The interaction of protein protease inhibitors (for a review see Reference 3) with proteinases they inhibit is attractive for the study of kinetics and thermodynamics of protein-protein interaction. Among many advantages of these systems are: very simple stoichiometry (frequently 1:1), high specificity, and very high association constant near neutral pH. Furthermore, loss of enzymatic activity upon complex formation provides an additional monitoring technique for following the association, in addition to the generally applicable ones, such as change in molecular weight.

Recent findings have increased this list of advantages. It has been shown that proteinase inhibitors have on their surface a small cluster of amino acid residues, the reactive site, which comes into intimate contact with the active site of the enzyme in the stable enzyme-inhibitor complex (4). Within this reactive site there is a specific peptide bond, the reactive site peptide bond, which is specifically hydrolyzed (or resynthesized) by the enzyme. This leads to an equilibrium between virgin inhibitor (reactive site peptide bond intact) and modified inhibitor (reactive site peptide bond hydrolyzed) (5). The equilibrium constant is given by

$$K_{\text{hyd}} = \frac{[I^*]}{[I]}$$

where $I$ and $I^*$ are the virgin and modified inhibitors, respectively.

Both virgin and modified inhibitors interact with the enzyme to form the same stable complex, $C$ (6, 7), which in turn can dissociate to either the free enzyme and virgin inhibitor or to free enzyme and modified inhibitor. These statements can be summarized by the minimal mechanism of interaction for trypsin inhibitors

$$T + I \xrightarrow{k_1} L \xrightarrow{k_2} C \xrightarrow{k_3} K + I^*$$

where $T$ is the free trypsin. Such a mechanism is, however, clearly too simple, since it demands that covalent changes involving the reactive site peptide bond occur as a consequence of simple, second order steps without the intervention of noncovalent intermediates.

The realization that proteinase inhibitors are specific proteolytic substrates greatly improves the prospects of understanding of the mechanism of interaction, since kinetic data obtained by following the virgin or modified inhibitor conversion by catalytic amounts of enzyme can be combined with data on the rate of
complex formation and dissociation obtained under conditions, where the two reactant proteins are present in equimolar or near-equimolar amounts. Furthermore, the equilibrium constant, $K_{\text{hydr}}$ (Equation 1) can be independently measured as can the over-all association constant, $K_{\text{assoc}}$ (8, 9)

$$K_{\text{assoc}} = \frac{\Sigma(C)}{(T) (T)+(T^*)}$$

(3)

where the summation term in the numerator denotes all possible associated species of the stoichiometry $T·I$ and $T·I^*$ that exist in equilibrium. It is shown later in this paper that at least two additional species other than the stable complex, $C$, should be considered.

In view of the interest in the system we have embarked on a program of detailed characterization of the interaction of bovine $\beta$-trypsin and soybean trypsin inhibitor (Kunitz) over a broad pH range with the aims of (a) formulation of an acceptable minimal mechanism of interaction; (b) determination of all of the kinetic parameters appearing in such a mechanism; (c) independent determination of all of the values of the two equilibrium constants, $K_{\text{hydr}}$ and $K_{\text{assoc}}$; and (d) comparison of the values of these equilibrium constants with those calculated from the kinetic parameters.

The comparison described in d was already carried out on the basis of the rather rough preliminary values of all the parameters available in our laboratory in the fall of 1970 (2). It was found that the "kinetic" and "thermodynamic" values of both $K_{\text{hydr}}$ (Equation 1) and of $K_{\text{assoc}}$ (Equation 3) agree within a factor of 3 to 5 over a relatively broad pH range. It should be appreciated that this comparison involves several kinds of data obtained by a variety of experimental techniques and approaches. Several workers in our laboratory are now refining and improving upon the various data sets. We are planning to publish the "final" data as soon as they are available in a series of papers, each dealing with data acquired by the use of a single experimental technique. This is the first paper of such a series.

In this paper we are concerned with rate of formation of the stable complex, $C$, from $\beta$-trypsin and from either virgin or modified soybean trypsin inhibitor (Kunitz) over a pH 4.5 to 8.0 range and also with the over-all rate of dissociation of the stable complex in the pH 1.7 to 3.5 range.

**EXPERIMENTAL PROCEDURE**

**Choice of Experimental Method**—In studying the association and dissociation reactions we have chosen to limit the pH ranges to those where the respective reactions are practically irreversible. This imposed a lower limit of pH 4.5 on the association reaction since below this pH, $K_{\text{assoc}}$ is too low (8) to insure complete association. Similarly an upper limit of pH 3.5 was selected for the dissociation reactions, since above that pH dissociation is incomplete.

In the chosen pH ranges both the association and dissociation reactions are very fast and therefore stopped flow spectrophotometry was a natural choice for their study. Feinstein and Feeney (10) have already reported on a preliminary stopped flow study of the association of turkey ovomucoid with bovine trypsin. In that study, as well as in several previous studies on the interaction of small substrates with serine esterases the association process was monitored by displacement of dyes such as proflavine or thionine from the enzyme by the bound substrate or inhibitor (11-14). This approach provides large, readily measurable signals at a convenient wave length. However, it suffers from a variety of complications characteristic of all-in

direct techniques. Complications can be envisaged if the enzyme-substrate (14) or enzyme-inhibitor complex or the inhibitor itself bind the dye. Furthermore, difficulties are encountered due to the fact that, in our laboratory, we have found that $K_{\text{assoc}}$ (Equation 1) can be independently measured as can the over-all association constant, $K_{\text{assoc}}$ (8, 9) 

$$K_{\text{assoc}} = \frac{\Sigma(C)}{(T)(T)+(T^*)}$$

(3)

where the summation term in the numerator denotes all possible associated species of the stoichiometry $T·I$ and $T·I^*$ that exist in equilibrium. It is shown later in this paper that at least two additional species other than the stable complex, $C$, should be considered.

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small amount of trypsin in these preparations was removed as trypsin-inhibitor complex by Sephadex chromatography near neutral pH (20). In earlier work trypsin was left in the preparations and solutions of modified inhibitor produced a sizeable amount (20 to 30%) of virgin inhibitor due to re-equilibration (5) upon adjustment to neutral pH. This led to complications in interpretation of kinetic data (see "Results"). The molar concentration of the inhibitor was obtained by absorbance measurement at 279 nm assuming 1.1 (21) as the optical factor and a concentration of the inhibitor was obtained by absorbance measurement at 279 nm assuming 1.1 (21) as the optical factor and a molecular weight of 22,000.

Soybean inhibitor complexes with either α- or β-trypsin were prepared by mixing roughly equimolar quantities of tryptic and of inhibitor and then isolating the complexes by Sephadex chromatography (20). Most of these materials were prepared by utilizing the effluents from the stopped flow apparatus after the completion of a trypsin-inhibitor association experiment.

All of the experiments were carried out in 0.50 M KCl, 0.05 M CaCl₂ in deionized, glass-distilled water. This is the solven system used in most trypsin-inhibitor interaction studies reported from our laboratory. The reactant solutions were buffered by acetate (pH 4.5 to 5.6), 2-(N-morpholino)ethanesulfonic acid (pH 5.4 to 6.9), piperazine-N,N'-bis(2-ethanesulfonic acid) (pH 6.9 to 7.5), and N'-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.4 to 8.0) (Sigma Chemical Corp.) buffers.

For all association measurements below pH 6 the pH values of both reactant solutions were adjusted to be equal. The pH of the effluent after the completion of the stopped flow experiment was measured and was found to be within 0.05 pH from the starting pH. This final pH value is the one reported. At pH values above 6, complications arose due to autolysis of concentrated tryptic solutions. To circumvent this the inhibitor solution was buffered at the desired pH, the tryptic solution was left unbuffered at pH 3.5. After the final pH of the effluent was read and recorded. No significant variation in over-all reaction rates was observed when this precaution was not followed, the principal effect was diminution of signal strength as the tryptic solutions were maintained above pH 6 for prolonged periods of time (hours).

Measurements—Protein concentrations and difference spectra were obtained with either Cary 14 or Cary 15 Spectrophotometers.

The rate measurements were made on a Durrum-Gibson stopped flow spectrophotometer equipped with interchangeable 2- and 20-mm path length Kcl-F observation cells and a calibrated zero-offset control.

Since very small signals were monitored in this study great care was taken to eliminate even small extraneous signals. One cause of such extraneous signals was the use of the instrument's water thermostat, especially when the thermostat was operated at temperatures significantly below or above ambient. These extraneous signals apparently arise because the mixing chamber and observation cell are thermostated while the reactant syringes are not. To eliminate this source of errors the instrument was operated at the ambient temperature of the air-conditioned laboratory (21 ± 1°) without the water thermostat.

All protein solutions were freshly prepared from lyophilized powders before each series of experiments. Dust was minimized by Millipore filtration.

To maximize the signals the 100% transmittance baseline was set for a solution of reaction products rather than, as is commonly done, for pure solvent. This was accomplished by mixing the reactants several times in the stopped flow apparatus, before interpretable data were taken. After the last of these preliminary mixings the output voltage of photomultiplier was adjusted to 1.000 volt. A slit of 4 mm was generally used. The photomultiplier voltage ranged from 550 volts for the most concentrated solutions to 350 volts for the most dilute ones. The noise filter was adjusted to 100 times less than the full time sweep.

After the reactants were mixed, per cent transmittance as a function of time was recorded on a Tektronix storage oscilloscope. The trace was photographed and digitized, occasionally by simply reading the photograph but normally with the aid of a Larr Y Coordinatograph which produced punched cards for computer use. Typically 40 to 200 points were recorded from each photograph.

In dissociation measurements closely similar procedures were followed except that one of the syringes was filled with a solution of desired trypsin-inhibitor complex at pH of approximately 5.5, and the other with 0.5 M KCl, 0.05 M CaCl₂ adjusted to very low pH with HCl. The pH equilibration upon mixing was assumed to be instantaneous and the decrease in transmittance on dissociation was recorded. The interpretation of data is described under "Results." Non-linear least squaring was carried out on CDC6500 computer, according to methods described by Deming (22).

RESULTS

Static Difference Spectra—Static difference spectra between trypsin-inhibitor complex in the sample cell and the two reactants in separate compartments of the reference tandem double cell are shown in Fig. 1. The results are in agreement with those reported earlier by Dononayl and Trouwbridge (15) and Eidelhoch and Steiner (16). It is clear that a signal for following complex formation kinetics is available, but it is very weak. In order to choose a wave length at which to carry out the kinetic measurements we have also plotted these results as Δλ/Δη, where Δλ is obtained from Fig. 1A and η is the molar absorbance of the complex at the specific wave length (Fig. 1B). From inspection of these plots we have concluded that the most satisfactory wave length for kinetic measurements is 260 nm, since this valley in the difference spectrum is quite broad and therefore least affected by the very broad slit (4 mm corresponding to 16-nm spectral band width) which was used in the kinetic measurements.

Order of Complex Formation Reaction—Equimolar solutions of soybean trypsin inhibitor and of bovine β-trypsin were mixed in the stopped flow apparatus and transmittance at 260 nm was monitored as a function of time after mixing. As expected transmittance rose (absorbance fell). The magnitude of the signal (see below) was approximately what had been expected from the static difference spectra. Furthermore, a few experiments at 293 nm (see Fig. 1) showed that the transmittance fell (absorbance rose) at that wave length, again as expected. These preliminary experiments indicated that complex formation was indeed being monitored.

In order to determine the order of the reaction the starting concentration of reactants was varied over the broadest experimentally accessible range (10⁻⁴ to 10⁻¹ M) and the half-times were determined. The results are presented as the logarithm of half-time versus the logarithm of concentration in Figs. 2. It is clear that the order with respect to concentration switches from second order to first order in the concentration range examined. Strikingly similar results were obtained both for slowly reacting modified inhibitor (upper curve) and for rapidly reacting virgin inhibitor (lower curve). The switching from second to first order was observed at all pH values investigated.
FIG. 1. A, difference in molar absorptivity between $\beta$-trypsin-soybean trypsin inhibitor complex and the free reactants. Data were obtained in tandem double cells (1-cm path length of each compartment). Sample cell: Compartment 1, $3.01 \times 10^{-5}$ M trypsin-inhibitor complex + $0.51 \times 10^{-5}$ M inhibitor; Compartment 2, solvent. Reference cell: Compartment 1, $3.01 \times 10^{-5}$ M trypsin; Compartment 2, $3.51 \times 10^{-5}$ M inhibitor. All at pH 6.00 in 0.5 M KCl, 0.05 M CaCl$_2$, and 0.1 M 2-(N-morpholino)ethanesulfonic acid. $B$, the data of $A$ divided by molar absorptivity of $\beta$-trypsin-soybean trypsin inhibitor complex at the appropriate wave length.

The order with respect to time can be obtained by examination of the oscilloscope traces. Two such traces are shown in Fig. 3. It can be seen that the upper one (low concentration) is largely second order while the lower one (high concentration) is largely first order. Thus, the order with respect to time also switches from second to first as the concentration of reactants is raised.

Formulation of Model—The observed behavior clearly suggests that at least two processes are sequentially involved in stable complex formation, a second order process followed by a first order one. We have already pointed out in the introduction that the minimal mechanism given in Equation 2 seems unsatisfactory, since it postulates that bond-breaking and bond-making events occur in simple, second order steps. The simplest way to account for the experimental observations on order switching and to overcome the objections to the mechanism of Equation 2 is to extend the minimal mechanism to

$$T + I \xrightarrow{k_1} L \xrightarrow{k_2} C$$

where $L$ and $L^*$ are loose, noncovalent complexes $T-I$ and $T-I^*$ respectively.

Fortunately, all of the steps in this mechanism need not be considered simultaneously in order to deal with the complex formation experiments. As already pointed out above pH 4.5 the equilibrium constant for complex formation, $K_{complex}$ is so large (8) that complex formation is effectively irreversible. Therefore, the complex dissociation steps $k_2$ and $k_3$ in Equation 4 need not be considered. This reduces the mechanism for virgin inhibitor-trypsin association to

$$T + I \xrightarrow{k_1} L \xrightarrow{k_2} C$$

An analogous equation can be written for modified inhibitor-trypsin association.

These $\Delta \epsilon/\epsilon_0$ values indicate the fractional change in molecular absorptivity occurring on complex formation at each wave length.
p-trypsin, 2.00;

4.65; 2-mm path length. The system, where small in comparison to $k_z$. If $k^{-1} \ll k_z$ Equation 5 describes a simple set of data. To do this it is necessary to know, whether $k^{-1}$ is large or with the evaluation of the parameters from the experimental rate-limiting process is always the possibility we must also consider a new complication, which represents the infinite time value of the transmittance (i.e. 100% T). (This was determined by allowing the % T axis deflection to reach a constant value.)

However, this simplification is still not sufficient to proceed with the evaluation of the parameters from the experimental data. To do this it is necessary to know, whether $k^{-1}$ is large or small in comparison to $k_z$. If $k^{-1} \gg k_z$ Equation 5 describes a system, where $T + I$ rapidly come to equilibrium with $L$ and the rate-limiting process is always the $L \rightarrow C$ conversion. On the other hand if $k^{-1} \ll k_z$ Equation 5 describes a simple set of consecutive reactions, where at low reactant concentration the $T + I \rightarrow L$ rate is rate-limiting and at high concentration the rate-limiting step is $L \rightarrow C$. In deciding between these two possibilities we must also consider a new complication, which arises from the introduction of the intermediates $L$ and $L^*$. Static difference spectra provide us only with the difference in absorbance between $T + I$ and $C$; however, this difference could arise either on the formation of $L$ or on conversion of $L$ to $C$. Obviously, it could also be a combination of these possibilities; however, one is hopefully dominant and the somewhat simplistic all or nothing decision will be attempted.

If $k^{-1} \ll k_z$ and the absorbance change occurs on the $T + I \rightarrow L$ step the observed transmittance changes would be second order both with respect to time and to concentration at all concentrations studied. Thus, this possibility can be immediately rejected. If $k^{-1} \ll k_z$ and the absorbance change occurs in the $L \rightarrow C$ step, the required second order to first order switching will be observed. However, at intermediate concentrations, where the half-times of $L$ formation and its decay to $C$ are approximately equal, there will be a pronounced sigmoidity (initiation period) in the absorbance versus time curves, since shortly after mixing the formation of $C$ is slow, because only little $L$ has formed. This qualitative conclusion has been confirmed by computer simulation. Careful examination of all of the oscilloscope traces at a variety of concentrations showed no sigmoidity. Therefore, this possibility was rejected.

Thus, we are left with $k^{-1} \gg k_z$, i.e. rapid pre-equilibration, to form $L$. This possibility should be favored anyway by analogy to many enzymatic reactions, where the first substrate-binding step is generally regarded as very rapid and readily reversible. The later finding that the constants $K_L$ and $K_{I_C}$ are equal and independent of pH further supports this choice.

In this case, if the absorbance change arose on the $T + I \rightarrow L$ reaction and since this reaction is too rapid to monitor directly, large time-dependent absorbance changes would be observed only at low concentrations where only a small fraction of the reactants combines to form $L$. This fraction is proportional to $[T][I]$ if $[T]_0 = [I]_0$ and the rate of formation of $C$ is proportional to $[L]$, hence second order kinetics for the over-all process. On the other hand at high concentrations the combination of $T + I$ to give $L$ (and the signal) is essentially complete before recording begins. Thus in the first order concentration range the reaction could not be monitored. This possibility can be eliminated since signals remained strong well into the first order concentration range.

The last possibility, that $k_{-1} \gg k_z$ and absorbance changes occur entirely on the $L \rightarrow C$ step is taken as correct. A quantitative consequence of this statement is that the kinetic difference spectrum, $\Delta A_{260}$, the difference in molar absorbivity at 260 nm between the products (i.e. the system a long time after mixing) and the reactants (i.e. the system immediately after mixing) should be independent of starting reactant concentration and equal to the static difference spectrum (see Fig. 1). In making the last comparison however, it should be borne in mind that the static difference spectra were determined with a narrow wave length band width (less than 1 nm), while the kinetic ones were determined with a very broad band width (16 nm) and therefore the absolute magnitude of the kinetic difference spectra should be expected to be smaller than of the static difference spectra owing to slit broadening.

The data shown in Fig. 4 are a compilation of the kinetic difference spectra obtained in this research. Each point in the upper part of the figure represents an average of all $\Delta A_{260}$ values obtained at the pH of interest, generally at a variety of concentrations. In order to avoid a possible bias, data obtained with virgin and with modified inhibitor and data obtained in different buffer systems are distinguished. In the lower part of the figure the points are averages of all the data obtained at a single concentration (generally at a variety of pH values).

While the data are greatly scattered no clear trends in pH or concentration dependence are apparent and the kinetic difference spectra for mixing of either virgin or of modified inhibitor with trypsin appear approximately the same. Furthermore, the magnitude of the values is similar to (although lower) than the value of static difference spectra indicated as solid bars in the figures. Thus, within the large experimental error, the assumption that the signal arises predominantly in the $L \rightarrow C$ step ap-

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**Fig. 3.** Oscilloscope traces of the change in transmittance at 260 nm with time. Conditions: upper photograph, $2.00 \times 10^{-4}$ m p-trypsin, $2.00 \times 10^{-4}$ m virgin soybean trypsin inhibitor in 0.5 m KCl, 0.05 m CaCl$_2$, and 0.05 m acetate (final concentrations); pH 4.65, 20-mm path length. Lower photograph, $3.00 \times 10^{-4}$ m p-trypsin, $3.00 \times 10^{-4}$ m virgin soybean trypsin inhibitor in 0.5 m KCl, 0.05 m CaCl$_2$, and 0.05 m acetate (final concentrations); pH 4.65, 2-mm path length. The upper trace in each photograph represents the infinite time value of the transmittance i.e. 100% T. (This was determined by allowing the % T axis deflection to reach a constant value.)
Evaluating Parameters—In order to obtain an expression for $C$ as a function of time we define equilibrium constants

$$K_L = \frac{\left[T\right]_0}{[T]} = \frac{k_{-1} + k_2}{k_1}$$

and

$$K_L^* = \frac{\left[T^*\right]/[T]}{[T]/\left[T^*\right]} = \frac{k_4 + k_{-3}}{k_4}$$

For the special cases of interest here of equal starting reactant concentrations, $[T]_0 = [I]_0 = a_0$. It is convenient to define concentration variable $a = a_0 - [C]$. Then at any time, $T = (T_a - a)$, and substitution into Equation 6 and solving the quadratic equation yields

$$[L] = \frac{K_L}{4} \left( \sqrt{1 + \frac{4a}{K_L}} - 1 \right)^2$$

Since

$$\frac{d(C)}{dt} = -\frac{d[a]}{dt} = k_2[L]$$

an expression for $a$ as a function of time is obtained by inserting Equation 8 into Equation 9 and integrating. This yields

$$k_2t = -2 \left[ \ln \left( 1 + \frac{4a}{K_L} \right) - 1 \right] - \frac{1}{\sqrt{1 + \frac{4a}{K_L} - 1}}$$

A strictly analogous expression arises for the reaction with modified inhibitor with $k_{-3}$ replacing $k_2$ and $K_L^*$ replacing $K_L$.

If the initial concentration of reactants is low (i.e. $4a_0/K_L \ll 1$), this relation reduces to a simple second order expression

$$k_2t = -2 \left[ \ln \frac{1}{a_0} - 1 - \frac{1}{a_0} \right]$$

with a second order rate constant $k_2/K_L$ and in this concentration region the half-time is given by

$$t_{1/2} = \frac{K_L}{k_2a_0}$$

Thus, the slope of the logarithm of $t_{1/2}$ versus logarithm of $a_0$ (starting concentration) (see Fig. 2) is minus one and the value of log $t_{1/2}$ when log $a_0 = 0$ ($a_0 = 1$) is log $K_L/k_2$.

If the initial concentration of the reactants is high (i.e. $4a_0/K_L \gg 1$) then Equation 10 reduces to the familiar first order expression

$$k_2t = -\ln \frac{a}{a_0}$$

and a half-time given by

$$t_{1/2} = \frac{1}{k_2 \ln 2}$$

Obviously, the plot of logarithm of $t_{1/2}$ versus the logarithm of $a_0$ in this concentration range is a horizontal straight line and its intercept on the log $t_{1/2}$ axes allows for direct evaluation of $k_2$.

The discussion above shows that, provided data similar to those shown in Fig. 2 were available for both the virgin inhibitor and trypsin and modified inhibitor and trypsin over a broad concentration range at each pH of interest, the values of the parameters $k_2$ and $K_L$ and of $k_{-3}$ and $K_L^*$ could be read from such plots virtually by inspection.

However, for a variety of reasons this superficially simple procedure was relatively little utilized in this work, and was relegated mainly to checking of the parameters calculated by other methods. The reasons are as follows:

1. The concentration range required to obtain switching from virtually pure second order to virtually pure first order behavior is very broad and hard to attain experimentally. It should be noted that this concentration range is far broader than the more familiar one for switching from first to zero order behavior in the classical Michaelis-Menten system.

2. The method places maximal stress on very high and very low concentration data. These are experimentally the least reliable data, while the most reliable data in the middle of the concentration range are not utilized at all.

3. Using each photograph to evaluate only a single $t_{1/2}$ is wasteful since the trace contains additional information.

It was clear that a procedure was needed in order to utilize the data in the middle concentration range and to use the full...
time course of the association reaction. This was accomplished by nonlinear least-squares fitting of the digitized data to the expression given by Equation 10. It should be noted that in such fitting there are four possible parameters to vary: starting absorbance, final absorbance, and $k_2$ and $K_{L}$ (or $k_{-3}$ and $K_{L*}$ in the modified inhibitor-trypsin association). It may seem surprising that starting and final absorbances are variables since the final absorbance was set to be zero (see "Experimental Procedure") and in case of large signals starting absorbance should be obvious. However, as an inspection of Fig. 3 will show the signals measured here were small and therefore the starting absorbance was not obvious and small 100% transmittance line shifts had an important effect on the data.

Unfortunately, convergence could not be obtained if all four parameters were allowed to vary, and the value of $K_{L}$ or of $K_{L*}$ had to be guessed at and fixed in the fitting procedure. Thus the remaining three parameters (starting absorbance, final absorbance, and $k_2$) were allowed to vary around original guesses, and convergence was obtained. This procedure was carried out for all the data obtained at each pH value (typically about 25 runs (five each at five initial reactant concentration spanning a 30-fold initial concentration range)). The procedure was repeated with different assumed values of $K_{L}$ and the results showing the smallest concentration dependence of $k_2$ were judged to be correct. This generally allowed for an evaluation of $pK_{L}$ (or $pK_{L*}$) to ±0.2, about the same error band as the one obtained from the graphical procedure of Fig. 2 analyzed as described above. The difference between final absorbance and initial absorbance (corrected for reactant concentration and path length) was converted to $-\Delta A_{530}$ and these data are shown in Fig. 4.

In another variant of the computing procedure the expected difference between the final and initial absorbance was calculated from an assumed value of $-\Delta A_{530} = 825$ and kept constant. Thus, only $k_2$ and initial absorbance were allowed to vary. The effect of this variant was not dramatic and average values of $k_2$ and of best $pK_{L}$ (or $K_{L*}$) were closely similar to those obtained from other methods. In order to show what can be learned about $K_{L*}$ from the analysis of the time dependence of a single trace at an intermediate concentration, we present in Table I an analysis of a single trace at pH 6.30. It is clear from the results that Equation 10 with appropriate $K_{L*}$ value fits the time course very much better than either a first order or a second order reaction.

On the basis of examination of all of the data described above we have concluded that the best value for $pK_{L}$ and $pK_{L*}$ is $pK_{L} = pK_{L*} = 4.65 \pm 0.2$. Within the stated experimental error these values are independent of pH over the whole 4.5 to 8.0 pH range. This conclusion is in disagreement with our preliminary data (2) published earlier. We stated there that $pK_{L}$ was 4.6 and independent of pH and that $pK_{L*}$ was 4.7 below pH 6 but that it dropped sharply to 3.5 as the pH was raised above 6. It now appears that this error arose from mistakes in data, not in calculation. In the earlier experiments the catalytic amounts of trypsin present in modified inhibitor preparation were not removed (see "Materials") therefore when solution of predominantly modified inhibitor were raised to pH values near neutrality re-equilibration could take place (5) and after several hours as much as 20 to 30% of the modified inhibitor could be converted to the virgin form. Since virgin inhibitor reacts with trypsin at a much higher rate than the modified inhibitor does, the reaction traces obtained show a fast phase followed by a slower one i.e. second order character even at high concentrations. Elimination of trypsin eliminates the chance of re-equilibration and traces obtained at high initial reactant concentration are essentially first order.

**Table I**

<table>
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<th>Reduced fractional time</th>
<th>First order calculated</th>
<th>Calculated from Equation 10</th>
<th>Second order calculated</th>
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<td>5</td>
<td>13.1</td>
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</table>

**Fig. 5.** The dependence of the logarithm of the first order rate constants, $k_2$ and $k_{-3}$, for conversion of loose complexes, $L$ and $L^*$, to the stable complex, $C$. $\blacklozenge$, log $k_2$ ($L \xrightarrow{k_2} C$); $\blacktriangle$, log $k_{-3}$ ($L^* \xrightarrow{k_{-3}} C$) stopped flow data; $\blacktriangleleft$, log $k_{-3}$ data of Reference 7.
$k_2/K_L$ and $k_{-2}/K_{L*}$ should be largely, although not entirely, free of systematic errors due to possible $K_L$ and $K_{L*}$ misassignment, since the errors in the two parameters largely compensate.

An important possible source of errors in the pH dependence of the data could arise from buffer effects. In all the pH ranges, where two buffers overlapped experiments were carried out at a single pH value with two different buffer systems. No important differences were noted.

**Comparison of α and β Trypsin.** In all of the experiments described thus far β-trypsin was exclusively employed, however the isolation procedure yielded equal amounts of α-trypsin. A single set of experiments was carried out on the association of virgin inhibitor with α-trypsin at pH 4.65 and the results are compared with those for virgin inhibitor-β-trypsin in Fig. 6. It appears from these data that the values of the first order rate constant $k_2$ (for the $L \rightarrow C$ reaction) are the same for both systems but that the loose complex, $L$, between α-trypsin and virgin inhibitor is almost an order of magnitude weaker than that formed with β-trypsin, i.e. $K_{L*} \cong 10K_{L*}.$

This result is consistent with the finding that the α-trypsin forms less stable complexes than β with a variety of trypsin inhibitors: soybean trypsin inhibitor (Kunitz) (2, 24), pancreatic trypsin inhibitor (Kunitz) (24), and chicken ovomucoid (25). These results point out again the necessity of carrying out of all quantitative measurements on bovine trypsin with purified preparations of β-trypsin and re-emphasizes the great importance of the contribution of Schroeder and Shaw (17). On the other hand this restriction increases the labor involved in research a great deal.

**Rates of Dissociation of Stable Complexes—**The use of the difference spectral monitor allowed us to carry out an additional set of experiments, which would not have been possible by the use of the indirect proflavine or thionine probes, since these dyes do not bind to free trypsin at very low pH values. Solutions of complex were mixed with a strongly acidic solvent. It was assumed that the attainment of the final pH value was very fast and that the time dependence of the absorbance at 293 nm monitored the expected dissociation of complex. As expected absorbance at 293 rose with time (on association the absorbance falls) and calculated $\Delta A_{650}$ was approximately 800 (see Fig. 4 for the absorbance values for association). In a single experiment at 293 nm absorbance fell, again the opposite of association. The observed traces were strictly first order with respect to time and the measured rate constants were independent of initial complex concentration. The values of the observed rate constants as a function of pH are shown in Fig. 7. The collection of data was terminated at the low pH end of the range because the observed rates became too fast to measure with our equipment. It was terminated at the high pH end (around pH 3.8) because increasing the pH to pH values higher than 3.8 does not cause complete dissociation of the complex (8). In this work the lack of complete dissociation above this pH value was confirmed, since when experiments at higher pH values were attempted the observed $\Delta A_{650}$ was smaller than 800, indicating incomplete dissociation.

The data obtained in this experiment were quite easy to interpret and the values shown in Fig. 7 are probably highly reliable. However, the assignment of the rates of dissociation must be based solely on the basis of the stopped flow measurements, since no other kinetic and thermodynamic data on the trypsin-soybean trypsin inhibitor system are available in this low pH range. The only additional item of proof available is that these data can be merged moderately well with dissociation rate constants obtained at much higher pH values by more direct techniques (2).

The dissociation rate constant, $k_D$, in the mechanism given by Equation 4 is given by

$$k_D = k_{-2} + k_3$$

The alternate explanation that the rate $k_D$ is related to $k_1$ and $k_4$ is highly unlikely (see "Discussion"). Kinetic control dissociation experiments carried out on the soybean trypsin inhibitor (Kunitz)-bovine β-trypsin system at pH 2 (or at other very low pH values) (2, 7) show that virtually pure virgin inhibitor is obtained upon dissociation and, therefore, for this system $k_{-2} >> k_4$. From this and Equation 15 it follows that

$$k_D = k_2$$

Similar relation ($k_{-2} >> k_3$) appears to hold for several other proteins.

**Fig. 6.** Comparison of the dependence of the logarithm of the association half-time, $t_{1/2}$, on the logarithm of the reactant concentration for the association of β-trypsin (O) and α-trypsin (△) with virgin soybean trypsin inhibitor (Kunitz); pH 4.65; 0.5 m KCl, 0.05 m CaCl₂, and 0.05 m acetate; $T = 21^\circ$.

**Fig. 7.** Dependence of the logarithm of the rate constant of the dissociation of the trypsin-inhibitor complex on pH. O, complex made from α-trypsin and soybean trypsin inhibitor; 1.87 × 10⁻² m, $\Delta$, complex made from β-trypsin and soybean trypsin inhibitor; 3.75 × 10⁻¹ m, $\lambda = 290$ nm. The complex was initially at pH 5.00 in 0.5 m KCl, 0.05 m CaCl₂.
tein proteinase inhibitor-serine proteinase systems (for a review see Reference 3), but it is not as yet clear whether it is universally applicable to all such systems. The pH dependence of $k_{-a}$ is striking, since the rate of dissociation does not appear to level out even at pH 1.4. The conventional interpretation of such a finding is that the dissociation of complex is accelerated by protonation of a group with a pK of 1 or lower. Alternately, one can reject the usual assumption about the rapid attainment of ionization equilibrium and argue that the rapid dissociation process involves a rate-determining slow protonation of a critical group.

The rate of dissociation of complexes made from inhibitor and $\alpha$-trypsin is a factor of 3 slower than that of complexes made from inhibitor and $\beta$-trypsin over the entire pH range studied here. Superficially, this is inconsistent with stronger association between $\beta$-trypsin and protein inhibitors. However, as pointed out in the previous section the rate of formation of $\beta$-trypsin-inhibitor complex ($k_1/K_{1a}$) is slower than ($k_1/K_{1b}$), by a larger factor, thus bringing all observations into harmony.

The major objection to the data given in this section is that they may represent not the rate of dissociation of the complex but a rate of some conformation change either in the complex or in one of the products after dissociation. Similar objections may be raised with regard to other parts of this paper.

In order to lessen, but unfortunately, not eliminate these objections we have carried out a number of control experiments. Both trypsin and inhibitor solutions were diluted in the stopped flow apparatus at constant pH and more importantly both protein solutions were subjected to pH jump experiments similar to those carried out on trypsin-inhibitor complex. In the case of soybean trypsin inhibitor, no time-dependent absorbance changes in the time range of interest to us here were observed. On the other hand in the case of trypsin, such changes were frequently detected but were always too fast for us to measure and thus probably complete before significant amount of reactions of interest to us took place. Bellon and Delage (26) have shown large time-dependent changes in ultraviolet absorbance of bovine trypsin, trypsigen, and chymotrypsigenin in the millisecond time scale corresponding to major conformational transitions in these proteins. However, these authors also point out that these transitions speed up greatly with increases in ionic strength and in temperature. Their measurements were carried out commonly at 5 and 10° and at ionic strength range of 0.01 to 0.1. Our measurements were carried out at 21° and at ionic strength of 0.65 (or higher due to additional contribution of acids and buffers). Therefore, the results appear quite consistent.

**DISCUSSION**

**Formal Mechanism**—This paper is a proposal for an expansion of the formal, minimal mechanism for trypsin-soybean trypsin inhibitor interaction by inclusion of nonequivalent, intermediate complexes $L$ and $L^*$. The expanded, minimal mechanism is inherently plausible. It is analogous to the usual mechanisms written for reversible hydrolysis of substrates by serine proteinase (in presence of a large concentration of product nucleophile to allow for appreciable reversal) (27, 28). The only formal difference is that the typical reversible mechanism allows for the dissociation of the nucleophile by the acyl enzyme (analogous to $C$ in our mechanism), while nothing is released in the inhibitor association mechanism, since inhibitors are cyclic substrates and, therefore, form only a single product.

The mechanism is fully consistent with the finding of Haynes and Feehey (29) that the trypsin-soybean trypsin inhibitor association is not diffusion-controlled, since an increase in solvent viscosity does not decrease the second order association rate constant. $k_{-a}$ will be discussed below, $T + I \xrightarrow{k_1} L$ is probably a diffusion-controlled process, but an increase in solvent viscosity should decrease both $k_1$ and $k_{-a}$ equally and thus leave $K_{1a}$ unaffected.

The present mechanism overcomes the serious intuitive objection to the previously proposed (6, 30) minimal mechanism since it does not require that covalent bonds be altered in a single second order step. In replacing one minimal mechanism by another somewhat more elaborate mechanism one wonders whether the new mechanism is now elaborate enough. The answer is obviously that it is not. As soon as methods with a better resolving power are applied to the problem, the existence of additional intermediates will be recognized. Alternately, such new intermediates can be deduced even without direct experimental evidence for their existence, by analogy to other systems involving hydrolysis of peptide bonds. We have resisted this second approach, and, therefore, we have not introduced $L$ and $L^*$ before we had direct evidence for them, even though their involvement was intuitively obvious. Similarly, we are not introducing any additional unproven intermediates in this paper.

The next question to be asked is whether the mechanism of Equation 4 is unique to the trypsin-soybean trypsin inhibitor system or is general for all proteolytic enzyme-protein inhibitor associations. Aside from the inherent plausibility, another argument in favor of generality is that in preliminary experiments we have shown that the same mechanism applies to the interaction of trypsin with virgin and with modified (Arg-Ala reactive site bond hydrolyzed (20)) chicken ovomucoid. The rates of association were monitored at pH 4.0 in a high precision recording pH meter by following the proton release upon formation of complex (8, 9). The order of the association reactions switched from second to first and the data could be fitted with $pK_L = pK_{L^*} = 4.5$.

It should be obvious that in suggesting generality we do not mean to imply that the phenomenon of order switching will be observable for all proteolytic enzyme-protein inhibitor pairs. The observability of this phenomenon requires that experiments be performed at reactant concentrations similar to $K_L$ and $K_{L^*}$. If in some systems $K_L$ and $K_{L^*}$ values are high it may not be possible to reach sufficiently high concentrations to observe the effect. This last conclusion is related to the fact that even though several highly competent investigators have already studied the kinetics of trypsin-inhibitor association they have always found the reaction to be strictly second order. The reason is simple. Since the former workers utilized conventional (slow) equipment, they carried out their experiments at very low reactant concentrations (much lower than $K_L$) in order to slow down the association. Under these conditions second order behavior is expected and found.

**Values of Parameters**—Since the association reaction of virgin inhibitor and trypsin is not rate-determined (even at low concentrations) by the second order rate constant, $k_1$, for the formation of the loose complex, $L$, the value of $k_1$ must be about an order of magnitude greater than that of the actually observed second order rate constant, $k_1/K_{1a}$. The maximal value of that rate constant (between pH 7 and 8) is $6 \times 10^4$ M$^{-1}$ s$^{-1}$ and there-

1 W. R. Finkenstadt, unpublished experiments.
fore $k_2$ should be of the order of $10^6 \ \text{m}^{-1} \ \text{s}^{-1}$. Since this value is rather close to the usual estimate of the upper limit of diffusion-controlled reactions, it is highly probable that the $T + I \rightarrow L$ reaction is diffusion controlled. The estimate of $k_2$ coupled with a measured value of $K_z$ leads to the conclusion that the first order rate constant for dissociation of the loose complex, $L$, ($L \rightarrow T + I$) is $10^8 \ \text{s}^{-1}$ or greater. Therefore, this rate constant contributes little to the observed kinetics of dissociation of the stable complex, since the highest observed value of $k_0$ is $10^3 \ \text{s}^{-1}$ (see Fig. 7).

Since over a broad pH range the values of $K_L$ and of $K_{L*}$ are equal, it seems natural to assume that $k_1 \sim k_{-1}$ and $k_1 \sim k_L$. It should be pointed out that while $K_z$ and $K_{L*}$ do not depend on pH in the pH 4.5 to 8.0 region they must necessarily increase (formation of $L$ and $L*^e$ becomes less favorable) as the pH is lowered, since at low pH values potentiometrically determined $K_{\text{Bovine}}$ values are less than $1/K_L$ (8, 9) and the potentiometric technique clearly measures $L$ as complex.

The parameter, $k_{C--I}$, the rate of conversion of $L*^e$ to $C$, was measured by another technique by Hisson and Laskowski (7). The points are shown in Fig. 5. In these experiments equimolar solutions of modified inhibitor and of trypsin were mixed rapidly at the pH of interest, allowed to incubate for a short time interval and then subjected to a rapid pH drop (to pH 2) in order to affect kinetic control dissociation. Inhibitor was then isolated, freed from trypsin, and the fraction of virgin inhibitor determined by disc gel electrophoresis. This fraction rose in a first order manner from essentially zero to essentially one as a function of time of incubation (time between mixing of the reactants and the pH drop).

Since it was shown before that kinetic control dissociation of the stable complex, $C$, produces essentially pure virgin inhibitor (2, 6, 7, 23), Hisson and Laskowski (7) equated the first order rate of conversion of modified to virgin inhibitor in their system with the $L*^e \rightarrow C$ rate.

It is highly gratifying that these entirely independent data are in reasonable agreement with the stopped flow results, since it alleviates our major concern about a possibility of assignment of the observed time dependent absorbance changes to an entirely wrong chemical event.

Unfortunately, the other parameters obtained in this paper ($k_2, K_L$, and $K_{L*}$) cannot be directly compared with the results of others, since all other workers have reported second order behavior. However, the values of $k_0/K_L$ and $k_{C--I}/K_{L*}$ can be compared with the reported second order rate constants.

The value of $k_0/K_L$ reported here at the high pH plateau (pH 7 to 8) is $6 \times 10^4 \ \text{m}^{-1} \ \text{s}^{-1}$. The results reported by others for a similar constant obtained by various conventional techniques are: Green (31), $2 \times 10^4 \ \text{m}^{-1} \ \text{s}^{-1}$, Haynes and Feeney (29), $8.2 \times 10^4 \ \text{m}^{-1} \ \text{s}^{-1}$, Hisson (32), 5 to 6 $\times 10^4 \ \text{m}^{-1} \ \text{s}^{-1}$. It should be noted that all of the literature results were obtained with an unspecified mixture of $\alpha$ and $\beta$ (and possibly other trypsins) and so for Hisson’s (32) results, under solvent conditions different from our own. In view of these differences the agreement is surprisingly good.

Values of second order rate constants for association of virgin and modified inhibitor with commercial bovine trypsin were measured by Hisson et al. (33) by potentiometric monitoring of proton release upon complex formation at pH 5.0 (in presence of benzamidin to decrease the rates to experimentally accessible range). Their values are $k_2/K_z = 5 \times 10^3 \ \text{m}^{-1} \ \text{s}^{-1}$ and $k_{C--I}/K_{L*} = 1 \times 10^4 \ \text{m}^{-1} \ \text{s}^{-1}$. The present values at pH 5 are $1.5 \times 10^4 \ \text{m}^{-1} \ \text{s}^{-1}$ and $4.5 \times 10^4 \ \text{m}^{-1} \ \text{s}^{-1}$, respectively. Since the potentiometric experiments were done in the same solvent system as the present ones, it seems surprising that the results are 3 to 4 times slower. However, a simple explanation is at hand. The potentiometric data were obtained at relatively high reactant concentrations ($10^{-5}$ to $10^{-4}$ M) where switching of reaction order is expected. The results were assumed to be second order, and clearly a second order rate constant calculated from the observed half-time (see Fig. 2) in this concentration range should be lower than the true value.

If the analogy between the mechanism proposed here and the mechanism of irreversible serine esterase hydrolysis of substrates is to be regarded seriously, then another set of comparisons can be made. Since in such a comparison $L$ corresponds to the noncovalent enzyme-substrate complex $E-S$, and $C$ to an acyl enzyme, $pK_z$ should be compared to $pK_L$ and $k_L$ to the catalytic rate constant for acylation. Thus, the comparison should be made with kinetic data for acylation limited substrates: amides, peptides, and especially peptide bonds in native proteins. Unfortunately, such data are scarce in the literature, especially for the last case of native proteins. Comparison of some available data is made in Table II. It is obvious that $pK_L$ indicates much more favorable noncovalent binding of trypsin with STI than with any of the other substrates, and further, $k_L$, if it is an acylation rate, is much greater than the other listed values of acylation rate constants for peptide and amide substrates. On the other hand, a very large dependence of both $pK_L$ and $k_L$ on sequence of surrounding residues, charge, and presumably conformation is apparent. Thus, it is not unlikely that a normal substrate with $pK_L \sim pK_z$ of the inhibitor and $k_L$ of the inhibitor will be found. It is clear, however, that if soybean trypsin inhibitor behaves like a substrate in the formation of a noncovalent enzyme-substrate complex $E-X$, and $C$ to an acyl ester leads to the conclusion that the $pK_L$ is listed as $pK_m$ and $k_L$ as $k_{\text{cat}}$. This is done for the sake of comparison only.

### Table II

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$pK_m$</th>
<th>$k_{\text{cat}}$</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Benzoyl-L-arginine amide...</td>
<td>2.6</td>
<td>2.8</td>
<td>34</td>
</tr>
<tr>
<td>Val(Ala) Lys Leu Val Gly ...</td>
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<td>7.0</td>
<td>35</td>
</tr>
<tr>
<td>Val(Asp) Lys Leu Val Gly ...</td>
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<td>2.8</td>
<td>35</td>
</tr>
<tr>
<td>GlyGlyValGlyGlyProAlaNH_2 ...</td>
<td>2.7</td>
<td>1.6</td>
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<tr>
<td>Oxidized arginine-vasopressin</td>
<td>3.3</td>
<td>3.6</td>
<td>37</td>
</tr>
<tr>
<td>Bovine trypsinogen</td>
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<td>$2.5 \times 10^{-2}$</td>
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</tr>
<tr>
<td>Bovine chymotrypsinogen</td>
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<td>$1.8 \times 10^{-3}$</td>
<td>35</td>
</tr>
<tr>
<td>A</td>
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<td>1.0</td>
<td>35</td>
</tr>
<tr>
<td>B</td>
<td>4.3</td>
<td>1.8</td>
<td>35</td>
</tr>
<tr>
<td>Soybean trypsin inhibitor</td>
<td>4.7</td>
<td>140</td>
<td>This work</td>
</tr>
</tbody>
</table>

* Consult the references for detailed reaction conditions, these differ considerably so that only gross comparisons of these data are valid. When a choice of comparable data was available those at optimum pH (near 8) and for Arg-X rather than Lys-X bond are listed (since the reactive site of soybean trypsin inhibitor is Arg-Lle).

A derivative of chymotrypsinogen A with 13 carboxylates substituted with glycine ethyl ester (35).

$pK_L$ is listed as $pK_m$ and $k_L$ as $k_{\text{cat}}$. This is done for the sake of comparison only.
loose complex and in the acylation step, then it is an excellent substrate indeed.

pH Dependence—The pH dependence of $K_L$ and $K_{L,b}$ in the 4.5 to 8 range appears to be consistent with generally small pH dependences of $K_*$ and $K_3$ values for binding of substrates to serine esterases.

The pH dependence of log $k_1$ and log $k_{-3}$ (Fig. 5) shows a broad plateau in each case and then a decline when pH is lowered beyond 7. It appears that the pH of the controlling group is 7.0 for the $k_3$ data and 6.7 for the $k_{-3}$ data (this difference may not be significant in view of the experimental error). This feature is an expected one if $k_3$ is the rate constant for acylation and should be interpreted as ionization of the catalytically important histidyl in trypsin. Analogies to such behavior with simple substrates are so numerous that listing them would be prohibitive. It is highly likely that a similar high plateau and a linear decline with decreasing pH commencing near pH 7 will be observed for many other trypsin-trypsin inhibitor pairs, when log $k_3$ and log $k_{-3}$ data for these pairs are determined. However, as the pH is lowered below 6 a striking and surprising phenomenon is seen. On the basis of the simple model the linear plateau of log $k_3$ and log $k_{-3}$ would be expected to continue. Instead there is a pronounced plateau in the log $k_3$ data and a less pronounced but still clear one in the log $k_{-3}$ data. We have been puzzled by these plateaus for some time. We think it highly improbable that they are a result of experimental error.

Before the plateaus between pH 6 and 5 can be explained it will be necessary to determine whether this phenomenon is general for the association of protein trypsin inhibitors with trypsin or only a peculiarity of the soybean trypsin inhibitor-trypsin system. In favor of generality is the determination of Putterm (38) of the second order rate constant for association of pancreatic trypsin inhibitor (Kunitz) with trypsin. The plot of a logarithm of this constant against pH also shows a broad plateau at low pH values.

On the other hand our studies of the pH dependence of the association equilibrium constant, $K_{assoc}$, show that while the pH dependences for pancreatic trypsin inhibitor (Kunitz)-trypsin and chicken ovomucoid-trypsin are almost identical, the pH dependence of $K_{assoc}$ for soybean trypsin inhibitor-trypsin differs appreciably from the other two. These data suggest that in the soybean inhibitor-trypsin interaction one additional group (i.e. a group not involved in trypsin-ovomucoid or trypsin-pancreatic inhibitor association) with $pK_1$ or $pK_2$ 5 is strongly perturbed. Involvement of such an additional group might well be the explanation of the plateaus. Therefore, we do not expect to attempt an explanation of the plateaus until the pH dependences of $k_3$ and $k_{-3}$ is determined for another trypsin-inhibitor pair.

The striking feature of the mechanism proposed here and of the pH dependence of the rate constants is the symmetry with respect to $T + I \rightarrow C$ and $T + I^* \rightarrow C$ reactions. The formal mechanism is totally symmetrical, the values of $k_2$ and $K_{L,b}$ are identical. While the values of analogous rate constants $k_3$ and $k_{-3}$ are quite different, their pH dependence is closely similar. This similarity in pH dependence extends also to the analogous pair of dissociation rate constants $k_{-3}$ and $k_2 (2)$ to be reported on in subsequent papers. The strong symmetry suggests (but does not prove) that closely similar chemical events occur upon formation of $C$ from virgin and from modified inhibitor and therefore that enzymatic catalysis is involved in both reactions.

Note Added in Proof—Vincent and Lazdunski (39) have recently reported on the second order rate constant for the association between bovine trypsin and bovine pancreatic trypsin inhibitor (Kunitz). The pH dependence of this rate constant parallels the pH dependence of the second order rate constant for the association of soybean trypsin inhibitor (Kunitz) with bovine $\beta$-trypsin, $k_3/K_L$, even more closely than the earlier data of Putterm (38). It thus appears that the plateau in the pH 5 to 6 region is general for the association of trypsin inhibitors with trypsin and not peculiar to soybean trypsin inhibitor (Kunitz) alone.

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Detailed Mechanism of Interaction of Bovine $\beta$-Trypsin with Soybean Trypsin Inhibitor (Kunitz): I. STOPPED FLOW MEASUREMENTS
James A. Luthy, Melvin Praissman, William R. Finkenstadt and Michael Laskowski, Jr.


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