the available protons would originate from the free carboxyl of aspartic acid a high degree of specificity would obtain. Detailed arguments for this view are reviewed by Leach (1955). The high yield of aspartic acid–free peptides obtained from ribonuclease has been reported (Schultz, 1961). Greater insight into the various degrees of nonspecific cleavage taking place at 105° in 0.03 N HCl is offered by the data presented here on three proteins of known amino acid sequence.

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Studies of Soybean Trypsin Inhibitor. I. Physicochemical Properties*

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Crystalline soybean trypsin inhibitor (STI) was found to be fairly homogeneous by ultracentrifugal measurements and by gradient chromatography on diethylaminoethyl cellulose (DEAE-cellulose). Its partial specific volume in 1 M KCl at 20.0° and its Archibald molecular weight are 0.698 ± 0.006 ml/g and 21,500 ± 800 g/mole respectively. A hydrogen ion titration curve of STI has been obtained in 1 M KCl at 25.0°. The ionizing groups and their intrinsic pK values found are 35 carboxyl (4.21), 2 imidazole (6.45), 1 α-amino (7.8), 11 ϵ-amino (9.9), and 4 tyrosyl (9.5). The guanidyl groups affect the titration curve only through the net charge, Z, of STI, because of their high intrinsic pK, the large negative charge of the protein at high pH, and the large value of the electrostatic factor, w. The pK values for the carboxyl groups are abnormally low; this suggests the presence of local interactions involving these groups. Empirical values of the electrostatic factor, w, are found to be 0.026 for carboxyl groups and 0.060 for tyrosyl groups in 1 M KCl. This increase in w suggests that the conformation of the molecule may be dependent on pH, a conclusion which will be discussed in a forthcoming paper in connection with optical rotation and ultraviolet difference spectra measurements.

Theoretical and experimental studies of pH-dependent, reversible denaturation in dilute solution offer the possibility of providing us with information about the nature of side-chain interactions and conformation in protein molecules (Scheraga, 1960, 1961a,b). As a further experimental test of current ideas about denaturation, a study of crystalline soybean trypsin inhibitor (STI) was undertaken. This protein was selected since Kunitz (1947) had reported its denaturation to be reversible.

This paper is a report of various physicochemical experiments on STI, the results of which are necessary for the interpretation of the denaturation data to be reported in the forthcoming paper (Wu and Scheraga, 1962).

Experimental

Materials.—The STI used was obtained from Worthington Biochemical Corp., Freehold, N. J. (five-times crystallized, Lot No. SI5433). Reagent grade diethylaminoethyl cellulose (DEAE-cellulose), with an exchange capacity of 0.85 meq per g, was purchased from Brown Co., Berlin, N. H. Two-times crystallized trypsin, containing

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approximately 50% $MgSO_4$, was obtained from Worthington Biochemical Corp. (Lot No. 590). The $MgSO_4$ was not removed, since its concentration was very low at the high dilutions used here. Chromatographically pure benzoyl-L-arginine ethyl ester hydrochloride (BAEe) was obtained from Mann Research Laboratories, Inc., New York.

Bromobenzene (Distillation Products Industries, Rochester, N. Y.; b.p. 154–155°) and Esso kerosene were used to prepare density-gradient columns. These reagents were washed three times (with shaking) with an equal volume of conductivity water, dried over calcium chloride, and saturated by shaking at 20.0° with an aqueous solution which was 0.5 M with respect to both KBr and KCl.

Carbon dioxide–free KOH was prepared according to the method of Kolthoff (1922). The KOH was standardized against dried potassium acid phthalate, and HCl was standardized against the KOH. Beckman *p*H 4, 7, and 12.45 buffers were used to standardize the *p*H meter at 25.0°. All other materials were of reagent or analytical grade. Conductivity water was used throughout.

Stock Solutions.—An excess of STI was put in 1 M KCl, the ratio being 0.15 g STI to 14 ml of 1 M KCl. After standing in the refrigerator (3°) overnight, the mixture was warmed to room temperature (~25°), centrifuged at 1610 R.C.F. for 15 minutes, and filtered through filter paper which had previously been washed with 1 M KCl.

The concentration of STI was determined by absorption at 280 mμ at 25.0° in a Beckman DU spectrophotometer with photomultiplier attachment; 1-cm cells were used. The optical density was found to be linear up to at least 1.5 with STI concentration. On the basis of micro-Kjeldahl analysis (performed by Dr. S. M. Nagy of Massachusetts Institute of Technology) and the Kunitz (1947) nitrogen factor of 16.74%, an optical density of unity corresponded to 1.059 mg/ml of STI; the optical density was independent of acidity from *p*H 2.2 to 8. Calibrated Carlsberg and other volumetric pipets were used throughout.

The trypsin stock solution was prepared by dissolving 20 mg of the enzyme preparation in 1 ml of 0.001 M HCl. Dilution of 0.1 ml of this stock solution with 4 ml of 0.001 M HCl gave a solution which contained 0.322 mg/ml of trypsin, as determined from its optical density at 280 mμ with the aid of the factor 1.44 OD/mg reported by Davie and Neurath (1955). The concentrated stock solution was stored at −10°.

Gradient Chromatography.—The stock solution of STI was analyzed by gradient chromatography by the procedure of Rackis *et al.* (1959). DEAE-cellulose was equilibrated with 0.01 M potassium phosphate starting buffer at *p*H 7.6 and packed in a 2.2-cm diameter column (26 cm height) with a 1-mm hole at the bottom. Approximately 15 ml of a solution of STI (concentration 3.5 mg/ml) was introduced on the column and eluted with an exponential gradient of salt (0 to 0.3 M NaCl) in starting buffer. Samples of 10.5 ml were collected by a Technicon fraction collector with a photoelectric drop-counting device. The effluent was analyzed for optical density at 280 mμ and by the ninhydrin method of Moore and Stein (1954) at 570 mμ.

Activity Analysis.—To determine the rate of hydrolysis of BAEe by trypsin (in the presence of varying amounts of STI), KOH uptake at *p*H 7.9 was measured with an automatic titration assembly (Radiometer type TTT la Titrator and SBR 2a Titrigraph from Welwyn International Incorporated) at 25.0°. A radiometer GK 2021 B combined glass and calomel electrode was used for the measurement. Beckman *p*H 4 and *p*H 7 buffers were used to standardize the *p*H meter.

A constant amount of the trypsin stock solution (*p*H 3) (0.1–0.2 ml) was introduced into the titration cell with a Carlsberg pipet and a varying amount of STI (0.015 to 0.2 ml) was then added (*p*H 7.6). The proteins were allowed to mix *gently* with magnetic stirring.[1] Ten ml of 0.0005 M BAEe in 0.2 M $CaCl_2$ (*p*H 5.8) was then pipetted into the titration cell after 30 seconds.[2] The magnetic stirrer was then started to mix the solution effectively from this time on. The *p*H of the solution was adjusted to slightly above 7.9 with the addition of a few drops of 0.015 and 0.005 N KOH, for rough and fine adjustment respectively. The automatic titration assembly was then started. KOH (0.0385 N) was used in the 0.5-ml syringe buret with a micrometer graduated in 1250 divisions. The rate of base uptake was plotted on the chart of the recorder.

Partial Specific Volume.—The partial specific volume of STI was determined at 20.002 ± 0.002° by the density-gradient method of Linderstrøm-Lang and Lanz (1938). Potassium chloride and sodium chloride were used to prepare solutions of standard density, the values of which were obtained from the International Critical Tables (Vol. III, pp. 79, 87). The outside of the tip of the 1-μl Carlsberg pipet was coated with silicone.

Molecular Weight.—The molecular weight of STI was determined by the Archibald method with a Spinco Model E ultracentrifuge with RTIC unit and phase plate. The temperature was controlled at 20.0 ± 0.1° at all times during the experiment. The concentration, c_0, was determined in a 4°, 12-mm synthetic boundary cell with 1 M KCl and 1% STI in 1 M KCl in the cup and centerpiece, respectively. The Archibald runs were made in 4°, 12-mm analytical cells with 1% STI in 1 M KCl. In the preliminary runs, about 0.1 ml of Dow-Corning No. 555 sili-

[1] Vigorous stirring at this stage will result in some loss of trypsin activity.

[2] It is important to add BAEe *after* trypsin and STI are added; otherwise a linear relation between volume of STI solution added and rate of KOH uptake is not obtained.

cone fluid was introduced into the analytical cell to make the bottom boundary appear. The angle of the schlieren diaphragm was set at 70° in all runs. Kodak metallographic plates were used. A total magnification of more than 54 times was obtained after the plate was projected onto a screen from which tracings were made.

Amino Acid Analysis.—The amino acid composition of STI was determined by two commercial laboratories (Oxford Laboratories, Redwood City, Calif., and Analytica Corporation, 118 E. 28th St., New York).

For the Oxford analysis 0.5 ml of 1% STI in 1 M KCl was hydrolyzed with 0.5 ml concentrated HCl in a sealed, evacuated (10–15 mm Hg) tube for 22 hours at 110°. The hydrolyzed product was placed in an evacuated desiccator over NaOH pellets overnight and dissolved in 5.0 ml of *p*H 2.2 sodium citrate buffer (0.2 N). Aliquots of 2.0 ml each were analyzed on the 150-cm and 15-cm, respectively, Amberlite IR-120 columns of the Spinco automatic analyzer.

The Analytica analysis was obtained by ion-exchange chromatography and quantitative ninhydrin analysis of a hydrolysate prepared by treatment of 1% STI in 1 M KCl with 200 volumes of constant boiling hydrochloric acid at 115 ± 1° for 16 hours in a sealed, evacuated pyrex tube. Excess hydrochloric acid was removed under vacuum, and the hydrolysate was adjusted to pH 2.5 and filtered through sintered glass. Aliquots of this hydrolysate were then taken for ion-exchange chromatography on 15-cm and 150-cm columns of Amberlite IR-120. For some amino acids, a 72-hour HCl hydrolysate was used. The tryptophan value was determined by ion-exchange chromatography and quantitative ninhydrin analysis of the hydrolysate prepared by treatment of 1% STI in 1 M KCl with 200 volumes of 3.5 N $Ba(OH)_2$ at 100 ± 1° for 10 hours in a sealed, evacuated pyrex tube. Barium was removed under warm conditions as the carbonate, and the *p*H of the filtrate was adjusted to 2.5 for chromatography on a 15-cm column of Amberlite IR-120. The cysteic acid and methionine sulfone values were obtained by performic acid oxidation of 1% STI in 1 M KCl by the procedure of Schram *et al.* (1954). Cysteic acid and methionine sulfone were chromatographed on a 150-cm column of Amberlite IR-120 and quantitatively determined by spectrophotometric analysis after reaction with ninhydrin. Sulfhydryl sulfur was determined as *S*-carboxymethylcysteine by the method of Cole *et al.* (1958). Total amide nitrogen was determined by 2-hour hydrolysis with 1 N H_2SO_4 at 100° as described by Laki *et al.* (1954). Total nitrogen was determined by the Kjeldahl method. The digest was boiled for 7 hours after the second addition of 30% hydrogen peroxide. Three analyses were made, and the average deviation from the average was 0.53%. The total sulfur content was determined by the colorimetric method of Vecera and Spevak (1956). Two analyses were performed, and the average deviation from the average was 21%.

Titration Apparatus and Titration Procedure.—An automatic titration assembly (Radiometer, type TTTla, recorder SBR2a from Welwyn International Inc.) and Radiometer GK 2021B combined glass and calomel electrode were used to obtain titration curves of STI. Ten ml of protein solution (0.32–0.50%) was brought to *p*H 2.0 by addition of HCl and titrated with automatic recording with 1 N KOH. The reversibility of the titration was checked by bringing the solution to *p*H 11.8 by addition of KOH and titrating with 1 N HCl. Similar titrations were carried out on the solvent, the titration curve being obtained by subtraction of the solvent curve from that of the protein solution. The details are the same as reported by Cha and Scheraga (1960).

Spectrophotometric Titration.—Ultraviolet absorption measurements were made on a Beckman DU Spectrophotometer with photomultiplier attachment; 1-cm cells, covered with glass stoppers to avoid absorption of CO_2, were used. The temperature in the cell was maintained at 23.4° ± 0.1°. The protein concentration was 0.04–0.06%. A series of buffers from *p*H 7 to 13.75 were prepared; the final salt concentrations after STI was added were 0.23 M KCl + 0.015 M borate or phosphate. The optical density of the protein solution was read against the corresponding buffer at each *p*H. Measurements were made at 288 and 295 mμ. The change in molar extinction coefficient was considerably larger at 295 mμ, and the values at this wave length are reported. The *p*H measurements for the spectrophotometric titration were made on a Beckman model GS *p*H meter; the A scale was used. The *p*H meter was standardized with Beckman *p*H 7 and 12.45 buffers.

Results

Gradient Chromatography.—Although no heterogeneity appeared in the ultracentrifuge measurements described below, there did appear to be a small fraction (2–5%) of inactive material in the elution diagram of Figure 1. Outside the region of this small shoulder, the ninhydrin color and activity of STI superimposed on the optical density curve at 280 mμ. Rechromatography of the main peak gave an identical pattern with a similar amount of inactive material, irrespective of whether the material in the main peak had been lyophilized or not prior to rechromatography. This inactive form may appear either in the desalting dialysis procedure or on the column itself. No further investigation of this point was carried out. On the basis of the relatively small amount of inactive component in the stock solutions, it was concluded that the latter were sufficiently pure for physicochemical studies. Therefore, no further purification of the STI was carried out.

Activity Analysis.—Figure 2 shows the volume

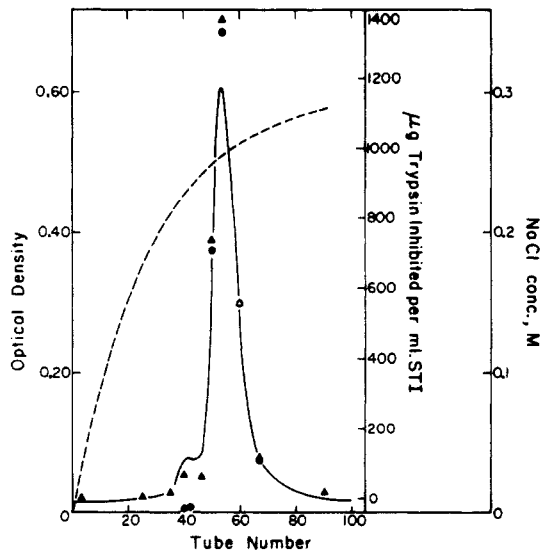

FIG. 1.—Gradient elution diagram of Worthington five-times-crystallized soybean trypsin inhibitor. The supernatant of STI in 1 M KCl was dialyzed against 0.01 M potassium phosphate buffer, *p*H 7.6, applied to the column, and eluted with 0.3 M NaCl in 0.01 M potassium phosphate buffer, *p*H 7.6 (exponential gradient). The DEAE-cellulose column had an I.D. of 2.2 cm and was 26 cm high. The temperature was 25°, and each tube contained 10.5 ml. For preparing the salt gradient a mixing chamber of 300 ml volume was used. The solid line is the OD at 280 mμ, the triangles are $^1/_2$ of the OD at 570 mμ (ninhydrin color), and the circles are the activity of STI. The salt gradient is shown as the dashed curve.

of STI (0.229 mg/ml) added to a fixed amount of trypsin (0.152 ml at 0.321 mg/ml) *vs.* relative rate of base uptake. The rate[3] decreases linearly as more STI is added.

The mole ratio of STI to trypsin *vs.* relative rate of base uptake is shown by the dashed line. Complete inhibition will occur at a ratio of 0.69 to 1. This value is lower than 1 to 1 because of impurities in the trypsin preparation. Several authors have determined the purity of twice-crystallized salt-free trypsin of Worthington Biochemical Corp. Cole and Kinkade (1961) found that the Worthington trypsin of different batches was about 77% pure. Perrone *et al.* (1959) reported a purity of 80%, and Liener (1960) indicated a purity of 50 to 65%. Our ratio of 0.69 is thus not unexpected.

The solid curve of Figure 2 was used as a calibration. The activity of STI per ml in the unknown sample was calculated from the slope of a curve similar to that of Figure 2. A standard curve like that of Figure 2 was determined each day whenever the activity of unknown STI samples was needed. This was necessary because the trypsin stock solution decreased in activity on storage in the refrigerator. The STI solution used for the standard curve was made by dissolving STI in 0.01 M potassium phosphate buffer, *p*H 7.6. The phosphate concentration in the final analysis solution was negligible. The method is reproducible to within 2%, as shown by the duplicate points of Figure 2.

Partial Specific Volume.—The apparent specific volume was calculated by the method of Schachman (1957). Since the apparent specific volume is independent of STI concentration in this case, the average value for different concentrations is equal to the partial specific volume, $\bar{v}$. A value of 0.698 ± 0.006 was obtained as the partial specific volume of STI in 1 M KCl at 20.0°.

Molecular Weight.—The molecular weight of STI from ultracentrifugal studies is given by equation (1) (Schachman, 1957).

$$M = \frac{RT}{(1 - \bar{v}\rho)\omega^2} \frac{(dc/dx)_m}{x_m c_m} = \frac{RT}{(1 - \bar{v}\rho)\omega^2} \frac{(dc/dx)_b}{x_b c_b} \quad (1)$$

The molecular weight at the top and bottom of the cell was calculated for each speed in each of the *preliminary* runs. The ratio of the molecular weight at the meniscus to that at the bottom, M_m/M_b, was calculated for each speed (7928 to 11,250 rpm),[4] and this ratio was either slightly more or slightly less than unity (0.94 to 1.04). No trend was observed for this ratio as the speed varied; therefore, the similarity of molecular weights at the meniscus and at the bottom of the cell, within experimental error, indicated that STI is homogeneous in its molecular weight. For the final runs, from which the molecular weight was calculated, no silicone fluid was used and only the top of the cell was analyzed. At least two photographs were used for each speed, including the synthetic boundary run.

An average M_m of 21,500 ± 100 was obtained from these data. We believe that this average is accurate to 4%. A value of 21,600 ± 800 was obtained for M_m and 21,800 ± 800 for M_b in the preliminary runs with silicone fluid. Only one

[3] The volume of base added *vs.* time is a straight line, and the slope of this line is the rate. The rate of base uptake is independent of the amount of BAEe present, and is directly proportional to the concentration of trypsin in the range of concentrations used here.

[4] When a stopwatch was used for about 10 minutes to check the time needed for 100 revolutions of the counter, the speed control setting of the ultracentrifuge at 11,573 rpm was found to be in error by 2.6%, whereas the settings at 7,928, 8,766, 9,945, and 10,589 were correct to better than 0.05%. Our molecular weight measurement at the highest speed agrees with those at the lower speeds only if our calculated speed of 11,250 is used. Our inquiry to Spinco confirms our finding that the setting is indeed in error at 11,573. The 11,573 setting should be approximately 11,250 and the 74,070 setting should be approximately 72,000 rpm (letter of Nov. 13, 1959, from W. T. Gray of Spinco to H.A.S.). This error in setting is apparently a general one in all Model E ultracentrifuges; therefore, we wish to call this to the attention of other users.

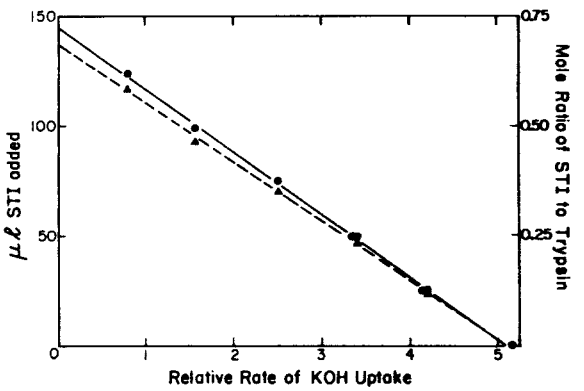

FIG. 2.—Inhibition of tryptic activity by STI at 25.0° (solid line). An aliquot of the STI solution was added to 152 μl of the stock trypsin solution. Ten ml of 0.0005 M BAEe was used for each sample, and the rate of 0.0385N KOH uptake was measured at *p*H 7.9. STI concentration (in the stock solution) was 229 μg/ml, and trypsin concentration (in the stock solution) was 321 μg/ml. The mole ratio of STI to trypsin *vs.* relative rate of KOH uptake is shown by the dashed line. The two lines do not coincide, since both ordinates were chosen arbitrarily.

picture was used for each speed in those runs.

Our value for the molecular weight is compared in Table I with others reported in the literature.

Amino Acid Composition.—The results of the Oxford and Analytica amino acid analyses are shown in Table II. They were computed on the basis of a molecular weight of 21,500. A molecular weight of 21,700 was obtained from the "Best Estimate" column of Table II.

The following correction factors were applied to the raw data in calculating the results shown in Table II.

AMMONIA (DIRECT HCl HYDROLYSATE).—The value was obtained by subtracting from the total NH_3 of the hydrolysate an amount of NH_3 corresponding to the calculated amount of decomposition of serine and threonine (Hirs *et al.*, 1954).

THREONINE AND SERINE.—The original value of threonine was divided by 0.95 and that of serine by 0.90 to compensate for the loss of these amino acids during hydrolysis (Rees, 1946).

METHIONINE (DIRECT HCl HYDROLYSATE).—The original value was divided by 0.95 to correct for chromatographic loss of methionine (Moore *et al.*, 1958).

TABLE I

COMPARISON OF MOLECULAR WEIGHTS OF STI

Method	*M*	Reference
Osmotic pressure	24,000 ± 3,000	Kunitz (1947)
Light scattering	20,000 ± 1,000	Steiner (1954)
Ultracentrifugal runs on trypsin-STI compound	21,000	Sheppard and McLaren (1953)
Archibald	21,500 ± 800	This paper

TABLE II

AMINO ACID COMPOSITION OF STI (Number of Groups per 21,500 g of Protein)

	Oxford	Analytica	Kunitz (1947)	Best Estimate
Lysine	11.8	10.7		11
Histidine	2.4	2.0		2
Arginine	10.4	9.3		9
Aspartic acid	28.9	28.9		29
Threonine	8.1	8.3		8
Serine	12.2	13.3		13
Glutamic acid	21.3	21.2		21
Proline	11.6	10.2		10
Glycine	18.1	18.4		18
Alanine	9.4	9.2		9
½ Cystine	4.1	4.0		4
Valine	14.8	11.6		12
Methionine	2.7	2.6		3
Isoleucine	15.3	13.8		14
Leucine	16.3	15.6		16
Tyrosine	4.6	4.4	4.8	4
Phenylalanine	10.2	9.0		9
Tryptophan		1.7	2.3	2
Amide NH_3		13.6		
½ Cystine[a]		4.5		
Methionine[b]		1.3		
Cysteine[c]		0.0		
Total S		6.3	6.5	
Total N		251.1		
Ammonia	17.2	15.8		16

[a] Determined as cysteic acid. [b] Determined as methionine sulfone. [c] Determined as *S*-carboxymethylcysteine.

CYSTEIC ACID.—The original value was divided by 0.92 to compensate for hydrolysis losses of cysteic acid (Schram *et al.*, 1954).

Since there are some small discrepancies between the Oxford and Analytica data, we used these data as a first approximation, as far as the ionizable groups are concerned, and obtained "final" values by fitting the experimental titration curve with a theoretical one, as outlined in the next section.

The number of carboxyl groups is equal to the sum of aspartic acid, glutamic acid, and one terminal carboxyl (Neurath and Davie, 1954) minus the ammonia value. For 21,500 g of STI this gives a value of 34.0 from the results of Oxford, 35.3 from those of Analytica, and 37.5 from the amide NH_3 value of Analytica. The experimental titration curve gives a value of 35, which is just about the average of the three values above.

We believe that the amino acid analysis of Analytica Corporation is more accurate for two reasons. First, it agrees better with the experimental titration results. Second, it gives a % N value that is close to that of the Kjeldahl analysis and to that of Kunitz (1947). Kunitz reported a value of 16.74% N; the results of Analytica gave 16.56% from amino acid analysis and 16.35% from Kjeldahl analysis. The results of Oxford, however, gave 17.68% N. When the

amino acid values of the titration curve were used together with the rest of the Oxford analysis, a value of 16.92% N was obtained.

There was a major discrepancy between the methionine values determined by direct chromatography of the HCl hydrolysate and by chromatography of methionine sulfone. The possibility exists that some resistant peptide remains in the 16-hour hydrolysate and coincidentally elutes from the 150-cm column together with free methionine. Therefore, the methionine content in a 72-hour HCl hydrolysate was determined. The decrease in methionine after 72 hours of hydrolysis was 31% and was considerably greater than the usual decrease of 5–10% observed for this amino acid between 16 and 72 hours. This finding supports the hypothesis that a resistant peptide fragment was eluted together with the methionine in the 16-hour hydrolysate.

Kunitz (1947) reported that STI contained 4.0% tyrosine, 2.2% tryptophan, and 0.97% sulfur by weight. This gives 6.5 moles of sulfur per 21,500 g of STI and is in excellent agreement with the results of direct chromatography of the HCl hydrolysates of both Oxford and Analytica. The tryptophan value determined by Kunitz is considerably higher, and that of tyrosine is in fair agreement with the other values.

Direct Titration.—A molecular weight of 21,500 was used in the computations. Kunitz (1947) reported an isoelectric point of 4.5 for STI, based on cataphoretic mobility. Since the *p*H of STI in 1 M KCl was found to be quite near 4.5, we assumed that there was no chloride binding. This assumption is supported by the fact that STI solutions in 0.3 M, 0.5 M, and 1 M KCl all have the same *p*H. We thus obtained a value of Z_{max} = 23, which is consistent with the number of basic groups present in the STI molecule as shown in Table II.

Figure 3 shows the titration curve of STI in 1 M KCl at 25.0°. It was obtained from the average of seven duplicate or triplicate runs with STI and the corresponding runs on blanks. The deviation from this average curve was calculated for each run at each *p*H, and the resulting average deviation was 0.17 group. Two of the runs were from high *p*H to low *p*H, and they showed no difference from those titrated from the opposite direction; the whole *p*H range was thus shown to be reversible. The points of Figure 3 do not level off at the low *p*H end, and the error becomes larger at both extremes of the *p*H range studied. The curve of Figure 3 was calculated with the parameters in Table III, and the method of calculation is given in the discussion section. Guanidyl groups affect the titration curve only through the net charge, *Z*, of STI, in this case because the large negative charge and *w* values at high *p*H make the effective *pK* of these groups too high for them to be observed (at *p*H 11.5, 0.868 wZ = −1.30).

Spectrophotometric Titration.—The spectrophotometric titration curve for the tyrosyl groups in buffers at 23.4° is shown in Figure 4. There was some time dependence as the *p*H was raised above 13, and the points shown are the initial values extrapolated to zero time. The reverse curve was obtained by adding KOH to the stock STI solution so that its *p*H was above 12. After a few minutes its *p*H was lowered by addition of HCl. Buffer solutions at different *p*H levels were then added to aliquots of this solution for measurements. It can be seen in Figure 4 that the titration is reversible. The high negative charge and a larger *w* (lower ionic strength of the buffer used), and possibly the interference of tryptophan groups, may prevent the attainment of the leveling off position at high *p*H (Fig. 4).

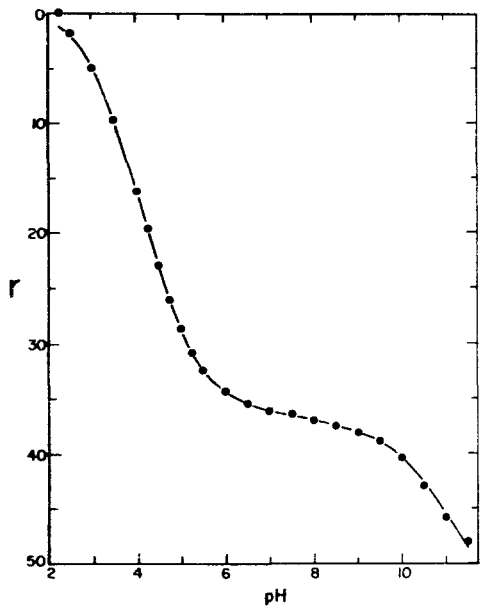

FIG. 3.—Titration curve of STI in 1 M KCl at 25°. *r* is the number of H^+ ions dissociated per molecule of STI. Circles are experimental points; line is the theoretical curve.

TABLE III

PARAMETERS USED TO CALCULATE TITRATION CURVE OF FIGURE 3

Group	Number of Group Found	*pK*°
Carboxyl	35	4.21
Imidazole	2	6.45
α-Amino	1	7.8
ε-Amino	11	9.9
Tyrosyl	4	9.5
Guanidyl	Does not enter calculation except through its effect on net charge, *Z*	
w = 0.026 (*pH* 2.25 to 7); w = 0.060 (*p*H 7.5 to 11.5)		

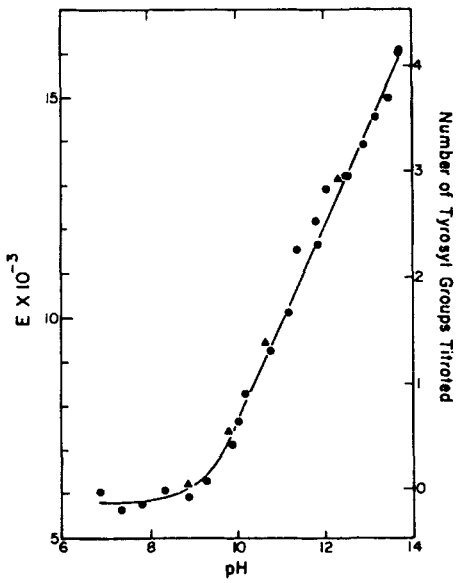

FIG. 4.—Spectrophotometric titration curve of STI in borate and phosphate buffers containing 0.23 M KCl and 0.015 M borate or phosphate. E is molar extinction coefficient at 295 mμ. ●, Direct titration; ▲, reverse titration.

DISCUSSION

Of the physicochemical data presented, it is necessary at this point to discuss only those from the titration experiments.

The reversible titration data may be interpreted by equation (2), for each type of ionizable group (Tanford, 1955), where x is the fraction of the groups of the given kind which have dissociated protons at the given pH, and $pK°$ is the negative logarithm of the intrinsic dissociation constant for the corresponding groups.

$$p\mathrm{H} - \log \frac{x}{1-x} = pK° - 0.868\, wZ \quad (2)$$

The ordinate of the titration curve is given by equation (3), where n_i is the number of groups for each type and the subscript i refers to the same

$$r = \sum n_i x_i \quad (3)$$

type of ionizing group. The term $0.868\, wZ$ arises from the electrostatic interaction between the proton and protein molecule with net charge Z. The quantity w may be regarded as an experimental parameter and would be expected to be constant at a given temperature and ionic strength over a pH range in which the size, shape, and permeability of the molecule do not change.

The value of w may also be calculated from the theory of Linderstrøm-Lang (1924), in which the protein is regarded as a spherical ion of radius b with the charge distributed uniformly over the surface. The theoretical value of w is then given by equation (4) (Tanford, 1955), where $a = b + 2.5$A and is the radius of exclusion, ϵ is the electronic charge, D is the dielectric constant, k is Boltzmann's constant, T is absolute temperature, and κ is the Debye-Hückel parameter.

$$w = \frac{\epsilon^2}{2DkT}\left[\frac{1}{b} - \frac{\kappa}{1+\kappa a}\right] \quad (4)$$

Since the value of Z and the number of groups are not very certain, we rely on a fit between experimental and theoretical curves. The values of Z were obtained by taking the isoionic point as 4.5 and assuming no binding of chloride or potassium ions. The data for the carboxyl groups were plotted according to equation (2), with a series of n_i used for each Z_{max}. Values of n_i used were 32, 34, 35, 36, 38, while those of Z_{max} were 22, 23, and 24. From the shape of these plots many combinations could be quickly ruled out. The combination of $Z_{max} = 23$ and $n_i = 35$ gave the best straight line, whereas others curved either up or down considerably at one or both ends. This combination is shown in Figure 5, from which

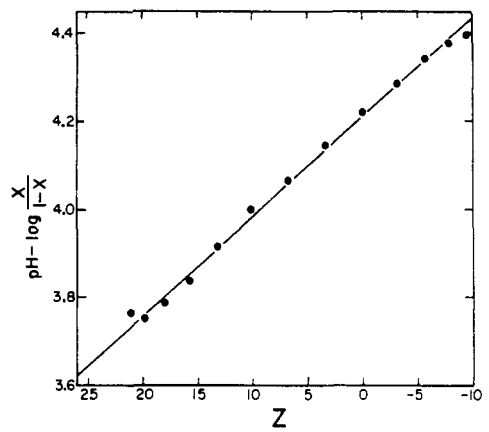

FIG. 5.—Logarithmic plot for the dissociation of carboxyl groups.

w and $pK°$ were determined from the slope and intercept at $Z = 0$. For tyrosyl groups we have used trial values of 9,600, 10,600, and 12,000 as the total change in molar extinction coefficient when tyrosyl groups are ionized and values of 5,800, 5,900, 6,000, 6,100, and 6,200 as the molar extinction coefficient at low pH when tyrosine is not ionized. Figure 6 is a plot of data for tyrosyl groups according to equation (2) with $x = (E - 6{,}100)/9{,}600$, where E is the molar extinction coefficient at any pH. The points of Figure 6 were taken from the smoothed curve of Figure 4. An average value of 9.5 was obtained for the $pK°$ and 0.085 for w at 0.243 ionic strength. In view of the long extrapolation to $Z = 0$, the uncertainty of the experimental points in Figure 6, and the relatively small change in $pK°$ when the total change in molar extinction coefficient is varied, we have used the $pK°$ and w values for tyrosyl groups only as a first approximation; they were subsequently refined as discussed below. The uncertainty in

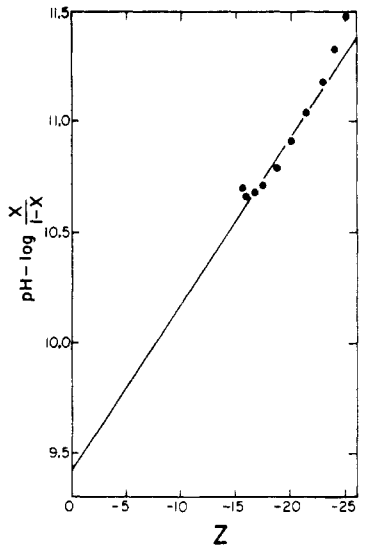

FIG. 6.—Logarithmic plot for the dissociation of tyrosyl groups. The straight line was computed by the method of least squares neglecting the first and last two points.

the data for the tyrosyl groups may be a result of interference from the tryptophan groups present in the STI molecule.

The values of w calculated from equation (4) for spherical ions of radius 19 to 19.7 A are 0.042 to 0.040 for an ionic strength of unity, and 0.059 to 0.055 for an ionic strength of 0.243. When the value of w from the tyrosyl groups is corrected for the difference in ionic strength, a value of about 0.060 is obtained for w in 1 M KCl.

It is evident that a much larger w is required to fit the titration curve in the region of ionization of tyrosyl groups compared with carboxyl groups. With this information, an attempt was made to fit the titration curve with 4 and 5 tyrosyl groups, with 11 and 12 lysyl groups, and with different $pK°$ and w values. One α-amino and one α-carboxyl group were used in the calculation because there are one carboxyl-terminal group and one amino-terminal group in STI (Neurath and Davie, 1954). The region of ionization of histidyl groups was fitted last, and the theoretical titration curve was then calculated. The $pK°$ and w values of the tyrosyl group obtained agree with the experimental spectrophotometric curve of Figure 4, and the theoretical titration curve agrees with the experimental one as shown in Figure 3.

From Table III it is seen that the carboxyl groups have an abnormally low $pK°$, while imidazole, amino, and tyrosyl groups all appear to be normal. Several different kinds of local interactions could account for the abnormal $pK°$ of the carboxyl groups (Scheraga, 1961c). The constancy of w between pH 2 and 7, indicated by the titration data, is compatible with optical rotation and ultraviolet difference spectra data in the same pH range (Wu and Scheraga, 1962). The increase[5] in w at high pH is accompanied by a change in the pH-induced ultraviolet difference spectrum (Wu and Scheraga, 1962). Further discussion of the optical rotation and ultraviolet spectral data will be presented in paper II (Wu and Scheraga, 1962), along with a discussion of the effect of temperature on the conformation of STI.

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[5] The average of the experimental values of w in 1 M KCl, 0.026 and 0.060 (namely, 0.043) is the same as the value calculated (0.041) from equation (4). This is good agreement, considering the crudeness of the model on which equation (4) is based.