the product peptides (Tanford, 1962). These data represent the mean value ± the standard deviation for three determinations.

*Steady-State Kinetics of SOL-T- and SphB-T-Catalyzed Proteolysis of Human Plasma $HDL_3$.* The initial concentration of $HDL_3$ influenced the $V_0$ of proteolysis by SOL-T as assessed from the tangent of the pH-stat titration curve intersecting the origin (Figure 3A). The $V_0$ value for an $HDL_3$ protein concentration of 8 mg/mL was approximately 3-fold higher than that observed at 1.5 mg/mL (0.015 mg/mL active trypsin initially added). A similar effect of $HDL_3$ concentration on the $V_0$ of proteolysis by SphB-T was also observed (Figure 3B). Note, however, that a 10-fold higher SphB-T concentration (0.15 mg/mL) was needed to obtain proteolysis rates comparable to those exhibited by SOL-T (Figure 3A).

The studies on the dependence of $HDL_3$ concentration on the $V_0$ of human $HDL_3$ proteolysis by SphB-T or SOL-T in 0.15 M NaCl at pH 7.4 and 37 °C are summarized in Table I. With SphB-T, the $V_0$ increased approximately 4.7-fold within an $HDL_3$ protein concentration range between 1.5 and 12 mg/mL. Furthermore, the $V_0$ was proportional to enzyme concentration. For example, the $V_0$ values for the proteolysis of $HDL_3$ (8 mg of protein/mL) by two different levels (10-fold range in enzyme concentration) of SphB-T were within experimental error when corrected for the amount of enzyme added (Table I). A Lineweaver–Burk plot of these data was linear (Figure 3B, inset). From the linear regression analysis of these data (Table III), a $K_m$ value (slope × intercept$^{-1}$) of 7.1 ± 1.0 mg of protein/mL and a $V_{max}$ (intercept$^{-1}$) of 3.5 ± 0.5 μmol of $H^+$ produced $min^{-1}$ (mg of active trypsin)$^{-1}$ were calculated.

The $V_0$ of $HDL_3$ proteolysis of SOL-T increased approximately 7-fold for an $HDL_3$ concentration range between 0.8 and 15.0 mg of protein/mL (Table I). In addition, at equivalent $HDL_3$ concentrations, the $V_0$ values were higher with SOL-T than with SphB-T. From the linear Lineweaver–Burk plot of the $V_0$ data of $HDL_3$ proteolysis by SOL-T (Figure 3A, inset), the $K_m$ value (7.5 ± 1.0 mg/mL) was quite similar to that determined with SphB-T (Table III). However, the $V_{max}$ [52.9 ± 5.0 μmol of $H^+$ produced $min^{-1}$ (mg of active trypsin)$^{-1}$] was approximately 15-fold higher than the value obtained with SphB-T (Table III).

*Steady-State Kinetics of Proteolysis of Human Apo $HDL_3$ by SOL-T and SphB-T.* In the case of apo $HDL_3$ at a concentration of 1.6 mg/mL, the $V_0$ of proteolysis by SOL-T was approximately 2.3-fold higher than at 0.4 mg/mL protein (Figure 4A) when in the presence of the same concentration of active SOL-T (0.006 mg/mL). Likewise with SphB-T, a 2.6-fold increase in the $V_0$ of apo $HDL_3$ hydrolysis was observed within the same initial apo $HDL_3$ concentration range (Figure 4B). Nevertheless, the magnitudes of the initial slopes from the pH-stat titration curves with SOL-T were higher than the corresponding ones with SphB-T, even though the con-

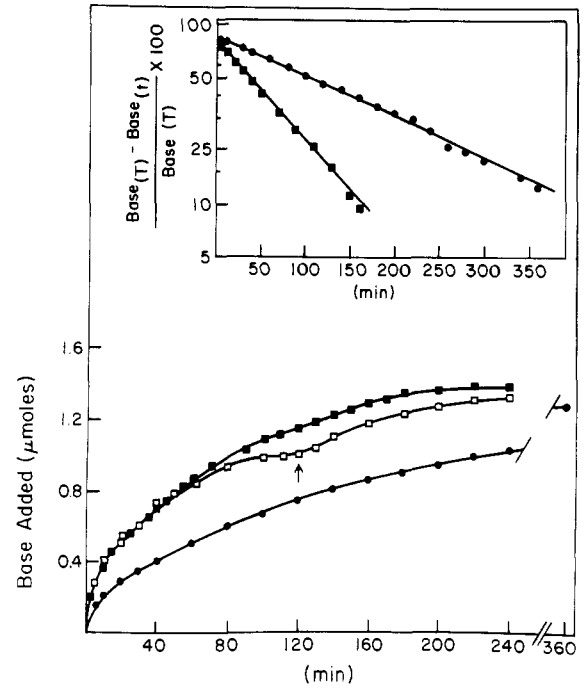


FIGURE 2: Effect of prolonged incubation of human $HDL_3$ with equivalent active concentrations of SOL-T or SphB-T in the absence of $Ca^{2+}$. A time course of the base uptake at 37 °C in 0.15 M NaCl in the absence of $Ca^{2+}$ was conducted. The pH was maintained at 7.4, and the proteolysis was monitored by pH-stat titration as described under Experimental Procedures. The initial $HDL_3$ concentration was 1.5 mg of protein/mL. SOL-T or SphB-T was added as follows: (●) 0.15 mg of active SphB-T/mL added only initially; (□) 0.15 mg of active SOL-T/mL added initially followed by an additional aliquot of SOL-T (70% of the amount initially added) after 120 min as indicated by the arrow; (■) 0.15 mg of active SOL-T/mL added initially followed by repeated additions of SOL-T (25% of the amount initially added) every 25 min. (Inset) Semilog plot of the percent of total base added vs. time of proteolysis of human $HDL_3$ by equivalent active concentrations of SOL-T (■) or SphB-T (●). $[Base]_{(T)}$ refers to the total amount of base added upon completion of the hydrolysis reaction, and $[base]_{(t)}$ refers to the amount of base added at a given time. The experimental first-order rate constants were calculated from the slope of these data by linear-regression analysis (i.e., $k_{exp}$ = −slope).

Table II: Dependence of the Initial Rate of Human Apo $HDL_3$ Proteolysis Catalyzed by Sepharose-Bound or Soluble TPCK-Treated Bovine Trypsin on the Initial Concentration of the Substrate

| trypsin | initial [apo $HDL_3$] (mg of protein/mL) | [trypsin] (mg of protein/mL)[a] | [apo $HDL_3$]/[trypsin] (wt ratio) | initial rate of proteolysis[b,c] [$\mu$mol of $H^+$ produced $min^{-1}$ (mg of active trypsin)$^{-1}$] |
|---|---|---|---|---|
| Sepharose bound | 0.4 | 0.012 | 33 | 3.30 ± 0.30 |
| | 0.8 | 0.012 | 67 | 6.09 ± 0.50 |
| | 1.6 | 0.012 | 133 | 8.45 ± 0.60 |
| | 4.4 | 0.012 | 367 | 14.94 ± 1.60 |
| soluble | 0.4 | 0.006 | 67 | 17.78 ± 1.5 |
| | 0.8 | 0.006 | 133 | 34.48 ± 1.7 |
| | 1.6 | 0.006 | 267 | 41.20 ± 3.0 |
| | 7.2 | 0.006 | 1200 | 46.60 ± 2.5 |

[a-c] The same assumptions and reaction conditions as in Table I were used. These data represent the mean value ± the standard deviation for three determinations.

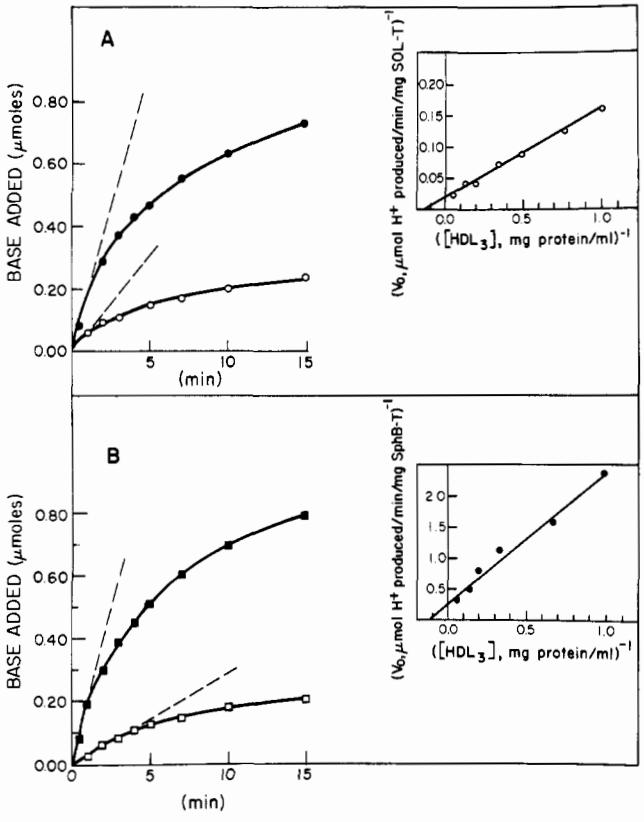


FIGURE 3: Dependence of the initial rate of human $HDL_3$ proteolysis on the initial $HDL_3$ concentration. The initial rate studies were conducted in 0.15 M NaCl at pH 7.4 and 37 °C in the pH-stat titration as described under Experimental Procedures. The initial rates were determined from the slope of the tangent to the titration curve which intersected at the origin. (A) Initial rate curves of $HDL_3$ proteolysis with SOL-T. The SOL-T concentration was 0.015 mg of active trypsin/mL. The initial $HDL_3$ concentration varied: (○) 1.5 mg of protein/mL; (●) 8.0 mg of protein/mL. (Inset) Lineweaver–Burk plot for the dependence of the initial rate on the initial $HDL_3$ concentration by SOL-T (○). (B) Initial rate curves of $HDL_3$ proteolysis by SphB-T. The SphB-T concentration was 0.15 mg of active trypsin/mL. The initial $HDL_3$ concentration was as follows: (□) 1.5 mg of protein/mL; (■) 8.0 mg of protein/mL. (Inset) Lineweaver–Burk plot [as in (A) inset] for reactions catalyzed by SphB-T (●).

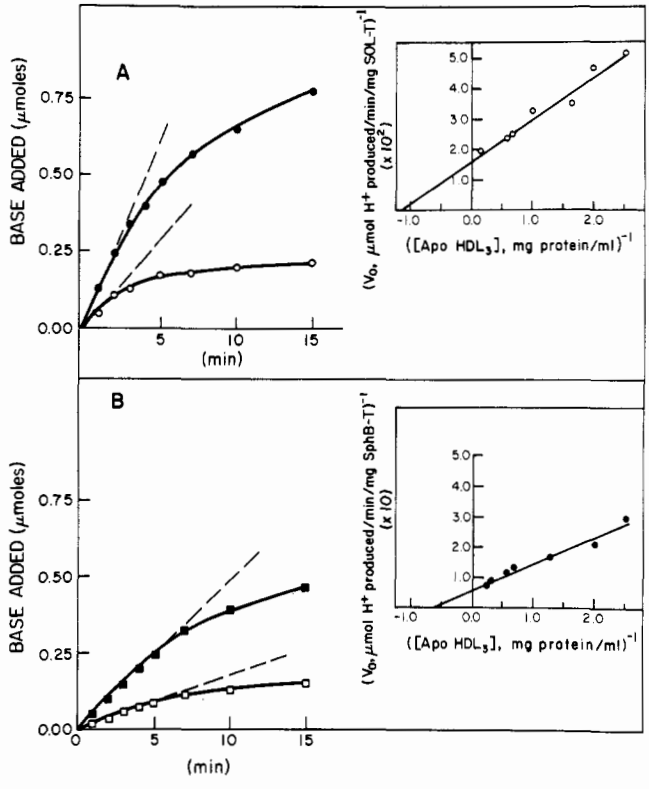


FIGURE 4: Dependence of the initial rate of human apo $HDL_3$ proteolysis on the initial apo $HDL_3$ concentration. The initial rate studies were conducted in 0.15 M NaCl at pH 7.4 and 37 °C in the pH-stat titration as described in the legend to Figure 3. (A) Initial rate curves of apo $HDL_3$ proteolysis with SOL-T. The SOL-T concentration was 0.006 mg of active trypsin/mL, and the initial apo $HDL_3$ concentration was the following: (○) 0.4 mg of protein/mL; (●) 1.6 mg of protein/mL. (Inset) Lineweaver–Burk plot for the dependence of the initial rate on the initial apo $HDL_3$ concentration by SOL-T (○). (B) Initial rate curves of apo $HDL_3$ proteolysis with SphB-T. The SphB-T concentration was 0.012 mg of active trypsin/mL, and the initial apo $HDL_3$ concentration was the following: (□) 0.4 mg of protein/mL; (■) 1.6 mg of protein/mL. (Inset) Lineweaver–Burk plot [as in (A) inset] for reactions catalyzed by SphB-T (●).

centration of active SphB-T (0.012 mg/mL) was 2-fold higher than that of SOL-T shown in Figure 4A.

When the apo $HDL_3$ protein concentration was raised from 0.4 to 4.4 mg/mL, the $V_0$ of proteolysis with SphB-T increased approximately 4–5-fold (Table II). A Lineweaver–Burk plot (Figure 4B, inset) was linear. The $K_m$ value of apo $HDL_3$ proteolysis by SphB-T (1.6 ± 0.3 mg of protein/mL, Table III) was significantly lower than that obtained with $HDL_3$ (7.1 ± 1.0 mg of protein/mL, Table III). Furthermore, the $V_{max}$ of apo $HDL_3$ hydrolysis by SphB-T [17.6 ± 2.5 $\mu$mol of $H^+$ produced $min^{-1}$ (mg of active trypsin)$^{-1}$, Table III] was significantly higher than with $HDL_3$ [3.5 ± 0.5 $\mu$mol of $H^+$ produced $min^{-1}$ (mg of active trypsin)$^{-1}$].

At apo $HDL_3$ concentrations between 0.4 and 7.2 mg/mL (Table II), the $V_0$ of hydrolysis by SOL-T increased approximately 2.5–3.0-fold. From the Lineweaver–Burk plot of these data (Figure 4A, inset), a $K_m$ value of 0.9 ± 0.1 mg of pro-

Table III: Steady-State Kinetic Parameters of Human $HDL_3$ [a] or Apo $HDL_3$ [b] Proteolysis Catalyzed by Sepharose-Bound or Soluble TPCK-Treated Bovine Trypsin

| trypsin | substrate | $K_m$ (mg of protein/mL) | $V_{max}$ [μmol of $H^+$ produced $min^{-1}$ (mg of active trypsin)$^{-1}$] |
|---|---|---|---|
| Sepharose bound | $HDL_3$ | 7.1 ± 1.0 | 3.5 ± 0.5 |
| | apo $HDL_3$ | 1.6 ± 0.3 | 17.6 ± 2.5 |
| soluble | $HDL_3$ | 7.5 ± 1.0 | 52.9 ± 5.0 |
| | apo $HDL_3$ | 0.9 ± 0.1 | 60.4 ± 6.0 |

[a] The kinetic parameters were obtained from Lineweaver–Burk plots. The $HDL_3$ concentration ranged from 0.8 to 15.0 mg of protein/mL. The initial rates were calculated as in Table I to correct for proton yield and to express values in terms of the active trypsin concentration which ranged from 0.015 to 0.030 mg/mL. The kinetic experiments were conducted at 37 °C in 0.15 M NaCl and maintained at pH 7.4 by pH-stat titration with 0.01 M NaOH. The values listed are the mean ± the standard deviation for three separate experiments. [b] As in *a*, except that the apo $HDL_3$ concentration ranged from 0.4 to 4.4 mg of protein/mL and the active trypsin concentration ranged from 0.006 to 0.012 mg/mL.

tein/mL was calculated (Table III). With SOL-T, the corresponding $V_{max}$ value was 60.4 ± 6.0 μmol of $H^+$ produced $min^{-1}$ (mg of active enzyme)$^{-1}$ (Table III). As with SphB-T, the $K_m$ value of apo $HDL_3$ hydrolysis with SOL-T was lower than that observed when $HDL_3$ was the substrate (Table III). In contrast, there was no major difference between the $V_{max}$ value of $HDL_3$ and apo $HDL_3$ hydrolysis by SOL-T.

Prolonged hydrolytic reactions of apo $HDL_3$ by SOL-T or SphB-T proceeded much more rapidly than the corresponding reactions with $HDL_3$ under similar incubation conditions. As in the case with $HDL_3$, the proteolysis reaction was first order for the majority of the reaction when substrate concentrations were lower than the $K_m$ value. For example, in Figure 4A (open circles) the first-order reaction ($T_{1/2}$ = 1.44 $min^{-1}$) was nearly complete within 10 min as indicated by the plateau of base uptake.

*Electrophoretic Separation of the Peptides Generated from the Incubation of Human $HDL_3$ with SOL-T or SphB-T.* Figure 5A shows the $NaDodSO_4$–polyacrylamide gel electrophoretic patterns of control human $HDL_3$ and of $HDL_3$ (1.5 mg of protein/mL) incubated with SOL-T (0.015 mg of active trypsin/mL) for 15 min at 37 °C in 0.15 M NaCl at pH 7.4 maintained by pH-stat titration. Gels 1 and 2 (controls) indicate the relative mobilities of apo A-I and apo A-II in the absence and presence of β-mercaptoethanol (ME), respectively. Within 15 min of incubation with SOL-T, $HDL_3$ exhibited an additional band, of apparent molecular weight 10 000–12 000, below that of apo A-II (gel 3). As assessed by the decreased intensity of the bands, both Apo A-I and apo A-II were susceptible to hydrolysis by SOL-T. In the presence of ME (Figure 5A, gel 4), besides a residual apo A-I band, there was a diffuse band representing peptides in the molecular weight range between 9000 and 12 000. None of the apo A-I products migrated in the position of unreduced apo A-II (Figure 5A, compare gels 3 and 4). Furthermore, none of the apo A-I fragments migrated in the 17 500 molecular weight region occupied by apo A-II (unreduced) regardless of the time of incubation of $HDL_3$ with either SOL-T or SphB-T. The identification of the proteolytic products deriving from either apo A-I or apo A-II was facilitated by independent experiments using $^{125}$I-apo A-I ($HDL_3$) or $^{125}$I-apo A-II ($HDL_3$) as substrates (Figure 5B). A single band of radioactivity was observed after the electrophoretic separation of $^{125}$I-apo A-I

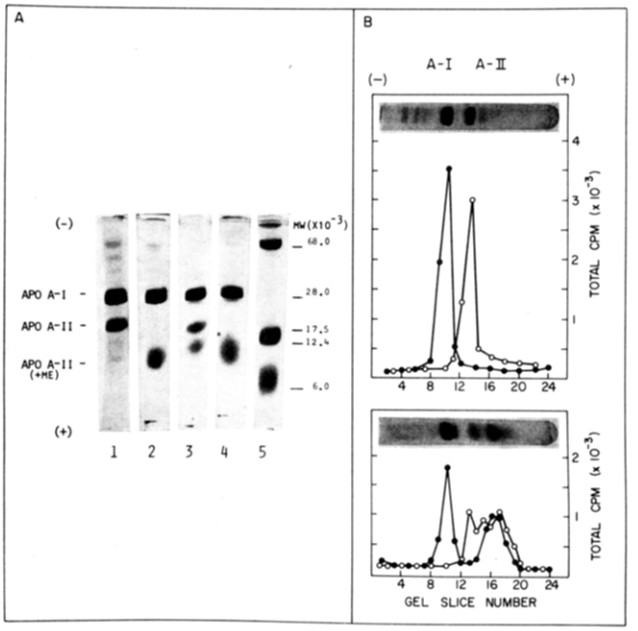


FIGURE 5: $NaDodSO_4$–polyacrylamide gel electrophoretic patterns of control human $HDL_3$ hydrolyzed by SOL-T or SphB-T. (A) Human $HDL_3$ (4 mg of protein/mL) in 0.15 M NaCl, maintained at pH 7.4 by pH-stat titration, was incubated at 37 °C in the presence or absence of SOL-T (0.015 mg of active trypsin/mL) for 15 min, followed by the addition of DFP ($5 \times 10^{-3}$ M final concentration) to arrest the proteolysis. Aliquots were incubated with 0.1 volume of 10% $NaDodSO_4$ in the presence or absence of 2% ME at 37 °C for 1 h. The electrophoresis was carried out on 35-μg samples. Gel 1, control $HDL_3$; gel 2, control $HDL_3$ + ME; gel 3, $HDL_3$ + SOL-T; gel 4, $HDL_3$ + SOL-T + ME; gel 5, bovine serum albumin (22 μg, $M_r$ 68 000), horse heart cytochrome *c* (25 μg, $M_r$ 12 400), and human insulin (22 μg, $M_r$ 6000) + ME. (B) Electrophoresis of $^{125}$I-apo A-I ($HDL_3$) or $^{125}$I-apo A-II ($HDL_3$) before and after incubation with SphB-T. Human $^{125}$I-apo A-I ($HDL_3$) or $^{125}$I-apo A-II ($HDL_3$) (2 mg of protein/mL, $4 \times 10^5$ cpm/mL) in 0.15 M NaCl maintained at pH 7.4 by pH-stat titration was incubated at 37 °C in the absence (top) or presence (bottom) of SphB-T (0.12 mg of active trypsin/mL) for 30 min. The reaction was stopped, and samples were prepared for electrophoresis as described in (A) in the absence of ME; 30 μg was applied to each gel. At the end of the run, gels were sliced into 3-mm segments, and the distribution of radioactivity was determined. Duplicate gels of $^{125}$I-apo A-I ($HDL_3$) (●) or $^{125}$I-apo A-II ($HDL_3$) (○) were compared to a third gel which was stained with Coomassie blue. Direction of migration was from left (cathode) to right (anode).

($HDL_3$) or $^{125}$I-apo A-II ($HDL_3$) control samples (Figure 5B, top panel). When $^{125}$I-apo A-I ($HDL_3$) (2 mg of protein/mL, $4 \times 10^5$ cpm/mL) in 0.15 M NaCl, pH 7.4, was hydrolyzed by SphB-T (0.12 mg of active trypsin/mL) for 30 min at 37 °C, two major radioactive bands were detected by counting gel slices (Figure 5B, bottom panel, closed circles). Approximately 49% of the initial apo A-I radioactivity coincided with the band of intact apo A-I and a component migrating slightly faster than it (gel slices 9–11). The remaining radioactivity was in a broad molecular weight range of 8000–14 000 (gel slices 15–19). Incubation of $^{125}$I-apo A-II ($HDL_3$) with SphB-T, under conditions identical with those of $^{125}$I-apo A-I ($HDL_3$), produced three major radioactive bands (Figure 5B, bottom pannel, open circles). Approximately 38% of the initial $^{125}$I-apo A-II coincided with that of intact apo A-II (gel slices 12–14), and the remainder coincided with components of 12 000–16 000 and 8000–12 000 molecular weight (gel slices 15–19). From these data, we concluded that the proteolysis of apo A-I and apo A-II in $HDL_3$ could be followed most readily by the decrease in the intensity of the bands corresponding to intact apo A-I and apo A-II on $NaDodSO_4$–polyacrylamide gel electrophoresis under nonreducing conditions, since both apo A-I and apo A-II hydrolytic products

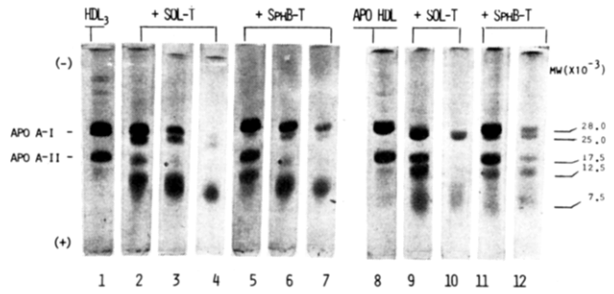


FIGURE 6: $NaDodSO_4$–polyacrylamide gel electrophoretic patterns of human $HDL_3$ or apo $HDL_3$ after prolonged proteolysis by equivalent active concentrations of SOL-T and SphB-T. Gel 1, control $HDL_3$. For gels 2–4, human $HDL_3$ (1.5 mg of protein/mL) in 0.15 M NaCl, maintained at pH 7.4 by pH-stat titration, was incubated at 37 °C in the presence of SOL-T (0.15 mg of active trypsin/mL). At time intervals, 0.05-mL aliquots were removed into tubes containing 0.05 mL of 0.02 M sodium barbital, pH 8.3, followed by the immediate addition of DFP ($5 \times 10^{-3}$ M final concentration) to arrest the proteolysis. Samples were prepared for electrophoresis as described in the legend to Figure 5A in the absence of ME; 30 μg of protein was applied to each gel. $HDL_3$ was incubated with SOL-T for 3 (gel 2), 30 (gel 3), and 120 min (gel 4). For gels 5–7, $HDL_3$ was incubated with SphB-T (0.15 mg of active trypsin/mL). At timed intervals, 0.075-mL aliquots as suspensions (see Experimental Procedures) were removed into tubes containing 0.025 mL of 0.04 M sodium barbital, pH 8.3, followed by the immediate addition of DFP ($5 \times 10^{-3}$ M final concentration) to arrest the proteolysis. Supernatants from these tubes were prepared for electrophoresis as described for gels 2–4. $HDL_3$ was incubated with SphB-T for 3 (gel 5), 120 (gel 6), and 240 min (gel 7). Gel 8, starting apo $HDL_3$. For gels 9 and 10, apo $HDL_3$ (1.5 mg of protein/mL) in 0.15 M NaCl maintained at pH 7.4 by pH-stat titration was incubated at 37 °C with SOL-T (0.005 mg of active trypsin/mL). At timed intervals, 0.05-mL aliquots were removed from the digestion mixture, SOL-T activity was arrested, and samples were prepared for electrophoresis as described for gels 2–4; 30 μg was applied to each gel. Apo $HDL_3$ was incubated with SOL-T for 1.5 (gel 9) and 15 min (gel 10). For gels 11 and 12, apo $HDL_3$ was incubated with SphB-T (0.005 mg of active trypsin/mL). At time intervals, 0.075-mL aliquots were removed from the digestion mixture, SphB-T activity was arrested, and samples were prepared for electrophoresis as described for gels 5–7. Apo $HDL_3$ was incubated with SphB-T for 5 (gel 11) and 30 min (gel 12).

migrated in the 10 000–12 000-dalton area.

The gel patterns of the proteolytic fragments of $HDL_3$ (1.5 mg/mL) by SOL-T (0.15 mg of active enzyme/mL) after prolonged incubation are shown in Figure 6 (gels 2–4). Within 3 min (Figure 6, gel 2), there was a distinct band with a molecular weight between 10 000 and 12 000 representing fragments from both apo A-I and apo A-II. There was also a major band migrating slightly faster than apo A-I with an apparent molecular weight of 25 000. From these results, we estimated that within 3 min (Figure 6, gel 2) SOL-T hydrolyzed approximately 35% of apo A-I. After 30 min (gel 4), 70% was hydrolyzed. In contrast, 70% of the initial apo A-II was hydrolyzed within 3 min and more than 95% within 30 min of incubation. Thus, on a percentage basis, SOL-T hydrolyzed apo A-II significantly faster than apo A-I.

Qualitatively, similar gel patterns were obtained with $HDL_3$ digested by SphB-T, but significant quantitative differences were noted (Figure 6). Within 3 min, the $HDL_3$ incubated with either SOL-T or SphB-T exhibited a 10 000–12 000 molecular weight band containing hydrolytic products from apo A-I and apo A-II (gels 2 and 5). A minor band migrating slightly faster than apo A-I ($M_r$ 25 000) became visible within 7 min of incubation with SphB-T (data not shown). However, approximately the same amount of this 25K fragment was present also after 10, 30, and 120 min (gel 6) of incubation with SphB-T but was 3-fold lower than that observed during the digestion of $HDL_3$ by SOL-T. As estimated by gel scanning, when SphB-T was used, approximately 10–20% and 50% of the intact A-I was hydrolyzed within 3 (gel 5) and 120 min (gel 6) of incubation, respectively. In contrast, 35% and 95% of apo A-II was hydrolyzed within 3 and 120 min of incubation, respectively. These results indicate that on a percentage basis SphB-T, as well as SOL-T, hydrolyzed apo A-II significantly faster than apo A-I.

*Electrophoretic Patterns of Human Apo $HDL_3$ by SOL-T and SphB-T.* After 1.5 min of incubation of apo $HDL_3$ (1.5 mg of protein/mL) with SOL-T (0.005 mg of active trypsin/mL), nearly all protein was hydrolyzed (Figure 6, gel 9) with the formation of a major 25 000 molecular weight band. In addition, there was a band having a molecular weight of 12 000–13 000 which we attributed as deriving from apo A-I since the mobility was not affected by ME (data not shown). Additional hydrolytic products deriving from both apo A-I and apo A-II were found in the diffuse band in the molecular weight range of 7000–9000. Within 15 min of incubation with SOL-T (gel 10), all apo-II and the 12–13K products were completely hydrolyzed whereas significant amounts of the 25K fragment and 7–9K products were still present.

In the case of SphB-T, under identical conditions of initial apo $HDL_3$ and enzyme concentration, the rates of proteolysis of both apo A-I and apo A-II were significantly less than those by SOL-T. After 5 min of incubation (Figure 6, gel 11), only about 20% of apo A-I was cleaved, and only a small amount of the 25K band was present (approximately 10% of the initial apo A-I). Furthermore, apo A-II was hydrolyzed approximately as fast as intact apo A-I (gel 12). These results indicate that SOL-T hydrolyzed apo A-I and apo A-II faster than SphB-T and thus generated a higher yield of hydrolytic products per unit of time.

## Discussion

The results of the kinetic studies have provided a basis for comparing the proteolytic action of SOL-T and SphB-T on human $HDL_3$ and apo $HDL_3$. In the case of $HDL_3$, since the activities of SOL-T and SphB-T during the first few minutes of incubation were nearly equal to the activities at the beginning of the reaction, steady-state kinetics of $HDL_3$ proteolysis could be examined without the addition of $Ca^{2+}$ ions which were needed to stabilize the esterase activity of SOL-T.

When two or more enzyme forms catalyze the same reaction against a common substrate (S), differences in $V_0$ values can be readily described according to the Michaelis–Menten equation:

$$V_0 = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

Since the $K_m$ values for $HDL_3$ proteolysis by SOL-T were similar to those by SphB-T but the $V_{max}$ by SOL-T was about 15-fold greater (see Table III), it follows that for the same $HDL_3$ concentration, the $V_0$ of $HDL_3$ proteolysis by the soluble enzyme was faster than that exhibited by the immobilized counterpart. With apo $HDL_3$ as a substrate, the $K_m$ values with both SphB-T and SOL-T were lower than the corresponding ones with native $HDL_3$ (see Table III). In addition, the $V_{max}$ obtained from the hydrolysis of apo $HDL_3$ by SphB-T was significantly greater than that with $HDL_3$. In contrast, similar $V_{max}$ values were obtained when $HDL_3$ or apo $HDL_3$ was hydrolyzed by SOL-T (Table III). Therefore, at a given apo $HDL_3$ concentration, the $V_0$ of proteolysis by SphB-T was greater than that found with $HDL_3$ because of lower $K_m$ and greater $V_{max}$ values as compared to $HDL_3$. In contrast, as a result of only the lower $K_m$ with the delipidated substrate, the $V_0$ values of apo $HDL_3$ hydrolysis by SOL-T were greater than those with $HDL_3$. Furthermore, the $V_0$ values of apo $HDL_3$

Table IV: Advantages and Disadvantages of Sepharose-Bound vs. Soluble Trypsin as an HDL Structural Probe

| trypsin | advantage | disadvantage |
|---|---|---|
| Sepharose bound | (1) nonpenetrating to the lipoprotein surface | (1) covalent attachment via lysine groups may disallow potential interactions with substrates due to conformational constraints |
| | (2) may serve as a model for proteolysis resulting from lipoprotein-cell interactions | (2) catalytic efficiency is lower with most protein substrates (Silman & Katchalski, 1966); activity of modified vs. native trypsin should be evaluated with macromolecular substrates |
| | (3) activity remains relatively constant and is not readily susceptible to autolysis (Silman & Katchalski, 1966) | |
| | (4) enzyme is readily removed from digestion mixtures for product characterization | |
| soluble | (1) enzyme is not artificially restricted in conformation | (1) exposed vs. buried proteolysis sites may not be distinguishable |
| | (2) catalytic efficiency is maximum | (2) enzyme has a propensity toward inactivation by autolysis in the absence of $Ca^{2+}$ and requires constant assessment of activity |

hydrolysis by SOL-T were larger than those catalyzed by SphB-T due mainly to the comparatively higher $V_{max}$ seen in the presence of SOL-T.

A study of the prolonged hydrolysis of $HDL_3$ with essentially equivalent active concentrations of SOL-T and SphB-T in the absence of $Ca^{2+}$ was made possible by multiple additions of SOL-T to the reaction mixture. A similar replenishment procedure with SphB-T was unnecessary since SphB-T activity was stable for at least 6 h even in the absence of $Ca^{2+}$. At the same initial concentrations of $HDL_3$ (1.5 mg/mL protein) and trypsin (0.15 mg of active trypsin/mL), the proteolysis of $HDL_3$ followed pseudo-first-order kinetics with either SOL-T or SphB-T throughout most of the reaction. Under these conditions, the first-order rate constant ($k_{exp}$) was 2.5-fold higher with SOL-T than with SphB-T (Figure 2, inset). For $[S] \ll K_m$, the Michaelis–Menten equation simplifies to

$$V_0 = \frac{V_{max}}{K_m}[S] \quad (2)$$

Furthermore, the velocity ($V_t$) at any time of the reaction under these conditions obeys the rate equation

$$V_t = \frac{-d[S]}{dt} = k[S] \quad (3)$$

where $k$ is the first-order rate constant. Rearranging eq 3 and integrating between [S] at $t = 0$ and [S] at any other time yield the expression

$$\log \frac{[S]_0}{[S]} = \frac{k}{2.303}t \quad (4)$$

where the value $k/2.303 = k_{exp} = V_{max}/K_m$ relates the data of the prolonged incubation to the initial rate experiments. Since $V_{max}/K_m$ and $k_{exp}$ were determined by examining the kinetics within a widely different time period, the data obtained provided a means for comparing activities of soluble and immobilized enzymes during early and prolonged times of incubation. From the initial rate data, the ratio $(V_{max}/K_m)_{SOL\text{-}T}/[(V_{max}/K_m)_{SphB\text{-}T}]$ related the kinetic parameters obtained with SOL-T and SphB-T. This ratio was calculated to be 2.9 for the $HDL_3$ substrate, a value which is in good agreement with the ratio of the first-order rate constants $k_{exp(SOL\text{-}T)}/k_{exp(SphB\text{-}T)} = 2.5$ calculated for the prolonged digestion of $HDL_3$ by SOL-T and SphB-T. The initial rate studies also indicated that the $K_m$ values of $HDL_3$ proteolysis were the same for both SOL-T and SphB-T. Therefore, the relatively higher rate of $HDL_3$ proteolysis by SOL-T during the initial and prolonged incubation period appears to be primarily due to the higher $V_{max}$ value observed with $HDL_3$. This agreement between the initial rate and prolonged kinetic parameters also suggests that the substrate region which was initially recognized by either SOL-T or SphB-T was the same as that recognized during the course of prolonged proteolysis. However, our studies do not allow to establish whether SOL-T and SphB-T had the same affinity for $HDL_3$ since the $K_m$ values were taken simply to express the concentration of $HDL_3$ which gave 50% of the maximal attainable proteolysis rate ($V_{max}$). Since $HDL_3$ contains a heterogeneous set of proteins, each having several arginine and lysine residues as potential sites of cleavage, the $K_m$ and $V_{max}$ parameters derived from these studies can only reflect average values.

The analysis by $NaDodSO_4$–polyarylamide gel electrophoresis of the proteolytic products indicated that in $HDL_3$ both apo A-I and apo A-II were hydrolyzed by either SOL-T or SphB-T and that on a percentage basis apo A-II was cleaved faster than apo A-I. The differences observed between the gel patterns of the products obtained from the hydrolysis of $HDL_3$ and apo $HDL_3$ by SOL-T or SphB-T were mainly quantitative; however, some qualitative differences were noted. With apo $HDL_3$, products of molecular weight of 25 000, 12 000–13 000, and 7000–9000 were observed; in contrast, with $HDL_3$, the 7000–9000 products were seen with neither enzyme. The reasons for these differences are under investigation.

The results of the kinetic and the electrophoretic data invite several comments. The higher value of $V_{max}$ for the hydrolysis of apo $HDL_3$ by SphB-T as compared to that of native $HDL_3$ may reflect the difference in the lipid content between the two substrates and/or changes in the conformation of the two apoproteins, apo A-I and apo A-II. These differences may also depend on the fixed conformation of the immobilized enzyme as suggested by the similar values of the $V_{max}$ exhibited by SOL-T when assayed against apo $HDL_3$ and native $HDL_3$. The difference in the physical state between the two enzymes may also explain why apo $HDL_3$ was hydrolyzed by SOL-T faster than by SphB-T, suggesting an effect of enzyme immobilization on its kinetic properties. This view is in keeping with other reports in the literature which indicate that enzymes when covalently attached to a solid support act on macromolecular substrates less efficiently than expected, based on their activity against low molecular weight synthetic substrates (Metz & Summaria, 1961; Bar-Eli & Katchalski, 1963; Levin et al., 1964; Silman & Katchalski, 1966; Knights & Light, 1974). The reason which is most commonly given to explain the differences in the kinetic properties between immobilized and soluble forms of a given enzyme is that steric hindrance caused by the carrier matrix influences the interaction of the

enzyme with the substrate. However, the chemical modification attending the attachment of the enzyme to a matrix may also affect this interaction. A more precise interpretation of the data will be possible after we establish the chemical nature of the proteolytic fragments obtained from the action of both soluble and immobilized enzyme on either $HDL_3$ or apo $HDL_3$. Such studies, which we are currently carrying out, should also increase our knowledge of $HDL_3$ structure and the precise cleavage points by the two enzyme forms and perhaps also give us an insight as to whether proteolytic events of the kind noted in vitro may also occur in vivo. We already know that apo A-II, when in native HDL, is selectively cleaved in vitro by a proteolytic activity associated with human granulocytes (Ritter & Scanu, 1980; Polacek & Scanu, 1982). It has also been shown that the apo E of hypertriglyceridemic very low-density lipoprotein (VLDL) is hydrolyzed into two major fragments by thrombin (Gianturco et al., 1982). Such a specificity of action may be taken to suggest that these kinds of cleavages may be of physiological importance.

Although apo A-II is generally considered to bind more tightly to HDL than apo A-I, the former apoprotein is hydrolyzed more rapidly than the latter by the soluble or immobilized enzyme. Thus, there does not appear to be a relationship between the binding affinity of the apoproteins and the proteolytic phenomenon.

It is apparent that SOL-T and SphB-T each offer advantages and disadvantages when used as structural probes for the study of the surface topology of the apoproteins in HDL. From the information summarized in Table IV, we conclude that soluble and immobilized trypsin can yield important complementary information on $HDL_3$ structure provided that the activity of each enzyme form is carefully assessed and the kinetic differences are recognized. It is also apparent that the trypsin–$HDL_3$ model can be extended to the study of other proteolytic enzymes and their interaction with other lipoprotein particles.

## Acknowledgments

We thank Maria Garcia of the Program Project Core for samples of $HDL_3$ and apo $HDL_3$, Dr. Donald Steiner for the gift of human insulin, and Rose E. Scott for her help in preparing the manuscript.

**Registry No.** Trypsin, 9002-07-7; Sepharose, 9012-36-6; Ca, 7440-70-2.

## References

Bar-Eli, A., & Katchalski, E. (1963) *J. Biol. Chem. 238*, 1690–1698.

Camejo, G. (1969) *Biochim. Biophys. Acta 175*, 290–300.

Chase, T., Jr., & Shaw, E. (1967) *Biochem. Biophys. Res. Commun. 29*, 508–514.

Cuatrecasas, P. (1970) *J. Biol. Chem. 245*, 3059–3065.

Cunningham, L. W., Jr. (1954) *J. Biol. Chem. 211*, 13–19.

Edlestein, C., Kezdy, F. J., Scanu, A. M., & Shen, B. W. (1979) *J. Lipid Res. 20*, 143–153.

Edelstein, C., Halari, M., & Scanu, A. M. (1982) *J. Biol. Chem. 257*, 7189–7195.

Foreman, J. R., Karlin, J. B., Edelstein, C., Juhn, D. J., Rubenstein, A. H., & Scanu, A. M. (1977) *J. Lipid Res. 18*, 759–767.

Gianturco, S. H., Karlin, J. B., Prasad, S., Gotto, A. M., & Bradley, W. A. (1982) *Circulation 66*, II-11.

Granda, J. L., & Scanu, A. M. (1966) *Biochemistry 5*, 3301–3308.

Jacobsen, C. R., Leonis, J., Linderstron-Lang, K., & Ottesen, M. (1957) *Methods Biochem. Anal. 4*, 171–210.

Jeng, I., Steelman, R., Reilly, P., Jeng, Y., & Schonfeld, G. (1980) *Biochem. Biophys. Res. Commun. 80*, 876–882.

Karlin, J. B., Juhn, D. J., Starr, J. I., Scanu, A. M., & Rubenstein, A. H. (1976) *J. Lipid Res. 17*, 30–37.

Kezdy, F. J., Jaz, J., & Bruglands, A. (1958) *Bull. Soc. Chim. Belg. 67*, 687–706.

Knights, R. J., & Light, A. (1974) *Arch. Biochem. Biophys. 160*, 377–386.

Levin, Y., Pecht, M., Goldstein, L., & Katchalski, E. (1964) *Biochemistry 3*, 1905–1913.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Markwell, M. A. K., Haas, S. N., Bieber, L. L., & Tolbert, N. E. (1978) *Anal. Biochem. 87*, 206–210.

Metz, M. A., & Summaria, L. J. (1961) *Nature (London) 189*, 576–578.

Noble, R. P. (1968) *J. Lipid Res. 9*, 695–700.

Polacek, D., & Scanu, A. M. (1982) *Trans. Assoc. Am. Phys. 95*, 86–93.

Ritter, M. C., & Scanu, A. M. (1980) *J. Biol. Chem. 255*, 3763–3769.

Scanu, A. M. (1966) *J. Lipid Res. 1*, 295–306.

Scanu, A. M., & Edelstein, C. (1971) *Anal. Biochem. 44*, 570–575.

Scanu, A. M., Reader, W., & Edelstein, C. (1968) *Biochim. Biophys. Acta 160*, 32–45.

Schonfeld, G., & Pfleger, B. (1974) *J. Clin. Invest. 54*, 236–246.

Schonfeld, G., Pfleger, B., & Roy, P. (1975) *J. Biol. Chem. 250*, 7943–7950.

Schonfeld, G., Chen, J., McDonnell, W. F., & Jeng, I. (1977) *J. Lipid Res. 18*, 645–655.

Schonfeld, G., George, P. K., Miller, J., Reilly, P., & Witztum, J. (1979) *Metabolism 28*, 1001–1010.

Segal, I. H. (1975) *Enzyme Kinetics Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, Wiley-Interscience, New York.

Shen, B. W., Scanu, A. M., & Kezdy, F. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 837–841.

Shore, V. G., Sae, A. S.-W., & Shore, B. (1978) *Biochim. Biophys. Acta 529*, 319–330.

Silman, I. H., & Katchalski, E. (1966) *Annu. Rev. Biochem. 35*, 873–908.

Tanford, C. (1962) *Adv. Protein Chem. 17*, 69–165.

Verdery, R. B., III, & Nichols, A. P. (1975) *Chem. Phys. Lipids 14*, 123–134.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.