THE KINETICS OF TRYPsin DIGESTION*

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The present study of a typical proteolytic enzyme extends the quantitative investigations previously reported from this laboratory on the effects of pressure on protein systems in vivo (1) and in vitro (2). This paper presents new data on the self-digestion of trypsin and the digestion of casein by trypsin; a forthcoming paper will discuss the effects of temperature and hydrostatic pressure on these processes. This particular system was chosen because of classical interest, because of the commercial availability of crystalline trypsin and purified casein, and also because some investigations of the kinetics of digestion of casein by trypsin are already to be found in the literature (3).

As is frequently the case, the rate law suggested by the interpretation of the data has a complex integrated form not easily tested by the usual methods. A simple graphical technique (which we have called the slide-fit technique) has proved useful in handling kinetic data in general (Powell, unpublished data). With this technique it is quite as easy to test the agreement of data with complex rate laws as with the simple classical rate laws.

Section I of this paper presents an analysis of trypsin self-digestion data. The rate of this process was studied to find out whether it might proceed rapidly enough to introduce any significant error into the studies of casein digestion. The data, while not intended for kinetic analysis, are presented as a simple illustration of the slide-fit technique and because the kinetic process, second order digestion complicated by reversible inhibition of the enzyme by its digestion products, appears completely parallel to the casein digestion.

Section II presents new data covering a wide range of the essential variables of casein-trypsin digestion and interprets them similarly in terms of known properties of the components.

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I. Slide-Fit Technique and Trypsin Self-Digestion

Experimental—Conditions in the trypsin experiments were exactly like those for casein-trypsin (Section II), except that samples were taken from a single stock. A freshly made solution of 3 mg. per ml. of crystalline trypsin (Worthington Laboratory, assay 50 per cent) in very dilute hydrochloric acid (pH 1.96) was mixed in the proportion 1.1 to 1.5 with 0.1 M phosphate buffer of pH 7.3, and the mixture (pH 7.1, chosen to duplicate the casein conditions which, in turn, approximate Kunitz' conditions) was stirred mechanically at 35°. Part (2.6 ml.) of each sample was precipitated with 5 per cent trichloroacetic acid (TCA) and the optical density of the filtrate determined at 280 mμ, and part (1.1 ml.) was allowed to digest 1.5 ml. of 1 per cent casein in the same buffer under standard conditions (35°, 20.0 minutes) as a measure of its residual enzymatic activity. The amount of casein digested was determined by measuring the optical density of a TCA filtrate at 280 mμ, and correcting for the optical density of the first part of the sample, i.e., the contribution of self-digested trypsin (Table I).

Calibration—A similar 3 mg. per ml. solution of trypsin was diluted with varying amounts of the dilute hydrochloric acid so that successive samples contained amounts differing by 10 per cent increments. Each sample was then diluted (1.1 ml. + 1.5 ml.) with the phosphate buffer (to reproduce the self-digestion conditions above) and 1.1 ml. of this solution was added to 1.5 ml. of 1 per cent casein and incubated; the optical density of a TCA filtrate was determined. The resulting per cent of active trypsin versus the optical density of the casein digested is shown in Fig. 1.

The data for Sample 3-T0 (Table I) were then converted to per cent of

![Fig. 1. Activity of trypsin measured by casein digested in 20 minutes under standard conditions. Ordinate, optical density; abscissa, amount of trypsin charged as per cent of normal charge (see the text).](http://www.jbc.org/)

TABLE I

Trypsin Self-Digestion

<table>
<thead>
<tr>
<th>Time, min.</th>
<th>Self-digest, optical density</th>
<th>Casein digest, optical density</th>
<th>Corrected* optical density</th>
<th>Per cent undigested</th>
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<td>Sample 3-7 (initial concentration, 1.27 mg. per ml. of 50% trypsin)</td>
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Sample 1-7 (initial concentration 1/2 of that above)

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*Correction = 11/26 (optical density of self-digest - 0.067) where 0.067 is the blank.
† The figures in this column are from Fig. 1.
‡ Points omitted in Fig. 3.
§ The figures in this column are from Fig. 2.

Fig. 2. Trypsin calibration. Ordinate, optical density at 280 μμμ from partially self-digested trypsin; abscissa, per cent active or undigested trypsin as determined from activity in digesting casein under standard conditions. □, blank experiment.
active trypsin by use of Fig. 1, and these values, as determined by casein digestion ability, are plotted against the optical density (Table I) of partially digested trypsin in Fig. 2. This is a direct calibration which can be used (as with the data for Sample 1-T0) to determine the amount of digested trypsin in a solution without recourse to the cumbersome procedure of casein digestion.

DISCUSSION AND RESULTS

**Simple Orders of Reaction**—Trypsin is known to exist in dynamic equilibrium with a reversibly denatured form of itself (4); it is presumably this form of the protein which is digested by the active enzyme. Thus

\[ \text{T} + \text{T}_d \rightleftharpoons (\text{T}_a, \text{T}_d) \xrightarrow{k} \text{T}_a + \text{product (P)} \]  

(1)

Since the denaturation equilibrium (4) and complex formation equilibrium (theory of absolute reaction rates) are attained very rapidly, their constants may be combined with the specific rate constant, and the trypsin considered to be a single reacting species, T, giving the rate equation

\[ \frac{dP}{dt} = -\frac{dT}{dt} = k'(T_a)(T) = k(T)^a \]  

(2)

If \( \alpha \) is the degree of reaction and \( T_0 \) the original charge of trypsin, then

\[ P = \alpha T_0 \text{ and } T = (1-\alpha) T_0 \]

Substituting, rearranging, and integrating formally give

\[ \int_0^\alpha \frac{d\alpha}{(1 - \alpha)^3} = T_0 k \int_0^t dt \]

We may combine the constants and take the logarithm of both sides

\[ \log \int_0^\alpha \frac{d\alpha}{(1 - \alpha)^2} = \log k' + \log t \]

After integrating, we may then choose a purely arbitrary set of values for \( \alpha \), covering any range desired, and obtain a corresponding set of values for the log of the integral without recourse to any data whatsoever. This is a purely mathematical evaluation of the general second order reaction for given values of \( \alpha \). (Derivations for other orders are made similarly.)

1 Throughout the paper the following terminology is used. T_a and C_a refer to initial concentrations; a 2C-T experiment is one with 2 times the “standard” casein concentration (see “Experimental”); C-xT refers to a set of experiments with varying \( T_0 \); T_a is active trypsin; T_d is reversibly denatured trypsin; T_a-T_d, etc., are the intermediate complexes; \( K_i, K_p \) are equilibrium constants and the k's are rate constants of the digestion reactions; P is unspecified reaction product; C is unreacted casein; C_d is digested casein.
A plot of $\alpha$ against these values of its integral is, from the above equation, the equivalent of a plot of $\alpha$ against $\log k' + \log t$. Since $\log k'$ is a constant, it is obvious that $\log t$ must describe the shape of the curve and $\log k'$ only its position.

We may now take any data presumed to follow the above rate law and plot the obtained values of $\alpha$ against $\log t$. If the reaction mechanism is not correct, the plot of $\alpha$ against $\log t$ will give, in general, a curve of different shape which cannot be superimposed on the hypothetical curve by a shift of the abscissa. If they do fit, the position along the abscissa determines $k'$.

**Comparison of Data to Simple Orders of Reaction**—A set of theoretical curves for zero through the third order for the slide-fit is shown in Fig. 3 with the per cent of undigested trypsin (rather than $\alpha$) plotted against the log of the integral. The data are fitted by plotting the theoretical curve and the data on identical paper and shifting the two sheets horizontally, always keeping the $x$ axis superimposed. The data are then traced in the position of best fit and the reaction $k$ is determined by noting the value of the theoretical curve for unit time of the data. To illustrate (Fig. 3), the data of Lauffer and Price (5) for the demonstrated first order ($\delta$, 2) thermal denaturation of tobacco mosaic virus and the data of Hughes and Ingold (6) for the second order decomposition of phenethyl ammonium hydroxide in water have been used. In contrast, the data for trypsin self-digestion (and casein data) are seen to show a rate drop even faster than third order.

**Inhibition by Reaction Products**—Returning to the original simple reaction mechanism (Equations 1 and 2), we may write the expression for the
activated complex equilibrium

\[ K_i = \frac{(T_a \cdot T)}{(T_a)(T)} \]  

or \[ K_i(T) = \frac{(T_a \cdot T)}{(T_a)} \]  

\[ (3) \]

If we now assume that the active trypsin is inhibited by the products in a simple reversible manner (cf. (7)), the following equations result:

\[ T_a + P \xrightarrow{K_p} T_a \cdot P, \quad K_p = \frac{(T_a \cdot P)}{(T_a)(P)} \]  

or \[ K_p(P) = \frac{(T_a \cdot P)}{(T_a)} \]  

\[ (4) \]

The presumption that the inhibition is reversible is likely from the corresponding reaction of the casein-trypsin system which is discussed below. The total amount of undigested trypsin present is

\[ (T) = (T_a) + (T_a \cdot T) + (T_a \cdot P) \]

If we now add the above equilibrium expressions, Equations 3 and 4, and add 1 to each side of the resulting equation

\[ 1 + K_i(T) + K_p(P) = \frac{(T_a)}{(T_a)} + \frac{(T_a \cdot T)}{(T_a)} + \frac{(T_a \cdot P)}{(T_a)} \]

or

\[ (T_a) = \frac{(T_a) + (T_a \cdot T) + (T_a \cdot P)}{1 + K_i(T) + K_p(P)} = \frac{(T)}{1 + K_i(T) + K_p(P)} \]

The rate equation

\[ - \frac{dT}{dt} = k(T) \]

may now be rewritten by substituting for \( T_a \)

\[ - \frac{dT}{dt} = k \frac{(T)^a}{1 + K_i(T) + K_p(P)} \]

Actually in both this and the casein-trypsin system comparison of the alternatives with the data has shown that the amount of \( T_a \) not combined with the product is arithmetically negligible compared to that which is in the form \((T_a \cdot P); \ i.e., 1 + K_i(T) \ll K_p(P)\). Although the development may be carried through with the above form, it is simpler to introduce this fact and to reintroduce the variable \( \alpha \) for the degree of reaction. Then

\[ T_o \frac{d\alpha}{dT} = k \frac{(1 - \alpha)^2(T_o)^2}{K_p(\alpha T_o)} \]

and after rearrangement and integration

\[ \frac{\alpha}{1 - \alpha} - \ln \frac{1}{1 - \alpha} = \frac{k}{K_p} t \]  

\[ (5) \]
This single parameter expression for the self-inactivation or self-digestion of trypsin would obviously be difficult to test by ordinary methods, since it cannot be solved simply for $\alpha$. By the slide fit technique, however, it may be treated exactly the same as the above examples. Arbitrary values for $\alpha$ are chosen, substituted, and plotted against the corresponding evaluation of the integral, as before, on semilogarithmic paper. This leads to the curve of Fig. 4.

It can be seen from Equation 5 that if the hypothesis is correct the rate of self-digestion should be independent of the starting concentration. When the results of experiments at two different concentrations are plotted on the same type of paper with the per cent which has reacted (or not reacted, actually) against time and the points fitted to the above theoretical curve, the fit is striking, as may be seen in Fig. 4. The rate constant (value of the theoretical curve for time = 1 minute) is 0.0052 (off scale as drawn).

Other Hypotheses—In addition to the two factors deleted in the derivation of Equation 5, various other theories were considered. The hypotheses and unfavorable results were parallel to those described for casein-trypsin digestion in Section II.

II. Digestion of Casein by Trypsin

Experimental—The standard experiment comprises the digestion at 35.0° of 1 per cent Difco “isoelectric” casein by approximately two-thirds its volume of 1 mg. per ml. of crystalline trypsin at pH 7.10 (chosen to parallel Kunitz’ and Northrop’s conditions). This is referred to as a C-T experiment. An experiment with twice as much initial casein is a 2C-T or with half as much trypsin a C-1/2T experiment.
The casein is prepared by shaking 1.000 gm. of Difco isoelectric casein into 75 to 80 ml. of 0.1 M disodium hydrogen phosphate, stirred mechanically as rapidly as possible, without engendering foaming. After adjustment to pH 7.30 at 25° with 0.1 M potassium dihydrogen phosphate (about 12 to 13 ml.), the solution is made up to 100 ml. in a volumetric flask with 0.1 M disodium hydrogen and potassium dihydrogen phosphates mixed to pH 7.30 at 25°. This solution will keep 3 to 4 days in the refrigerator.

The trypsin solution used is 1 mg. per ml. (Worthington Laboratory) "once purified crystalline trypsin" (assay 50 per cent) in refrigerated hydrochloric acid of pH 1.96. The solution must be kept iced and should be used immediately.

Since the ultimate aim of these experiments was to study the reaction under pressure, the following procedure was designed to keep the enzyme and substrate separate until used under pressure and at various temperatures. With semiautomatic, syringe pipettes (to speed the procedure and avoid changes in the trypsin) a set of test-tubes is charged with 3.0 ml. each of 5 per cent TCA. To each of a set of stainless steel bombs (Johnson, Baylor, and Fraser (2)) are added the above casein solution, a stainless steel messenger, and 5 drops of hexadecane (Paragon). (The hexadecane (m.p. 18°) may be replaced by tetradecane for experiments at lower temperature.) Both have the advantages of inertness, availability, and lack of effect on the optical density when removed as described. Hexadecane melts and allows mixing within 1 minute, even at 25°, and in glass tubes.) The bombs are chilled in ice until the hexadecane is frozen (3 to 5 minutes) and meanwhile the trypsin solution is prepared. Then 1.1 ml. of the fresh trypsin solution are added and enough hexadecane to fill the bomb (2 to 5 drops). A rubber dam is placed on each bomb, the top is screwed in place, and the group is immersed in the bath at the desired temperature. After 1 minute the set of bombs is removed, inverted several times to allow the messengers to mix substrate and enzyme, giving a final pH of 7.1, and then returned to the bath. At the desired time intervals the bombs are removed, and the contents dumped into the TCA solution and poured back and forth. After at least 1 hour the mixture is again chilled to solidify the hexadecane and filtered, and the optical density is determined at room temperature at 280 mλ in the Beckman quartz spectrophotometer. The optical density is converted to per cent of casein undigested by using the calibration curve of Fig. 5, the preparation of which is described below.

Calibration—Kunitz, using the optical density of a TCA filtrate as above, devised a method for the determination of trypsin activity by measuring the casein digested in a given time (8). Here, however, we must know the amount or fraction of casein digested. To relate casein digested to
optical density, varying amounts were digested completely (under the stated conditions this requires about 3 hours, but the digestions were actually run for 18 to 18.5 hours to insure completion) and the optical density of the filtrate was measured. To compensate for the varying charges of casein half the usual volume of 10 per cent (instead of 5 per cent) TCA solution was used for quenching and an equal volume of buffer (which has no effect on the optical density) containing enough casein to make up the charge differential was added before the experimental sample.

Completion of casein digestion implies partial subsequent digestion of the trypsin by itself. If the same amount of casein is digested completely with varying amounts of trypsin, the variations in optical density must be due to the self-digested trypsin (since undigested trypsin has no effect on the blank). Hence, by extrapolation back to zero concentration of trypsin, one can estimate the optical density which would have been produced from casein alone. This was done with the results shown in Fig. 5, and the resulting intercepts were used to make the calibration of Fig. 6.

Since the zero intercept for casein did not quite coincide with the blanks prepared by adding trypsin and casein separately to TCA, the straight line of Fig. 6 was drawn for the best fit to these points and the blank average. The error possibly introduced in this compromise is insignificant in all except the C-T experiments.

Discussion of Methods—In considering the results of experiments in which the kinetics are based on determination of some property which varies with the time of reaction, it must always be questioned whether the
quantity measured is a true indication of the disappearance of the reactant. In other words, we are determining here the rate of appearance of a somewhat arbitrarily defined product and considering it as a measure of the disappearance of casein. In justification three observations may be pointed out: First, it has been found that the optical density curve (Fig. 6) follows Beer's law; i.e., it is linear. Secondly, the opalescence produced by TCA with most digested casein disappears at the same time that the optical density of the filtrate reaches a maximum. Since the precipitate is an indication of undigested casein or large fragments, this means that no precipitable, undigestible fragments are piling up; on the other hand, once the precipitate disappears, the optical density, which is our measure of casein disappearance, no longer increases. Thirdly, in the preparation of the calibration of trypsin self-digestion (Figs. 1 and 2), the drop in digestive activity was followed simultaneously with the appearance of the optical density of the filtrate. The two processes were completely parallel within the limits of experimental error; i.e., if the per cent of active trypsin determined by ability to digest casein is plotted against the optical density of the self-digested filtrate, a straight line is obtained which passes through the blank. The increase in optical density is thus a measure of the disappearance of the protein trypsin as manifested by its enzymatic activity.

In previous studies of casein-trypsin digestion various properties have been used as the measure of the amount digested. Some (e.g. Van Slyke amino nitrogen or formol titration) actually measure the number of bonds cut; others (e.g. viscosity) measure protein disappearance, but are difficult to interpret. The criterion of a satisfactory property is that it measures the appearance or disappearance of a substance on one or the other side.
of the rate-determining step and that other kinetic processes do not excessively complicate its increase or decrease. The present assumption is that TCA precipitation discriminates between molecules of the size of casein and considerably smaller particles which are either the direct result of the rate-determining step or which are formed very rapidly thereafter. This presumption is strengthened not only by the above observations but also by the reasonable nature of the results.

DISCUSSION AND RESULTS

Although the digestion of casein by trypsin has been extensively studied, no satisfactory picture of the process has appeared. In view of the conflicting results in the literature, it seems necessary to start by showing that the casein-trypsin digestion cannot be explained by a simple classical rate law, and then to proceed to more elaborate considerations.

The simplest picture of casein-trypsin digestion is one in which trypsin self-digestion is negligible; i.e., the trypsin is a true catalyst. A set of appropriate plots for the C-T data shows that by the usual techniques of kinetic analysis the data fit either the first or second laws with a little straining. In each case, however, the best straight line fails to pass through the zero intercept by a considerable margin. When the initial trypsin concentration is varied, a rather clear linear dependence on concentration can be seen. But when the starting concentration of casein is varied, the conclusion is reached that the reaction is apparently zero order for casein. Since this is completely inconsistent with the results of individual runs and with the results of enzymatic kinetics in general, it is obvious that this interpretation is faulty.

The lack of agreement of the simple kinetic models with the data can be more clearly shown by use of the slide-fit technique, and it may be seen from the casein digestion points of Fig. 3 that the rate falls off even faster than the most improbable third order.

Role of Trypsin Self-Digestion—The most striking feature of all of the data (Figs. 7 and 8) is the extremely rapid dropping of the rate as the reaction proceeds. This aspect of casein-trypsin digestion has been noted before (9) by other investigators and various reasons have been assigned to it.

Since it became known that trypsin exists in dynamic equilibrium with a reversibly denatured trypsin (4) and that the active form is capable of digesting the denatured form, it has frequently been assumed that the complexities of trypsin digestions are due in part, at least, to concomitant self-digestion of the enzyme. Northrop has shown (10) that casein digestion at 0° is a first order process and it was believed that this was because of the lack of self-digestion at this temperature. The data for trypsin self-
digestion (Section I) show that the rate is slower than the casein-trypsin reaction by about an order of magnitude in the concentration range which we are considering. It is *a priori* unlikely, therefore, that enzymatic self-digestion is important in the rate decline of casein-trypsin digestion. Ac-

- **Fig. 7.** C-T raw data. Varying amounts of casein digested with the same amount of trypsin. Ordinate, per cent undigested; abscissa, time in minutes (logarithmic scale). C-T (Δ, Δ); 1/4 C-T (□); C-T (○); 2C-T (△). The solid symbols are the corresponding controls.

- **Fig. 8.** C-xT raw data. The same amount of initial casein digested with varying amounts of trypsin. Ordinate and abscissa as in Fig. 5. C-1/10T (Δ); C-1/2T (□); C-T (○) (same points as in Fig. 5); C-2T (△); C-3T (◇). The solid symbols are the corresponding controls.

...tually the rate change introduced even by assumption of trypsin self-digestion (at its normal rate in the absence of casein) turns out to be inconsiderable, even at extreme degrees of reaction (see the dotted curve in Fig. 9). The possibility of the rate drop being due to self-digestion of the enzyme can, therefore, be dismissed.
Inhibition by Reaction Products—In the literature on casein-trypsin, mention has frequently been made of the inhibiting effect of the products of the reaction. Although later kinetic studies seem to have neglected this factor, Northrop (7) has investigated this effect, using partially purified trypsin, and has shown that the inhibitor combines reversibly with the trypsin and that the ratio is probably 1:1.

Assumption of the mechanism

\[
T_a + C \overset{K_o}{\underset{k}{\rightleftharpoons}} (T_a \cdot C) \overset{K_p}{\rightarrow} C_d + T_a \overset{K_p}{\rightleftharpoons} (T_a \cdot C_d)
\]

allows substitution in the rate equation

\[
-\frac{dC}{dt} = k(T_a \cdot C) = kK_o(T_a)(C)
\]

in a manner completely parallel to that used in the trypsin system. We need not consider the equilibrium between \(T_a\) and reversibly denatured trypsin because this is rapidly attained (4) and has no effect on the kinetics of the digestion process. Trypsin self-digestion is also disregarded because it has been shown above to be too slow to be of significance. In the substitution for \(T_a\), the following equation is obtained (cf. Section I).

\[
(T_a) = \frac{(T_a \cdot C_d) + (T_a) + (T_a \cdot C)}{1 + K_p(C_d) + K_o(C)}
\]

Actually by comparing the complete integrated form with the data, even assuming extreme cases, we have found that the amount of \(T_a\) not combined with the product is arithmetically negligible compared to that in the form \(T_a \cdot C_d\). To simplify the development this factor is introduced and the variable \(\alpha\), the degree of reaction, is substituted, as was done in the trypsin derivation. Then

\[
(T_a) \frac{d\alpha}{dt} = kK_o(T_a)(1 - \alpha)(C_o)
\]

which on integration gives

\[
\ln \left(\frac{1}{1 - \alpha}\right) - \alpha = \frac{kK_o(T_a)}{K_p(C_o)} t
\]

This cannot be solved for \(\alpha\) and tested in the usual manner, but comparison of this rather complicated relationship with the data may easily be made by means of slide-fit technique.

Comparison of Data and Theory—The raw data are plotted in two sets (Figs. 7 and 8) to emphasize the dependence on the starting concentrations of casein and of trypsin and to avoid confusion of the points. It can be
seen from Equation (6) that the data for all experiments should, if the hypothesis is correct, be representable by a single curve, provided that the time values for various experiments are displaced by the appropriate values of $T_0$ and $C_0$. $T_0$ cannot, because of the instability of trypsin solutions, be simply calculated from the dilution of the stock. A control digestion with standard $T_0$ and $C_0$ is therefore run with each experiment and the correct $T_0$ value determined by the best fit of the control points (see Figs. 7 and 8) to the curve of the standard run, which is taken arbitrarily as having unit charges.
The standard (C-T) data are then transferred to another sheet of graph paper; the new sheet is moved horizontally by the corrected \( T_0 : C_0 \) ratio for each experiment and the points for this experiment traced. The results are again shown in Figs. 9 and 10 for clarity.

Now the assembled data can be fitted to the theoretical curve obtained in the above derivation by superposition of the sheets and sliding along the \( x \) axis. It can be seen (Figs. 9 and 10) that the fit of the data is fairly good, especially considering the wide range of the reactant ratio, and much better than that obtained by any previous hypothesis. The discrepancies occur in the early points which lie below the theoretical curve, and, in general, it seems that the fit might be better if the curve were somewhat flatter.

*Second Order Inhibition*—The most obvious effect which would result in a flatter curve is the assumption of a higher order of inhibition. Actually the curve for second order inhibition is not greatly different from the above theoretical curve and results in a better fit to some of the data. Such a hypothesis does not seem readily interpretable mechanistically. Moreover, this is so clearly inconsistent with the trypsin self-digestion data and with Northrop's experience (7) with the inhibitor that it was abandoned.

*Other Inhibition Effects*—In the above derivation several factors were omitted. If we consider that active trypsin may be present in appreciable amount or that an arithmetically significant amount may be bound by itself and by undigested casein, corresponding integrated equations may be obtained, including as many such factors as desired. All such equations, however, lead to theoretical curves steeper than those of Figs. 9 and 10, and, in all cases, a less satisfactory fit by far, thus justifying their omission in the above derivation.

In passing it may be noted that several other hypotheses were tested to be sure that the data could not be equally well fitted. They include assumption of a varying order of inhibition, depending on the ratio of casein to trypsin, assumption of second order inhibition with trypsin or unchanged casein, assumption of higher orders of inhibition with the product, and assumption of a sum of first and second order inhibition with the product. The failure of any of these, even in extreme cases, to give at all satisfactory fits lends confidence to the view that the above explanation is valid.

*Comparison with Previous Work*—Not all the data which were obtained by Northrop on the digestion of casein are comparable, since much of the early work was done with partially purified enzyme and led to anomalous results. With crystalline trypsin Northrop (9) digested casein under conditions similar to ours, determining the digested casein by TCA precipitation and nitrogen analysis of the supernatant fluid. In Fig. 11 Northrop's data from the three experiments at different concentrations of casein.
digested at 35° have been fitted to the same theoretical curve which was used in Figs. 9 and 10. The fit lends confidence to both the present theory and analytical method.

**Inhomogeneity of Casein**—One of the points which it was of interest to consider in connection with the anomalies noted in casein-trypsin digestion by previous workers was the known inhomogeneity of casein. Examination of casein electrophoretically has indicated that so called purified casein, prepared by isoelectric precipitation, as was the casein used in the present work, consists of two main fractions in the ratio 81:19 and is subject to small variations depending on the source (11).

The data of Figs. 9 and 10 lend themselves readily to the assumption that the casein is not pure, in the sense that a minor component has been digested much more rapidly than the main fraction. It may be seen that the data tend to extrapolate to about 85 per cent instead of 100 per cent. (This value was actually determined by means of a log versus log plot, which shows the effect much more clearly.) If this is true, the early part of the digestion should show characteristic behavior and drop rapidly from 100 per cent to the lower curve. In Fig. 10 (dotted curve) and Fig. 12 the assembled data have been fitted to a theoretical curve, each point of which is 85 per cent of the corresponding ordinate of the curve of Figs. 9 and 10. In addition, in Fig. 12 further data are included, covering the first part of the reaction and showing that the fit to the original curve is reasonably satisfactory. With the curve of 85 per cent pure casein, however, the behavior is exactly that which would be expected. It is of interest to note that these data were obtained in a different laboratory by

![Diagram](http://www.jbc.org/pdf/fig11.png)

**Fig. 11. Data of Northrop fitted to the curve of Figs. 9 and 10. Same ordinate and abscissa.**

*These additional data were obtained by Mr. A. C. Knight, Department of Chemistry, University of California.*
another investigator using different casein and a new sample of trypsin (from the same original manufacturer's lot) and using an altered technique in which the samples were taken from a single batch stirred at 35°. Considering that these samples could include no controls to link them to the previous data, the fit and overlap are excellent.

A theory to cover the whole range could be constructed and handled by the slide-fit technique, but would be of little value since it would involve the introduction of additional assumptions and several new parameters which could be evaluated only roughly, because of the relatively minor importance of the rapidly digested component and the relative closeness of its rate to that of the major component.

![Graph](image)

**Fig. 12.** Data of Figs. 9 and 10 plus further data covering the early stages of digestion fitted to a curve which assumes casein to contain 15 per cent of a more rapidly digested component. The solid symbols are the xC-T data, the open symbols are the C-zT data, the solid circles the added data. Same ordinate and abscissa.

Qualitatively, however, it may be said that casein behaves as a mixture and that the proportions of the components determined kinetically are strikingly close to those obtained independently from electrophoretic measurements, probably within the variations in the sources. The minor component digests approximately 5 times as fast as the major component. The approximate rate constant for the major fraction is 0.018. It must be remembered that these are portmanteau constants and include several equilibrium constants, as well as the specific rate constant of the digestion.

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data included in Fig. 12. Particular appreciation is due Miss Rosamond S. Baker for conscientious and capable technical assistance.

SUMMARY

Preliminary data for the self-digestion of trypsin and more complete data for the digestion of purified casein by crystalline trypsin are presented and analyzed. Both reactions have been studied in phosphate buffer, pH 7.1, at 35°, and the casein data cover a range of 97 per cent completion and a concentration ratio of 160 (casein to trypsin). The data are representable by a single parameter expression in each case on the assumption that the reaction involves 1 molecule of trypsin and 1 of casein (or trypsin), but that the enzymatic activity of trypsin is inhibited by a reversible equilibrium with the products of digestion. A closer fit to the casein data is obtained by the further assumption that the casein consists of two components digested at different rates; the proportions obtained from the present kinetic data agree well with the known composition of casein. Comparison of the data to the theory is made by a graphical method of analysis which is generally applicable to kinetic data and most useful in cases in which the integrated rate law is complex.

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