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Research Article

TRYPSIN INHIBITORS OF HUMAN SERUM. I. STANDARDIZATION, MECHANISM OF REACTION, AND NORMAL VALUES [1]

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Camus and Gley (1) appear to have been the first to observe that human serum inhibits trypsin, and Ascoli and Bezzola (2) and Brieger and Trebing (3), the first to report that this trypsin inhibiting activity may be increased in some diseases. Since that time the inhibition of trypsin by the serum and plasma of normal and diseased persons has been the subject of several investigations. Grob (4) and Jacobsson (5) have reviewed the earlier literature.

A variety of substrates and trypsin preparations have been used in measuring the inhibition of trypsin by serum and in studying the mechanism of this reaction. The determination of inhibitor levels has received the most attention and results have often been expressed in arbitrary units or referred to the amount of inhibition produced by normal serum. Concentrations in normal human serum have been expressed in terms of micrograms of crystalline trypsin inhibited per ml. of serum by Christensen (6), Peacock and Sheehy (7), Shulman (8), and Jacobsson (5). The average values were 655, about 1,400, 2,200, and 752, respectively. In each case the assay method involved preincubation of trypsin and serum in an alkaline medium. Christensen and Jacobsson, following the suggestion of Kunitz (9), standardized their trypsin preparations by titration with crystalline soybean inhibitor.

Hussey and Northrop (10) and Grob (4), using crude trypsin preparations, found the inhibition by human plasma to be reversible. On the other hand, Shulman (11) and McCann and Laskowski (12) using crystalline trypsin preparations, the former with human serum and the latter with rat plasma, found the inhibition to be stoichiometric and irreversible. It had been recognized that there are two components in human plasma which inhibit trypsin, and Jacobsson (13) showed that these could be demonstrated by zone electrophoresis to be components of the α_1- and the α_2-globulin. He subsequently found (5), using the Anson hemoglobin assay for trypsin, that both inhibitors react reversibly with trypsin, and that the dissociation constant for the inhibitor in the α_1-globulin was 6×10^{-10} and that for the inhibitor in the α_2-globulin, 3×10^{-10}. Having found that urea makes the otherwise irreversible inhibition of trypsin by soybean inhibitor appear to be reversible (5, 14), Jacobsson suggested that the reaction with serum trypsin inhibitors might be irreversible in the absence of urea.

The present report is concerned with some modifications of the casein method of Kunitz (9) for the determination of tryptic activity, with the question of the reversibility of the reaction between trypsin and serum inhibitor, and with certain other factors bearing on the standardization of methods for the determination of trypsin inhibitor levels in serum.

METHODS AND MATERIALS

Estimation of the concentration of trypsin and inhibitor. Salt-free trypsin and soybean trypsin inhibitor (Worthington Biochemicals), twice crystallized and five times crystallized, respectively, were used. Stock solutions of trypsin and of soybean inhibitor were prepared in 0.0025 N HCl and the absorbance was determined at 280 mμ in a Beckman Model DU spectrophotometer. The factors 0.585 for trypsin and 1.10 for soybean inhibitor, given by Kunitz (9), were used to convert absorbance to mg. protein per ml. solution. Working solutions of both enzyme and inhibitor were prepared as indicated below.

Substrate. Three Gm. vitamin-free casein (Pfanstiehl) was suspended in 100 ml. of 0.17 M tris (hydroxymethyl) aminomethane brought to pH 7.6 with HCL. The suspension was heated in a boiling water bath for 15 min-

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[2] Taken, in part, from a thesis submitted in partial fulfillment of the requirements for the degree of Master of Science, Graduate School, University of Southern California.

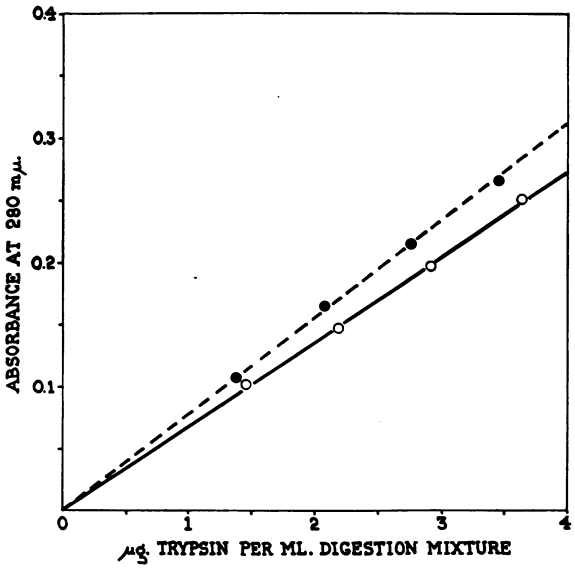

FIG. 1. STANDARDIZATION OF TRYPSIN DIGESTION OF CASEIN

The absorbance of the supernatant from the trichloroacetic acid precipitate of the digest as compared to the trypsin concentration: ○—○, in the absence of added Ca^{++}; ●- - -●, with Ca^{++} added to 4×10^{-3} M in the preincubation mixture before adding substrate.

utes to dissolve the casein. The solution had a final pH of 7.0.

Determination of trypsin activity. The casein digestion procedure of Kunitz (9) was used with some modifications. Since serum inhibitory activity disappears rapidly on exposure to a pH of less than 6, all dilutions were made with 0.17 M tris buffer, pH 7.6, rather than with 0.0025 N HCl as in the original method of Kunitz. A working trypsin solution was prepared by diluting the stock solution 1:10 with the buffer. To serial volumes of 0.1 to 0.8 ml. of the working trypsin solution, sufficient buffer was added to bring the total volume in each tube to 2 ml. These trypsin dilutions and the casein substrate were allowed to incubate separately for 15 minutes at 35° C. in order to attain temperature equilibrium. After this time 1 ml. of 3 per cent casein was added to each of the trypsin dilutions and the mixtures were allowed to incubate at 35° C. Digestion was stopped after 20 minutes by the addition of 6 ml. of 2.5 per cent trichloroacetic acid. Blanks were prepared by adding the trichloroacetic acid to the casein and then mixing with the trypsin dilutions. It was found to be important to allow about one and a half hours after addition of trichloroacetic before removing the precipitate by centrifugation. The absorbance of each supernatant was measured at 280 mμ with a Beckman Model DU spectrophotometer. Absorbance, corrected for the blank value, was plotted against trypsin protein concentration as shown in Figure 1.

The original method of Kunitz employs a 0.5 per cent casein concentration in the final digestion mixture. It was found that the rate of reaction decreased during the 20 minute digestion period, but that a linear rate could be maintained by increasing the final casein concentration to 1 per cent. Under these circumstances the rate also becomes linear with trypsin concentration over a greater range of trypsin concentrations.

Using the higher substrate concentration, some difficulty was encountered with the release of digestion products from the precipitate produced by adding 3 ml. of 5 per cent trichloroacetic acid to the digestion mixture. More satisfactory results were obtained by using 6 ml. of 2.5 per cent trichloroacetic.

These changes, together with the change to preincubation at pH 7.6, have the disadvantage that our specific enzyme activities are not comparable with those obtained by the original method. However, the reliability is improved by employing conditions in which zero order kinetics are obtained. Specific enzyme activity is defined as the increase in absorbance per minute per μg. of trypsin per ml.

Determination of inhibitor activity. A working soybean inhibitor solution was prepared by diluting the stock

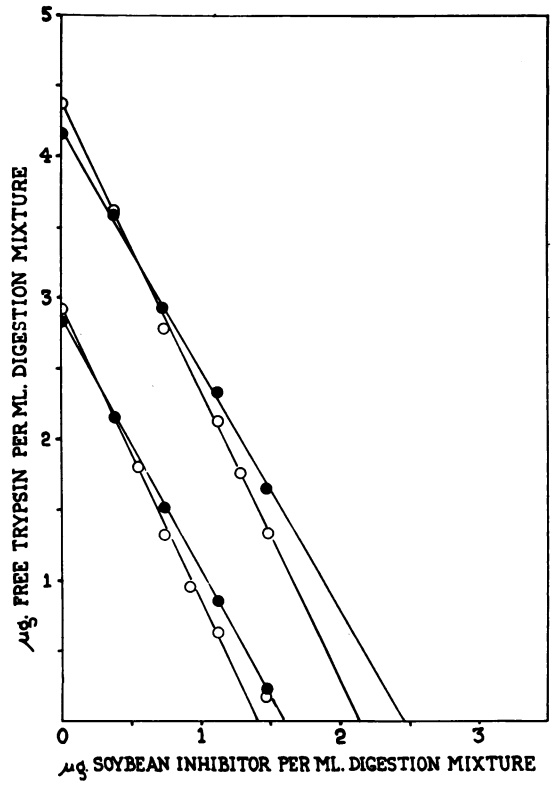

FIG. 2. EFFECT OF CALCIUM ON THE INHIBITION OF TRYPSIN BY SOYBEAN INHIBITOR

Residual trypsin as a function of increasing amounts of soybean inhibitor for two different trypsin concentrations: ○—○, in the absence of added Ca^{++}; ●- - -●, Ca^{++} added to a concentration of 4×10^{-3} M in the preincubation mixture before the addition of substrate.

solution 1:10 with the tris buffer. Serial volumes of this working inhibitor solution were added to a fixed volume of the working trypsin solution and the final volumes adjusted to 2 ml. with tris buffer. Residual trypsin activity was determined by adding 1 ml. of 3 per cent casein and completing the determination of activity described above.

In determining inhibition by serum, the serum was diluted 1:100 or 1:200 with the tris buffer, and serial volumes of these dilutions were added in place of the soybean inhibitor.

Blanks were prepared for the lowest and highest concentration of serum used and intermediate blank values were calculated by interpolation. This correction was not required for soybean inhibitor, which contained no appreciable material absorbing at 280 mμ which was not precipitated by trichloroacetic acid.

Additions of ethylenediaminetetracetate and calcium. Ethylenediaminetetracetate (EDTA) was added as the disodium salt and calcium as $CaCl_2$. The concentrations indicated subsequently are those in the preincubation mixture, before the addition of substrate.

RESULTS

Inhibition of trypsin by soybean inhibitor

The effect of Ca^{++}. Since Ca^{++} has been shown to increase the stability of trypsin at an alkaline pH (15, 16) and to increase the esterase and amidase activity of trypsin (17), it was decided to investigate the effect of this cation on the proteolysis of casein by trypsin and on the observed combining ratio of trypsin and soybean inhibitor. Results are shown in Figure 1 and Figure 2. As indicated in Figure 1, the addition of Ca^{++} to a concentration of 4×10^{-3} M increases the specific activity of the trypsin from 3.4×10^{-3} to 3.9×10^{-3}. The intercept obtained by extrapolating the curves in Figure 2 to zero free trypsin gives the amount of inhibitor which should completely inhibit the total amount of trypsin added. Combining ratios can be calculated from these values. Combining ratios obtained in this manner are CT/I [3] = 2.10 in the absence of Ca^{++}, and CT/I = 1.80 for Ca^{++} added to 4×10^{-3} M. Thus Ca^{++} appears to have increased the activity of this sample of trypsin by about 15 per cent and the observed combining ratio has decreased correspondingly.

Effect of ethylenediaminetetracetate (EDTA). The results of similar experiments showing the effects of Ca^{++}, EDTA and Ca^{++} plus EDTA on the specific activity and on the observed combining ratio with soybean inhibitor of two trypsin preparations are summarized in Table I. The trypsin preparations represent the same original crystalline trypsin but since the experiments were performed two years apart, they are considered here as different samples. Ca^{++} seems to exert a maximum effect at 10^{-3} M. In each case, the decrease in combining ratio parallels the increase in specific activity. Low concentrations of EDTA decrease the specific activity of trypsin in the absence of added Ca^{++}. This may indicate the presence of traces of Ca^{++} in the reagents, or of tightly bound Ca^{++} or other cations in the trypsin molecule. As was observed by Green and Neurath (17), higher concentrations of EDTA have an activating effect. Although EDTA at lower concentrations may cause a 28 per cent decrease in trypsin activity, the combining ratio with soybean inhibitor remains unchanged. EDTA completely counteracts the effect of added Ca^{++}.

Table II summarizes the results of similar experiments performed with serum.[4] The findings

TABLE I

The effect of Ca^{++}, ethylenediaminetetracetic acid and ethylenediaminetetracetic acid plus Ca^{++} on two trypsin samples and on their reaction with soybean inhibitor

Added Ca^{++} *M/L.*	EDTA* *M/L.*	Specific enzyme activity $\times 10^3$	CT† / I
	Trypsin sample 'a'		
0	0	3.4	2.10
4×10^{-3}	0	3.9	1.80
4×10^{-3}	10^{-2}	2.7	2.16
	Trypsin sample 'b'		
0	0	3.2	2.28
5×10^{-5}	0	3.4	
10^{-3}	0	3.7	
4×10^{-3}	0	3.7	1.88
10^{-2}	0	3.7	
0	10^{-4}	2.9	
0	10^{-3}	2.3	2.29
0	10^{-2}	2.7	
2.25×10^{-3}	2.5×10^{-3}	2.8	2.31

* Ethylenediaminetetracetic acid.
† Micrograms of trypsin inhibited by 1 μg. soybean inhibitor.

[3] CT/I = Micrograms of trypsin inhibited by 1 μg. soybean inhibitor.

[4] The sera used in these experiments were samples of pooled sera obtained from the Chemistry Laboratory of the Los Angeles County General Hospital.

were qualitatively similar to those obtained in experiments with soybean inhibitor.

The specific activities for both Tables I and II were calculated from trypsin concentrations based on the absorbancy of the trypsin solutions. Values of CT/I for Table I and of the amount of trypsin apparently inhibited per ml. of serum (Table II) were calculated on the same basis. The corrected values for the inhibition of trypsin by serum, which are given in the last column of Table II, were calculated on the assumption that soybean inhibitor combines with trypsin on an equimolar basis under the same conditions. This basis for standardization will be considered in more detail in the discussion.

Effect of preincubation time

Since it is known that trypsin undergoes autolysis in alkaline solution and that Ca^{++} increases the stability of trypsin at an alkaline pH (7, 8), our results indicated that Ca^{++} was exerting a protective action during the alkaline preincubation period. This possibility was investigated by determining trypsin activity after varying periods of preincubation. Working trypsin solutions were prepared by dilution of the stock solution with 0.0025 N HCl or 0.0025 N HCl plus 4×10^{-3} M Ca^{++}. 1.8 ml. of the tris buffer or 1.8 ml. of the tris buffer plus 4×10^{-3} M Ca^{++} were allowed to come to 35° C. and 0.2 ml. portions of the working trypsin solution were added. At various intervals 1 ml. of 3 per cent casein was added and proteolytic activity was determined by adding 0.2 ml. of the working trypsin solution to a mixture of buffer and casein. Subsequent activities were calculated as per cent of initial activity. Results are shown in Figure 3. As was expected, trypsin undergoes a loss of activity at pH 7.6. The initial rate of decay is quite rapid but gradually levels off. Ca^{++} does indeed afford partial protection against this loss in activity. After 15 minutes preincubation at 35° C., about 70 per cent of the original activity remains in the absence of added Ca^{++}, while 85 per cent remains when Ca^{++} is added.

TABLE II

The effect of Ca^{++} and ethylenediaminetetracetic acid on trypsin and its inhibition by serum

Added Ca^{++} M/L.	EDTA* M/L.	Specific enzyme activity $\times 10^3$	Trypsin inhibited: Apparent mg./ml. serum	Trypsin inhibited: Corrected† mg./ml. serum
		Pooled serum sample A		
0	0	2.5	2.38	1.00
4×10^{-3}	0	3.9	1.66	1.11
0	10^{-4}	2.2	2.26	0.95
		Pooled serum sample B		
0	0	2.9	2.61	1.24
4×10^{-3}	0	3.9	1.90	1.27
0	10^{-3}	2.1	2.64	1.26

* Ethylenediaminetetracetic acid.
† Corrected on the basis of inhibition by soybean inhibitor under the same conditions (see text).

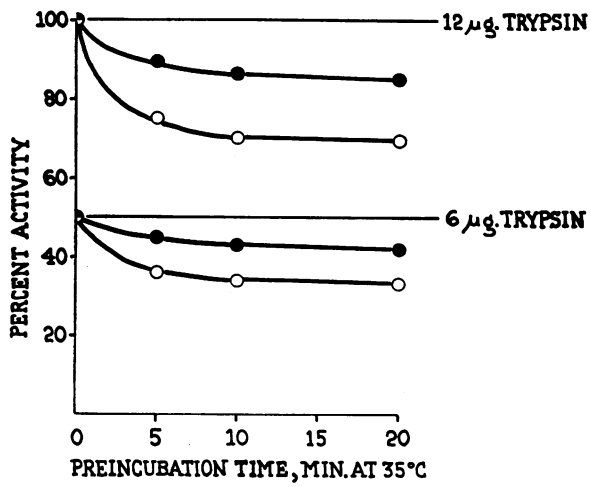

FIG. 3. THE EFFECT OF PREINCUBATION OF pH 7.6 AND 35° C. ON THE ACTIVITY OF TRYPSIN

Expressed as the per cent of initial activity for 12 µg. trypsin: ○—○, in the absence of added Ca^{++}; ●- - -●, in the presence of 4×10^{-3} M Ca^{++} in the preincubation mixture.

Dissociation of trypsin-serum inhibitor complex

The following experiments were carried out with preincubation for 15 minutes at pH 7.6, in the presence of 4×10^{-4} M Ca^{++}. Under these conditions, 1 mg. of crystalline soybean inhibitor was found to inhibit 1.8 mg. of our trypsin preparation. Since, as indicated in the discussion, 1 mg. of soybean inhibitor should inhibit 1.2 mg. of trypsin, apparent trypsin concentrations were corrected by a factor of 1.2/1.8.

The effect of increasing amounts of serum on the tryptic proteolysis of casein is shown in Figure 4a. Departure from stoichiometric inhibition with higher concentrations of serum indicates dis-

sociation of a trypsin-inhibitor complex. The same data are plotted in Figure 4b as per cent inhibition by increasing amounts of serum, showing that the inhibition is proportional to the amount of serum added up to about 70 per cent inhibition. This assumes significance in the determination of serum trypsin inhibitor levels, since only values obtained at less than 70 per cent inhibition yield a proportionality between inhibition of activity and the amount of inhibitor present.

Assuming the affinities of the two inhibitors in serum for trypsin are approximately the same, as was found by Jacobsson, that 1 M of each inhibitor combines with 1 M of trypsin, and that the inhibition is noncompetitive,[5] a dissociation constant was calculated from the equation:

$$K_{dis} = \frac{FT\ FI}{CT},$$

where FT and FI represent free trypsin and free inhibitor, respectively, and CT represents combined trypsin. FT was obtained by reference to the activity curve and CT by subtracting FT from the total trypsin added. If the assumptions enumerated above are correct, the ml. of serum obtained by extrapolation of the curves in Figure 1a contain a total number of moles of inhibitor equal to the total moles of trypsin added. FI is then obtained by subtracting CT from the total amount of inhibitor for each serum concentration. All concentrations were changed to moles per liter, using 24,000 as the molecular weight of trypsin (13). The dissociation constant calculated in this manner has the value 8×10^{-10} M. In Figure 5, the molar concentration of combined trypsin is plotted against the molar concentration of inhibitor. The solid line represents the theoretical curve calculated from the dissociation constant 8×10^{-10} M.

[5] This assumption may not be true in the strict sense of the word. However, a threefold decrease in substrate concentration had no effect on the calculated K_{dis}.

Serum trypsin inhibitor levels in normal persons

Serum trypsin inhibitor levels were determined for 40 apparently normal persons between the ages of 20 and 50 years. These were laboratory personnel, medical students, and professional blood donors. The mean for males was found to be 1,025 and for females 1,037; therefore both sexes were considered together. The mean was found to be 1,032 μg. trypsin bound per ml. serum, with a standard deviation of plus or minus 127 and a standard error of the means of plus or minus 6.3 μg. per ml. serum.

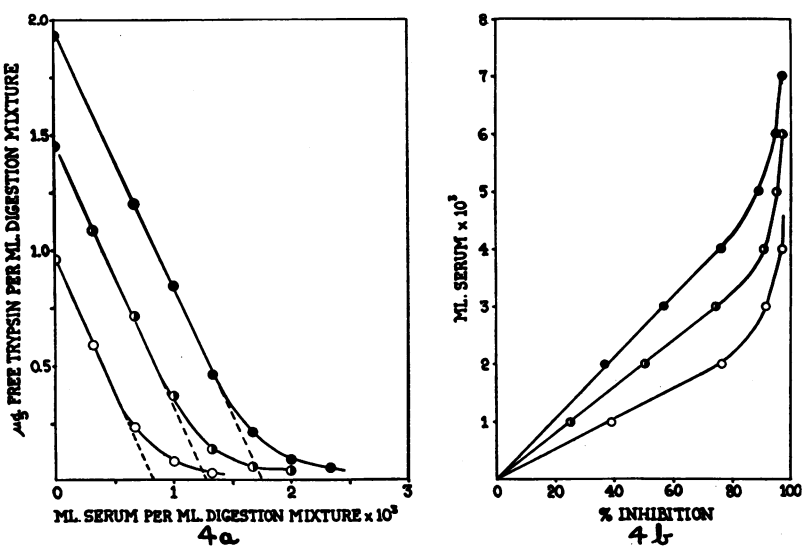

FIG. 4. INHIBITION OF TRYPSIN BY INCREASING CONCENTRATIONS OF HUMAN SERUM

Three trypsin concentrations, plotted in 4a as free trypsin as compared to the quantity of serum; and the same data in 4b as per cent inhibition as a function of the amount of serum added.

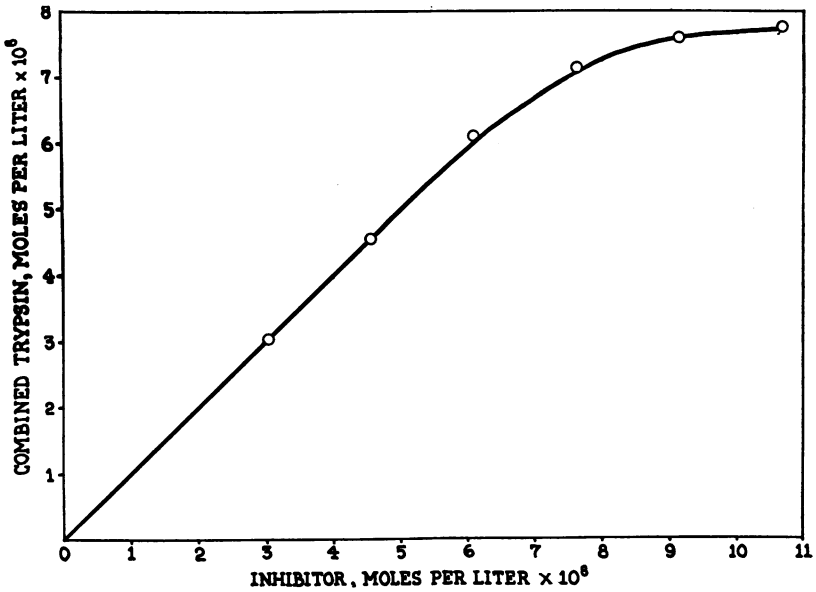

FIG. 5. DISSOCIATION OF TRYPSIN-SERUM INHIBITOR COMPOUND

The points represent experimental values for combined trypsin as a function of increasing total serum inhibitor. The curve is calculated assuming that trypsin and serum inhibitor combine reversibly in equimolar quantities.

Analysis of the differences between duplicate determinations gave a standard deviation for single determination of plus or minus 1.6 per cent and the standard deviation between duplicate analyses on different days was plus or minus 1.0 per cent. At the mean normal value these would be plus or minus 16 and plus or minus 10 μg. trypsin inhibited per ml. of serum. Since determinations were always done in duplicate, the observed standard deviation of plus or minus 127 μg. from the mean normal value is largely a reflection of the variation within the population rather than of errors in the determinations.

DISCUSSION

The instability of trypsin above pH 5 complicates the determination of trypsin inhibitor in serum, since this inhibitor undergoes inactivation at a pH below 6. Although Ca^{++} stabilizes trypsin in alkaline solution, it does not completely prevent the loss of activity. Consequently, when trypsin is preincubated with serum inhibitor at pH 7.6 the trypsin activity decreases as a result of inactivation as well as from combination with inhibitor.

It would be desirable to express inhibitor concentrations in serum in terms of the amount of trypsin inhibited per unit volume of serum. In the present study we have determined trypsin concentrations by measuring the absorbance of the trypsin stock solution at 280 mμ, but such a measure of trypsin concentration is subject to considerable error because of the presence of protein impurities, denatured trypsin, or trypsin autolysis products in the trypsin preparation. Kunitz (9) suggested that trypsin preparations be standardized by the reaction with soybean inhibitor. Jacobsson (5) has done this in his study of trypsin serum inhibitor. This basis for standardization seems well justified, since highly purified soybean inhibitor is commercially available and it has been established that 1 M of trypsin combines with 1 M of inhibitor (18–20). Kunitz had originally taken the weight combining ratio to be 1, but taking the molecular weight of soybean inhibitor to be 20,000 (21), the theoretical value of CT/I would be 1.2. This is considerably lower than the lowest value, 1.80, which we obtained (Table II). If the values of CT/I obtained with and without added Ca^{++} are corrected by assuming that the loss of activity during preincubation at pH 7.6 also represents loss of trypsin able to combine with inhibitor, the corrected values of CT/I become essentially the same in both cases, and average 1.63. This would sug-

gest that some 26 per cent of the absorbance of our trypsin stock solution at 280 mμ was due to inert material.

It might be argued that essentially the same results could be accomplished by standardizing results according to the specific activity of the trypsin. Assuming that both soybean and serum inhibitor combine only with active trypsin, the results obtained with and without added calcium can be harmonized in this way, but this method fails in the experiments with added EDTA. If we take the behavior in the presence of Ca^{++} as a base, trypsin sample 'a' (Table I) has a specific activity of 3.9×10^{-3} and gives a value of 1.80 for CT/I. In the absence of added Ca^{++}, the specific activity is 3.4×10^{-3} and gives a value of 2.10 for CT/I. In the absence of added Ca^{++}, the specific activity is 3.4×10^{-3} or 13 per cent less. Taking this to mean that there is 13 per cent less trypsin than in the presence of Ca^{++}, the observed value of CT/I of 2.10 should be reduced to 1.83, in good agreement with the value of 1.80. However, in the presence of EDTA, the specific activity is reduced by 31 per cent to 2.7. This would lead to a correction of the observed value of CT/I from 2.16 to 1.50. Similar results are obtained with serum inhibitor. The results given in Table II, if corrected for specific activity, would give the same values for mg. trypsin per ml. serum for measurements made with and without added Ca^{++}, but a different value in the presence of EDTA.

The results obtained with serum, measured with or without aded calcium or EDTA, do give the same result if based on the amount of the trypsin available for combination with soybean inhibitor under the same conditions. The corrected values given in the last column of Table II were obtained by assuming that the value of CT/I for soybean inhibitor is 1.2 under all conditions. For example, having an observed value of CT/I of 1.80 for soybean inhibitor in the presence of Ca^{++}, it was assumed that the trypsin concentration was 1.20/1.80 or 0.67 as great as indicated by the spectrophotometric determination on the stock trypsin solution. Consequently, rather than having 1.66 mg. of trypsin inhibited by 1 ml. of serum, only 0.67×1.66, or 1.11 mg. was actually inhibited.

The behavior in EDTA remains unexplained, however. It would be logical to assume that the reduced specific activity in the presence of lower concentrations of EDTA was due to the sequestering of traces of Ca^{++} or other activating or stabilizing ions. However, the effects on observed values of CT/I are not consistent with those obtained by adding Ca^{++}.

The results which have been presented indicate that the inhibition of trypsin by human serum is reversible and that although the dissociation is slight, it should be considered in the determination of serum trypsin inhibitor levels. The value for the dissociation constant is probably correct only within an order of magnitude, since it was calculated from very low residual trypsin activities. The calculation also involves certain assumptions which are probably not strictly true, namely that both inhibitors in serum have the same affinity for trypsin and that the inhibition is noncompetitive. However, the satisfactory agreement between observed and theoretical results indicates that any difference in the affinities of the two inhibitors for trypsin is less than an order of magnitude and that if the inhibition is competitive, it must be of the type postulated by Green (22) for soybean trypsin inhibitor and pancreatic trypsin inhibitor.

The average value of 1,030 μg. trypsin per ml. serum for the inhibitor level in normal, human serum is somewhat higher than those reported by Christensen, 655, and by Jacobsson, 752, and lower than those obtained by Peacock and Sheehy, about 1,400, and by Shulman, 2,200. The latter two estimates failed to take into account the autolysis of trypsin during the alkaline preincubation period, which would contribute to the high values obtained. Christensen standardized his trypsin preparation with soybean inhibitor. However in Christensen's method of assay, the highest dilution of serum which allowed a clot to be formed in the presence of trypsin, thrombin and fibrinogen was assumed to contain a concentration of serum inhibitor equivalent to the amount of trypsin used in the test. Thus his measurements were made in the region of dissociation of the trypsin-inhibitor complex and would be expected to yield low values. Jacobsson also standardized his trypsin with soybean inhibitor using the casein digestion method, but his serum inhibitor determinations were carried out with hemoglobin as the substrate. Using this procedure for soybean inhibitor, he reported an apparent combining ratio of 1.54 Gm. trypsin per Gm. soybean inhibitor, rather

than 1.85 when the casein substrate was used. We believe that the combination with soybean inhibitor should be measured under precisely the same conditions as the serum measurements for which it will serve as a standard. On this basis, and using a theoretical value of 1.2 Gm. trypsin as we have, rather than the value of 1 Gm. trypsin per Gm. soybean inhibitor which was used by Jacobsson, his average value becomes 1,080. This is in good agreement with our value of 1,032.

SUMMARY

The Kunitz casein method for the measurement of trypsin activity has been modified by increasing the substrate concentration and changing the conditions for precipitation of undigested substrate (9). The modifications result in zero order kinetics.

Using this assay procedure, the measurement of trypsin inhibitor activity in serum has been examined with respect to standardization. During the required preincubation of inhibitor and trypsin at pH 7.6, there is a loss of trypsin activity which is initially rapid, but becomes essentially zero after 15 to 20 minutes. The presence of Ca^{++} decreases this rate of decay, but does not afford complete protection.

The decrease in trypsin activity during preincubation produces an equivalent decrease in the amount of trypsin available for combination with both soybean and serum trypsin inhibitors, as judged by the behavior with or without added Ca^{++}. In the presence of EDTA the specific enzymatic activity of the trypsin may be further reduced, but this does not result in a comparable reduction in the amount of trypsin apparently able to combine with either serum or soybean inhibitor.

The results suggest that the soundest basis for standardization of trypsin inhibitor values in serum is the assumption that the amount of trypsin available for binding is the same as that which combines with crystalline soybean inhibitor under the same conditions that are employed for the measurement of the serum inhibitor.

The inhibition of trypsin by human serum has been shown to be reversible, and the significance of this in the determination of serum trypsin inhibitor levels has been pointed out. A dissociation constant of 8×10^{-10} M has been calculated for the trypsin-inhibitor complex. Certain assumptions involved in the calculation have been discussed.

It has been found that 1 ml. of normal serum will inhibit 1.03 ± 0.13 mg. of trypsin, and shown that this can be harmonized with Jacobsson's results. Reasons are presented for the differences from other reported values.

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