CRYSTALLINE SOYBEAN TRYPSIN INHIBITOR

II. General Properties

By M. KUNITZ

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, New Jersey)

(Received for publication, October 21, 1946)

The isolation of a crystalline trypsin inhibitor from soybean has been reported in previous publications (1). This paper deals with some of the properties of the new crystalline protein and with the mechanism of its inhibiting action on trypsin and chymotrypsin.

The soybean inhibitor is a protein of the globulin type. It is precipitated by trichloracetic acid and is non-diffusable through collodion or cellophane membranes. Its light absorption spectrum is that of a typical protein with a maximum at 280 mμ and a minimum at 252 mμ. The protein contains less than 0.01 per cent phosphorus and is free of carbohydrate. It acts as an inhibitor only when it is in its native state; denaturation of the soy protein by heat, acid, or alkali is accompanied by a loss in its inhibiting power.

The action of the native soybean protein as a trypsin inhibitor is due to its combination with trypsin to form an irreversible stoichiometric compound. The combination is apparently instantaneous.

The soy protein inhibits slightly the proteolytic action of chymotrypsin, but unlike that of trypsin the inhibition is due to the formation of a loose reversible compound of the type described by Northrop (2) for the combination between pepsin or trypsin with crude inhibitors. The reaction between chymotrypsin and the soybean inhibitor was found to agree with the law of mass action, for a reversible uni-unimolecular reaction.

Crystalline soybean protein, if denatured, is readily digestible by pepsin, by chymotrypsin, or by trypsin.

Crystalline soybean inhibitor has no inhibiting effect either on the proteolytic activity or on the milk-clotting ability of pepsin.

EXPERIMENTAL

Test of Purity of Crystalline Soybean Trypsin Inhibitor[1]

1. Effect of Recrystallization.—The principal steps in the procedure for the

[1] For the sake of brevity the terms "soy inhibitor" and "soy protein" are frequently used in the text instead of the full expression "crystalline soybean trypsin inhibitor."

isolation of the crystalline soybean inhibitor are the following: (1) Extraction of soybean meal in 0.25 N H_2SO_4. (2) Adsorption of the inhibitor from the acid extract on bentonite and elution with 5 per cent solution of pyridine in water. The pyridine is removed by dialysis. (3) Precipitation of the inhibitor in amorphous form at pH 4.65. This step is repeated twice. (4) Crystallization at pH 5.0 and 35°C. The extent of purification during the various stages of preparation is shown in Table I.

The material reaches its highest purity after two crystallizations, as shown by measurements of inhibiting activity and also by the Molisch test for carbohydrate impurities. The specific activity of the second mother liquor no longer differs from that of the second crystals. Further crystallization does

TABLE I

Effect of Crystallization on the Purity of Soybean Inhibitor

1000 gm. Nutrisoy XXX flakes.

Preparation	Volume	Total yield	Specific activity	Molisch test for carbohydrate
	ml.	*units of trypsin inhibitor*	*units per mg. protein*	
Acid extract	5,000	10,000	0.38	++++
Dialyzed bentonite elute	475	8,000	0.85	
2nd amorphous precipitate pH 4.65	8–10 gm.	3,000	1.00	
1st crystals	4 gm.	1,500	1.03	?
1st mother liquor			0.88	+
2nd crystals			1.05	−
2nd mother liquor			1.02	−

not have any significant influence on the specific activity of the crystalline protein.

2. Solubility Test for Purity.—The purity of a sample of several times crystallized soybean inhibitor was tested by measuring the solubility of the material in 0.1 M acetate buffer pH 4.6 at 5°C. in the presence of increasing amounts of solid protein in suspension.

The usual procedure of stirring increasing amounts of crystals in a definite volume of solvent until equilibrium is reached was found unsuitable for the soy inhibitor, since stirring brought about gradual denaturation of the protein. It was found more satisfactory to use solutions of various concentrations of the protein and then to bring about saturation by adjusting the pH to that of the isoelectric point of the material. Rapid equilibrium is thus established between the solid phase in form of amorphous precipitate and the saturated solution. The details of the procedure are as follows:

Experimental Procedure.—5 gm. of three times crystallized soy inhibitor were dissolved in 50 ml. 0.1 M sodium acetate at 5°C. The protein was then precipitated in

amorphous state by adding rapidly 50 ml. 0.1 M acetic acid; the precipitate was filtered with suction on hardened paper at about 5°C. The filter cake was redissolved in 50 ml. 0.1 M sodium acetate and reprecipitated again with 50 ml. 0.1 M acetic acid. The precipitation was repeated once more. The protein concentration of the second and third filtrates was determined and found to be practically identical and equal in each case to about 1.5 mg. per ml. The final precipitate was resuspended at 5°C. in 100 ml. of a mixture of equal parts of 0.1 M sodium acetate and 0.1 M acetic acid. Various amounts of suspension, from 0.1 to 5 ml., were then distributed in test tubes containing 5 ml. of cold 0.1 M sodium acetate. This brought about complete solution of the precipitate. The clear solutions were then poured back and forth into test tubes containing 5 ml. cold 0.1 M acetic acid. A precipitate of amorphous protein was immediately formed in every tube except in those containing very small amounts of the original suspension. Samples of 1 ml. were taken for total protein determination and the remaining material was centrifuged at 5°C. The protein concentration of the supernatant solutions was then determined.

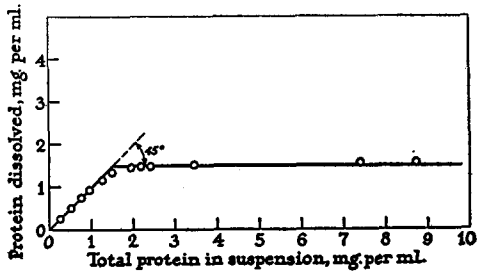

FIG. 1. Solubility curve.

The results are shown in Fig. 1. The solid lines represent the theoretical phase rule curve for a pure substance. According to the phase rule the solubility of a pure substance is independent of the amount of solid phase of the substance present in suspension. The first four points fall on the 45° line since there was complete solution in the mixtures corresponding to those points. The other points lie close to the theoretical horizontal line except for those near the intersection of the straight lines. This irregularity has been observed frequently in the solubility curves of a number of other crystalline proteins and it may be due to the presence of small amounts of denatured protein formed during the equilibration process. The solubility experiment as a whole shows that the material is free of any impurities which can be removed by washing. It does not preclude however the possibility of the presence of an impurity which has a tendency to form a solid solution with the bulk of the material.

Reaction between Crystalline Soybean Inhibitor and Crystalline Trypsin

Addition of increasing amounts of soy inhibitor to a solution of trypsin decreases the proteolytic activity of the trypsin in direct proportion to the amount of soy inhibitor added. Pure soy inhibitor counteracts approximately an equal

weight of pure trypsin. The inhibition is apparently instantaneous and is independent, within a wide range, of the pH of the solution.

The quantitative relationship between the amount of soy inhibitor added and the amount of trypsin inhibited is shown in Figs. 2 and 3.

The amount of trypsin inhibited is directly proportional to the amount of inhibitor used and is independent of the total concentration of trypsin in the inactivation mixture.

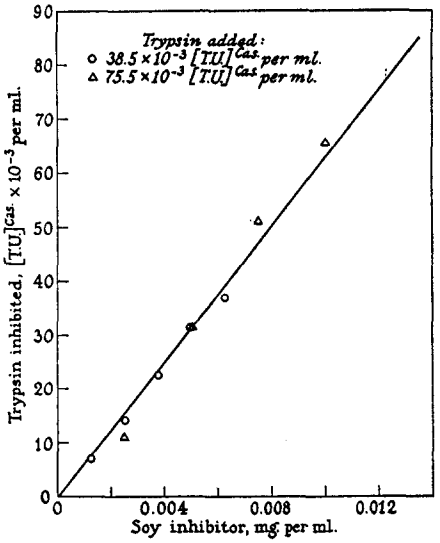

FIG. 2

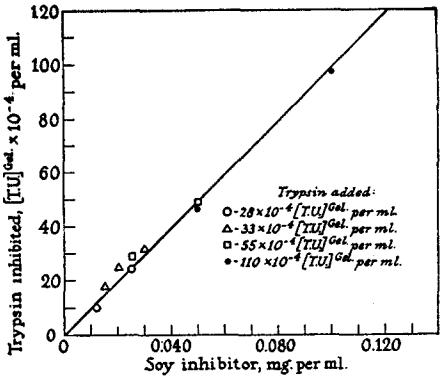

FIG. 3

FIG. 2. Effect of soy inhibitor on the digestion of casein by trypsin.
FIG. 3. Effect of soy inhibitor on the digestion of gelatin by trypsin.

Experimental Procedure.—1 ml. samples of a solution of 50 γ crystalline trypsin per ml. 0.0025 M HCl were mixed with 1 ml. samples containing increasing amounts of soy inhibitor dissolved in 0.0025 M HCl. The amount of inhibitor varied from 0 to 50 γ per ml. in steps of 10 γ. 1 ml. of each mixture was then added to 1 ml. samples of 1 per cent casein pH 7.6 and the tryptic activity was determined as described in the section on Methods. The same experiment was repeated with samples of a stock solution containing 25 γ of trypsin per ml. The results of the two experiments are given in Fig. 2.

The direct proportionality between the amount of inhibitor used and the amount of trypsin inhibited, independently of the total concentration of trypsin in solution, is also shown in Fig. 3. In this case the inactivation mixture was at pH 7.6 and the inhibition was measured by the gelatin formol titration method (see Methods).

The amount of trypsin inhibited per unit weight of inhibitor, when expressed in tryptic units, is independent of the purity of the preparation of trypsin used

and it corresponds approximately to a weight of pure trypsin equal to the weight of inhibitor used. It appears that the reaction between soy inhibitor and trypsin is of the ionic type similar to neutralization of H ion by OH ion. The reaction cannot be reversed either by dilution or by change of pH.

Isolation of a Crystalline Compound of Trypsin and Soybean Inhibitor

A crystalline protein has been isolated from a solution containing crystalline trypsin and crystalline soy inhibitor. The new protein is composed of about equal weights of trypsin and inhibitor proteins. It is inert when added to a solution of casein or gelatin, but it does show either tryptic or inhibitory activity when denatured selectively. The method of isolation of the compound and a description of some of its properties are given in the subsequent paper.

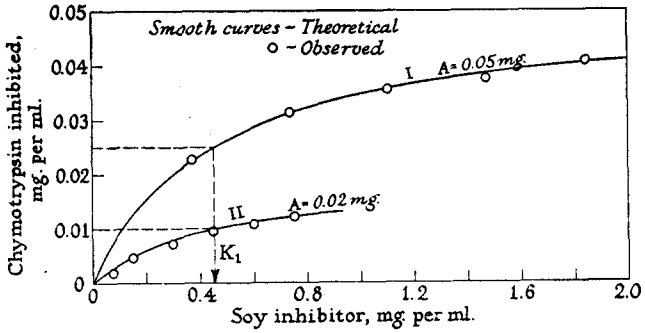

FIG. 4. Effect of soy inhibitor on clotting of milk by chymotrypsin.

Reaction between Chymotrypsin and Soybean Inhibitor

Soy inhibitor exerts a slight inhibiting effect on the proteolytic and the milk-clotting activities of chymotrypsin. The relationship between the amount of inhibitor used and the amount of chymotrypsin inhibited as tested on the ability of chymotrypsin to clot milk is shown in Fig. 4. The plotted curves differ strikingly from those obtained for trypsin (Figs. 2 and 3). The amount of chymotrypsin inhibited per unit weight of inhibitor is small compared to that of trypsin and it decreases rapidly with the relative proportion of total inhibitor and chymotrypsin mixed. The data on the amount of chymotrypsin inhibited when 20 γ per ml. were used fall on a lower curve than the data for 50 γ chymotrypsin per ml. The lack of proportionality between the amount of chymotrypsin inhibited and the soy inhibitor used holds true also for the effect on the digestion of casein, as shown in Fig. 5.

The type of curves obtained is similar to that of the curves obtained by Northrop (2) in his studies of the effect of crude inhibitors on pepsin and trypsin and suggests the same mechanism, namely, that the reaction between the soy inhibitor and chymotrypsin is of the reversible type obeying the law of mass

action so that there is always an equilibrium between the concentration of the product of the reaction and the concentrations of the reactants in solution.

An analysis of the data is simplified by the fact that the total amount of the inhibitor in all the solutions used is large compared with the amount of inhibitor combined with chymotrypsin so that the concentration of free inhibitor equals approximately that of the total inhibitor taken. It is assumed here, as in the case of Northrop's experiments, that the reaction is uni-unimolecular so that one molecule of chymotrypsin combines reversibly with one molecule of inhibitor to form one molecule of an addition compound.

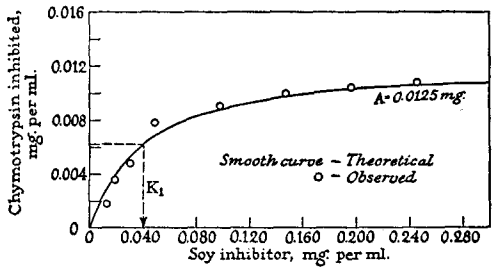

FIG. 5. Effect of soy inhibitor on the digestion of casein by chymotrypsin.

Let M_a and M_b be the molecular weights of chymotrypsin and the soy inhibitor proteins respectively. Let also A and B equal total weights and a and b equal weights of the free chymotrypsin and inhibitor in solution in volume V, then at equilibrium we have, in accordance with the law of mass action for a reversible reaction,

$$\frac{a}{M_a V} \times \frac{b}{M_b V} = K \frac{A - a}{M_a V} \tag{1}$$

$\frac{A - a}{M_a V}$ being the concentration of the compound formed which is numerically the same as the concentration of the inhibited chymotrypsin.

K = equilibrium constant.

Since $b = B$ (approximately) Equation 1 becomes

$$\frac{a}{A - a} = \frac{V}{B} K_1 \tag{2}$$

where $K_1 = KM_b$ and is equal numerically to the value of $\frac{B}{V}$ at 50 per cent inhibition, *i.e.*, when $\frac{a}{A - a} = 1$. Equation 2 can be also written as

$$\frac{A - C}{C} = \frac{VK_1}{B}$$

Solving for C we get

$$C = \frac{AB}{VK_1 + B} \tag{3}$$

Equation 3 was used to calculate the values of C for the theoretical curves given in Figs. 4 and 5 for the relationship between C = the weight of chymotrypsin combined and B = the total weight of inhibitor used, V being equal to 1, since the weights given

TABLE II

Calculation of the Theoretical Curves for the Inhibition of Chymotrypsin by Soybean Inhibitor

Curve	A	K_1	B	C Calculated	C Observed	Equation
	γ		γ	γ	γ	
Fig. 4, I	50	450	367	22.5	22.7	
			734	31.0	31.2	
			1100	35.5	35.5	$C = \frac{50B}{450 + B}$
			1470	38.2	37.4	
			1840	40.2	40.6	
Fig. 4, II	20	450	75	2.9	2.0	
			150	5.0	4.6	
			300	7.0	7.2	$C = \frac{20B}{450 + B}$
			450	10.0	9.1	
			600	11.4	10.5	
			750	12.5	12.1	
Fig. 5	12.5	40	12.2	2.9	1.7	
			18.3	3.9	3.5	
			30.5	5.4	4.8	
			49.0	6.9	7.8	$C = \frac{12.5B}{40 + B}$
			98	8.9	9.1	
			147	9.9	10.0	
			196	10.4	10.5	
			245	10.7	10.8	

were expressed per unit volume. The value of K_1 was read in each case at $C = 0.5\,A$ on a preliminary smooth curve drawn between the experimental points in the same region.

The calculated values of C are given in Table II. They are identical, within experimental error, with the observed data given in the same table. The experimental results are in agreement with the theoretical assumption that the mechanism of inhibition of chymotrypsin by soybean inhibitor consists in the formation of a uni-unimolecular compound in equilibrium with free chymotrypsin and soy inhibitor in solution. Equation 3 shows that the amount of chymotrypsin inhibited per unit weight of soy inhibitor is proportional to the total

amount of chymotrypsin in solution and is decreased with the increase in amount of inhibitor used and with dilution; in the case of trypsin, the amount of trypsin inhibited per unit weight of soy inhibitor is constant and is equal approximately to the weight of inhibitor used, independent of the total concentration of trypsin or inhibitor.

It is to be noticed that the value of K_1 while identical in Fig. 4 for curve I and curve II, differs from the value of that constant given in Fig. 5. The concentrations of the reactants given in all cases have been expressed in weights per milliliter of the inactivation mixtures, without considering the further dilution and further changes caused on addition of samples of 1 ml. of the mixture to the substrates used for activity measurements. The sample was added

TABLE III

Effect of Soy Inhibitor on Clotting of Milk by Pepsin

Pepsin-inhibitor mixtures					
Pepsin 4 γ per ml. 0.1 M acetate buffer pH 5.0, *ml.*....	0.5	0.5	0.5	0.5	0.5
Soy inhibitor 1 mg. per ml. acetate buffer pH 5.0, *ml.*.	0	0.2	0.3	0.4	0.5
Made up to 1 ml. with 0.1 M acetate buffer pH 5.0. Mixtures left in room for 10 minutes, then 0.5 ml. of each added to 2.5 ml. of 20 per cent Klim milk in 0.1 M acetate buffer pH 5.0 at 36°C. and time of clotting observed.					
Clotting time, *min*.....	3.5–4.9	3.5	3.5	3.3	3.0
Repeated with same pepsin inhibitor mixtures after standing at 25°C. for 3 hrs.					
Clotting time, *min*.....	3.5	3.1	2.9	2.9	2.8

to 2.5 ml. of 20 per cent solution of dry Klim milk of pH 5.8 in the experiments on milk clotting or to 1 ml. of 1 per cent solution of casein pH 7.6. Hence the difference in the equilibrium constant in the two cases.

The thoretical Equation 2 has been checked directly by substituting the experimental data and solving for K_1; approximately constant values of K_1 were obtained equal to those given in Table II.

Effect on Pepsin

Soy inhibitor has no inhibiting effect on pepsin, either on its proteolytic activity at pH 2.0 or on its ability to clot milk at pH 5.8. At pH 2.0 soy inhibitor is digestible by pepsin. The experiment on clotting of milk is given in Table III.

The Globulin Nature of Soy Protein

A globulin is defined as a protein which in its native state has a minimum solubility at the isoelectric point; the solubility increases in the presence of

salt. In accordance with this definition, the soy inhibitor protein is a typical globulin being least soluble at its isolectric point in the absence of salt. Addition of salt however increases its solubility.

(*a*) *Solubility and pH. Isoelectric Point.*—

Experimental Procedure.—Samples of 0.1 ml. of a stock suspension of 10 mg. of crystals of soy inhibitor per ml. of H_2O of pH about 4.5 were added to 10 ml. 0.02 M acetate buffers of varied pH. The pH and turbidity of the various mixtures were measured after standing for several hours at about 25°C. The cataphoretic mobility of the crystals in the same mixtures was measured the same day. The results are given in Table IV.

TABLE IV

Isoelectric Point of Crystalline Soybean Trypsin Inhibitor and Solubility

pH (quinhydrone electrode)	4.15	4.27	4.49	4.70	4.81	4.87	5.00	5.12	5.34	5.50	5.65	5.80
Light absorption measured at 600 mμ												
Optical density	0.095	0.150	0.196	0.195	0.135	0.070	0.045	0.043	0.040	0.034	0.038	0.032
Cataphoretic mobility: 1 extra drop of concentrated suspension of crystals added to the solution above pH 4.80												
Mobility in μ per sec. per volt per cm.	+1.75	+0.88	− trace	−1.17	−1.45	−1.82	−2.20	−2.20	−2.9	−3.1		

TABLE V

Effect of Salts on Solubility of Soy Inhibitor at pH 4.5

3 ml. samples of a stock suspension of 100 mg. of soy inhibitor in 20 ml. water at pH 4.5 were mixed with 3 ml. of various salt solutions in 0.02 M acetate buffer pH 4.5.

Final salt solutions	0.01 M acetate buffer pH 4.5	0.1 M acetate buffer pH 4.5	0.1 M NaCl	0.05 M Na_2SO_4	0.05 M $MgSO_4$	0.1 M $MgSO_4$
			All in 0.01 M acetate buffer pH 4.5			
Suspensions left 18 hrs. at 10°C., then 1 hr. at 25°C. Centrifuged clear at 2500 R.P.M. for 10 min.						
Mg. protein per ml. supernatant	0.73	0.95	1.57	1.51	1.565	2.01
(Determined by measuring optical density at 280 mμ)						

The crystals of the soy inhibitor are least soluble at the point of minimum cataphoretic mobility, the isoelectric point, which is at pH 4.5.

(*b*) *Effect of Salts on the Solubility of Soy Inhibitor at the Isoelectric Point of the Protein.*—The solubility of the crystals of soy inhibitor at its isoelectric point is considerably increased in the presence of salts. This is shown in Table V. The crystals are more soluble in the presence of magnesium ions than in the presence of an equivalent concentration of sodium ions.

Stability and Denaturation of Soy Inhibitor

Crystalline soy inhibitor is stable in the range of pH of 1–12 when dissolved in dilute buffer solution and stored at temperatures below 40°C. At higher

temperatures and in stronger acid or alkaline solutions the protein is gradually denatured, as evidenced by a decrease in its solubility at the isoelectric point or in salt solutions. The gradual denaturation of the protein is accompanied by a corresponding loss in its ability to inhibit the action of trypsin.

1. Denaturation in 0.1 M NaOH.—

A solution of 0.1 M NaOH containing 2.5 mg. soy inhibitor per ml. was left at 36°C. 2 ml. samples were taken after various intervals of time, neutralized with 2 ml. of 0.1 M HCl, and analyzed for denatured protein and inhibiting activity. The amount of denatured protein was determined by mixing 2 ml. of the neutralized solution with 4 ml. of 0.1 M acetate buffer pH 4.5 and centrifuging the precipitate formed after

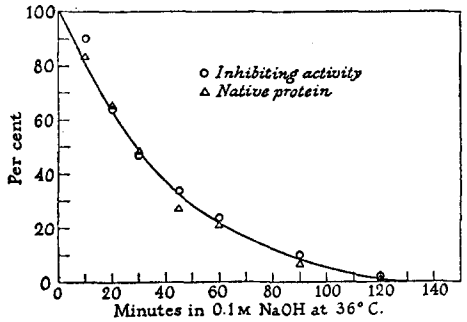

FIG. 6. Denaturation of soy inhibitor in 0.1 M NaOH at 36°C.

standing about 1 hour at room temperature. The precipitate consisted of denatured protein while the supernatant solution contained native protein. The concentration of protein in the supernatant solution was measured spectrophotometrically at 280 mμ. The inhibiting activity was determined on samples of the neutralized solution without addition of acetate buffer.

The results of the experiment are given in Fig. 6. The gradual loss in native protein is accompanied by a corresponding percentage loss in inhibiting activity.

Denaturation in 0.1 M HCl at 50°C. or higher gave results similar to those obtained on denaturation in 0.1 M NaOH.

2. Reversible Denaturation by Heat.—Soy inhibitor protein like many other proteins becomes denatured when heated in dilute acid or alkaline solution at temperatures above 40°C. The denaturation in the absence of salts is not accompanied by any visible precipitation of denatured material. The denatured protein is readily precipitable on addition of salt or on adjusting the pH of the heated solution to that of the isoelectric point. The denaturation is reversed on cooling. Prolonged heating however brings about permanent denaturation. The following experiments show that denaturation on heating and also the reversal of the denaturation on cooling as well as irreversible denaturation are accompanied by a corresponding loss or gain in the inhibiting activity.

(*a*) *Denaturation (Reversible) at 70°C.*—Samples of 2 ml. 0.1 per cent solution of soy inhibitor of pH about 3.0 (in 0.0006 M HCl) were placed in a water bath at 70°C., removed at various times, cooled to about 5°C., and mixed with 4 ml. of 0.15 M acetate buffer pH 4.5. The precipitates formed were centrifuged after standing 1 hour in the room. The supernatant solutions were analyzed for protein and inhibiting activity.[2] The results are shown in Fig. 7.

(*b*) *Reversal of Denaturation at 30°C.*—25 ml. of a 0.25 per cent solution of crystalline soy inhibitor in 0.0025 M HCl were heated at 80°C. for 5 minutes, then transferred to a water bath at 30°C. Samples of 1 ml. were mixed at various times with 5 ml. 0.06 M acetate buffer pH 4.5 and treated as described in (*a*). The results are shown in Fig. 8.

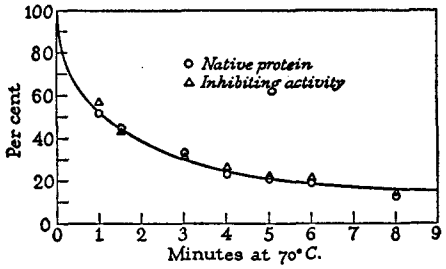

FIG. 7. Reversible denaturation of soy inhibitor at 70°C. and pH 3.0.

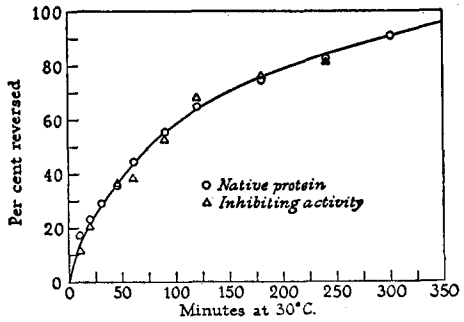

FIG. 8. Reversal of denaturation.

(*c*) *Irreversible Denaturation at 90°C.*—Samples of 1 ml. solution of 0.5 per cent crystalline soy inhibitor in 0.0025 M HCl were heated at 90°C. for various lengths of time and stored at 20°C. for 18 hours. Each sample was then mixed with 5 ml. 0.06 M acetate buffer pH 4.5 and treated as described in (*a*).

The results are shown in Fig. 9.

The denaturation by heat and also the reversal of denaturation on cooling proceed at a measurable rate. At temperatures above 40° and below 60°C. denaturation and the reversal of denaturation proceed until a point of equi-

[2] Activity measurements when done on the uncentrifuged suspension gave higher values, possibly because of reversal of denaturation in the digestion mixture at pH 7.6.

librium is reached between the amount of native and denatured protein in solution. The equilibrium values depend on the temperature and the pH of the solution. Studies of the kinetics and the thermodynamics of reversible denaturation of crystalline soybean inhibitor protein will be described in a separate paper.

Digestion of Soy Inhibitor Protein by Proteolytic Enzymes

1. Digestion by Pepsin.—Crystalline soy inhibitor protein, if denatured, is readily digestible by pepsin in slightly acid solution. Native soy inhibitor protein is hardly affected by pepsin at pH 3.0. However, in more acid solution, even native soy protein is gradually digested, though only at a rate of less than $\frac{1}{500}$ of that of denatured soy protein.[3] The gradual digestion of native soy inhibitor protein by pepsin at pH 2.0 is accompanied by a proportional loss in trypsin-inhibiting activity, so that no significant and definite change in the specific activity of the soy protein is brought about by treatment with pepsin.

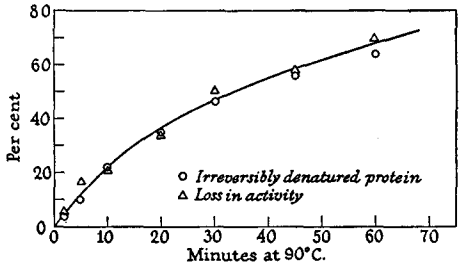

FIG. 9. Irreversible denaturation at 90°C.

Experimental Procedure.—(*a*) Digestion at pH 3.0 of denatured soy inhibitor: A tube containing 9 ml. of 0.25 per cent solution of soy inhibitor in 0.0025 M HCl was heated for 5 minutes at 90°C. and cooled for 2 minutes at 5°C. One ml. of 0.1 per cent solution of crystalline pepsin in 0.0025 M HCl was added and the mixture left at 30°C. Samples of 1 ml. were taken at various times and added to 5 ml. of boiling hot 5 per cent (0.3 M) trichloracetic acid. The precipitate was centrifuged after standing for about 1 hour at room temperature. The protein digest content of the clear supernatant was determined by measuring the optical density at 280 mμ. The results are given in Fig. 10, curve I.

(*b*) Digestion of native soy inhibitor at pH 3.0: Same procedure as in (*a*) except that the soy inhibitor solution had not been heated at 90°C. The results are given in Fig. 10, curve III. No digestion was observed during 6 hours.

(*c*) Digestion of soy inhibitor which was first denatured and then reversed: Same procedure as in (*a*), except that the soy solution after it had been heated to 90°C. was allowed to stand for 18 hours at 25°C. before the pepsin was added. See Fig. 10, curve II. The slight initial rise in curve II may be due to the presence of a small amount of irreversibly denatured protein, which was rapidly digested, the digestion then stopped.

[3] It is possible that at pH 2.0 or lower the protein becomes gradually denatured to a slight extent, and it is the denatured protein that is digestible by the pepsin.

(*d*) Digestion at pH 2.0: Same procedure as in (*a*) and (*b*), except that the solution of soy inhibitor was in 0.01 M HCl. The results are shown in Fig. 11.

(*e*) Loss of inhibiting activity on digestion of soy protein by pepsin at pH 2.0: Same procedure as in (*b*) except that samples of the pepsin digestion mixture were also tested for trypsin-inhibiting activity, in addition to those tested for loss in protein.

The results given in Fig. 12 show that the loss in activity is parallel to the gradual digestion of the soy protein by pepsin.

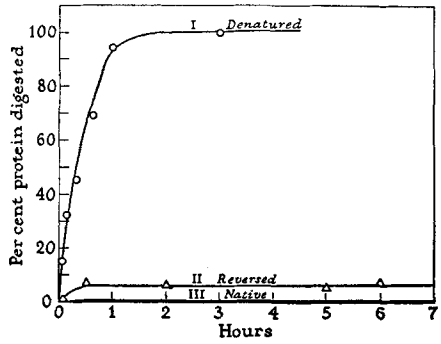

FIG. 10. Digestion of soy inhibitor by pepsin at pH 3.0.

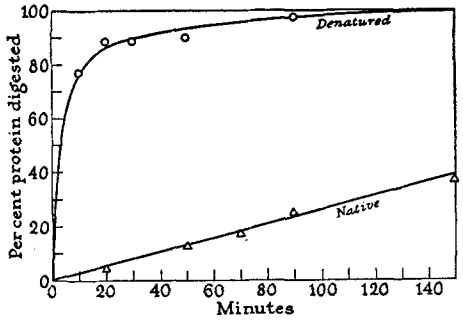

FIG. 11. Digestion of soy inhibitor by pepsin at pH 2.0.

2. Digestion of Soy Inhibitor Protein by Trypsin and Chymotrypsin.—Soy inhibitor, if denatured, is digestible by trypsin and chymotrypsin. However, in order to become susceptible to digestion by these enzymes the soybean protein has to be denatured more vigorously than when tested for pepsin digestion; the range of pH favorable for the action of trypsin and chymotrypsin is also favorable for the rapid reversal of denaturation of the soy inhibitor with the resulting inhibition of the proteolytic enzymes. It was found necessary to heat soy protein in 0.1 M NaOH for 10 minutes at 100°C. in order to make the protein susceptible to the digestive action of small amounts of trypsin or chymotrypsin. High concentrations of these enzymes undoubtedly digest soy protein even when less vigorously denatured. The measurement of digestion in the presence of relatively high concentrations of the enzymes is complicated by the autolysis of the enzymes, so that the measurements reflect

not only the amount of substrates digested but also the digestion of the enzymes themselves.

Experimental Procedure.—Stock solution of 0.5 per cent of soy inhibitor in 0.1 M NaOH was heated for 10 minutes at 100°C., and cooled.

Digestion Mixture.—5 ml. of heated stock solution + 5 ml. 0.1 M HCl + 1 ml. 0.5 M phosphate buffer pH 7.4 + 1 ml. trypsin (0.2 mg.) in 0.0025 M HCl, or 1 ml. chymo-

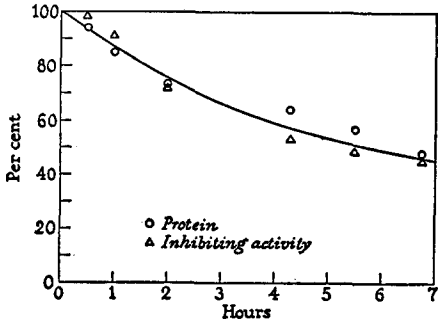

FIG. 12. Loss of trypsin-inhibiting activity of the soybean protein when digested by pepsin.

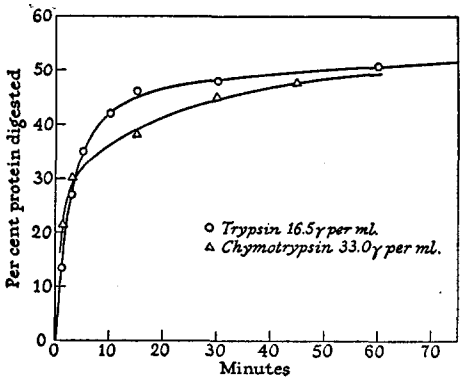

FIG. 13. Digestion of denatured soy inhibitor protein by trypsin and chymotrypsin.

trypsin (0.4 mg.). The mixture was left at 25°C. Samples of 1 ml. were mixed with 5 ml. of 5 per cent trichloracetic acid and centrifuged after standing several hours. Optical density of supernatant solutions was measured at 280 mμ. Corrections were made for the density reading of a blank in which a sample was mixed with trichloracetic acid before addition of trypsin or chymotrypsin. The results are shown in Fig. 13.

The rate of digestion was rapid in the initial stage of the reaction and slowed considerably at about 50 per cent digestion. The slowing of the digestion may be partly due to partial reversal of the substrate to native state at pH 7.6., enough to inhibit the proteolytic action of the enzymes, especially that of trypsin. It may also be due to a hydrolytic change brought about in the soy protein on heating in 0.1 M NaOH at 100°C., as evidenced by an increase in cor-

rection for the blank (before addition of trypsin) over that of an unheated sample.

Chemical and Physical Properties of Soy Inhibitor

A summary of some of the chemical and physical properties of the soy inhibitor protein is given in Table VI.

TABLE VI

Chemical and Physical Properties of Crystalline Soybean Trypsin Inhibitor

Elementary analysis in per cent dry weight*	C.....	51.95
	H.....	7.16
	N.....	16.74
	S.....	0.97
	P.....	0.00
	Ash....	0.10
Tyrosine, *per cent dry weight*‡		4.0
Tryptophane, *per cent dry weight*§		2.2
Free amino nitrogen, *per cent total N*‖		4.0
Total Cu-phenol reagent color value, *mg. tyrosine equivalents per mg. protein*¶		0.21
Optical rotation $[\alpha]_{Na}^{D}$ per gm. protein per ml. at pH 3.0		−105.0
Extinction coefficient at 280 mμ and at pH 3.0. Density per mg. protein per ml		0.91
Isoelectric point		pH 4.5
Molecular weight, by osmotic pressure measurement**		24000 ± 3000
Diffusion coefficient‡‡		0.07 − 0.08 cm.² per day at 24°C.

* Analysis carried out by Dr. A. Elek of The Rockefeller Institute, New York.

‡ Kindly determined by Miss Jean Grantham in the laboratory of Dr. E. Brand in the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, following NaOH hydrolysis by the method of Brand, E., and Kassell, B., *J. Biol. Chem.*, 1939, **131**, 489.

§ Colorimetric method of R. W. Bates (4). 1 ml. soy inhibitor containing about 5.0 mg. protein + 0.2 ml. 2.5 per cent $NaNO_2$ in H_2O + 0.5 ml. 5.0 per cent *p*-dimethyl-aminobenzaldehyde in 10 per cent H_2SO_4 + 15 ml. concentrated HCl.

Mixture left for 15 minutes in room then made up to 50 ml. with 50 per cent alcohol. Color was compared with that of 2.5 mg. of chymotrypsinogen treated in the same manner. The tryptophane content was calculated on the basis of 0.055 mg. tryptophane per milligram of chymotrypsinogen as determined by Brand and Kassell (5).

‖ Determined by formol titration (6).

¶ Method of Herriot (7). 1 ml. containing approximately 1 mg. protein + 0.0025 M $CuSO_4$ + 8 ml. 0.5 M NaOH + 3 ml. dilute Folin-Ciocalteau's phenol reagent (3) (1 part + 2 parts of water). The reagent is added drop by drop at a rapid rate. The color developed is compared with a similar mixture containing 0.205 mg. of tyrosine. The undiluted Folin-Ciocalteau's reagent is supplied by Hartman-Leddon Co., Philadelphia.

** Method of Northrop and Kunitz (8). Measurements in 0.5 M and 1 M NaCl pH 4.5 also in 0.5 M $MgSO_4$ pH 4.8.

‡‡ Method of Northrop and Anson (9).

Methods

1. Estimation of Trypsin and Trypsin-Inhibitor Activities.—

Trypsin activity was measured either by the method of formol titration of gelatin or by digestion of casein.

(*a*) *Gelatin-Formol Method.*—The method is essentially the same as described before (10).

Digestion Mixture.—1 ml. of trypsin solution containing 0.01 to 0.05 mg. is mixed in a 50 ml. pyrex tube with 5 ml. of 5 per cent gelatin dissolved in 0.1 M phosphate buffer pH 7.6 and left at 35°C. for 20 minutes. The following reagents are then added in this order:

1 ml. formaldehyde, Merck Reagent

0.5 ml. 0.1 per cent phenolphthalein in 95 per cent alcohol

2 ml. 0.1 M NaOH

The mixture is titrated with 0.02 M HCl to the color of a standard.

The Color Standard.—

5 ml. 5 per cent gelatin

1 ml. formaldehyde

3 ml. H_2O

1 drop of 0.1 per cent phenolphthalein

Several drops of 1 M NaOH to maximum pink color

A blank is prepared in the same way as the "digestion mixture" except that the formaldehyde is added to the gelatin before addition of trypsin.

It is preferable to adjust the pH of the stock of 5 per cent solution of gelatin with 5 M NaOH so that the blank should require a titration of about 3 to 4 ml. of 0.02 M HCl. The range of concentration of trypsin used is such that the highest concentration of trypsin requires a titration of 0.5 to 1 ml. 0.02 M HCl.

The acid formed in the digestion mixture equals the blank titration value minus the titration value for the digestion mixture.

The method of calculation of tryptic activity is the same as that described for the casein method. One $[T.U.]^{Gel.\ F.}$ = 1 milliequivalent acid formed per minute in the 6 ml. digestion mixture.

(*b*) *Casein Digestion Method.*—A stock solution of casein is made by suspending 1 gm. of casein (preferably "Hammarsten") in 100 ml. 0.1 M Sorensen's phosphate buffer pH 7.6. The suspension is heated for 15 minutes in boiling water, thus bringing about complete solution of the casein. The solution, designated as 1 per cent casein, is stored in the refrigerator and is stable for about a week or longer. Samples of 1 ml. of 1 per cent casein are pipetted into 15 ml. pyrex test tubes and placed in a water bath at 35°C. for about 5 minutes before being used.

The Trypsin Standard Curve.—One ml. samples of crystalline trypsin dissolved in 0.0025 M HCl or in a suitable buffer solution are added to samples of 1 ml. casein at intervals of about 1 minute, mixed well, and left at 35°C. for 20 minutes. The solutions are then poured back and forth into tubes containing 3 ml. of 5 per cent trichloracetic acid. The precipitates formed are centrifuged after standing 1 hour or longer at about 25°C. The concentration of split products in the supernatant solutions is determined either by the Cu-phenol reagent method as described in footnote ¶ (Table

VI) on page 305 of this paper or by measuring the optical density of the solutions at 280 mμ. The optical density method is simpler and has been used throughout the present studies. The readings are corrected for blank solutions which are prepared by mixing 1 ml. of 1 per cent casein solution with 3 ml. of 5 per cent trichloracetic acid and then adding 1 ml. of the highest concentration of trypsin tested or 1 ml. of the buffer solution used in making up the trypsin dilutions. The corrections for blanks for the intermediate concentrations of trypsin are calculated by interpolation. The readings (corrected for blanks) are plotted as shown in Fig. 14. The plotted curve can be used for the determination of tryptic activity of any sample of material by reading the milligrams of trypsin corresponding to the corrected optical density reading of the sample. The activity is then expressed in terms of the sample of crystalline trypsin used for drawing the standard curve. A more general way is to calculate the specific activity of the trypsin and then replot the curve in terms of density *vs.* tryptic units.

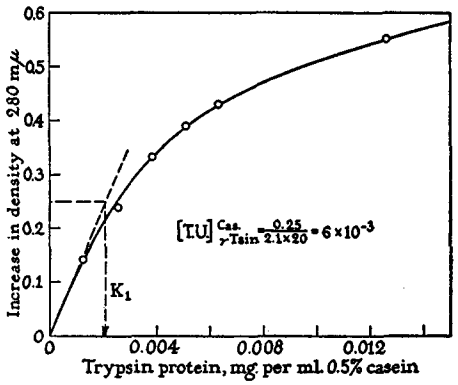

FIG. 14. Standard curve for digestion of casein by trypsin. Optical density at 280 mμ plotted *vs.* milligrams of trypsin protein.

The tryptic unit is defined as the activity which gives rise, under the conditions described, to an increase of one unit of optical density at 280 mμ per minute digestion, and is designated as $[T.U.]^{Cas.}$ The specific activity of the sample of trypsin used is obtained by drawing a straight line tangent to the first part of the curve. In Fig. 14, the slope $\frac{0.25}{2.1\gamma}$ (indicated by the dotted lines) divided by 20 minutes is the specific activity of the given material, *i.e.* the activity per microgram trypsin protein, *i.e.* $[T.U.]^{Cas.} = \frac{0.25}{2.1 \times 20} = 6 \times 10^{-3}$.

A new curve is then plotted (Fig. 15), the ordinates of which are identical with those in Fig. 14, while the abscissae are expressed in tryptic units, one γ being equal to 6×10^{-3} $[T.U.]^{Cas.}$ The data for Fig. 15 are conveniently obtained by reading the densities corresponding to 1γ, 2γ, 4γ, 6γ, etc., off the smooth curve of Fig. 14 and then plotting these values as ordinates against $6, 12, 24, 36 \times 10^{-3}$ [T.U.] as abscissae. The data on the new curve are independent of the purity of the sample of trypsin used and

hence it can be employed as a general standard curve for determination of tryptic activity, provided the same stock of casein is used and under the same experimental conditions of pH, temperature, etc.

The proteolytic activity of chymotrypsin is determined in the same way as that of trypsin.

(*c*) *Trypsin Inhibitor Activity Measurements.*—Inhibitor activity is expressed in terms of units of trypsin inhibited, and the measurement consists simply in comparing the tryptic activity of two samples of trypsin, one containing a definite amount of inhibitor and the other sample being free of inhibitor. The difference in the tryptic activity of the two samples of trypsin, provided the inhibitor is not in excess, expressed

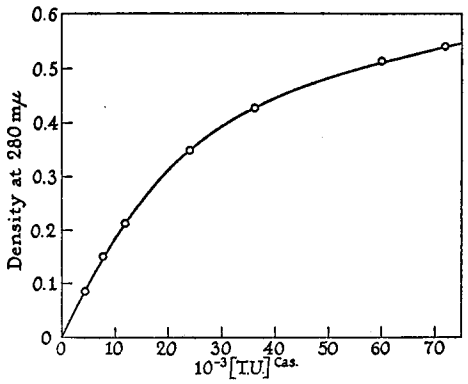

FIG. 15. Standard curve for digestion of casein by trypsin. Optical density at 280 mμ plotted *vs.* tryptic units.

in [T.U.] or in weight of pure trypsin divided by the weight of the inhibitor used is a measure of its specific activity.

Experimental Procedure.—Samples of 1 ml. containing 50 γ trypsin dissolved in 0.0025 M HCl were mixed with 1 ml. containing various amounts of soy inhibitor dissolved in 0.0025 M HCl. 1 ml. of each mixture added to 1 ml. of 1 per cent casein pH 7.6 was digested 20 minutes at 35°C., then mixed with 3 ml. 5 per cent trichloracetic acid and treated as described before. The measurements and the calculations are given in Table VII. The average specific activity of the inhibitor is about 1.0 when expressed in terms of weight of pure trypsin inhibited.

Crystalline soybean inhibitor because of its stability and purity can be used as a convenient standard for assaying samples of trypsin. The reaction with trypsin is independent of the method used for measuring the proteolytic activity of trypsin.

2. Protein Determination.—

(*a*) *Total N by Kjeldahl.*—The protein concentrations used in this paper were based on the total nitrogen determined by a semi-micro Kjeldahl method, 1 mg. of nitrogen being equivalent to 6.0 mg. of soybean protein.

Digestion Mixture.—1 ml. sample containing 2 to 5 mg. protein + 1 ml. concentrated H_2SO_4 + 1 drop selenium oxychloride + 0.25 gm. K_2SO_4 + several alundum chips. Digested 5 to 10 minutes in 100 ml. pyrex Kjeldahl flask, cooled, and 5 ml. H_2O added,

then steam distilled in a ground-glass-jointed outfit, in the presence of 5 ml. 30 per cent NaOH.

The distillate is received in a flask containing 5 ml. 0.02 M HCl and is titrated with 0.02 M NaOH from a burette graduated to 0.01 ml. using methyl red as indicator.

(*b*) *Colorimetric Method by Means of Cu-Phenol Reagent According to Herriott as Described in Footnote* ¶ (*Table VI*), *page 305*.—The color developed is measured in a colorimeter or spectrophotometer at 600 mμ. The protein concentration is read on a standard curve obtained by plotting colorimeter or density readings *vs.* known concentrations of protein as determined by the Kjeldahl N method.

(*c*) *Optical Density Measurement at 280 mμ.*—A very convenient way of estimating protein in clear solutions is by measuring the ultraviolet light absorption at 280 mμ. The density readings are proportional to the concentration of protein up to density

TABLE VII

Trypsin Inhibiting Activity of Soy Inhibitor

Soy inhibitor per ml. 0.5 per cent casein, γ	0	2.5	5.0	7.5	10.0	12.5
Optical density at 280 mμ (corrected for blank)	0.550	0.515	0.455	0.348	0.185	0.008
10^{-3} [T.U.]$^{Cas.}$ read on curve, Fig. 15	75.5	61.5	43.7	25.5	10.5	0
10^{-3} [T.U.]$^{Cas.}$ inhibited (by difference)	0	14.0	31.8	50.0	65.0	75.5
Specific activity 10^{-3} [T.U.]$^{Cas.}$ per γ inhibitor		5.6	6.4	6.7	6.5	6.0
Average	1 γ inhibitor $\doteq 6.2 \times 10^{-3}$ [T.U.]$^{Cas.}$ $\doteq$ 1.03 γ trypsin					

readings of almost 1.0. The proportionality constant varies, however, with different proteins.

The factors for calculating protein concentration from density measurement at 280 mμ are:

Soy bean inhibitor 1.10
Crystalline trypsin 0.585
Crystalline chymotrypsin 0.500.

The writer has been assisted in this work by Miss Barbara Brodsky.

SUMMARY

A study has been made of the general properties of crystalline soybean trypsin inhibitor. The soy inhibitor is a stable protein of the globulin type of a molecular weight of about 24,000. Its isoelectric point is at pH 4.5. It inhibits the proteolytic action approximately of an equal weight of crystalline trypsin by combining with trypsin to form a stable compound. Chymotrypsin is only slightly inhibited by soy inhibitor. The reaction between chymotryp-

sin and the soy inhibitor consists in the formation of a reversibly dissociable compound.

The inhibitor has no effect on pepsin.

The inhibiting action of the soybean inhibitor is associated with the native state of the protein molecule. Denaturation of the soy protein by heat or acid or alkali brings about a proportional decrease in its inhibiting action on trypsin. Reversal of denaturation results in a proportional gain in the inhibiting activity.

Crystalline soy protein when denatured is readily digestible by pepsin, and less readily by chymotrypsin and by trypsin.

Methods are given for measuring trypsin and inhibitor activity and also protein concentration with the aid of spectrophotometric density measurements at 280 mμ.

REFERENCES

1. Kunitz, M., *Science*, 1945, **101**, 668; *J. Gen. Physiol.*, 1946, **29**, 149.
2. Northrop, J. H., *J. Gen. Physiol.*, 1920, **2**, 471; 1922, **4**, 245.
3. Folin, O., and Ciocalteau, V., *J. Biol. Chem.*, 1927, **73**, 629.
4. Bates, R. W., *J. Biol. Chem.*, 1937, **119**, p. vii.
5. Brand, E., and Kassel, B., *J. Gen. Physiol.*, 1941, **25**, 167.
6. Northrop, J. H., *J. Gen. Physiol.*, 1926, **9**, 767.
7. Herriott, R. M., *Proc. Soc. Exp. Biol. and Med.*, 1941, **46**, 642.
8. Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1926, **9**, 354.
9. Northrop, J. H., and Anson, M. L., *J. Gen. Physiol.*, 1929, **12**, 543.
   Anson, M. L., and Northrop, J. H., *J. Gen. Physiol.*, 1937, **20**, 575.
10. Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1932, **16**, 313.