Potentiometric Measurement of Protein-Protein Association Constants. Soybean Trypsin Inhibitor–Trypsin Association*

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A potentiometric technique for determination of protein-protein association constants is described. The technique consists of measuring the average number of protons ($\bar{q}$) released when solutions of protein A and protein B (at the same pH) are mixed and thus allowed to form complex AB. Relations between $\bar{q}$ and the apparent association constant, K_{app}, were developed and employed. The association constants and $\bar{q}$ values were determined for trypsin–soybean trypsin inhibitor system at 20.0° in 0.5 M KCl, 0.05 M $CaCl_2$ in the pH range 3.75–5.75 (and, by extrapolation of $\bar{q}$, up to pH 8.30). The results are in satisfactory agreement with the few scattered data in the literature. An interesting kinetic phenomenon of "overshoot" in the soybean trypsin inhibitor–trypsin association was observed in this study. It was tentatively explained on the assumption that the association reaction is faster than mixing, whereas the dissociation reaction is measurably slow.

Protein-protein associations are of great biological importance. Antigen-antibody, enzyme-inhibitor, and proteolytic enzyme–substrate interactions belong to this important class of reactions. Further, studies of protein-protein association may shed light on the nature of forces responsible for maintaining the structure of native globular proteins (Waugh, 1954, 1959). However, thermodynamic parameters describing these reactions and particularly the pH dependence of the association constants (over wide pH ranges) are very scarce. This scarcity arises from the limitations of various physicochemical techniques thus far applied, such as light scattering, osmotic pressure, sedimentation, *etc.* In the pH range where the association constants are small, the change in the appropriate average molecular weight of the system can be followed by these techniques as a function of concentration. In the pH region where the association constants are large, these techniques are no longer applicable since dissociation occurs only at concentrations so low that no reliable data can be obtained. In the cases where enzyme-inhibitor associations are studied, very high equilibrium constants can be measured by enzymatic techniques (*e.g.* Green, 1953, 1957). However, the physicochemical and enzymatic techniques usually leave a wide range of pH values where the equilibrium constants are not known.[1] Further, many interesting protein-protein associations cannot be measured by the enzymatic technique. It is therefore of interest to develop a method which has applicability over the entire pH range and does not require enzymatic activity to measure the association.

A symbolic representation of a one-to-one protein-protein association can be written as[2,3]

$$AH_{n-\bar{h}_A} + BH_{m-\bar{h}_B} \rightleftarrows ABH_{n+m-\bar{h}_{AB}} + \bar{q}H^+ \quad (1)$$

where A, B, and AB are the two reacting proteins and the complex, H is hydrogen ion, n and m are the total number of ionizing sites on A and on B, and $\bar{h}_A$, $\bar{h}_B$, and $\bar{h}_{AB}$ are the average number of protons dissociated from A, B, and AB, respectively, at the pH of interest. Finally, $\bar{q}$, the average number of protons released on association, is given by

$$\bar{q} = \bar{h}_{AB} - \bar{h}_A - \bar{h}_B \quad (2)$$

The equilibrium constant for reaction (1) is frequently written as

$$K_{app} = \frac{[AB]}{[A][B]} \quad (3)$$

where the brackets denote the sum of the concentrations of all possible species of AB, A, and B respectively. It can be shown by the use of various relations in titration curve theory (Linderstrøm-Lang, 1946; Edsall and Wyman, 1958; Laskowski, 1961)[4] that

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[1] The enzymic technique may in special cases measure K_{app} over wide pH ranges (Green, 1957). However, this can be done only in special cases and even then the data obtained are not highly reliable.

[2] For the sake of simplicity, charges on the protein species are omitted.

[3] For details of notation see Edsall and Wyman (1958).

[4] This equation appears to be derived and rederived in different notations by numerous authors. Proper assignment of credit is impossible.

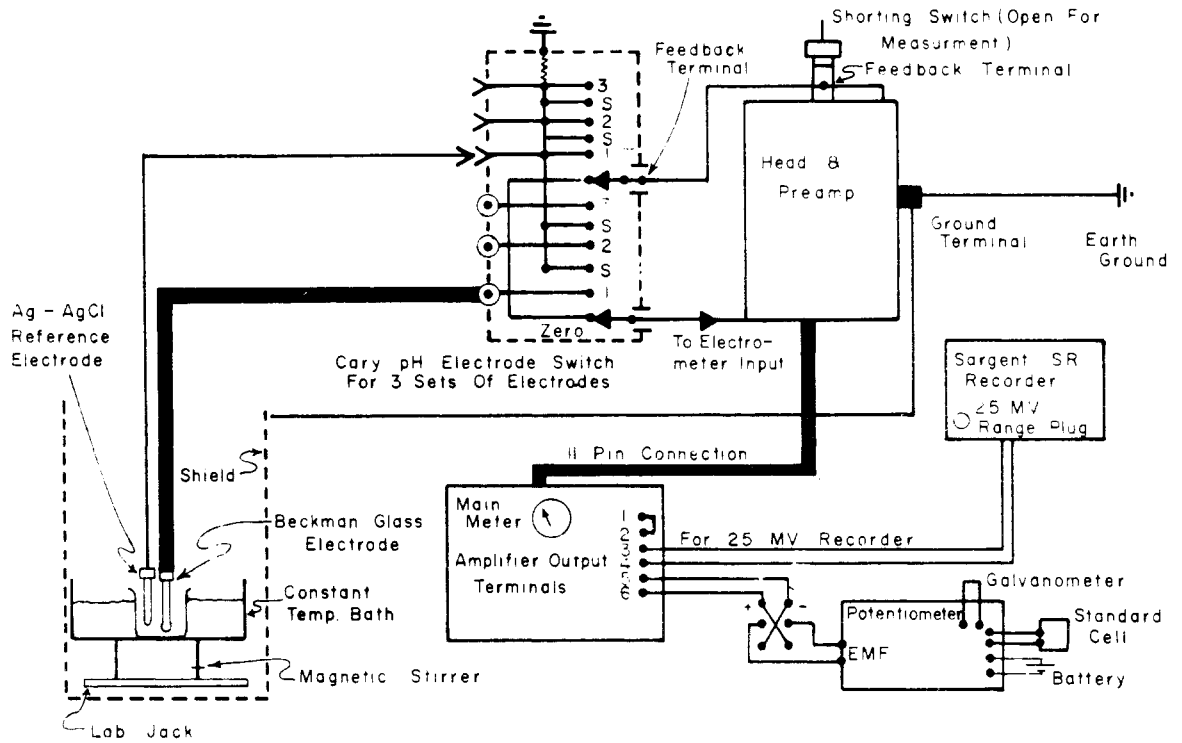

FIG. 1.—Circuit schematic for pH measuring system using Cary 31V vibrating reed electrometer.

$$\frac{d \log_{10} K_{app}}{d pH} = q \qquad (4)$$

This can easily be integrated to

$$(\log_{10} K_{app})_2 - (\log_{10} K_{app})_1 = \int_{pH_1}^{pH_2} \bar{q}\, d pH \qquad (5)$$

If one knows log K_{app} at pH_1, one can determine log K_{app} at any other pH by graphical integration of a plot of $\bar{q}$ versus pH. This study is an attempt to determine the usefulness of equation (5).

As a test system we have selected the one-to-one association of trypsin (T) and soybean trypsin inhibitor (STI) (Kunitz, 1947a), for which there appeared to be sufficient data in the literature (*e.g.* Laskowski and Laskowski, 1954) for comparison with our results.

EXPERIMENTAL

The procedure for measurement of $\bar{q}$ consisted of adjusting the trypsin and soybean trypsin inhibitor solutions to the same pH. Subsequent mixing caused a small pH drop, and the value of $\bar{q}$ was obtained by back-titration to the original pH. A prime requirement of the technique is the matching of solutions to the same pH values. This calls for a pH meter of exceptional stability and sensitivity. A system employing a vibrating reed electrometer as a null detector (Kraus *et al.*, 1950; Bates, 1954) fulfills these requirements.

pH Meter.—Figure 1 is a detailed schematic of the instrument. A Leeds and Northrup K-2 potentiometer is used to oppose the potential developed by the electrodes. The Cary 31V vibrating reed electrometer (used as a model 31) is employed as a null detector. The meter on the Cary 31V main amplifier and a Sargent SR recorder are used to follow the remaining imbalance. They were generally set for 1 MV (~0.02 pH) full scale. The electrodes are connected to the reed of the electrometer by a Cary pH transfer switch. The electrode assembly, sample cells, Manostat microburet, and constant temperature bath are shielded in a copper box (Faraday cage). The box has doors for easy access to various components. The electrodes and microburet are stationary; the sample cells, constant temperature bath, and magnetic stirrer are moved up and down by a laboratory jack. The microburet was controlled from the outside of the Faraday cage by means of a long, insulated extension handle.

Electrode System.—The Ag-AgCl reference electrode was employed since it is much more stable and adjusts more rapidly to temperature changes than the calomel electrode (Bates, 1954). The fiber liquid junction commonly used in reference electrodes shows poor stability even in simple salt solutions (Leonard, 1955) and especially in protein solutions because of adsorption of the proteins on the fiber. Therefore the liquid junction was changed (see Fig. 2) to a ground glass one. The junction employed was first suggested by L. Hammett (LaMer and Baker, 1922) and was shown to have a highly reproducible potential. The junction can be readily flushed and cleaned.

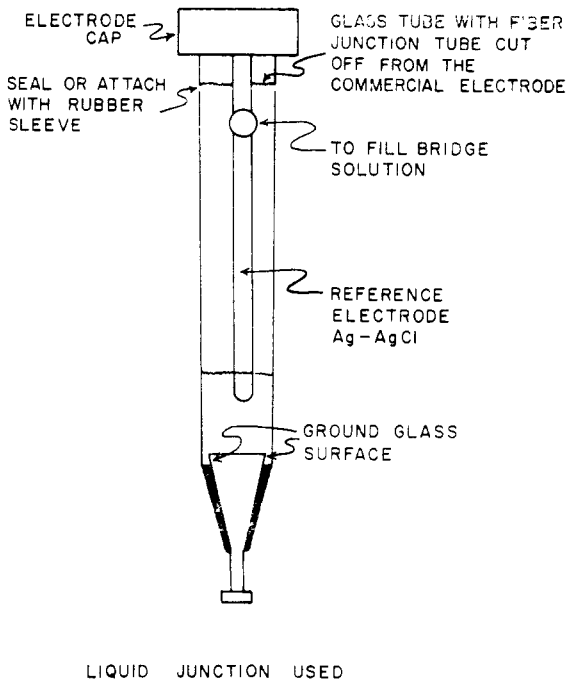

FIG. 2.—Ag-AgCl reference electrode with ground glass junction replacing fiber junction of a commercial Beckman electrode.

The leakage rate is very small; in order to reduce it further the hydrostatic head in the electrode was kept very low. In order to minimize instability due to protein systems, the salt concentration in the liquid junction was the same as in the protein solutions; *i.e.*, 0.50 M KCl and 0.05 M $CaCl_2$. Testing of several glass electrodes led to the use of preselected, general purpose Beckman glass electrodes (40498).

Testing the Instrument.—In order to test the accuracy of the pH meter we measured the difference between the pH 4.00 and pH 6.88 National Bureau of Standards buffers (Bates, 1954) at 20.0°. This was found to agree within ±0.001 pH with the expected value of 2.88. Since the pH of the buffers is defined to only ±0.01 pH, this result could be fortuitous. The difference between commercial pH 4.00 and 7.00 buffer solutions was sometimes found to be in error by as much as 0.03 pH.

Since we were interested in accurate measurement of pH changes smaller than 0.01 pH, another method of calibration had to be found. Addition of small quantities of KOH solution to equimolar mixtures of potassium acetate and acetic acid should produce pH changes given by

$$\Delta pH = 0.868 \frac{\Delta N_{OH^-}}{N_{ac^-}} \qquad (6)$$

where ΔN_{OH^-} is the number of moles of KOH added and N_{ac^-} the number of moles of potassium acetate present in the buffer sample. Although (6) is an approximation it should hold well for small ΔpH. By this procedure we have produced a series of calculated pH changes of 0.0010 pH. All of these were detected by the instrument and measured with an accuracy of ±0.0002 pH.

The instrument drift is exceptionally low; after temperature equilibration, pH readings on simple salt solutions only rarely drift as much as 0.003 pH per hour.

Materials.—The data of Figure 4 were obtained with five-times crystallized soybean trypsin inhibitor (Nutritional[5] No. 5378) and with several bovine trypsin samples (twice crystallized, salt-free Nutritional No. 4980, twice crystallized, salt-free, lyophilized Worthington[5] No. 607 and Worthington No. 6105). Except for these data all the rest were obtained with trypsin Worthington No. 6105 and with five-times crystallized, salt-free soybean trypsin inhibitor (Gallard-Schlesinger[5] No. 42535). This sample of STI was used because we were notified of its being chromatographically pure by Dr. Rackis (Rackis *et al.*, 1959; Rackis, 1961, personal communication); (the trypsin preparation used was the most active available in our laboratory; the more active sample, Worthington No. 607, was used up in the earlier study). Trypsin solutions were prepared by dissolving the enzyme in 0.05 M $CaCl_2$, 0.50 M KCl solution adjusted to pH 3.00. This stock solution was filtered, again adjusted to pH 3.00, and stored in the cold room at 3°. The STI solution was also prepared by dissolving the inhibitor in 0.50 M KCl, 0.05 M $CaCl_2$ solution at pH 5.00. This solution was centrifuged at 10,000 rpm for 40 minutes to remove undissolved protein. The stock solution was filtered and stored in the cold room at 3°. All pH adjustments were made by the use of 0.01 M KOH and 0.10 M HCl with KCl added to make the ionic strength of the acid and base solution 0.65, which is the ionic strength of the protein stock solutions. Standard solutions of KOH were quantitatively diluted for use in the microburet and adjusted with KCl to ionic strength of 0.65.

The concentrations of the protein solutions were determined at 280 mμ with a Beckman DU spectrophotometer. The optical factors used were: for trypsin 0.650 (Worthington, 1961), for STI 1.10 (Kunitz, 1947b). The molecular weight of STI was taken as 20,000 (Steiner, 1954) and that of trypsin as 24,000 (Kay *et al.*, 1961). The distilled water was deionized by a mixed bed column deionizer (Barnstead 0808), which was attached to the distilled water outlet. The resistivity was 1,000,000 ohms or greater. Primary standard potassium acid phthalate was used for the standardization of KOH solutions. National Bureau of Standards buffers were used for pH calibration. All other chemicals were reagent grade. To insure that protein solutions came in contact with glass surfaces only, special glass tips were made

[5] Nutritional Biochemicals Corporation, Cleveland, Ohio; Worthington Biochemical Corporation, Freehold, N. J.; Gallard-Schlesinger Chemical Corporation, Garden City, N. Y.

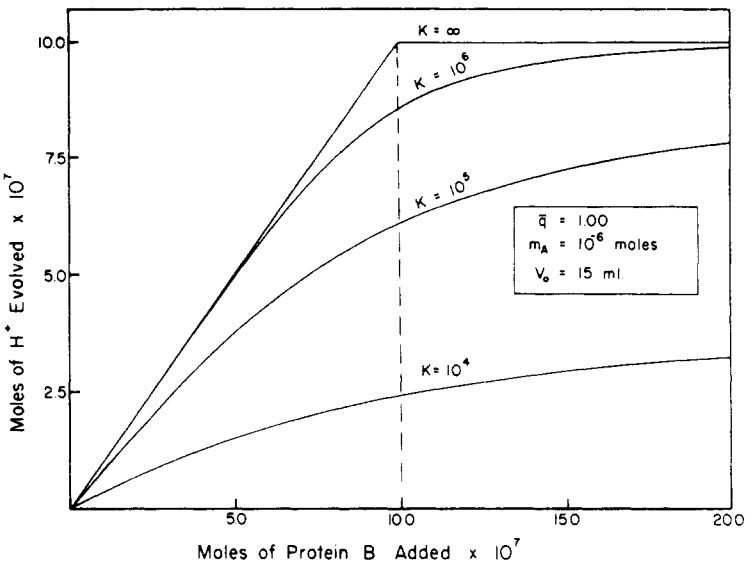

FIG. 3.—Theoretical curves of the number of moles of H^+ released as a function of the number of moles of protein B added for an A + B⇄AB association. The curves are given for a fixed number of moles of A($m_A = 10^{-6}$) in an initial volume V_0 (15×10^{-3} liters). Protein B is 10^{-4} M and $\bar{q}$ is 1.00.

for transfer hypodermic syringes. All measurements were carried out at 20.00 ± .02°.

Experimental Procedure.—The procedure employed was first to standardize the instrument with the National Bureau of Standards pH 4.00 buffer. The STI solution was then adjusted to the desired pH. Trypsin solution was subsequently adjusted to the same pH (±0.001) as the STI solution. An increment of the STI solution was then added to the trypsin solution[6] and the pH change (drop) upon association was observed. This change (arising from the release of protons on association) was titrated with base from the microburet until return to the null point, thus measuring the number of equivalents of hydrogen ion released. Successive increments were added until the reaction was complete and no further pH change was observed. In the cases where the reaction was not complete, increments were added until the pH changes became so small that their reproducibility was in question. This procedure leads to a plot of the number of moles of protons liberated versus the number of moles of protein added (see Fig. 3).

Interpretation of Data.—Upon addition of m_{STI} moles of STI to a fixed number of moles of trypsin, m_T, αm_{STI} moles of complex are formed and X moles of hydrogen ion are released. It is clear that

$$X = \alpha m_{STI} \qquad (7)$$

The value α is determined by the apparent association constant, K_{app}, at the pH of interest and by the concentration of trypsin present. Equation (3) can readily be rearranged to

$$K_{app} = \frac{\alpha m_{STI}/V}{(1 - \alpha)m_{STI}/V(m_T - \alpha m_{STI})/V} \qquad (8)$$

where V is the total volume (in liters). Combination of equations (7) and (8) yields

$$K_{app} = \frac{\bar{q}XV}{(qm_T - X)(qm_{STI} - X)} \qquad (9)$$

Since the initial volume is V_0, the volume after n additions of STI is $V_0 + n\Delta V$, where ΔV is the volume increment per addition. If Δm_{STI} moles of STI are added in each increment then (9) becomes

$$K_{app} = \frac{\bar{q}X(V_0 + n\Delta V)}{(qm_T - X)(qn\Delta m_{STI} - X)} \qquad (10)$$

Equation (10) thus allows calculations of the expected results, *i.e.*, curves of X vs. n. In actual practice, since the equation for X is quadratic and that for n linear, it is easier to calculate n(X) for various assumed values of the parameters. An electronic computer was used for this purpose.

The results can best be appreciated by referring to Figure 3, where we have plotted theoretical curves for number of moles of protons released as a function of the number of moles of soybean trypsin inhibitor added. For the sake of simplicity $\bar{q}$ was taken as unity and the association constant K_{app} was allowed to vary. It is seen that, when the association constant is extremely large, the reaction is stoichiometric and the plot consists of two straight line segments, the first rising with slope $\bar{q}$ and the second horizontal. Data obtained in the pH and concentration region

[6] This procedure can be readily reversed. Increments of T can be added to STI and essentially the same results are obtained.

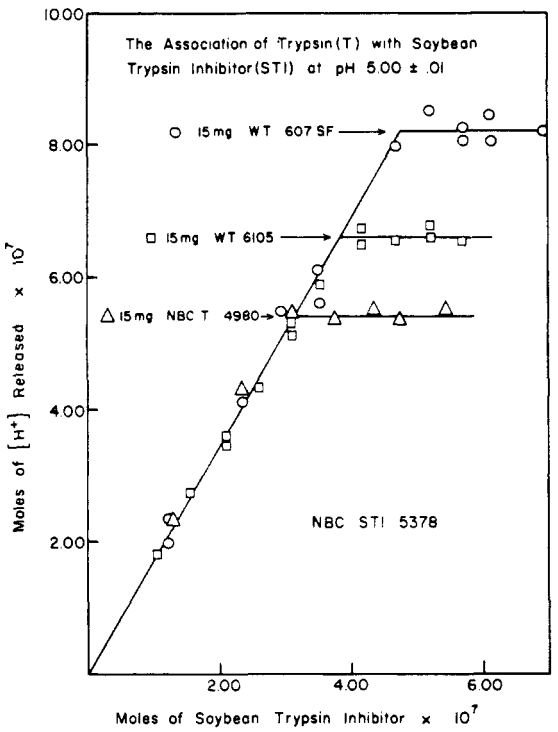

FIG. 4.—The association of three 15-mg samples of trypsin (T) with soybean trypsin inhibitor (STI) at pH 5.00 ± 0.01 (showing different activities of the trypsin samples) with Nutritional Biochemicals STI 5378. ○, Worthington trypsin 607 SF, 22% inactive, 1 mg STI inhibits 1.52 mg trypsin; □, Worthington trypsin 6105, 38% inactive, 1 mg STI inhibits 1.92 mg trypsin; △, Nutritional Biochemicals trypsin 4980, 49% inactive, 1 mg STI inhibits 2.35 mg trypsin.

where K_{app} (or more properly K_{app} c) is large yield simply values of $\bar{q}$ and of equivalence (the position of the break). It is essentially impossible to determine K_{app} directly since the small curvature in the equivalence region is far less than the experimental error (Fig. 4).

As K_{app} is allowed to fall the plots cease to resemble two straight line segments and become smooth, monotonically rising curves. It is much harder to estimate equivalence position from such a curve. On the other hand, the curvature now allows an evaluation of K_{app}, provided that $\bar{q}$ is known, by the simple use of equation (10). If the equilibrium constants are not too small $\bar{q}$ can be readily obtained from the *initial* slope of the graphs, since it is seen from Figure 3 that this slope is very closely similar to $\bar{q}$ (in the case of $K_{app} = 10^6$). K_{app} can then be computed from the rest of the data by the use of equation (10). The small errors made in the original identification of $\bar{q}$ and the initial slope can then be corrected by successive approximations. Once K_{app} is obtained at any given pH, and $\bar{q}$ values are known at other pH values, K_{app} at the other pH values can be evaluated by the use of equation (5).

Finally it is seen in Figure 3 that as K_{app} becomes very low (10^4 and 10^5) the initial slope is no longer a good approximation for $\bar{q}$. In such a case both $\bar{q}$ and K_{app} can *in principle* be evaluated from all of the data. One procedure for doing this is to assume various $\bar{q}$ values, compute K_{app} from every point, and check for constancy of K_{app}. *In principle* only one $\bar{q}$, K_{app} combination will yield satisfactory results. In practice, an analysis of this sort produces reasonably constant K_{app} for various assumed values of $\bar{q}$ except at high values of m_{STI}, where the experimental error of the method is greatest (see Figs. 7, 8). Thus such an analysis yields quite precisely K_{app} as a function of assumed $\bar{q}$, but does not allow a unique choice of $\bar{q}$ to be made. This choice must be made in some other manner. Several choices are available: (1) independent measurement of either q or of K_{app}; (2) extrapolation of $\bar{q}$ from the previously available $\bar{q}$ vs pH data; (3) application of equation (5) to known K_{app} at higher pH and assumed q at the pH of interest to yield another function K_{app} (q); the crossing point of this function and the one already at hand thus determines K_{app} and $\bar{q}$. Of the three procedures the last is probably the easiest and most reliable. It was, however, rejected on the ground that one of the major objectives of this study was a test of equation (5). If the third choice had been chosen all of the K_{app} values (save one at pH 4.25) would have been obtained by the use of equation (5) and thus the equation could not have been tested. Thus methods (1) and (2) were employed. Since the final results agreed quite closely with theory, essentially the same answers would have been obtained had method (3) been chosen.

RESULTS

Figure 4 shows a plot of the number of moles of hydrogen ion released against the number of moles of inhibitor added for three different samples of trypsin at pH 5.00. Since little curvature is seen at pH 5.00, the reaction is essentially stoichiometric. Each of the 15-mg samples of trypsin reacts with a different amount of the inhibitor, indicating that each of the samples contains a different amount of active trypsin. If we assume that STI is 100% active then the three trypsin samples are, respectively, 78, 62, and 51% active.[7] Identical results were obtained from simultaneous measurements of enzymatic activity of the various trypsin-inhibitor mixtures.[8] In the light of several recent investigations it is not surprising to find appreciable quantities of inactive protein in commercial samples of trypsin. Liener (1960), Cole and Kinkade (1961) and Maroux *et al.* (1962) have shown by different

[7] Some variation in these figures was encountered on different stock solutions.

[8] Assays were performed on *p*-toluenesulfonyl-L-arginine methylester as substrate by the pH-stat technique.

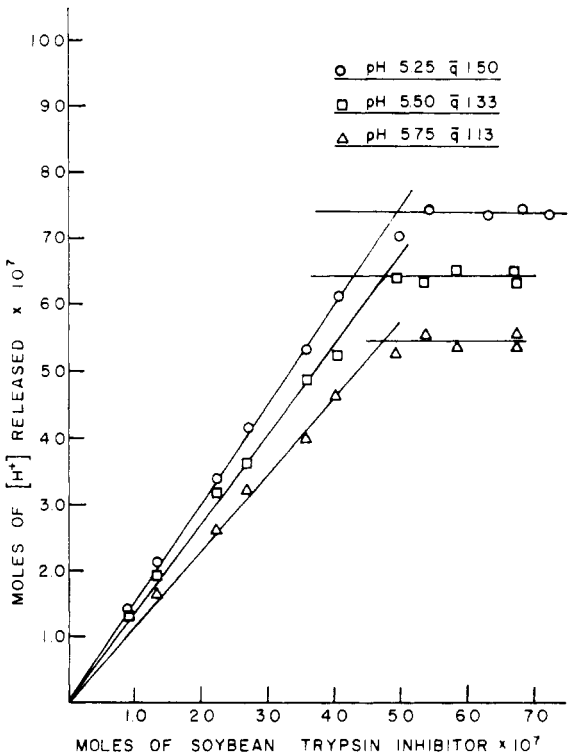

FIG. 5.—The association of trypsin with soybean trypsin inhibitor. *pH 5.25:* $V_0 = 15 \times 10^{-3}$ liters, $C_{STI} = 4.52 \times 10^{-5}$ moles/liter, and $m_T = 4.90 \times 10^{-7}$ moles. *pH 5.50:* $V_0 = 15 \times 10^{-3}$ liters, $C_{STI} = 4.49 \times 10^{-5}$ moles/liter, and $m_T = 4.82 \times 10^{-7}$ moles. *pH 5.75:* $V_0 = 15 \times 10^{-3}$ liters, $C_{STI} = 4.49 \times 10^{-5}$ moles/liter, and $m_T = 4.78 \times 10^{-7}$ moles. The data indicate the variation of the slope $\bar{q}$ with increasing pH and the essentially constant equivalence.

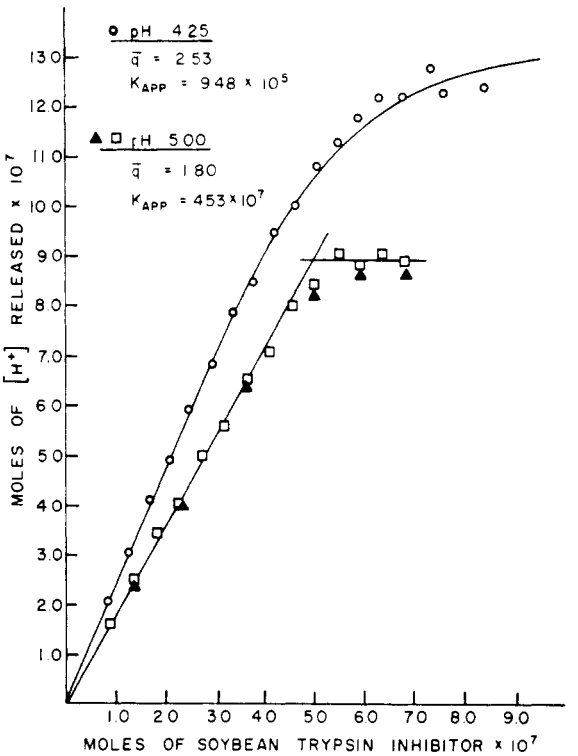

FIG. 6.—The association of trypsin with soybean trypsin inhibitor at the pH values indicated. *pH 5.00:* $V_0 = 15 \times 10^{-3}$ liters, $C_{STI} = 4.91 \times 10^{-5}$ moles/liter, and $m_T = 4.96 \times 10^{-7}$ moles. 1 mg STI inhibits 1.74 mg trypsin. *pH 4.25:* $V_0 = 15 \times 10^{-3}$ liters, $C_{STI} = 4.22 \times 10^{-5}$ moles/liter, and $m_T = 5.58 \times 10^{-7}$ moles. Smooth curve is theoretical for the $\bar{q}$ and K_{app} at pH 4.25 given in the figure. ▲, data obtained at pH 5.00 after 18-day storage of trypsin and inhibitor stock solutions at 3°.

chromatographic procedures that commercial preparations of trypsin contain only 50–80% of active protein. It is important to note in Figure 4 that the slope, $\bar{q}$, for each sample is the same, and therefore the inactive trypsin appears to play no role in the reaction. This is in complete agreement with the literature (Green, 1953; Laskowski and Laskowski, 1954). Since a substantial amount of trypsin is inactive, the concentration of active trypsin must be determined at pH 5.00 and this value employed at lower pH values, where equivalence cannot easily be obtained. This was done for all stock solutions prepared, and the equilibrium data presented are based on the assumption of 100% active inhibitor.

Figure 5 shows the determination of $\bar{q}$ for pH 5.25, 5.50, and 5.75. The reaction is stoichiometric with the slope $\bar{q}$ decreasing with increasing pH. As can be seen from the figure there is very little variation in the equivalence point for the three sets of data. Even though the calculated equilibrium constants at these pH values predict that the reaction should be essentially stoichiometric, a *very slight* curvature is apparent in the data. This must be due to small experimental errors. These errors might arise either from the presence of impurities or from slight changes in the liquid junction potential upon mixing of the protein solutions.

Figure 6 shows the data obtained at pH 5.00 and pH 4.25. It can be seen that there is considerably more curvature in the pH 4.25 data. This curvature is sufficient to allow a calculation of the association constant, K_{app}. Since the curvature is slight the initial slope is essentially $\bar{q}$. The application of equation (10) and $\bar{q}$ of 2.44 (initial slope) yield K_{app} of 1.02×10^6. Refinements by successive approximations give final values: $\bar{q} = 2.53$, $K_{app} = 9.48 \times 10^5$. The solid curve in Figure 6 is a theoretical one computed from equation (10) and the final parameters.

The extent of curvature at pH 4.50 and 4.75 was too small (intermediate between the pH 4.25 and pH 5.00) to allow accurate determination of the equilibrium constants. Of course, $\bar{q}$ values were readily determined for those pH values and the equilibrium constants were obtained by equation (5) (Table II).

Figure 7 shows the data obtained at pH 4.00. This is in the pH region where the equilibrium

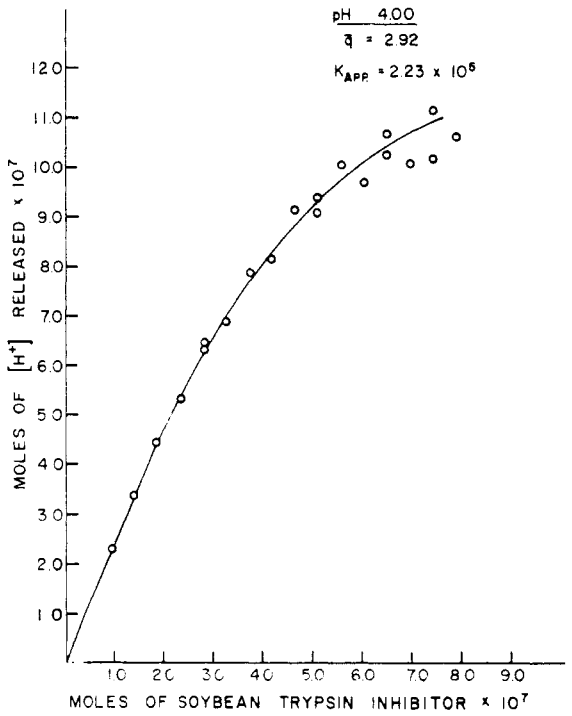

FIG. 7.—The association of trypsin with soybean trypsin inhibitor at pH 4.00. $V_0 = 15 \times 10^{-3}$ liters, $C_{STI} = 4.65 \times 10^{-5}$ moles/liter, and $m_T = 5.15 \times 10^{-7}$ moles. Smooth curve is theoretical for the $\bar{q}$ and K_{app} given in the figure.

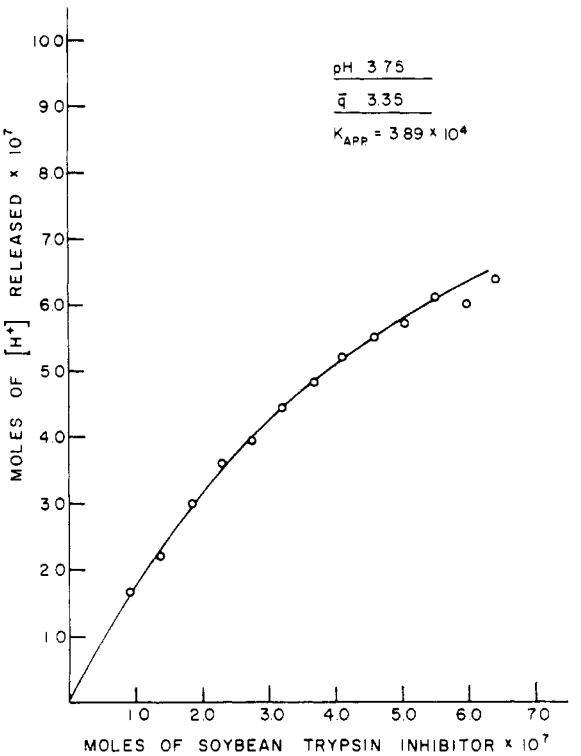

FIG. 8.—The association of trypsin with soybean trypsin inhibitor at pH 3.75. $V_0 = 15 \times 10^{-3}$ liters, $C_{STI} = 4.59 \times 10^{-5}$ moles/liter, and $m_T = 5.24 \times 10^{-7}$ moles. Smooth curve is theoretical for the $\bar{q}$ and K_{app} given in the figure.

constant is low enough so that the initial slope does not represent q. We first attempted to fit the experimental data by the successive approximation procedure outlined above, but the results did not converge on a single value of $\bar{q}$ and K_{app}. Excellent fits of the experimental data could be obtained for several widely different $\bar{q}$ values provided that corresponding values of K_{app} were chosen. It therefore became necessary to devise another procedure for the determination of $\bar{q}$. Performing the experiment at much higher trypsin concentration increases the extent of complex formation and the initial slope is more nearly $\bar{q}$. Since the initial slope $\bar{q}_{obs}$ is given by

$$\bar{q}_{obs} = \frac{K_{app}(T)}{1 + K_{app}(T)} \bar{q} \qquad (11)$$

simultaneous solution of $\bar{q}_{obs}$ obtained at two different trypsin concentrations yields both $\bar{q}$ and K_{app}. An experiment was carried out at a trypsin concentration five times greater than that of Figure 7 ($m_T = 2.58 \times 10^{-6}$) and $\Delta X/\Delta m_{STI}$ data were plotted against m_{STI}. Considerable (unexpected) curvature was obtained, presumably arising from complications due to the high trypsin concentration employed. Extrapolation of these data to zero m_{STI} yielded $\bar{q}_{obs} = 2.85$. Similar extrapolation of the data of Figure 7 yielded $\bar{q}_{obs} = 2.58$. Simultaneous solution yields $\bar{q}$ of 2.92 and K_{app} of 2.20×10^5. For $\bar{q}$ of 2 92 the best fit of the data in Figure 7 is obtained with K_{app} of 2.23×10^5. The very close agreement between the two methods of evaluation of K_{app} is regarded as satisfactory proof for validity of this procedure.

Figure 8 shows the data obtained at pH 3.75. It is seen here that the curvature is very great (K_{app} very low). An application of the procedure employed at pH 4.00 proved unsuccessful since the $\Delta X/\Delta m_{STI}$ curve could not be unambiguously extrapolated. Thus the value of $\bar{q}$ for this pH was obtained by an extrapolation of the available $\bar{q}$ vs pH data, which yielded a value of 3.35. With this value an excellent fit of the data in Figure 8 could be obtained with K_{app} of 3.89×10^4. These values are further supported by the agreement obtained between the values at this pH and at pH 4.00 and 4.25 by the use of equation (5) (see Table II).

In experiments carried out at or below pH 4.25, another interesting phenomenon (thus far not described) takes place. Above this pH, the reaction appears instantaneous. Upon addition of STI to a trypsin solution there is an instantaneous pH drop (seen as emf increase) (Fig. 9). Some minor variations in pH reading occasionally occur after the initial rapid drop, probably owing to small temperature adjustments, reequilibration of the electrodes, reaction to electrical disturbance,

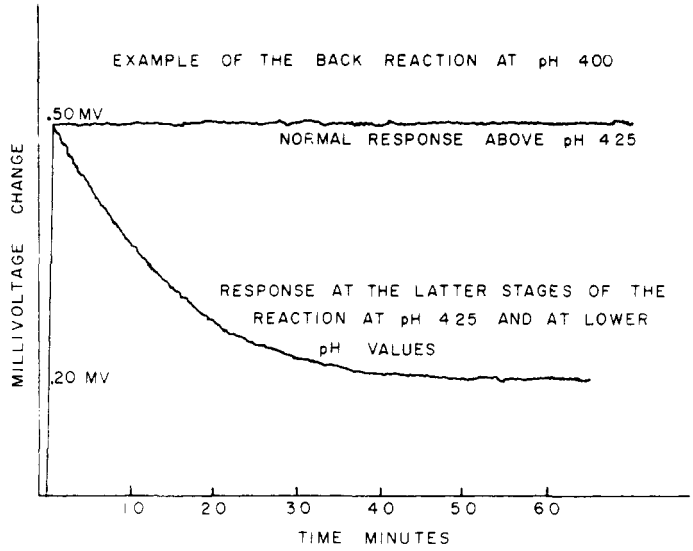

FIG. 9.—Recorded behavior of the 6th addition of STI (Table I) showing the kinetic behavior of the reaction at pH 4.00. The value for zero millivolts is not shown on the figure.

etc. These changes are never greater than 0.003 pH, generally in the direction of further pH drop, and are highly irreproducible. This behavior of the system depends appreciably on the characteristics of the electrodes employed. However, upon addition of STI to trypsin at pH 4.00 and 3.75 (and to trypsin-STI mixtures whose composition was close to equivalence at pH 4.25) the kinetic behavior of the pH reading is quite different. An instantaneous, large pH drop is reproducibly followed by a slow pH increase (Fig. 9). This backdrift phenomenon becomes more pronounced at lower pH values and closer to equivalence.

In Table I we have summarized the results of typical experiments at pH 4.00 and 3.75. It is seen that on addition of STI to trypsin a large number of protons are first released. Later some of these are bound by the proteins. A variety of explanations for these results can be considered. The simplest is to assume that the complex forms rapidly (with release of protons), and later isomerizes slowly to another form (with uptake of protons). This explanation is, however, completely untenable, as can be seen from examination of Table I, since initial addition at pH 4.00 produces almost no backdrift, whereas the later ones show that most of the initial change is later compensated by this effect.

An examination of data of Table I and of several other data at different protein concentrations led us to conclude that only one explanation is tenable. Upon initial addition, more complex forms than would be expected from simple equilibrium considerations (overshoot). The excess amount of the complex then decomposes, thus taking up some of the originally liberated protons. Such an explanation is consistent with the following observations: (1) no backdrift occurs at high pH,

TABLE I

INITIAL AND FINAL HYDROGEN ION RELEASE ON ASSOCIATION AT pH 4.00 AND 3.75

$m_{STI} \cdot 10^7$	ΔpH Initial[b]	$\left(\frac{\Delta X^c}{\Delta m_{STI}}\right)$ Initial	ΔpH Final	$\left(\frac{\Delta X}{\Delta m_{STI}}\right)$ Final
		pH 4.00[a]		
1.39	0.0344	2.58	0.0325	2.43
2.32	0.0189	2.46	0.0162	2.11
3.25	0.0151	2.15	0.0120	1.71
4.18	0.0132	2.08	0.0086	1.35
5.11	0.0114	2.05	0.0055	0.99
6.04	0.0086	1.61	0.0036	0.68
6.97	0.0069	1.49	0.0019	0.41
		pH 3.75[d]		
0.92	0.0186	2.54	0.0131	1.79
1.84	0.0148	2.52	0.0088	1.49
2.75	0.0115	2.08	0.0057	1.03
3.67	0.0108	2.33	0.0045	0.96
4.59	0.0088	1.88	0.0034	0.73
5.51	0.0076	1.89	0.0026	0.64
6.43	0.0065	1.44	0.0014	0.31

[a] A solution of 4.65×10^{-5} moles/liter STI was added to 5.15×10^{-7} moles of active trypsin, $V_0 = 15 \times 10^{-3}$ liters. [b] Some uncertainty exists in these data because of the difficulty in obtaining truly *initial* readings. [c] Computed on the assumption that over extremely short pH intervals titration curves are linear *i.e.* $\left(\frac{\Delta X}{\Delta m_{STI}}\right)$ initial $= \frac{\Delta pH \text{ initial}}{\Delta \text{ pH final}} \left(\frac{\Delta X}{\Delta m_{STI}}\right)$ final.

[d] A solution of 4.59×10^{-5} moles/liter STI was added to 5.24×10^{-7} moles of active trypsin, $V_0 = 15 \times 10^{-3}$ liters.

where the association constants are so large that the reaction is essentially stoichiometric, and thus no "excess" amount of the complex can be formed;

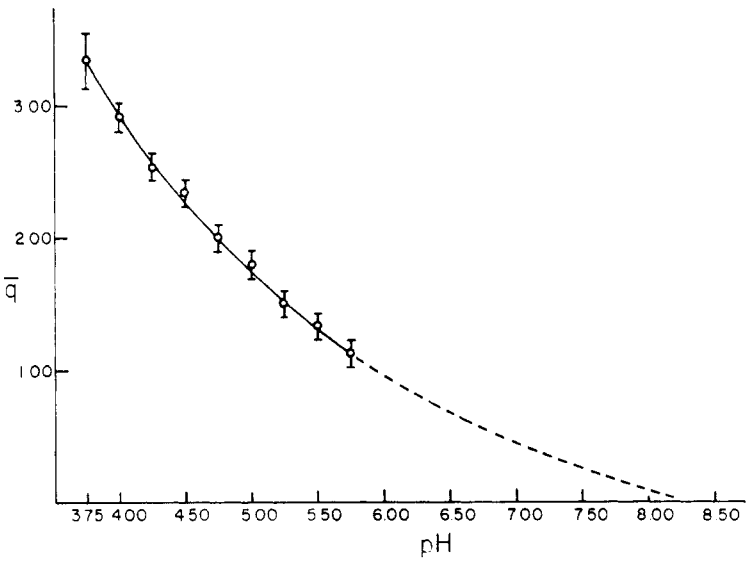

FIG. 10.—The variation of q̄ with pH. Smooth curve drawn through q values obtained and extrapolated to $\bar{q} = 0$. Area under this curve represents the change in log K_{app} with pH.

(2) more backdrift is encountered after later additions of STI, where more "excess" complex could be formed; (3) essentially no backdrift occurs when STI is added to very concentrated trypsin solutions, even at low pH.

The question now arises of how an overshoot in the reaction can occur. Simple kinetics of second order forward and first order reverse cannot permit an overshoot even if the association is very fast and the dissociation is slow. A proposed mechanism for the reaction is that when STI enters the reaction vessel there is a high concentration gradient of the inhibitor. The forward reaction or association takes place instantaneously in these high concentration regions; in other words, the kinetics of association is faster than the process of uniform distribution of the inhibitor, and an over-reaction occurs. This is compensated by a slow dissociation, when the complex is uniformly distributed. If the equilibrium constant is so large that any amount of STI can be accommodated, then the reaction is essentially stoichiometric, and no back-reaction or dissociation will occur. However, if the equilibrium constant is such that an appreciable fraction of the inhibitor should not react, then the conditions arise which may lead to a dissociation and the observed back-reaction. These are the conditions of experiments at low pH. At pH 4.25 the equilibrium constant is high enough so that no dissociation is observed until the later additions of STI, when α has fallen off sharply. When the pH is dropped to 4.00 the equilibrium constant is reduced by a factor of 4 and the conditions for over-reaction are present even on the first addition of STI. This becomes more pronounced at pH 3.75. One should expect that if the trypsin concentration were increased by a factor of five, as was the case in the high concentration experiments to determine $\bar{q}_{obs}$ at pH 4.00 and 3.75, no backdrift would occur because there would be sufficient trypsin to make α essentially 1. This is indeed the case.

The proposed mechanism requires the association reaction to be very fast. Green (1953) has shown that this is the case. Later measurements (Green, 1957) yielded a bimolecular rate constant of 2×10^7 liters/mole sec. at pH 7.8. This is indeed a very fast reaction. Whether the rate constant is sufficiently great at pH 4.00 is not known. Further, the mechanism requires that the dissociation of the STI-trypsin complex should be quite slow. This should lead to time-dependent titration of the complex. In recent experiments (Finkenstadt and Laskowski, 1962) it was found that sudden addition of acid to the complex at pH 5.00 (all associated) to drop the pH to 3.75 led to slow uptake of protons, with a half-time (~3 minutes) similar to that of the backdrift discussed above.

Throughout the rest of this paper, which is concerned with equilibrium constants, we have used the final equilibrium conditions, and only the final points of Table I were used in Figures 7 and 8.

DISCUSSION

Figure 10 is a plot of q̄ versus pH. The best smooth curve is drawn through the data and extrapolated (dashed curve) to $\bar{q} = 0$. The area bounded by this curve between any two pH values is the change in the logarithm of K_{app} between these two pH values (equation 5). The data given in Table II are calculated from Figure 10 (using the trapezoidal rule for integration over 0.25 increments in pH) and from the value of log K_{app} at pH 4.00. Table II shows the comparison

TABLE II
pH DEPENDENCE OF SOYBEAN TRYPSIN INHIBITOR–TRYPSIN ASSOCIATION[a]

pH	q	log K_{app} Calculated from eq. (5) and log K_{app} at pH 4.00	log K_{app} Calculated from eq. (10)
3.75	3.35	4.565	4.590
4.00	2.92		5.349
4.25	2.53	6.030	5.977
4.50	2.34	6.639	
4.75	2.00	7.182	
5.00	1.80	7.657	
5.25	1.50	8.070	
5.50	1.33	8.424	
5.75	1.13	8.732	
8.30[b]	0	9.832	

[a] In 0.50 M KCl, 0.05 M $CaCl_2$, at 20 ± 0.02°.
[b] Extrapolation of $\bar{q}$ *vs.* pH curve, Figure 10.

at pH 4.25 and 3.75 between the log K_{app} obtained experimentally by procedures already discussed, and the log K_{app} determined by the use of experimental $\bar{q}$ values in equation (5). Probably the best way of comparing these is to compare the Δ log K_{app} increments. For the interval from pH 3.75 to pH 4.00 the Δ log K_{app} calculated from equation (10) is 0.759, and the value obtained from equation (5) is 0.784; for the interval pH 4.00 to pH 4.25 the two values are 0.628 and 0.681 respectively. The agreement is quite good and verifies the internal consistency of the method. The agreement at pH 3.75 also supports strongly the extrapolation of the $\bar{q}$ versus pH curve to a $\bar{q}$ of 3.35. Poor agreement is obtained between theory and observed data if $\bar{q}$ does not rise between pH 4.00 and 3.75.

Table II shows that the equilibrium constant increases 14,700 fold from pH 3.75 to pH 5.75. It is seen in Figure 10 and much more dramatically in Figure 11, where log K_{app} is plotted versus pH, that the major change in K_{app} occurs between pH 3.75 and pH 5.75. The equilibrium constant changes only by a factor of 12.6 from pH 5.75 to pH 8.30, based on the dotted line extrapolation of Figure 10. We therefore felt that such an extrapolation was not overstepping the bounds of good judgment, and, since we were interested in a comparison of K_{app} wherever possible, this was a desirable feature of handling the data. It should be noted that pH 5.75 is not the limit of the potentiometric technique. Our instrument was not equipped for nitrogen atmosphere, and determinations of $\bar{q}$ at higher pH would be difficult without elimination of carbon dioxide. Of course, trypsin autolysis may become a serious factor as the pH is increased and it might destroy the possibilities of determining $\bar{q}$ in the basic pH region. However, we expect that the technique could be extended into the higher pH region with proper modifications. This will be attempted in the future.

Figure 11 shows a plot of our data (open circles) and of all other known equilibrium data for the STI-T association. It indicates clearly the utility of equation (5) and of the potentiometric technique for extending the determination of equilibrium constants outside the range of the usual physiochemical measurements for macromolecules. The potentiometric technique allows for measurement of arbitrarily large association constants provided only that for the system under study the association constant at some pH is low enough to be measured. In measurement of small equilibrium constants the technique suffers from the same difficulties as the conventional techniques, that the equilibrium constant must be large enough for measurable association to take place.[9] The log K_{app} determined at pH 7.8 by Green (1953) and represented by the open square is in good agreement with the extrapolated portion of our data. Grob (1949) suggested log K_{app} greater than 9 at this pH. At the low pH end the potentiometrically determined values fall between the rather widely scattered data obtained from the measurements of others. The equilibrium constants calculated by us from the sedimentation velocity experiments of Sheppard and McLaren (1953) and from the fluorescence depolarization studies by Steiner (1954) appear to have pH dependence similar to that observed in our work, although the sedimentation data are higher and fluorescence values lower. On the other hand the equilibrium constants obtained by Steiner (1954) by light scattering show a much smaller pH dependence. The discrepancy among these results probably arises from the difference in the experimental procedure employed. The sedimentation and fluorescence data were obtained at constant protein concentration, with the pH varied from the region where the proteins are completely dissociated to that where they are completely associated. The interpretation of such data is quite straightforward, and the results depend only slightly on the purity of the proteins employed. The light-scattering results were obtained at constant pH as a function of concentration. Such data are harder to interpret, since the results are heavily affected by the presence of inactive material. Another factor involved in the successive dilution procedure is that it causes small changes in the pH, and even though these changes are never more than 0.05 pH they may lead to a lowering of the equilibrium constant by a factor of 1.4 at pH 4.00. The large values of $\bar{q}$ in this pH region show that one must take extreme care with pH adjustments.

[9] It is possible that in some special situations the technique also has no lower limit. If the dissociation of the complex is slow, then $\bar{q}$ can be determined by comparison of rapid and slow titration curves even in pH regions where the association constant is too small for any appreciable association to take place. The use of this $\bar{q}$ in equation (5) would then allow calculation of K_{app}. Such experiments are now in progress in this laboratory.

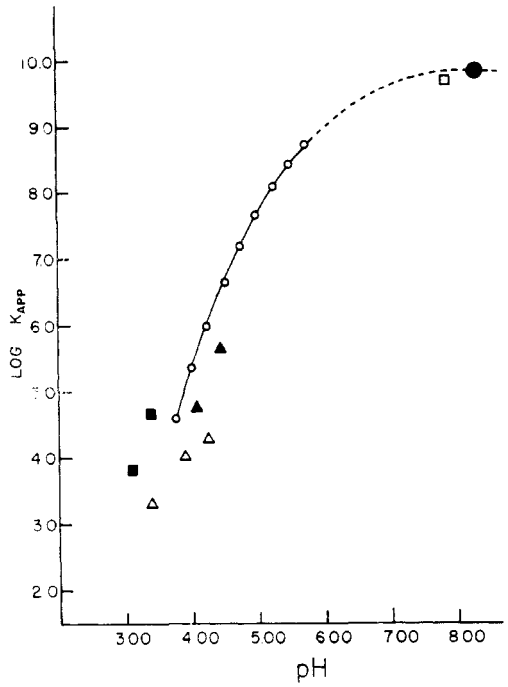

FIG. 11.—Variation of log K_{app} with pH. ○, data obtained by the potentiometric technique; ●, log K_{app} obtained from the extrapolation to $\bar{q} = 0$ of Figure 10; □, value obtained by Green (1953) by enzymatic technique at pH 7.8; △, values obtained by Steiner (1954) from light scattering measurements in 0.3 M KCl; ▲, values calculated by us from the polarization of fluorescence as a function of pH data of Steiner (Steiner, 1954, Fig. 6); ■, Calculated by us from the sedimentation data of Sheppard and McLaren (1953, Table III).

It must be borne in mind that the data of various investigators, summarized in Figure 11, were obtained under different conditions, which may well account for some differences. The measurements by others were not made in the same ionic medium as ours and different samples of proteins were used. By employing new chromatographic techniques one should be able to obtain purer preparations and refine the equilibrium data. The most serious flaw in all the equilibrium measurements, including our own, is the lack of an absolute measure of the tryptic activity. If a procedure were available for the absolute measurement of trypsin,[10] we would then be able to determine the active concentration of inhibitor and the true mole ratios. We have determined mole ratios based on the assumption of 100% active inhibitor. This is undoubtedly in error. The fact that the sample of STI used was shown to be chromatographically pure (Rackis, 1961) is very encouraging. Since we are in general agreement with the chromatographic studies on trypsin (previously mentioned), we feel that 10% is a reasonable maximum estimate of the inactive inhibitor. The assumption that the molecular weight of STI is 20,000 (Steiner, 1954) is most probably slightly in error. Such an error would affect all of the absolute values of $\bar{q}$ and thus all the theoretically computed equilibrium constants. If the error is less than 10% no substantial revision of our conclusions would be necessary.

The main aim of this study was to test and perfect the potentiometric technique for protein-protein association. This has been accomplished. It appears that the technique can be readily applied to any A + B protein-protein association where the molecular weights of the reacting species are known and provided that the association constants depend on pH. The measurement of equivalence and of q in the pH region where K_{app} is large is quite easy. The only requirement is a sufficiently sensitive and stable pH meter. On the other hand the evaluation of $\bar{q}$ and K_{app} from the curvature of the plots involves considerable difficulty in the detailed analysis of data. We think that, at present, the best use of the technique will be the coupling of K_{app} data obtained by conventional, macromolecular techniques in a pH range where such determinations are easy with potentiometrically determined $\bar{q}$ values in pH ranges where the reaction is stoichiometric. We did not choose to do so because we wanted to check the internal consistency of the technique.

[10] Dr. Myron Bender (personal communication, 1962) has recently devised such a procedure.

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