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Separation of Trypsin Inhibitors of Human Plasma on DEAE-Cellulose.* (31091)

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It has been recognized for some time that there are at least two trypsin inhibitors in human plasma. One of these is quite stable to temperatures around 60°C and at pH values around 4-5, while the other is quite unstable under these conditions. It has also been possible to demonstrate two components by electrophoresis on paper or starch, the minor component being an α_2-globulin and the major component an α_1-globulin which is unstable to heating and low pH values. The latter inhibitor has been obtained in a highly purified form(1,2,3) and shown to be a very effective inhibitor for chymotrypsin as well as trypsin. The nature of the more stable inhibitor has been in question, since it is present in relatively smaller amounts. The work of Shulman suggested that it may be an α_2-globulin with a molecular weight of about 16,500(4), but that conclusion was based primarily on the properties of material isolated from urine. More recently, a third component has been recognized(5) which combines with trypsin and chymotrypsin to give enzymatically active complexes which can no longer be inhibited by soybean trypsin inhibitor or the inhibitors from plasma. It now seems possible to identify this last component with α_2-macroglobulin(6), and with plasmin inhibitor(7), as well as with at least part of the α_2-trypsin inhibitor(8). In trypsin inhibitor assays it will appear as an inhibitor, since the activity of the trypsin (or chymotrypsin) complex with α_2-macroglobulin is less than that of the free enzyme.

The present work was initiated in an effort to obtain the α_1-trypsin inhibitor by as mild an isolation procedure as possible, and to separate it from other trypsin inhibitors in plasma. Although it has, in some measure, proved successful in this respect, it has also provided further information regarding the multiplicity of trypsin inhibitors in human plasma. It has been found that chromatography on DEAE-cellulose permits the separation of the trypsin-binding activity from the α_1-trypsin inhibitor, as well as the separation of a third inhibitor which is very stable to heat and low pH. The results of the fractionation of approximately one liter batches of ACD plasma will be described, together with some of the properties of the fractions. Since

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these fractions have been characterized by their reactivity with trypsin under specified conditions, they will be referred to as trypsin-binding protein, total trypsin inhibitor, and stable trypsin inhibitor.

Experimental procedure. Trypsin inhibitor and binding protein determinations. Trypsin activity was determined with benzoylarginine-p-nitroanilide as the substrate(9). A supersaturated, aqueous solution was prepared by heating 1 mg per ml of water at 80°C until solution was complete, cooling in water, and filtering through filter paper. Such solutions will usually remain free of precipitate for a month at room temperature, and without substantial hydrolysis if stored in the dark. Stock trypsin solution was prepared by dissolving 1 mg (Worthington, 3× crystallized, lyophilized, salt-free trypsin) per ml of 0.0025 N HCl and stored at 2°C. Working solutions, usually with a concentration of 50 μg per ml, were made by dilution with 0.0025 N HCl. Hydrolysis was carried out in 0.17 M Tris buffer containing 0.01 M $CaCl_2$, which was prepared by dissolving 26.6 g Tris-(hydroxymethyl)-aminomethane and 1.11 g $CaCl_2$ in about 700 ml of water, adjusting the pH to 7.7 at 25° with HCl, and diluting to 1 liter. Measurements of the hydrolysis of substrate were carried out by mixing 0.2 ml of working trypsin solution and 1.8 ml of Tris buffer in a test tube in a constant temperature block at 37°C. After 15 minutes, 1 ml of substrate solution which had been brought to 37° was added rapidly and the contents of the tube mixed. After 15 minutes (or a longer period if smaller amounts of tryptic activity were to be measured) the reaction was stopped by adding 1 ml of 5% phosphotungstic acid dissolved in 1 M sodium acetate-acetic acid buffer with a pH of 4.5. This also served to precipitate any protein which might be present in the trypsin inhibitor of trypsin-binding protein assays, as well as to precipitate unhydrolyzed substrate. After about 15 minutes the solutions were clarified by centrifugation and filtration through Whatman #1 filter paper, and the absorbancy of the p-nitroaniline measured at 383 mμ in a Beckman DU or Model B spectrophotometer. Blanks for zero incubation time were prepared by adding the phosphotungstate solution before substrate. The corrected increase in absorbancy at 383 mμ due to the amount of crystalline trypsin employed, on a weight basis or on the basis of the absorbancy of the trypsin solution, was then used to calculate apparent values for the trypsin inhibited or bound by various fractions. No correction has been applied to the results for the fact that crystalline trypsin may contain around 30% of inactive material(10,11).

Total trypsin inhibitor was measured by the same system, but replacing a part of the Tris buffer with the sample to be studied. Samples were diluted sufficiently in Tris buffer or dialyzed against the buffer to bring them to the pH of the assay system. Trypsin, sample and buffer to give a final volume of 2 ml were mixed and allowed to incubate for 15 minutes before adding substrate. Adjustments were made in the amount of inhibitor used so that between ⅓ and ⅔ of the trypsin would be inhibited. Under these circumstances, the decrease in absorbancy from the value for the standard is proportional to the amount of inhibitor present, unless the proportion of trypsin-binding protein in the sample is large.

Stable trypsin inhibitor was measured in the same way, except that samples were heated at 60°C and pH 4.1 for 20 minutes before the inhibitor activity was measured. Plasma, for example, was diluted to 10 volumes with 0.9% NaCl and a 0.5 ml sample of diluted plasma placed in a test tube in a constant temperature block at 60°C. The pH was reduced by adding 0.1 ml of 0.5 M sodium acetate-acetic acid buffer at pH 4.1 and heating was continued for 20 minutes, after which the tube was cooled in water. The tube was then placed in 37° constant temperature block and 1.2 ml of Tris buffer and 0.2 ml of working trypsin solution were added. After 15 minutes, substrate was added and the assay completed as indicated above. The final pH of the incubation mixture was not influenced significantly by as much as 0.5 ml of diluted serum or plasma, but the acetate buffer did reduce the pH of the final incubation mixture to pH 7.1. Consequently, results were referred to standard trypsin as-

says with the same amount of acetate buffer added, and hence the same pH.

Trypsin-binding protein was measured in the same way as total trypsin inhibitor, except that an excess of soybean trypsin inhibitor was added after the sample and trypsin had been allowed to react with each other for 15 minutes, and an additional 15 minutes was allowed before the substrate was finally added. In the usual assay with 15 μg of trypsin per tube, 20 μg of soybean trypsin inhibitor (Worthington, 3× crystallized) were added as a 50 μg/ml solution in Tris buffer. Although the results are somewhat dependent upon the time allowed for the mixture of binding protein, trypsin, and inhibitor to react, and are strongly dependent upon the order of the additions, these conditions give quite reproducible results which are satisfactory for the comparisons to which they have been applied.

Blood was purchased from a commercial blood bank, and had been collected with ACD solution as the anticoagulant. The specimens were those which had been rejected for use in transfusion because of positive serology or elevated bilirubin. To avoid the formation of fibrin on the column, fibrinogen and beta-lipoprotein were precipitated by adding 1 mg of sulfated rice starch per ml of ACD plasma (12). After 12 to 18 hours at 5°C, the precipitate was removed by centrifugation and the supernatant was dialyzed against the starting buffer to be used for chromatography.

Chromatography was carried out on DEAE-cellulose (Eastman) in a 4 ft length of 3 in. Pyrex pipe. Closures for the column were made from acrylic resin. In the present case they were 1 in. thick, with ½ in. of a diameter which would fit moderately closely inside the tube and ½ in. of a diameter about equal to that of the outside of the tube. The closures could then be clamped to the ends of the tube and made leak-proof with the standard gaskets and fittings normally used to join pipe sections. The closure at the top was fitted with a nylon pressure tubing connector (⅛ in. pipe thread to ¼ in. tubing) and the bottom closure was drilled to take a short length of hypodermic needle in a press fit, to which polyethylene catheter tubing could be fitted. A circular piece of plastic window screen was used to support a piece of filter paper on the top of the bottom closure, permitting the flow of column effluent across the upper surface of this closure to the outlet tube. With the top closure removed, DEAE was packed on top of the filter paper as a slurry, and under about 5 lb pressure. Packing was continued to within 5 cm of the top of the column, and required about 600 g of DEAE, with a mobile phase of about 4 liters. The top closure was then clamped on and connected with Tygon tubing (⅜ in. O.D., ⅛ in. I.D.), through a Milton Roy back pressure valve (240/C1) to a Milton Roy Minipump (MM-1-C-96R). The column was housed in a large refrigerator, but the pump, pressure valve, and a stainless steel service gauge (Marsh, Type 11S) to indicate column pressure were outside the refrigerator. All connections in the high-pressure side were made with ⅜ in O.D. × ⅛ in. I.D. Tygon tubing fitted with short sleeves to bring the diameter of the ends to ¼ in. to fit the Nylon pressure fittings. Reservoirs for buffers for elution were mounted above the pump, and where a linear gradient was employed this was formed by connecting two polyethylene bottles of the same size through tubulatures at the bottom and placing the mixing reservoir on a magnetic stirrer. Connection from the reservoirs to the pump was made through a stainless steel solenoid valve so that changes from one buffer source to another could be controlled by a timer (Industrial Timer Corp., model MC-12) which was also used to control other parts of the operation. The effluent from the column was taken through a Gilford Instrument Optical Density Converter attached to a Beckman DU monochromator for recording of the absorbancy at 280 mμ in flow cells with path lengths of 2 and 10 mm, and the effluent was then returned to the refrigerator which housed a fraction collector designed to deal with relatively large fractions (13). The flow rate was usually about 400 ml per hour at pressures between 4 and 8 lb per in.^{2}, and fractions were collected over ½- or 1-hour periods.

The DEAE-cellulose was washed with alkali and acid-alcohol and adjusted to approxi-

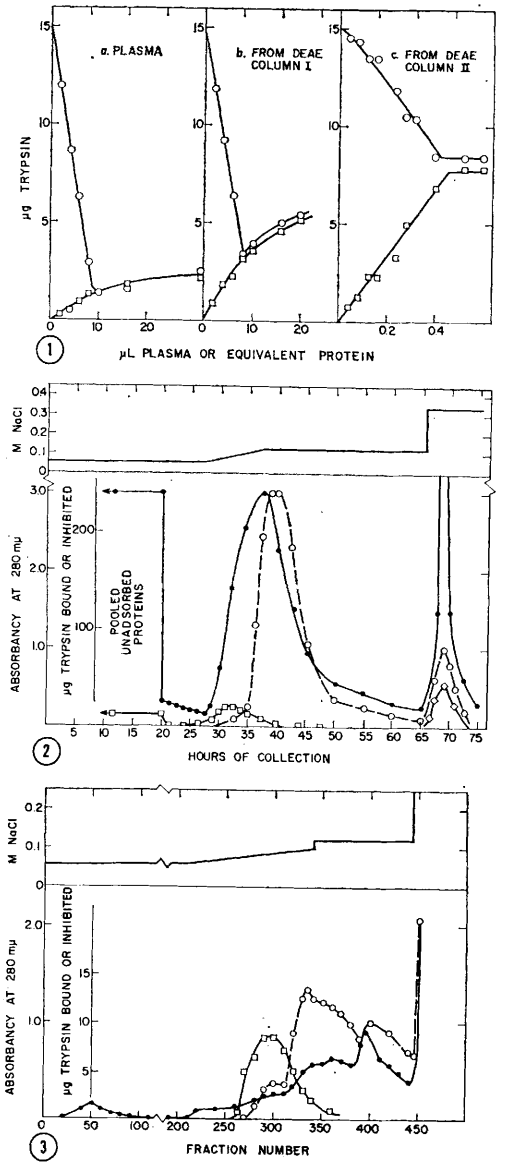

FIG. 1. Trypsin inhibitor, —○—, and trypsin binding protein, —□—, in 'a' plasma, 'b' trypsin-binding peak from chromatographic separation of plasma, 'c' trypsin-binding peak from rechromatography of trypsin-binding component from plasma. In both cases the apparent trypsin content is given as a function of the addition of increasing amounts of plasma or increasing amounts of the fractions with a protein content equivalent to the indicated amounts of plasma. In the trypsin-binding assay, an excess of soybean trypsin inhibitor (20 μg) has been added.

FIG. 2. Chromatography of human plasma on DEAE-cellulose at pH 7.7, in 0.02 M Tris buffer, with additional NaCl at the concentration indicated in upper part of Figure. Fractions are indicated in hours of collection at a flow rate of 390 ml per hour, and —●— gives protein concentration as indicated by the absorbancy at 280 mμ in a 1 cm cell. Amounts of trypsin inhibited, —○—, or bound, —□—, are indicated in μg per ml of fraction. Stable inhibitor, —△—, is given in the same way.

FIG. 3. Pooled trypsin-binding protein fractions from the original chromatography of plasma on DEAE-cellulose were rechromatographed under the same conditions. Symbols are the same as for Fig. 2.

mately the proper pH before packing the column. With the large amounts used, titration of the slurry of DEAE-cellulose in NaCl solution with NaOH is a good deal more economical than washing with buffer. Packing was then carried out with starting buffer, and the column washed until the pH and conductivity of the effluent were the same as those of the buffer applied. All buffers were made with 0.02 M Tris, pH 7.7 at 25°C, and the starting buffer contained 0.06 M NaCl in addition. Elution was carried out by increasing the NaCl concentration, with the same concentration of Tris, and the concentrations indicated below will refer to the NaCl.

Results. The results of typical determinations of total trypsin inhibitor and of trypsin-binding protein in plasma and in two fractions containing increasing proportions of trypsin-binding protein are shown in Fig. 1. As has been repeatedly demonstrated in the past, adding increasing amounts of diluted plasma or serum to a fixed quantity of trypsin results in a linear decrease in trypsin activity. The concentration of inhibitor was calculated from the linear region of this decrease. At some point, however, the inhibition reaches a maximum and trypsin activity begins to increase again. When an excess of soybean trypsin inhibitor is added, in the trypsin-binding protein assay, there is an initial increase in trypsin activity as increasing amounts of plasma are added. This increase is nearly linear at first, but soon falls off; and beyond the point where the minimum trypsin activity is reached in the inhibitor assay, the trypsin activity in the two assays is very nearly the same. Calculation of the amount of trypsin-binding protein is made on the basis of the initial rate of increase of activity with increasing amounts of plasma. In other words, the estimates of total trypsin inhibitor and of trypsin-binding protein must

both be made in a region where trypsin is in substantial excess of these two factors in plasma.

Results for stable inhibitor determinations are not shown, since these result in a linear decrease in trypsin activity with increasing additions of plasma. Both the trypsin-binding protein and the α_1-trypsin inhibitor are inactivated by the heating at low pH. In the case of plasma, the stable inhibitor values are 5 to 10% of the total inhibitor.

Initial chromatography of plasma. The conditions used for the chromatography of plasma were arrived at after exploring a number of conditions, and finding that the stable inhibitor required a substantially higher salt concentration for elution than was required for the major peak of total inhibitor. With the 0.02 M Tris buffer at pH 7.7, the α_1-inhibitor is eluted at about 0.1 M NaCl and the stable inhibitor at 0.18 to 0.2 M. A continuous gradient results in the elution of rather dilute fractions, of course, and unless the gradient is quite shallow a good deal of the major inhibitor trails into the stable inhibitor fraction. Consequently, it appeared better in the initial fractionation to elute the α_1-inhibitor with 0.12 M NaCl to obtain a relatively more concentrated peak, and then to continue washing with 0.12 M NaCl until the inhibitor and protein had dropped to low values. The NaCl concentration could then be increased to 0.35 M to remove the stable inhibitor in a concentrated peak. Both inhibitors are retained at 0.06 M, where a good deal of the total protein is not adsorbed, so this provides a suitable starting concentration of NaCl.

When the behavior of the binding protein was examined, it was found to be adsorbed at 0.06 M NaCl, and to be eluted at a somewhat lower salt concentration than the α_1-inhibitor. The separation of α_1-inhibitor and trypsin-binding protein required the use of a gradient, and still resulted in binding protein fractions which were contaminated with inhibitor. The conditions which were employed, then, were to start with the plasma (after treatment with sulfated rice starch) and the column both equilibrated with 0.06 M NaCl and 0.02 M Tris buffer at pH 7.7. The column was washed with this starting buffer until the unadsorbed protein had passed through the column and the absorbancy of the effluent was less than 0.2. This required about 4 liters of buffer after the sample was completely on the column. The NaCl concentration was then increased to 0.12 M in a linear gradient over a total volume of 6 liters. This was followed by 0.12 M NaCl for about 24 hours (9 to 9.5 l), when the absorbancy again dropped to less than 0.2. The NaCl concentration was then increased to 0.35 M and this was continued until the protein peak was eluted. The results of such a separation are given in Fig. 2. The absorbancy at 280 mμ in the 1 cm cell and μg of trypsin bound by trypsin-binding protein, by total trypsin inhibitor, and by stable inhibitor are plotted against hours of collection at a flow rate of 390 ml per hr. A substantial amount of trypsin-binding protein was lost with the unadsorbed fraction in this run. The trypsin-binding protein in this unadsorbed fraction was retained on the column at 0.06 M NaCl when it was re-chromatographed. Subsequent experience with the behavior of this fraction has indicated that the capacity of DEAE-cellulose is less than for most plasma proteins and that the column may be overloaded with respect to this component with some samples of plasma. The peak of the trypsin-binding protein which was eluted during the application of the gradient appeared a little ahead of the protein peak and the peak of total trypsin inhibitor somewhat behind the protein peak. The elution of inhibitor from the major peak trailed over a large volume after the peak had been passed, and even with the period of washing which was used, the stable inhibitor was substantially contaminated with α_1-inhibitor.

The recovery of trypsin-binding activity was rather low. Of an apparent total binding capacity for 125 mg of trypsin which was calculated for the plasma applied to the column in this particular case, only 20% was recovered in the collections for the 29th through the 36th hours. An additional 30% could be accounted for in the unadsorbed fraction. Although as much as 80% has been recovered

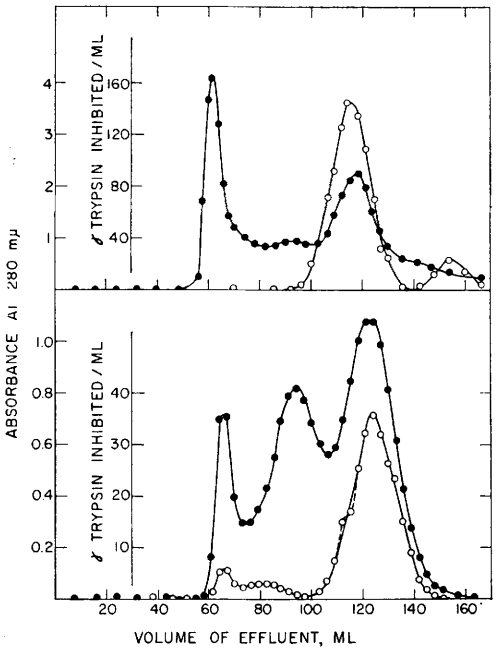

FIG. 4. Gel filtration of stable trypsin inhibitor fraction on Sephadex G-200 in 0.17 M Tris and 0.4 M NaCl, pH 7.7 in upper set of curves, in comparison with gel filtration of a plasma sample on the same column in the lower set. Protein, —●—, as indicated by absorbancy at 280 mμ and total trypsin inhibitor, —○—, in μg trypsin inhibited per ml. Inhibition in the first peak from plasma is due at least largely to α_2-macroglobulin. The next, rather broad peak does not contain trypsin-binding activity, nor does it correspond to either the α_1-trypsin inhibitor or the stable inhibitor, which appear together in the large peak of total inhibitor. These results indicate that the α_1-trypsin inhibitor has a molecular weight a little less than that of albumin, and stable inhibitor one which is a little greater.

in other runs, values in the range of 50 to 60% are more typical.

The recovery of α_1-trypsin inhibitor is more satisfactory. The fractions collected during the 37th through the 63rd hour contained inhibitor equivalent to 1170 mg trypsin. The total apparent inhibitor capacity of the plasma which was applied was 1560 mg trypsin, of which about 150 to 200 mg can be attributed to combination with stable inhibitor and trypsin-binding protein. An additional amount comes off very slowly, and is finally eluted with the stable inhibitor. In general, 85% or more appears to be accounted for, and about 60% can be recovered in relatively concentrated fractions.

The recovery of stable inhibitor is, again, less satisfactory. However, about 40% can be recovered in the more concentrated portions of the peak eluted with 0.35 M NaCl. To some extent, the low recoveries of this and of the trypsin-binding protein may be related to difficulties with the estimation of these fractions in the original plasma.

Rechromatography of the trypsin-binding protein fraction. Although all of the initial fractions are very impure, and substantially contaminated with each other, they provide suitable starting materials for further purifications. The trypsin-binding protein fraction can be greatly improved by a repetition of essentially the same chromatographic procedure, as seen in Fig. 3. Conditions were different only in that the gradient was run between 0.06 and 0.1 M NaCl, and this was then followed by 0.12 M NaCl at the end of the gradient; and that rechromatography was done on a smaller column with less total protein applied. The peak of total inhibition which is in the same position as the peak of trypsin-binding activity is due largely to the partial inhibition produced by the binding protein. The purification of this activity is substantial, as indicated by comparison of the assay of material from the chromatography of plasma (Fig. 1b) and the assay of pooled fractions from the second column (Fig. 1c). Some additional information has already been published on the behavior of this fraction in Sephadex gel filtration and starch gel electrophoresis, and its identification with alpha$_2$-macroglobulin(6).

Gel filtration and electrophoretic behavior of the stable inhibitor fraction. The stable inhibitor fraction from several batches of plasma was rechromatographed on DEAE under the same conditions, and the product which was more than 90% stable inhibitor was subjected to gel filtration on Sephadex G-200. The results obtained when this was carried out in 0.17 M Tris buffer plus 0.4 M NaCl are shown in Fig. 4. The behavior of total inhibitor in a sample of plasma is shown for comparison, and it is evident that the major component of the stable inhibitor is a moderately large protein. It is apparently somewhat larger than the main inhibitor peak which has been shown to be an α_1-globulin

with a molecular weight between 45,000 and 80,000(2,3). The minor component of the stable inhibitor is, however, a relatively low molecular weight protein.

Material from the major peak was concentrated and examined by electrophoresis on filter paper, with control samples of serum. Half of each filter paper strip was stained for protein and the remainder was cut to correspond with the position of the protein peaks and the pieces eluted and tested for inhibitor. The trypsin inhibitor from the major stable inhibitor peak migrated with the albumin fraction.

It should also be noted that the gel filtration pattern of plasma in Fig. 4 also shows inhibitor following the macroglobulin, and before either the α_1-inhibitor or stable inhibitor peaks. A peak of inhibitor is also present at this point in partially purified macroglobulin prepared from Cohn Fraction 111-0(6).

Discussion. Chromatography on DEAE-cellulose permits the separation of 3 fractions which inhibit trypsin. The first of these has the properties described by Haverbach *et al* (5), and which we have referred to as trypsin-binding protein. This has been shown to be an α_2-macroglobulin(6). Schultze and coworkers(8) have also shown that the α_2-trypsin inhibitor is a macroglobulin. The activity of the complex of trypsin with macroglobulin is substantial with such substrates as benzoyl-arginine-p-nitroanilide or tosylarginine methyl ester (TAME), but we have found that it is much less active against protein substrates. This α_2-macroglobulin is also an inhibitor of thrombin, and a similar difference has been found with respect to substrates by Lanchantin *et al*(14), as the thrombin esterase activity is essentially unchanged but conversion of fibrinogen to fibrin is inhibited. Steinbuch and associates have also found that α_2-macroglobulin combines with plasmin, inhibiting the fibrinolytic activity with little loss of esterase activity(15). On the basis of the greater residual activity of the trypsin-macroglobulin complex toward simpler substrates as compared to proteins, this component can probably also be identified with "trypsin inhibitor 1" which has been studied in the Institutes of Biochemistry and of Otolaryngology, Academy of Sciences of the Ukrainian SSR(16,17,18,19). In connection with the formation of enzymatically active complexes of α_2-macroglobulin with trypsin, Martin *et al*(20) have reported a very small residual activity of trypsin in the presence of excess soybean trypsin inhibitor when tosylarginine methyl ester (TAME) is the substrate, and that we have confirmed this observation(21).

The second trypsin inhibitor fraction in order of elution from DEAE is at least largely α_1-trypsin inhibitor, but has not been further investigated in this study.

The third inhibitor fraction is very stable to heating and to a relatively low pH. Although the amounts obtained have been so small as to preclude much characterization, it was found that the major portion of this material has a mobility about the same as that of albumin at pH 8.5, and the results of gel filtration on Sephadex G-200 indicate that it may be a little larger molecule than the α_1-trypsin inhibitor. If any part of this heat-stable component can be identified with the material studied by Shulman(4), it would have to be the much smaller part of this fraction shown in the separation by gel filtration in Fig. 4.

The behavior of trypsin inhibitors of human serum on DEAE-Sephadex has been reported by Lundblad(22). Gelatin and TAME were used as substrates, and in addition the latter substrate was used to measure the esterase activity of fractions without added trypsin. Fraction 1, eluted with 0.02 M phosphate, pH 6.6, was inhibitory with gelatin as substrate, but much less so with TAME. This fraction also contained the TAME esterase. It undoubtedly corresponds with the α_2-macroglobulin fraction, and we have confirmed the fact that these impure preparations of our macroglobulin contain TAME-esterase. Fractions collected with 0.1 or 0.2 M NaCl added to the phosphate buffer were strongly inhibitory with both gelatin and TAME as substrates. Our results would suggest that these fractions were largely α_1-trypsin inhibitor, though the heat-stable inhibitor might have been included in the second of these. Since Lundblad's experiments were carried out with step-wise elution, it is difficult to

know whether the phosphate buffer at a lower pH would offer any advantages.

In addition to these 3 fractions which are separated on DEAE-cellulose, there must be at least one additional inhibitor which differs in its behavior on gel filtration. Both the plasma (Fig. 4) and the lipid-poor euglobulin from Cohn Fraction 111-0 (Fig. 1 in ref. 6) contain an inhibitor which is intermediate in size between the α_2-macroglobulin and the major component of the heat-stable inhibitor or the α_1-trypsin inhibitor. The quantitative relationships among these inhibitors is not entirely clear, though the α_1-globulin is clearly the major component, and the α_2-macroglobulin would be the next larger if inhibition is measured with casein, hemoglobin, or gelatin as the substrate.

Summary. Chromatography of human plasma on DEAE-cellulose permits the separation of 3 fractions which inhibit trypsin. The first of these inhibits trypsin only partially, and is an α_2-macroglobulin. The second fraction represents most of the inhibitor in plasma, and must be largely that previously identified with the α_1-globulin fraction. The third fraction is very stable to heating at low pH, and is largely made up of material with a mobility very close to that of albumin. A fourth component can be shown to differ from these with respect to its behavior in gel filtration.

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