Detailed Mechanism of Interaction of Bovine β-Trypsin with Soybean Trypsin Inhibitor (Kunitz)

I. STOPPED FLOW MEASUREMENTS*

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SUMMARY

The rate of formation of the stable trypsin-inhibitor complex from bovine β-trypsin and either virgin or modified soybean trypsin inhibitor (Kunitz) was monitored by following the change in absorbance at 260 nm after mixing of equimolar reactants in a stopped flow apparatus. At low reactant concentrations both reactions are second order both with respect to concentration and to time. As the concentration of reactants is raised both the time and concentration dependence gradually switch to first order. These results coupled with other data suggest that the simplest possible mechanism of interaction is

$$\frac{k_1}{k_{-1}} \frac{k_2}{k_{-2}} \frac{k_3}{k_{-3}} \frac{k_4}{k_{-4}} \frac{1}{T + I*}$$

On the assumption that the equilibration to form the loose noncovalent complexes is rapid, values of $K_L = k_{-1}/k_1$ and $K_{I*} = k_3/k_4$ and of the rate constants $k_2$ and $k_{-2}$ were computed from the data. Within the limits of rather large experimental error $pK_L = pK_{I*} = 4.65$ independent of pH in the 4.5 to 8 range. The rate constants $k_2$ and $k_{-2}$ increase with increasing pH in this range and reach plateau values of 140 and 10 per s, respectively, near pH 8. Both rate constants show similar pH dependence with another plateau in the pH 5 to 6 range. The magnitude and pH dependence of these parameters is roughly consistent with $K_S$ values and catalytic rate constants for hydrolysis of excellent amide and peptide substrates.

Measurements of the dissociation of the stable complex between pH 1.7 and 3.5 were also made. The dissociation rate constant is very fast near pH 2 (approximately 100 per s) and declines sharply as the pH is raised. Preliminary measurements indicate significant differences in the rate of association of soybean trypsin inhibitor with α- and with β-trypsin as well as in the rate of dissociation of α-trypsin and of β-trypsin complexes.

The interaction of protein proteinase 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

$$K_{hyd} = \frac{(I^*)}{(I)}$$

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

$$T + I \rightarrow C \rightarrow T + I*$$

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==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, $K_{\text{hyd}}$ (Equation 1) can be independently measured as can the over-all association constant, $K_{\text{assoc}}$ (8, 9)

$$K_{\text{assoc}} = \frac{\Sigma C}{(I) [(I^*) + (I'^*)]}$$  

where the summation term in the numerator denotes all possible associated species of the stoichiometry $T-I$ and $T-I^*$ that co-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 with 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 the values of all the two equilibrium constants, $K_{\text{hyd}}$ and $K_{\text{assoc}}$; and (d) comparison 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 $K_{\text{hyd}}$ (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 indirect 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 from autolysis of trypsin (10). Our preliminary investigation of this system convinced us that sizable complexities would indeed be encountered.

On the other hand Bennett and Trowbridge (15) and Edelhoch and Steiner (16) have shown that there is a small but measurable difference spectrum between the trypsin-soybean trypsin inhibitor complex and the free proteins. We have chosen to employ this difference spectrum in our kinetic studies.

Because this difference spectrum is small and occurs in an inconvenient wave length range, its use as a monitoring signal obviously greatly lowers the quality of the data. In order to maximize the small signals we were restricted to mixing of equimolar quantities of reactants only. Thus, advantage could not be taken of pseudo-first order behavior obtained when one of the reactants is in large excess. As is seen in the body of the paper this restriction is rather severe. Nonetheless, we feel that the advantage of monitoring a direct signal rather than of a complicated indirect one far outweighs these disadvantages. Furthermore, the use of the difference spectrum allowed for measurement of the rates of dissociation of trypsin-inhibitor complexes at low pH values. These measurements are not possible with the proflavine or thionine probe.

**Materials**—Bovine $\alpha$- and $\beta$-trypsin were prepared from various lots of crystallized, salt-free trypsin (Worthington Biochemical Corp. and Boehringer and Sohne, GmbH, Mannheim) by a slightly modified chromatographic procedure of Schroder and Shaw (17). The SE-50 Sephadex column was operated at room temperature rather than 4° allowing for an increase in flow rate to about 40 ml per hour. Thus the separation could be completed in 21/2 days rather than in a week. Formic acid buffer, pH 2.0, was not used in the collection tubes. The fractions containing $\alpha$- and $\beta$-trypsin, respectively, were pooled and concentrated about 5-fold with an Amicon Diaflo apparatus. The pH of the resultant solutions was lowered to 2.8 to 3.0 by addition of 6 N HCl and the solutions were dialyzed in the same apparatus by passage of 8 to 10 volumes of $10^{-3}$ N HCl. The solutions were then lyophilized.

The relative activity of the final samples was determined by active site titration with p-nitrophenyl-$p'$-guanidinobenzoate (GdnBOm) at pH 8.3 according to the procedure of Chase and Shaw (18). Differential kinetic titrations were also carried out at pH 4 according to the method of Hruska et al. (19) in order to assess the presence of kinetically different forms. The molar concentration of trypsin was obtained from ultraviolet absorbance measurements at 280 nm. An optical factor of 0.651 (15) and a molecular weight of 24,000 were assumed. On this basis both the $\alpha$ and $\beta$ trypsin preparations were 90% or more active. The active portion of the $\beta$ preparation consisted of more than 85% of a kinetically fast component and 15% or less of a substantially slower component. The bulk of $\alpha$ preparations was kinetically slow. Soybean trypsin inhibitor (Kunitz) five times crystallized special grade Lots B7303 and C2905 was obtained from Gallard-Schlessinger Corp. These lots were especially selected from many tested commercial lots on the basis of their high inhibitor activity and homogeneity in disc gel electrophoresis. Modified inhibitor (Arg-$\beta$-Ile reactive site peptide bond hydrolyzed) was prepared by incubation of the virgin inhibitor at pH 3.0 with 2 mole % of trypsin. The amount of virgin inhibitor remaining in these preparations as monitored by disc gel electrophoresis (5) was less than 5%. The
small amount of trypsin in these preparations was removed as
tryptsin-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 measure-
ment 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 trypsin and
of inhibitor and then isolating the complexes by Sephadex chro-
matography (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 demineralized, glass-distilled water. This is the solvent
system used in most trypsin-inhibitor interaction studies re-
ported from our laboratory. The reactant solutions were buff-
ered 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-ethane-
sulfonic 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 generally found to be within 0.05 pH
from the starting pH. This final pH value is thus one reported.
At pH values above 6, complications arose due to autolysis of
concentrated trypsin solutions. To circumvent this only the
inhibitor solution was buffered at the desired pH, the trypsin
solution was left unbuffered at pH 3.5. Again 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 spec-
tra were obtained with either Cary 14 or Cary 15 Spectro-
photometers.

The rate measurements were made on a Durrum-Gibson
stopped flow spectrophotometer equipped with interchangeable
2- and 20-mm path length Kel-F observation cells and a cali-
brated 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 com-
monly 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 prelimi-
inary mixings the output voltage of photomultiplier was adjusted
up to 1000 volt. A slit of 4 mm was generally used. The photo-
multiplier voltage ranged from 550 volts for the most concen-
trated 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 oscillo-
scope. The trace was photographed and digitized, occasionally
by simply reading the photograph but normally with the aid of a
Larr V 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 solu-
tion 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
tryptsin-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 Bonnonyal and Troubridge (15) and
Eidelberg 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 Δε/εi,
where Δε is obtained from Fig. 1A and εi is the molar absorbance
of the complex at the specified wave length (Fig. 1B). From
inspection of these plots we have concluded that the most sat-
isfactory 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 nm corresponding to
10-nm spectral band width) which was used in the kinetic meas-
urements.

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 trans-
mittance 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 (absorb-
ance rose) at that wave length, again as expected. These pre-
liminary 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 experi-
mentally accessible range (10^-8 to 10^-4 m) and the half-times
were determined. The results are presented as the logarithm of
half-time versus the logarithm of concentration in Fig. 2. It
is clear that the order with respect to concentration switches
from second order to first order in the concentration range ex-
amined. 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.
A, difference in molar absorptivity between β-tryptsin-modified 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 × 10^{-5} M trypsin-inhibitor complex + 0.51 × 10^{-5} M inhibitor; Compartment 2, solvent. Reference cell: Compartment 1, 3.01 × 10^{-5} M trypsin; Compartment 2, 3.51 × 10^{-5} M inhibitor. All at pH 6.00 in 0.5 M KCl, 0.05 M CaCl₂, and 0.1 M 2-(N-morpholino)ethanesulfonic acid. 

B, the data of A divided by molar absorptivity of β-trypsin-soybean trypsin inhibitor complex at the appropriate wave length.

FIG. 1. A, difference in molar absorptivity between β-tryptsin-modified 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 × 10^{-5} M trypsin-inhibitor complex + 0.51 × 10^{-5} M inhibitor; Compartment 2, solvent. Reference cell: Compartment 1, 3.01 × 10^{-5} M trypsin; Compartment 2, 3.51 × 10^{-5} M inhibitor. All at pH 6.00 in 0.5 M KCl, 0.05 M CaCl₂, and 0.1 M 2-(N-morpholino)ethanesulfonic acid. 

B, the data of A divided by molar absorptivity of β-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

$$T + I \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} L \overset{k_2}{\underset{k_{-2}}{\rightarrow}} C \overset{k_3}{\underset{k_{-3}}{\rightarrow}} L^* \overset{k_4}{\underset{k_{-4}}{\rightarrow}} T + I^* \quad (4)$$

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, $K_{eq}$, is so large (8) that complex formation is effectively irreversible. Therefore, the complex dissociation steps $k_2$ and $k_4$ in Equation 4 need not be considered. This reduces the mechanism for virgin inhibitor-trypsin association to

$$T + I \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} L \overset{k_2}{\underset{k_{-2}}{\rightarrow}} C \quad (5)$$

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

These $\Delta \alpha/\alpha_i$ 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
data. To do this it is necessary to know, whether $k_1$ is large or
with the evaluation of the parameters from the experimental
the rate-limiting process is always the
the other hand if $k_1 \ll k_2$ Equation 5 describes a simple set of
arise either on the formation of
260 nm with time. Conditions:
KCl, 0.05
KCl, 0.05
CaCl$_2$, and 0.05 M acetate (final concentrations); pH
4.05; 2-mm path length. Lower photograph, 3.00 $\times$ 10$^{-4}$ M
KCI, 0.05 M CaCl$_2$, and 0.05 M acetate (final concentrations); pH
4.05; 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.)

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 $k_2$. If $k_1 \gg k_2$ 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 $k_1 \ll k_2$ Equation 5 describes a simple set of
consecutive reactions, where at low reactant 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 $k_1 \ll k_2$ 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 concen-
trations studied. Thus, this possibility can be immediately
rejected. If $k_1 \ll k_2$ 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 sigmoidicity
(initialization 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 oscil-
lopescope traces at a variety of concentrations showed no sigmoid-
ity. Therefore, this possibility was rejected.

Thus, we are left with $k_1 > k_2$, 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 $K_{iL}$ 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 propor-
tional to $[T][I]$ if 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 com-
bination of $T + I$ to give $L$ (and the signal) is essentially
complete before recording begins. Thus in the first order concen-
tration 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 $k_1 \gg k_2$ and absorbance changes
occur entirely on the $L \rightarrow C$ step is taken as correct. A quanti-
tative consequence of this statement is that the kinetic dif-
sion spectrum, $\Delta$trans, the difference in molar absorptivity 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 concentra-
tion 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 dif-
sion spectra should be expected to be smaller than the static
difference spectra owing to slit broadening.

The data shown in Fig. 4 are a compilation of the kinetic dif-
sion spectra obtained in this research. Each point in the
upper part of the figure represents an average of all $-\Delta$trans values
obtained at the pH of interest, generally at a variety of con-
centrations. 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 dif-
sion 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 assump-
tion that the signal arises predominantly in the $L \rightarrow C$ step ap-
FIG. 4. Kinetic difference spectra. A, the average values of difference in molar absorptivity at 260 nm between the complex and β-trypsin-virgin soybean trypsin inhibitor (○), and β-trypsin-modified soybean trypsin inhibitor (●) versus pH obtained from stopped flow data. Values from all the experiments performed at the stated pH (generally at different concentrations) are averaged. Conditions, 0.5 M KCl, 0.05 M CaCl₂, and 0.05 M buffer (pH 4.65 to 5.33 acetate; pH 5.8 to 6.72 N-(3-morpholino)ethanesulfonic acid; pH 7.0 to 7.3 piperazine-N,N'-bis(2-ethanesulfonic acid); pH 7.5 to 8.0 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid). B, the average values of difference in molar absorptivity between the complex and P-trypsin-virgin soybean trypsin inhibitor or β-trypsin-modified soybean trypsin inhibitor versus the logarithm of concentration obtained from stopped flow data. The averaging here involves repeat experiments under the same conditions. Conditions, 0.5 M KCl, 0.05 M CaCl₂, and 0.05 M buffer: ○, pH 4.65, STI, acetate; ●, pH 7.50, STI, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; □, pH 7.30, STI*, piperazine-N,N'-bis(2-ethanesulfonic acid).

pears to be confirmed. In formulating an expression for the absorbance as a function of time after mixing of reactants, this quantity can be set proportional to the time dependence of formation of the stable complex C.

Evaluation of Parameters—In order to obtain an expression for C as a function of time we define equilibrium constants

\[ K_L = \frac{[I][T]}{[L]} \]

and

\[ K_{L*} = \frac{[I^*][T]}{[L^*]} \]

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, \((T) = (T) - a = (L).\) Substitution into Equation 6 and solving the quadratic equation yields

\[ (L) = K_L/4 (\sqrt{1 + 4a_0/K_L} - 1) \]

Since

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

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

\[ k_2t = -2 \left[ \ln \sqrt{1 + 4a_0/K_L} - 1 - \frac{1}{1 + 4a_0/K_L} - 1 + \sqrt{1 + 4a_0/K_L} - 1 \right] \]

A strictly analogous expression arises for the reaction with modified inhibitor with \(k_{-3}\) replacing \(k_3\) 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

\[ \frac{k_2}{K_L} \frac{t_{1/2}}{a_0} \]

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

\[ t_{1/2} = \frac{k_2}{k_{-3}} \frac{1}{a_0} \]

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 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

\[ t_{1/2} = \frac{1}{2} \ln 2 \]

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 \(\log t_{1/2}\) axis 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 fit 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 \( k_2-k \) and \( K_{L,0} \) 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 \( K_{L,0} \) 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 general for an evaluation of \( pK_L \) (or \( pK_{L,0} \)) 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 \(-\Delta A_{560}\) 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 \(-\Delta A_{560} = 825\) 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 \( k_2-k \) and \( pK_{L,0} \)) were closely similar to those obtained from other methods. In order to show what can be learned about \( K_{L,0} \) 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 \( K_{L,0} \) 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 \( pK_{L,0} \) is \( pK_L = pK_{L,0} = 4.65 ± 0.2 \). 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 \( pK_{L,0} \) 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.

**pH Dependence**—Once the values of \( K_L \) and \( K_{L,0} \) are decided upon, \( k_2 \) and \( k_{-3} \) were evaluated from the stopped flow traces by use of Equation 10. A plot of the average values obtained at each pH studied is shown in Fig. 5. Also shown in this figure are values of \( k_{-3} \) obtained by an entirely different technique by Hinman and Lachowicz (7). These are considered further in the discussion.

The experimental scatter of data in Fig. 5 is surprisingly small; however, it should be pointed out that all of these data are subject to a systematic error due to the assignment of values of \( K_L \) and \( K_{L,0} \). This systematic error is probably of the order of ±0.15 in the log \( k_2 \) and log \( k_{-3} \) values. The assumption that the \( K_L \) and \( K_{L,0} \) values are entirely independent of pH may also introduce errors in the pH dependence of log \( k_2 \) and of log \( k_{-3} \), but we think it highly unlikely that such errors could be large enough to eliminate any of the features of the curves. Incidentally, the values of second order association rate constants

**Table I**

**Time course of absorbance change for association of modified soybean trypsin inhibitor (Kundz)**

Experimental condition: [STI'] = [T] = 5.5 × 10⁻⁶, pH 6.30, in 0.05 M KCl, 0.05 M CaCl₂, and 0.05 M 2-(N-Morpholino)ethanesulfonic acid. \( K_{L,0} = 2.3 × 10⁻³ \) \( (pK_{L,0} = 4.05) \) was assumed in the calculations from Equation 10.

<table>
<thead>
<tr>
<th>Reduced fractional time</th>
<th>First order (calculated)</th>
<th>Observed</th>
<th>Calculated from Equation 10</th>
<th>Second order (calculated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( t_{1/4}/t_{1/2} )</td>
<td>2</td>
<td>2.7</td>
<td>2.35</td>
<td>3</td>
</tr>
<tr>
<td>( t_{1/2}/t_{1/4} )</td>
<td>3</td>
<td>5.3</td>
<td>4.33</td>
<td>7</td>
</tr>
<tr>
<td>( t_{1/4}/t_{1/2} )</td>
<td>4</td>
<td>8.7</td>
<td>7.54</td>
<td>15</td>
</tr>
<tr>
<td>( t_{1/2}/t_{1/4} )</td>
<td>5</td>
<td>12</td>
<td>13.1</td>
<td>31</td>
</tr>
</tbody>
</table>
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 be-
cause 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 at-
ttempted the observed $\Delta_{900}$ was smaller than 800, indicating
incomplete dissociation.

The data obtained in this experiment were quite easy to inter-
pret 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 con-
stants obtained at much higher pH values by more direct tech-
niques (2).

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

$$k_D = k_{-2} + k_3$$  \hspace{1cm} (15)

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

$$k_D = k_2$$  \hspace{1cm} (16)

Similar relation ($k_{-2} \gg k_3$) appears to hold for several other
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 $k_{-2}$ 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 $r$-trypsin is a factor of 3 slower than that of complexes made from inhibitor and $p$-trypsin over the entire pH range studied here. Superficially, this is inconsistent with stronger association between $p$-trypsin and protein inhibitors. However, as pointed out in the previous section the rate of formation of $p$-trypsin-inhibitor complex $(K_1/K_2)_p$ is slower than $(K_1/K_2)_r$ 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 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 Dehaeghe (26) have shown large time-dependent changes in ultraviolet absorbance of bovine trypsin, trypsinogen, and chymotrypsinogen 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$^\circ$ and at ionic strength range of 0.01 to 0.1. Our measurements were carried out at 21$^\circ$ 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 esterases (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 Feekey (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. As will be discussed below, \[ T + I \rightarrow k_L L \] is probably a diffusion-controlled process, but an increase in solvent viscosity should decrease both $k_1$ and $k_{-1}$ equally and thus leave $K_L$ 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 in 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-31h 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, $k_L/K_L$. The maximal value of that rate constant (between pH 7 and 8) is $6 \times 10^8$ $\text{M}^{-1} \text{s}^{-1}$ and there-

1 W. R. Finkenstadt, unpublished experiments.
fore $k_3$ 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 $k_3$ coupled with a measured value of $K_L$ leads to the conclusion that the first order rate constant for dissociation of the loose complex, $L$, ($\frac{L}{T + I}$) is $10^6 \text{ s}^{-1}$ or greater. Therefore, this rate constant contributes little to the observed kinetics of dissociation of the stable complex, since the highest observed value of $k_p$ is $10^5 \text{ s}^{-1}$ (see Fig. 7).

Since over a broad pH range the values of $K_L$ and of $K_{LI}$ are equal, it seems natural to assume that $k_3 \sim k_4$ and $k_{-1} \sim k_4$.

It should be pointed out that while $K_L$ and $K_{c,0}$ do not depend on pH in the pH 4.5 to 8.5 region, they must necessarily increase (formation of $L^*$ and $L^{**}$ becomes less favorable) as the pH is lowered, since at low pH values potentiometrically determined $K_{metric}$ values are less than $1/K_L$ (8, 9) and the potentiometric technique clearly measures $L$ as complex.

The parameter, $k_{-3}$, the rate of conversion of $L^*$ to $C$, was measured by another technique by Hixon 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 diox 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, $L$, produces essentially pure virgin inhibitor and the fraction of virgin inhibitor determined by dye 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).

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 ($k_3$, $K_L$, and $K_{c,0}$) cannot be directly compared with the results of others, since all other workers have reported second order behavior. However, the values of $k_2/K_p$ and $k_{-3}/K_{c,0}$ can be compared with the reported second order rate constants.

The value of $k_2/K_p$ 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), $2 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$, Haynes and Feeney (20), $8.2 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$, Hixon (32), 5 to $6 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$. It should be noted that all of the literature results were obtained with an unspecified mixture of $a$ and $b$ (and possibly other trypsin) and save for Hixon’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 Hixon et al. (33) by potentiometric monitoring of proton release upon complex formation at pH 5.0 (in presence of benzamidine to decrease the rates to experimentally accessible range). Their values are $k_2/K_p = 5 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ and $k_{-3}/K_{c,0} = 1 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$. 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^{-2} 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 reversible 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, $pK_L$ should be compared to $pK_e$ and $k_2$ 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 $3T1$ than with any of the other substrates, and further, $k_2$, 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 $pK_e$ and $k_{cat}$ on sequence of surrounding residues, charge, and presumably conformation is apparent. Thus, it is not unlikely that a normal substrate with $pK_e \sim pK_L$ of the inhibitor and $k_{cat} \sim k_2$ of the inhibitor will be found. It is clear, however, that if soybean trypsin inhibitor behaves like a substrate in the formation of a stable complex, since the highest observed value of $k_p$ is $10^5 \text{ s}^{-1}$ (see Fig. 7).

![Table II](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$pK_e$</th>
<th>$k_{cat}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoyl-L-arginine amide...</td>
<td>2.6</td>
<td>2.8</td>
<td>34</td>
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<tr>
<td>Val(Ala), Lys-HoValGly</td>
<td>4.1</td>
<td>7.0</td>
<td>35</td>
</tr>
<tr>
<td>Val(Asp), Lys-IleValGly</td>
<td>2.5</td>
<td>2.8</td>
<td>35</td>
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<tr>
<td>GlyxValArgGlyProAlaNH$_2$</td>
<td>2.7</td>
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<td>Oxidized arginine-vaso...</td>
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<td>37</td>
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<td>Bovine trypsinogen</td>
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<tr>
<td>Bovine chymotrypsinogen</td>
<td>3.0</td>
<td>$1.8 \times 10^{-1}$</td>
<td>35</td>
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<tr>
<td>Bovine chymotrypsinogen A</td>
<td>3.3</td>
<td>1.0</td>
<td>35</td>
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<tr>
<td>Bovine chymotrypsinogen B</td>
<td>4.3</td>
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<td>35</td>
</tr>
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<td>Bovine chymotrypsinogen A</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-Arg).

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

‡ $pK_L$ is listed as $pK_m$ and $k_2$ as $k_{cat}$. 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 $K_{L*}$ in the 4.5 to 8 range appears to be consistent with generally small pH dependences of $K_a$ and $K_R$ values for binding of substrates to serine esterases.

The pH dependence of $\log k_2$ and $\log k_{-2}$ (Fig. 3) 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 $k_2$ data and 6.7 for the $k_{-2}$ data. This difference may not be significant in view of the experimental error. This feature is an expected one if $k_2$ is the rate constant for acylation and should be interpreted as ionization of the catalytically important histidyl in trypsin. Anologues 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 $\log k_2$ and $\log k_{-2}$ 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 decline of $\log k_2$ and $\log k_{-2}$ would be expected to continue. Instead there is a pronounced plateau in the $\log k_2$ data and a less pronounced but still clear one in the $\log k_{-2}$ 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 5 and 6 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-pancreatic trypsin inhibitor system. In favor of generality is the determination of Putter (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 (35) of the second order rate constant for association of trypsin system. In favor of generality is the determination of trypsin only a peculiarity of the soybean trypsin inhibitor-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 $pK \approx 5$ 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 dependence of $k_2$ and $k_{-2}$ 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 $K_{L*}$ are identical. While the values of analogous rate constants $k_2$ and $k_{-2}$ are quite different, their pH dependence is closely similar. This similarity in pH dependence extends also to the analogous pair of dissociation rate constants $k_{-2}$ 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, $k_2/K_L$, even more closely than the earlier data of Putter (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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