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Studies on Crystalline Trypsin, Soybean Trypsin Inhibitor and Inhibitor-Trypsin Compound with the Ultracentrifuge[1,2]

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A study of trypsin, soybean trypsin inhibitor and the inhibitor–trypsin compound with the ultracentrifuge is presented in which the stoichiometry of the reaction between trypsin and the inhibitor, the stability of the compound and the nature of the bonding between the components were investigated. It has been found that mixtures of trypsin and the inhibitor exhibit compound formation at *p*H 6.0 but not at *p*H 2.8 in 0.1 *M* phosphate buffer. Maximum compound formation occurs at *p*H 6.0 with mixtures containing approximately equal parts by weight of trypsin and inhibitor. The composition of the compound is shown to be constant. Sedimentation constants for trypsin and inhibitor are presented. The crystalline soybean trypsin inhibitor–trypsin compound was prepared and its sedimentation behavior was studied as a function of protein concentration, *p*H and ionic strength. The compound dissociates into its components below *p*H 3. The sedimentation constant, $s_{20,w}$, has an average value of 3.9 *S* from *p*H 3.6 to 10.4. Below *p*H 3.0, the $s_{20,w}$ equals 2.5 *S*, a value comparable to that for the trypsin and inhibitor. There is no evidence of dimerization or concentration dependence of the sedimentation constant for the three proteins in the concentration and *p*H range studied. The compound shows no evidence of dissociation in salt solutions as concentrated as 3 *M* even after standing 72 hours. It is also shown that the compound will form in the presence of the tryptic inhibitor benzoyl-L-arginine. The molecular weights of the crystalline inhibitor–trypsin compound, trypsin and inhibitor are discussed.

Kunitz[5] reported that the crystallized trypsin inhibitor which he isolated[6a,b] from soybean meal combines apparently instantaneously and irreversibly with crystallized trypsin over a wide *p*H range. Enzymatic activity studies indicated that soybean trypsin inhibitor (hereafter designated STI) counteracts the activity of approximately an equal weight of trypsin. The crystalline compound of inhibitor and trypsin (STI–T) was shown to be free of inhibitory or proteolytic activity in the native state and to consist of about equal weights of the two components.[7] The latter was deduced from an analysis of the "extensive" properties (tyrosine and tryptophan contents, ultraviolet light absorption and optical rotation per unit weight) of the compound and also of the two components. Based on the results of formol titrations, in which it was shown that there were fewer amino groups in the crystalline STI–trypsin compound than in either STI or trypsin alone, Kunitz postulated that the reaction is a "neutralization" of the free amino groups of trypsin by the free carboxyl groups of STI.

By means of dilatometric measurements, rate measurements and titration data on the STI–T system, McLaren[8] concluded that the compound is composed of molecules of one trypsin and one inhibitor molecule and is formed in a matter of seconds at *p*H 7.9, but does not form at *p*H 3.

Preliminary sedimentation studies on the STI–trypsin system[9] demonstrated that the STI–T compound was stable in the *p*H range of 3.6 to 10.4 but dissociated into its components below *p*H 2.9. When equal weights of pure STI and trypsin were mixed, it was found that compound formation was evident at *p*H 6.0 but not at *p*H 2.8. A more detailed ultracentrifugal analysis of the soybean trypsin inhibitor–trypsin system and the conditions under which the compound exists in solution is herein reported.

Experimental

Apparatus.—Sedimentation velocity determinations were performed with an air-driven ultracentrifuge of the turret type as designed by Pickels.[10] The instrument was equipped with the Philpot modification of the schlieren optical system.[11] This particular apparatus has been described by Stern.[12]

Ultraviolet absorption measurements were taken with a Beckman model DU quartz spectrophotometer. A Beckman model G *p*H meter was used for *p*H measurements.

Methods.—The proteolytic activity of crystalline trypsin and the inhibitory activity of crystalline STI was determined by the casein digestion method of Kunitz.[5] The protein concentrations of the trypsin and STI solutions were determined by measuring the ultraviolet light absorption at 2800 Å. The protein concentration is calculated by multiplying the optical density by the dilution and the appropriate factor (1.10 for crystalline STI and 0.585 for crystalline trypsin).[5]

The sedimentation constants were calculated from the dn/dx diagrams according to the methods of Svedberg and Pedersen.[13] A comparator was employed to measure the displacement of the peaks from the meniscus. Temperature measurements were recorded during the centrifugation with the aid of a copper–constantan thermocouple system. One junction of the thermocouple was placed 2 mm. from the slip stream of the rotor and a second junction was immersed in a water-bath contained in a dewar flask mounted on the panel board. In calculating the sedimentation constants, temperature corrections were made for each time interval between exposures. The mean sedimentation constants, $s_{20,w}$, have been corrected to a medium having the density and viscosity of water at 20°.

Materials.—Twice-crystallized trypsin (50% $MgSO_4$, trichloroacetic acid purified) was purchased from the Worthington Biochemical Laboratory, Freehold, N. J. STI, prepared according to the method of Kunitz,[6b] was three times crystallized and free of salt. The STI–T was prepared and twice crystallized according to the method of

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(5) M. Kunitz, *J. Gen. Physiol.*, **30**, 291 (1947).

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Kunitz.[7] Examination of the STI and STI-T compound under the microscope showed that both preparations were crystalline in appearance. We are indebted to Dr. H. Werbin for supplying us with a sample of the benzoyl-L-arginine. Reagent grade chemicals were used throughout.

Results

Sedimentation Velocity Studies on Mixtures of Crystalline STI and Crystalline Trypsin.—Two series of sedimentation velocity determinations, at *p*H values of 6.0 and 2.8, were performed with mixtures of STI and trypsin. For the first series, stock solutions of 0.91% STI, *p*H 6.0, and 0.67% trypsin, *p*H 6.1, were prepared in 0.1 *M* KH_2PO_4–Na_2HPO_4 buffer. For the second series, stock solutions of 0.95% STI, *p*H 2.9, and 0.97% trypsin, *p*H 2.7, were prepared in 0.1 *M* KH_2PO_4–H_3PO_4 buffer. The *p*H values for the solutions of mixtures containing increasing amounts of trypsin to a constant concentration of STI were *p*H 6.0 and 2.8, respectively. Samples of these mixtures were sedimented in the ultracentrifuge at speeds *ca.* 860 r.p.s. (180,000 *g*). The sedimentation data are recorded in Table I, and are plotted in Fig. 1

TABLE I

SEDIMENTATION CONSTANTS FOR MIXTURES OF STI AND TRYPSIN

Wt. ratio of trypsin:STI	Total protein concn., g./100 ml.	$s_{20,w}$ in Svedberg units
*p*H 6.0 series; 0.1 *M* KH_2PO_4–Na_2HPO_4		
0.22:1.00	0.56	2.6
.37:1.00	.63	3.0
.59:1.00	.73	3.3
.74:1.00	.80	3.6
1.00:1.00	.76	3.7
1.58:1.00	.80	3.7
2.00:1.00	.80	3.9
*p*H 2.8 series; 0.1 *M* H_3PO_4–KH_2PO_4		
0.10:1.00	0.53	2.4
.31:1.00	.63	2.5
.52:1.00	.73	2.6
.71:1.00	.83	2.5
.92:1.00	.92	2.7
1.01:1.00	.97	2.5

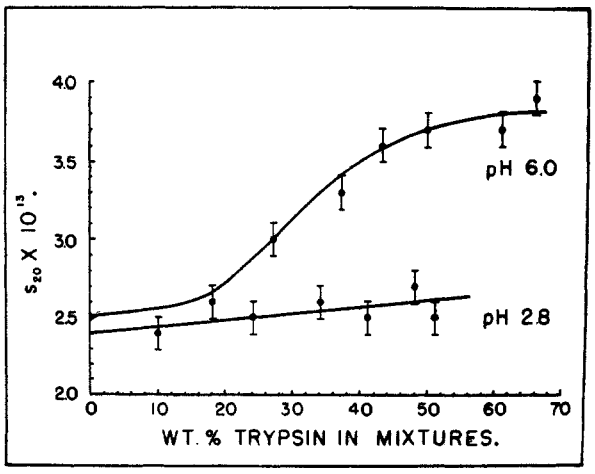

Fig. 1.—Variation of sedimentation constant, $s_{20,w}$, for mixtures of STI and trypsin at *p*H 2.8 and 6.0 as a function of the wt. % trypsin composition in the mixtures.

as $s_{20,w}$ *vs.* weight % trypsin in the mixtures at *p*H 6.0 and 2.8.

Control sedimentation determinations on solutions of STI and trypsin having protein concentrations comparable to the mixtures and at the two *p*H levels were also performed. The results for the controls appear in Table II. Representative photographs of the sedimentation patterns for the mixtures and controls are presented in Fig. 2.

TABLE II

SEDIMENTATION CONSTANTS FOR STI AND TRYPSIN CONTROLS IN 0.1 *M* PHOSPHATE BUFFERS

Protein concn., g./100 ml.	*p*H	$s_{20,w}$ in Svedberg units	Protein concn., g./100 ml.	*p*H	$s_{20,w}$ in Svedberg units
	Trypsin			STI	
1.0	2.7	2.4	1.0	2.9	2.6
0.7	2.7	2.6	0.7	2.9	2.4
.8	6.1	2.7	.8	6.0	2.6
.3	6.1	2.6	.6	6.0	2.6
			.4	6.0	2.4
			.2	6.0	2.4

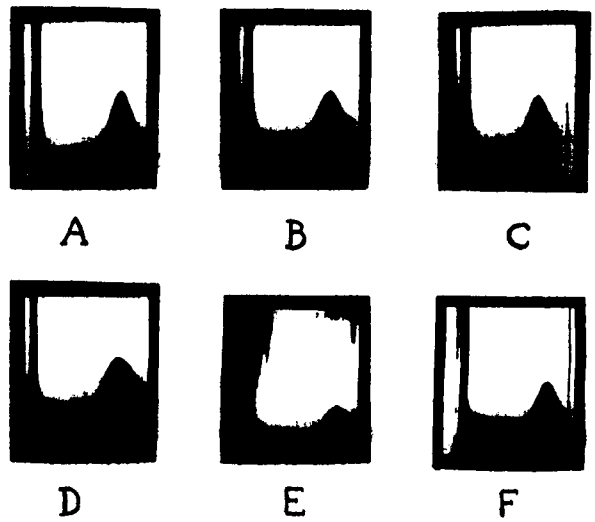

Fig. 2.—Sedimentation diagrams of STI–T mixtures and controls: A, STI–T mixture, *p*H 2.8, 0.8% protein, 41 wt. %T, $s_{20,w}$ = 2.5 *S*, 94 min. after full speed; B, 0.7% trypsin, *p*H 2.7, $s_{20,w}$ = 2.6 *S*, 91 min. after full speed; C, 0.7% STI, *p*H 2.9, $s_{20,w}$ = 2.4 *S*, 86 min. after full speed; D, STI–T mixture, *p*H 6.0, 0.7% protein, 37 wt. %T, $s_{20,w}$ = 3.3 *S*, 91 min. after full speed; E, 0.8% trypsin, *p*H 6.1, $s_{20,w}$ = 2.7 *S*, 72 min. after full speed; F, 0.6% STI, *p*H 6.0, $s_{20,w}$ = 2.6 *S*, 73 min. after full speed.

It will be noted that at *p*H 6.0, the sedimentation constant for mixtures of trypsin and STI increased from 2.6 to 3.7 as the concentration of trypsin approached the 50% level in the mixtures. Beyond the 1:1 weight ratio of trypsin to STI, the sedimentation constant did not change appreciably, not even when the mixture contained two parts of trypsin to one part STI. The sedimentation patterns for the mixtures at *p*H 6.0 showed a single slightly asymmetric peak whose trailing edge did not return to the base line. The control samples of trypsin, *p*H 6.1, and STI, *p*H 6.0, had average $s_{20,w}$ values equal to 2.6 and 2.5 *S*, respectively, in the

concentration range (0.2–0.8%) investigated. The sedimentation diagrams showed single, homogeneous peaks for these materials.

At *p*H 2.8, the sedimentation constants for the mixtures of trypsin and STI averaged 2.5 *S* and did not appreciably change in value as the concentration of trypsin was increased. The sedimentation patterns exhibited single symmetric peaks for the mixtures. The sedimentation determinations on the control solutions of trypsin, *p*H 2.7, and STI, *p*H 2.9, gave average $s_{20,w}$ values of 2.5 *S* for both proteins. The sedimentation diagrams showed single peaks with an indication of a small amount of heavy material having been precipitated at this acid *p*H.

Compound Formation in the Presence of Benzoyl-L-arginine.—An 0.37% protein solution containing equal parts of crystalline trypsin and STI in a one-half saturated benzoyl-L-arginine and *M*/15 phosphate buffer, *p*H 7.0, was centrifuged at 890 r.p.s. (188,000 *g*). A sedimentation velocity determination on an 0.78% trypsin solution in a one-half saturated benzoyl-L-arginine and *M*/15 phosphate buffer, *p*H 7.0, served as a control for this study.

It was found that compound formation between trypsin and STI occurred in the presence of one-half saturated benzoyl-L-arginine since the sedimentation constant s_{20} (corrected to solvent at 20°) was equal to 3.4 *S* for the mixture as compared to s_{20} of 2.4 *S* for the control solution containing only trypsin.

Sedimentation Velocity Studies on the Crystalline STI-T Compound.—In the light of the previous findings for mixtures of STI and trypsin which indicated that compound formation occurred at *p*H 6.0 but not at 2.8, the sedimentation behavior of solutions of the crystalline STI-T compound was initially studied at these *p*H values.

It was found that the sedimentation constants, $s_{20,w}$, for 0.4 and 0.7% solutions of STI-T compound in 0.1 *M* H_3PO_4–KH_2PO_4 buffer, *p*H 2.9, were 2.6 and 2.7 *S*, respectively, whereas at *p*H 6.2, 0.3, 0.5 and 0.7% solutions of STI-T compound in 0.1 *M* KH_2PO_4–Na_2HPO_4 buffer had $s_{20,w}$ values equal to 3.9, 4.0 and 4.1 *S*, respectively.

Since it was apparent that the inhibitor–trypsin compound dissociated into its components at the lower *p*H, the effect of *p*H on the stability of the STI-T compound was further investigated. Solutions containing 0.5% STI-T compound in 0.1 *M* phosphate buffers in the *p*H range of 1.7 to 10.4 were sedimented in the ultracentrifuge at speeds of 860 r.p.s. The sedimentation constants for these solutions are shown in Table III. Figure 3 represents a plot of $s_{20,w}$ *vs.* *p*H of the 0.5% solutions of STI-T compound. Representative samples of sedimentation diagrams for solutions at *p*H 1.7, 2.9, 6.2 and 10.4 are shown in Fig. 4.

The results of these investigations demonstrated that the STI-T was stable in 0.1 *M* phosphate buffers in the *p*H range of 3.6–10.4. Below *p*H 2.9, the compound completely dissociated into its components as judged by the sedimentation constants being equal to the constants for the STI and trypsin controls. The sedimentation diagrams for STI-T compound showed monodisperse, homogeneous peaks.

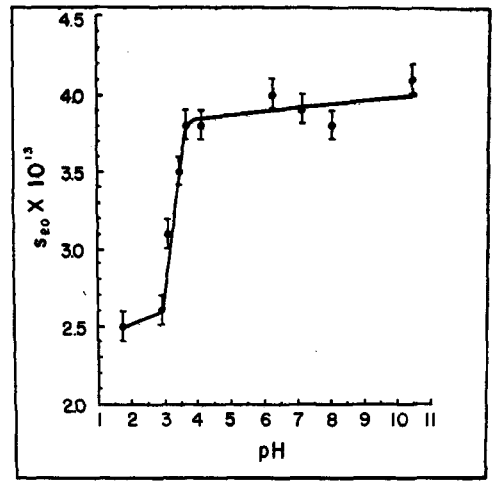

Fig. 3.—Variation of sedimentation constant, $s_{20,w}$, for 0.5% STI-T compound with *p*H.

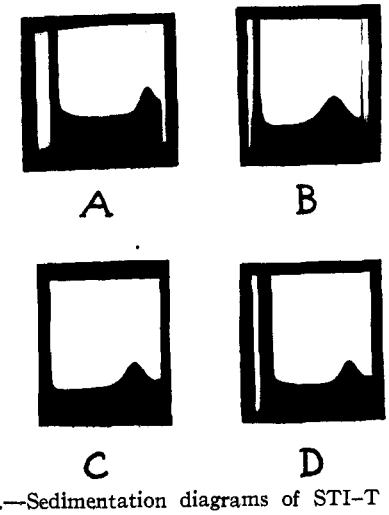

Fig. 4.—Sedimentation diagrams of STI-T compound: A, 0.5% STI-T, *p*H 1.7, $s_{20,w}$ = 2.5 *S* 61 min. after full speed; B, 0.7% STI-T, *p*H 2.9, $s_{20,w}$ = 2.7 *S* 86 min. after full speed; C, 0.5% STI-T, *p*H 6.2, $s_{20,w}$ = 4.0 *S* 53 min. after full speed; D, 0.5% STI-T, *p*H 10.4, $s_{20,w}$ = 4.1 *S* 53 min. after full speed.

TABLE III

SEDIMENTATION CONSTANTS FOR 0.5% SOLUTIONS OF STI-T COMPOUND IN 0.1 *M* PHOSPHATE SOLVENT AS A FUNCTION OF *p*H

*p*H of solution	Solvent composition (0.1 *M*)	$s_{20,w}$ in Svedberg units
1.7	H_3PO_4	2.5
2.9	H_3PO_4, KH_2PO_4	2.6
3.1	H_3PO_4, KH_2PO_4	3.1
3.4	H_3PO_4, KH_2PO_4	3.5
3.6	H_3PO_4, KH_2PO_4	3.8
4.1	H_3PO_4, KH_2PO_4	3.8
6.2	KH_2PO_4, Na_2HPO_4	4.0
7.1	KH_2PO_4, Na_2HPO_4	3.9
8.0	KH_2PO_4, Na_2HPO_4	3.8
10.4	Na_2HPO_4, Na_3PO_4	4.1

The effect of the ionic strength of solvent on the stability of the STI-T compound was also in-

vestigated. The data in Table IV show that 0.5% solutions of STI–T in 0.5, 1.0 and 2.0 *M* NaCl, centrifuged shortly after preparation, had an average $s_{20,w}$ value of 3.8 *S*. Slightly lower sedimentation constants were found for the STI–T solutions in 2.0 and 3.0 *M* NaCl that were centrifuged 48 and 72 hours after preparation. The sedimentation diagrams showed homogeneous, monodisperse peaks similar to the STI–T compound in 0.1 *M* phosphate buffer.

TABLE IV

SEDIMENTATION CONSTANTS FOR 0.5% SOLUTIONS OF STI–T COMPOUND IN NaCl SOLVENT

NaCl, *M*	0.5	1.0	2.0	2.0[a]	3.0[b]
$s_{20,w}$, *S* units	3.8	3.9	3.8	3.4	3.5

[a] Solution made up 48 hours prior to the run. [b] Solution made up 72 hours prior to the run.

Discussion

The sedimentation velocity studies on mixtures of STI and trypsin indicated *p*H dependency of compound formation since, at *p*H 2.8, the sedimentation constants did not increase for mixtures containing increasing amounts of trypsin and a constant amount of STI whereas at *p*H 6.0, there was an increase of $s_{20,w}$ from 2.6 to 3.7 *S*. In the latter series, it was also noted that a maximum sedimentation constant was reached when the weight ratio of STI to trypsin was approximately 1:1. Beyond this ratio of trypsin to STI, the sedimentation constants did not significantly increase any further, thus indicating that the compound has a constant composition.

The sedimentation diagrams of mixtures of STI and trypsin at *p*H 6.0 showed that the main component, namely, the compound, was not resolved from the residual lighter weight component in the centrifugal fields employed. This component, partially obscured by the faster sedimenting compound, contributed to the boundary a trailing edge which did not return to the base line. Thus, the observed peak was asymmetric.

At *p*H 2.8, where compound formation did not occur, the boundaries for mixtures of STI and trypsin are more symmetric. The trailing edges of the boundaries return to the base line, indicating the absence of a lighter component. The sedimentation constants for the series of mixtures at *p*H 2.8 averaged 2.5 *S*, which points to the fact that both components in this medium travelled together at the same velocity, as they independently did during the control determinations.

When the sedimentation behavior of the crystalline STI–T compound was investigated, it was found that it dissociated into its components at *p*H 2.9 as seen by its $s_{20,w}$ being equal to 2.6 *S*, whereas at *p*H 6.2, the $s_{20,w}$ was equal to 4.0 *S*. At the latter *p*H, the boundary was observed to be monodisperse and symmetric. In the concentration range (0.3–0.7%) studied, there was no evidence of concentration dependence of the sedimentation constant.

It is interesting to note that for mixtures of STI and trypsin at *p*H 6.0, the average maximum $s_{20,w}$ value was equal to 3.7 *S*, a value slightly lower than the average of 3.9 *S* for all the determinations of the crystalline STI–trypsin compound. Another apparent incompatibility is the gradual rise of the sedimentation constant to a maximum plateau (Fig. 1) with increasing amounts of trypsin in the mixtures at *p*H 6.0 in spite of the fact that compound formation occurs almost instantaneously in the trypsin–STI reaction. It would be assumed, therefore, that a peak corresponding to the maximum sedimentation constant should be apparent immediately.

These two points can be explained on the basis of a boundary anomaly. Johnston and Ogston[14] experienced a similar boundary anomaly when they studied binary mixtures of β-lactoglobulin, serum albumin and serum γ-globulin. McFarlane[15] and also Pedersen[16] reported an anomalous behavior of the sedimentation boundaries due to the different components in mixtures of proteins. There is an apparent increase of the amount of less rapidly sedimenting component with a corresponding decrease of the amount of the other as estimated from the refractive increments of the boundaries. Johnston and Ogston have shown that the effect is of the nature of a boundary anomaly caused by changes in the concentration of the slower component due to differences in its rates of sedimentation in the presence and absence of a faster component. This would result in an increase of the slower boundary with the corresponding decrease of the faster, as observed refractometrically with the schlieren optical system. In our studies, poor resolution of the two components, that is, the compound and the excess STI, produced a composite boundary exhibiting this anomalous sedimentation behavior. During the early stages of sedimentation, when for purposes of the sedimentation constant calculation numerous photographs are taken of the sharp boundaries before they become diffuse, the peaks are moving at an average rate which is the resultant of the two components.

Investigation of the *p*H dependence of stability of the crystalline compound gives credence to Kunitz' assumptions that the bonds involved in the formation of the stable compound are (at least partly) ionic in nature. This follows from the instability of the compound below *p*H 3. Below *p*H 3 the –COOH groups are non-ionized. This also explains the reason for the absence of compound formation in the mixtures at *p*H 2.8. The reaction of trypsin with STI probably involves an electrostatic attraction between suitably oriented ion pairs, but the number of pairs involved cannot be evaluated since the distance of approach is unknown.[8] Van der Waals binding between polar, non-ionic groups is probably also involved in compound formation since the compound is not dissociated by the addition of sodium chloride.

It is expected that at a high alkalinity, namely, above *p*H 11, the compound would dissociate as it does in the range below *p*H 3. However, no such study above *p*H 11 was made because the trypsin portion of the compound would have become selectively denatured and the sedimentation study

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would have been accordingly complicated. Titration data do indicate dissociation in solutions of high alkalinity, however.[8] It is interesting to note that the *p*H range of stability of the STI–T compound (3.6–10.4) is roughly limited by the isoelectric points of the proteins (*p*H 4.5 for STI[5] and *p*H 10.8 for trypsin[17]).

The ultracentrifugal studies of mixtures of STI and trypsin also indicated that compound formation occurred in the presence of one-half saturated benzoyl-L-arginine. It will be recalled that benzoyl-L-arginine is a product of tryptic hydrolysis of the synthetic substrate benzoyl-L-argininamide and in high concentrations has been shown to compete with the substrate for the active centers on the trypsin molecule.[18–20] Neurath and Schwert[21] believe that the active centers responsible for esterase and amidase activity of trypsin are the same. These centers may also be the sites for the compound formation.

Our findings indicate that either the STI has a greater affinity for the active centers of the enzyme than benzoyl-L-arginine, or else the multi-ionic linkages envelop the active centers of the trypsin molecule.

Dobry and Sturtevant[22] recently reported that the heat of reaction between STI and trypsin as determined calorimetrically at *p*H 2.7, 3.2 and 4.0 is 0 ± 1 kcal./mole of trypsin reacted. There was no *a priori* reason for working in this range of *p*H. In the light of our findings, zero heats of reaction are to be expected at *p*H 2.7 and 3.2 since the reaction does not occur. However, at *p*H 4.0, the reaction between STI and T may be retarded since the STI–T compound starts to dissociate just below this *p*H. The authors assumed that the irreversibility of the reaction indicates that it is accompanied by a large decrease in free energy and consequently a large increase in entropy. Since the reaction between STI and T has been shown to be reversible near this *p*H, additional heats of reaction must be obtained, particularly at *p*H values above 5, before the heat of the reaction can be considered certain.

Diffusion and sedimentation constants for the crystalline STI–T compound were also determined in Svedberg's laboratory in Upsala.[8] Using the Svedberg equation and a value of 0.73 for $\bar{V}$, a molecular weight equal to 41,000 was obtained for the crystalline STI–T compound. If Kunitz' value of 24,000 ± 3000 for the molecular weight of STI is accepted to be correct,[5] then by difference, the molecular weight of trypsin is around 17,000–20,000. Bergold[23] in 1946 reported a molecular weight for trypsin of 15,100. This is in accordance with the unpublished sedimentation data of Schwert[24] in which an $s_{20,w}$ at infinite dilution of 1.8 *S* for trypsin was found.

However, if we take into account the fact that nearly equal weights of trypsin and inhibitor are involved in compound formation, as shown here and by Kunitz, we obtain, from the molecular weight of the compound, a molecular weight of *ca.* 20,000 for trypsin, which is in agreement with the value 20,700 reported by Jansen and Balls.[25] This leaves *ca.* 21,000 for STI which is close to Kunitz' value.

This similarity in the molecular weights of trypsin and STI is substantiated by their identical $s_{20,w}$ values of 2.5 *S*. The sedimentation constant of 2.5 *S* for trypsin is in agreement with the value reported by Bier, *et al.*,[26] for trypsin and with the value reported by Neurath, *et al.*,[27] for the inactive diisopropylfluorophosphate derivative of trypsin. These authors observed aggregation of trypsin which was strongly temperature dependent. However, at constant *p*H and protein concentration, the sedimentation constant was found to be normal at 30°. We did not observe this phenomenon of aggregation perhaps since all the sedimentation determinations were performed near 30°. On the other hand, no indication of dissociation of either STI or trypsin was found in the concentration and *p*H range employed here. The sedimentation constants for trypsin, STI and STI–trypsin compound remained constant within the experimental error as the concentration was varied from 0.3 to 1.0% at *p*H 2.9 and 6.0. For our purposes, only a comparison of sedimentation constants of trypsin, STI and STI–T in the same concentration range was needed.

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