Kurtz, A. N., and Niemann, C. (1962), *Biochemistry 1*, 238.
Leete, E., Marion, L., and Spenser, I. D. (1955), *Can. J. Chem. 33*, 405.
Miles, J. L., Robinson, D. A., and Canady, W. J. (1963), *J. Biol. Chem. 238*, 2932.
Peterson, P. E., Wolf, J. P., III, and Niemann, C. (1958), *J. Org. Chem. 23*, 303.
Platt, A., and Niemann, C. (1963), *Proc. Natl. Acad. Sci. U.S. 50*, 817.
Reissert, A. (1897), *Ber. 30*, 1030.
Rydon, H. N., and Tweddle, J. C. (1955), *J. Chem. Soc.*, 3499.
Sanna, G. (1934), *Rend. Seminario Fac. Sci. Univ. Cagliari 4*, 28; *Chem. Abstr. 30*, 6363 (1936).
Shaw, K. N., McMillian, A., Gudmundson, A. G., and Armstrong, M. D. (1958), *J. Org. Chem. 23*, 1171.
Singer, H., and Shive, W. (1955a), *J. Org. Chem. 20*, 1458.
Singer, H., and Shive, W. (1955b), *J. Am. Chem. Soc. 77*, 5700.
Uhle, T. C. (1949), *J. Am. Chem. Soc. 71*, 761.
Wallace, R. A., Kurtz, A. N., and Niemann, C. (1963), *Biochemistry 2*, 824.

The Acyl-Enzyme Dimer of Chymotrypsin*

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ABSTRACT: Titration of the active sites of α- and β-chymotrypsin at *p*H values 2–4 by the specific substrate *N*-acetyl-DL-tryptophan *p*-nitrophenyl ester shows the presence of some catalytically inactive but rapidly activatable enzyme species, the percentage of which depends on the total protein concentration. Conversely, at *p*H 7–8 all of the enzyme is in the active form. The effect of the protein concentration on the active-inactive enzyme equilibrium indicates strongly that a dimer is formed. A number of pieces of evidence indicates that the dimer is an acyl-enzyme in which one molecule of enzyme acylates a second molecule: (1) the time dependence of the activation of the dimer; (2) the effect of *p*H on the monomer-dimer equilibrium; (3) inhibition of the dimerization by specific inhibitors of chymotrypsin; (4) the effect of protein concentration on the monomer-dimer equilibrium (see equation 6); and (5) the effect of *p*H and ionic strength on the rate of conversion of dimer to monomer which parallels that of a deacylation reaction. The dimer of chymotrypsin provides the ultimate acyl-enzyme, a naturally occurring compound consisting of two protein components.

Much evidence supports the hypothesis that chymotrypsin undergoes a reversible dimerization. This evidence is based mainly on physical measurements such as sedimentation, light scattering, diffusion, and the like, which determine an apparent molecular weight of the system (Schwert, 1949; Schwert and Kaufman, 1949; Schwert, 1951; Schwert and Kaufman, 1951; Smith and Brown, 1952; Frenkel, 1952; Steiner, 1954; Massey *et al.*, 1955; Egan *et al.*, 1957; Tinoco, 1957; Rao and Kegeles, 1958; Bethune and Kegeles, 1961; Winzor and Scheraga, 1964). A recent X-ray analysis of α-chymotrypsin crystals indicates that two enzyme molecules occupy one unit cell, strongly suggesting a crystalline dimeric form of the enzyme (Blow *et al.*, 1964).

This large mass of physical information does not, however, shed any light on the forces involved in the dimerization or on the mechanism of the reaction. From the point of view of enzyme chemistry, the most important point concerns the possibility of the involvement of the "active site" of the enzyme in the dimerization. On this subject contradictory evidence exists: some workers claim that neither chymotrypsinogen, DFP-inhibited chymotrypsin, nor photooxidized chymotrypsin dimerizes (Schwert, 1949; Egan *et al.*, 1957); others affirm the contrary (Schwert, 1951; Smith and Brown, 1952; Massey *et al.*, 1955). A kinetic investigation of the influence of dimerization on the catalytic activity of α-chymotrypsin did not result in a definitive mechanism of dimerization (Martin and Niemann, 1958).

The inability of the previous, mainly physical, methods to specify the mechanism of dimerization demanded a new method of approach to this problem. Such an approach was found accidentally during the titration of the active site of α-chymotrypsin by *N*-acetyl-DL-tryptophan *p*-nitrophenyl ester at *p*H values between 2 and 4 (Kézdy *et al.*, 1964). During such titrations we observed that an α-chymotrypsin solution at *p*H 4 contains some catalytically inactive but rapidly activatable enzyme species, the percentage of which depends on the total

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TABLE I: Titration of the Normality of Some Chymotrypsin Solutions Using *N-trans*-Cinnamoylimidazole.

Enzyme		$\frac{\text{Normality from Titration}}{\text{Normality from Weight}} \times 100\%$
Worthington 3× crystallized α-chymotrypsin	6032	74.5
	6077	75.5
	6079	83.3
	CDI6080-81R	76.1
	CDI6087-8	79.2
Worthington chromatographically pure α-chymotrypsin CDC 50A		74.4
Boehringer α-chymotrypsin 15139 6373510		61.6
Worthington β-chymotrypsin CDB 2604-05		56.7, 56.5, 56.5
	6001	71.1, 71.4
	5701	70.4
	CDB2203	69.4
Worthington δ-chymotrypsin	6030	78.7

protein concentration. On the other hand, all of the enzyme was found to be in the active form at *p*H 7–8. The observation of an inactive form of the enzyme, its activation by substrate, and the dependence of the inactive form on the protein concentration and the *p*H indicated to us that the inactive species was probably the dimer observed previously by physical methods.

Thus we had at our disposal an easy *chemical* method to determine the concentration of the dimer in solution. In addition, this approach allowed the measurement of the time dependence of the activation of the enzyme, yielding additional information about the mechanism of dimerization and its relation to the structure of chymotrypsin. In this paper we will present evidence that the inactive enzyme at low *p*H, as measured by titration of the enzyme active sites with *N*-acetyl-DL-tryptophan *p*-nitrophenyl ester, is in fact a dimer. Further, we will attempt to show that the dimer is very probably an acyl-enzyme formed by a mechanism similar to the formation at low *p*H of an acyl-enzyme from α-chymotrypsin and the carboxylic acid, *N*-acetyl-L-tryptophan (Kézdy and Bender, 1964). β-Chymotrypsin has a greater tendency to dimerize than does α-chymotrypsin. Since the dimerization of β-chymotrypsin is experimentally more accessible, the major part of this study concerns its dimerization, with a smaller portion describing the dimerization of α-chymotrypsin.

Experimental

Enzymes. Worthington 3-times-crystallized lyophilized α-chymotrypsin was used. Different batches of this enzyme behaved in an experimentally indistinguishable manner, in titration experiments as well as in dimerization studies. These samples were also indistinguishable from Worthington chromatographically pure α-chymotrypsin and Boehringer α-chymotrypsin. Worthington β-chymotrypsin was used.[1] A number of different batches behaved in an experimentally identical manner. Most of the experiments were performed with Worthington β-chymotrypsin No. 6001.

The analytical enzyme concentration was determined by spectrophotometric titration using *trans*-cinnamoylimidazole as substrate (Schonbaum *et al.*, 1961). The purity of the enzyme preparations is reported in Table I as the percentage of the enzyme normality obtained by titration of the active sites using *trans*-cinnamoylimidazole to the enzyme normality calculated on the basis of the weight of the protein and a molecular weight of 24,800. No provision was made to correct for the bound water in the enzyme preparations.

The chemicals, buffers, and the technique for the titration of the enzyme solution with *N*-acetyl-DL-tryptophan *p*-nitrophenyl ester at low *p*H values have been described previously (Kézdy *et al.*, 1964). The kinetic measurements were performed on a Cary Model 14 recording spectrophotometer equipped with a thermostated cell-compartment. Enzyme stock solutions were centrifuged at 15,000 rpm for 30 minutes after dissolving the weighed amount of enzyme in the appropriate buffer. Two types of kinetic measurements were carried out: (1) "*Stock*" *experiments.* In these experiments, the buffer and an acetonitrile solution of *N*-acetyl-DL-tryptophan *p*-nitrophenyl ester were mixed and the spontaneous hydrolysis of the substrate was recorded at 340 mμ for a few minutes. Then a small amount (5–100 μl) of the enzyme stock solution was added at a known time and recording of the reaction was started generally 10 seconds after addition. (2) *Equilibrium experiments.* In these experiments the buffer and the enzyme were mixed in the cell for a 600- to 800-second period of equilibration. Then the substrate was added and the recording of

[1] Several of the chymotrypsin samples were obtained from Dr. R. Egan of the Worthington Biochemical Corp., whom the authors wish to thank for his kindness.

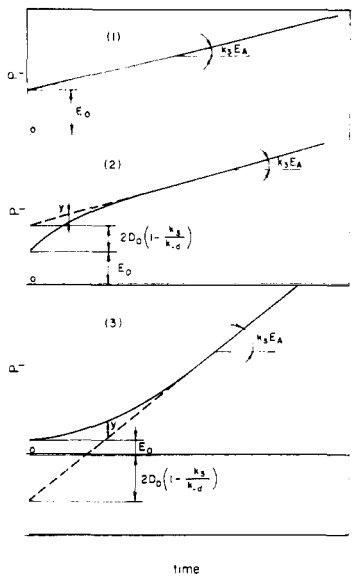


FIGURE 1: Hypothetical plots of equation (3) under three sets of conditions: (1) $k_3 = k_{-d}$; (2) $k_3 < k_{-d}$; (3) $k_3 > k_{-d}$. See text.

the spectral change commenced. In the "stock" experiments, an enzyme solution of any *p*H could be instantaneously titrated (before equilibrium occurred). In the equilibrium experiments, a titration under the particular equilibrium conditions necessarily took place.

Results

Before proceeding to a discussion of the experimental results, let us consider a system in which an active monomeric enzyme, E, is in equilibrium with its catalytically inactive dimer, D. If we add a high concentration of a rapidly reacting substrate to this equilibrium mixture of monomer and dimer, the active monomer, E,

$$2\,E \underset{k_{-d}}{\overset{k_d}{\rightleftharpoons}} D \qquad (1)$$

will disappear, perturbing the equilibrium; thus D will be decomposed into E with a rate-limiting step k_{-d}, until all the enzyme is activated. We have previously shown that the acylation of chymotrypsin by *N*-acetyl-DL-tryptophan *p*-nitrophenyl ester at low *p*H is essentially instantaneous (Kézdy *et al.*, 1964); further it may be shown that its K_m (app) $\ll k_{-d}/k_d$; therefore this reagent provides a ready instrument to perturb equilibrium (1). The *N*-acetyl-DL-tryptophan *p*-nitrophenyl ester substrate, S, will react with the enzyme following the known scheme (Kézdy and Bender, 1964):

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \qquad (2)$$
$$+P_1$$

For this reaction, it is known that $k_2 \gg k_3$ and the reaction was carried out under conditions that $S_0 \gg K_m$ (app) $= K_s k_3/(k_2 + k_3)$. Using these relationships and the fact that $E_A \ll S_0$, it can be shown that the totality of the enzyme will be transformed into ES′ and P_1 in an initial fast step, followed by a slower zero-order reaction of ES′ releasing P_2 and E, which will again yield ES′ and P_1 in the fast step.

If $k_{-d} \ll k_2$, the free enzyme, E, will react with the substrate much faster than the inactive dimer, D, will be activated. If this condition is satisfied, the inactive dimer, D, and the active monomer, E, may be treated mathematically as two different enzymes, acting independently of each other. Using these assumptions it can readily be shown that P_1, *p*-nitrophenol in this system, may be described by:

$$P_1 = \pi + k_3 E_A t + 2\,D_0[1 - (k_3/k_{-d})](1 - e^{-k_{-d}t}) \qquad (3)$$

where π is the initial (instantaneous) burst of *p*-nitrophenol and E_A is the analytical amount of enzyme in the solution (calculated as the monomer). A plot of P_1 versus t may be described by three qualitative shapes, depending on the value of the ratio k_3/k_{-d}, as seen in Figure 1.

(1) If $k_3/k_{-d} = 1$, $P_1 = \pi + k_3 E_A t$. This condition does not lead to any kinetic evidence for dimerization, the initial burst measuring the amount of active enzyme at the moment of the addition of the substrate, $\pi = E_0$.

(2) If $k_3 < k_{-d}$, the behavior of the system may be represented by curve 2 of Figure 1, which consists of three parts: (a) an instantaneous burst (at $t = 0$) which is a measure of E_0; (b) a linear portion at high values of $t(k_{-d}t \gg 1)$ whose slope is $k_3 E_A$. Since E_A is known, k_3 may be calculated from the slope. The extrapolated intercept of the linear portion equals $\pi + 2\,D_0[1 - (k_3/k_{-d})]$. Since π is known and since $E_A = \pi + 2\,D_0$, k_3/k_{-d} may be determined; (c) a curved portion at intermediate values of t. This curved portion permits the calculation of k_{-d} from the exponential portion of equation (3) since

$$y = P_1(\infty) - P_1(t) = 2\,D_0[1 - (k_3/k_{-d})]e^{-k_{-d}t} \qquad (4)$$

(3) If $k_3 > k_{-d}$, the behavior of the system may be represented by curve 3 of Figure 1, which again consists of three parts as above, but the curvature is convex instead of concave, with the result that the extrapolation of the linear portion of the curve leads to a negative intercept. Although curve 3 looks superficially different from curve 2, all the essential features and the methods of determination of the individual constants remain the same as above.

In addition to describing the kinetics associated with equation (1), let us also consider the equilibrium. If dimerization may be described mechanistically by an acyl-enzyme formation between two molecules of enzyme, then the process must be described by two steps, the first being the formation of a dimeric adsorptive complex, the Michaelis-Menten complex, followed by the formation of the dimeric acyl-enzyme. Since the formation and decomposition of the adsorptive com-

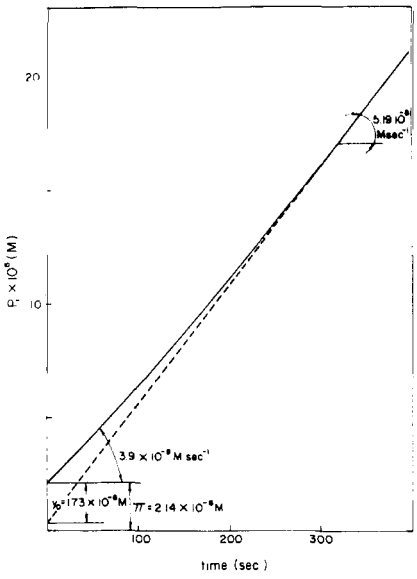


FIGURE 2: The β-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-DL-tryptophan *p*-nitrophenyl ester at *p*H 2.7 (0.05 M citrate buffer). $E_A = 5.03 \times 10^{-6}$ M, stock enzyme solution at *p*H 4.02; $S_0^L = 8.04 \times 10^{-5}$ M; 1.6% acetonitrile; 25 ± 0.5°. Measurements made at 340 mμ.

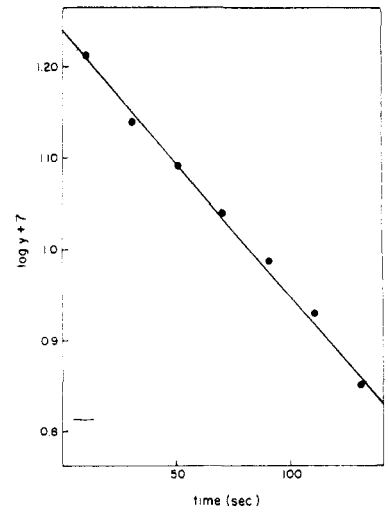


FIGURE 3: Plot of log *y* versus *t* for the reaction of β-chymotrypsin with *N*-acetyl-DL-tryptophan *p*-nitrophenyl ester. Data taken from the experiment described in Figure 2.

plex are controlled by very rapid, in some instances diffusion-controlled processes (Eigen and Hammes, 1963), the enzyme molecules in the form of adsorptive complexes will behave as free enzyme molecules in the reaction with *N*-acetyl-DL-tryptophan *p*-nitrophenyl ester. Hence D will measure only the amount of acyl-enzyme dimer.

At low *p*H values, where the carboxylic acid groups of the enzyme which may participate in the dimerization process are fully protonated, the mechanistic scheme involving a dimeric acyl-enzyme may be represented by:

$$2\,E \underset{}{\overset{K_s^E}{\rightleftharpoons}} C \underset{k_{-d}}{\overset{k_d}{\rightleftharpoons}} D \qquad (5)$$

where *C* represents the concentration of the adsorptive enzyme-enzyme complex. At equilibrium, equation (5) leads to

$$E_A/D_0 = 2(1 + K_d) + (K_d K_s^E/D_0)^{1/2} \qquad (6)$$

where $K_d = k_{-d}/k_d$. On the other hand, if the dimerization is a simple association process represented by

$$2\,E \overset{K}{\rightleftharpoons} D \qquad (7)$$

the equilibrium may be represented by

$$E_A/D = 2 + (K/D)^{1/2} \qquad (8)$$

Equations (6) and (8) differentiate between a simple association process and a dimerization involving an acyl-enzyme, for equation (8) demands that the intercept of a plot of E_A/D versus $1/D^{1/2}$ be 2, whereas equation (6) demands that this intercept be $2(1 + K_d)$.

The foregoing theoretical considerations will now be used in the treatment on two typical experiments, involving the interaction of β-chymotrypsin and α-chymotrypsin with *N*-acetyl-DL-tryptophan *p*-nitrophenyl ester.

β-Chymotrypsin. Figure 2 represents a typical reaction of β-chymotrypsin with *N*-acetyl-DL-tryptophan *p*-nitrophenyl ester, showing the production of *p*-nitrophenol with time in this reaction. The enzyme was equilibrated with the buffer for 600 seconds before the addition of the substrate (the *p*H of the enzyme stock solution was 4.02). The initial burst of P_1 is instantaneous with respect to the time scale of the experiment. After complete activation of the enzyme (*ca.* 400 seconds) the turnover of the enzyme yields a linear production of P_1, indicating that $S_0 \gg K_m$(app), similar to the result found with α-chymotrypsin (Kézdy and Bender, 1964). The entire curve is seen qualitatively to conform to the hypothetical curve of equation (3) with $k_3 > k_{-d}$ (see Figure 1). From the data of Figure 2, E_0 may be calculated from the initial burst, k_{-d} may be calculated from a plot of log *y* versus *t* (see Figure 3), k_3 may be calculated from the slope of the linear portion of the curve, and D may be calculated from the conservation of mass, knowing E_A and E_0. The values for these constants are given in Table II. The internal consistency of these data may be tested by using them to calculate the intercept $2\,D_0(1 - (k_3/k_{-d}))$ and comparing the calculated value with the experimental value. The agreement is better than 1% for this set of data. Table II indicates that $k_3 > k_{-d}$ for this system,

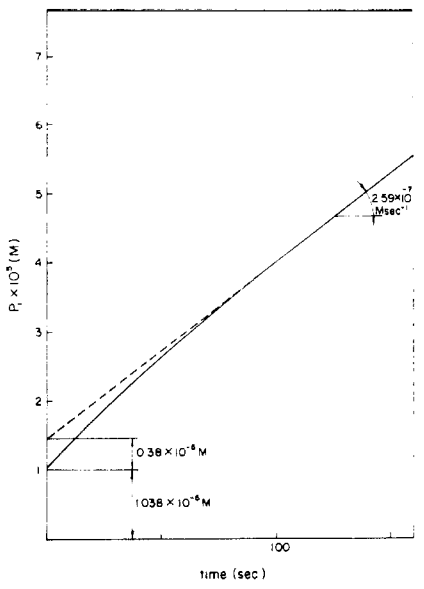


FIGURE 4: The α-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-DL-tryptophan *p*-nitrophenyl ester at *p*H 2.7 (0.05 M citrate buffer). $E_A = 2.29 \times 10^{-5}$ M, stock enzyme solution at *p*H 4.03; $S_0^L = 1.53 \times 10^{-4}$ M; 1.6% acetonitrile-water; 25.0 ± 0.5°. Measurements made at 340 mμ.

as stated previously. Thus the proposed kinetic scheme embodied in equation (3) describes the interaction of β-chymotrypsin with *N*-acetyl-DL-tryptophan *p*-nitrophenyl ester and is consistent with the active monomer–inactive dimer equilibrium of equation (1).

α-Chymotrypsin. Figure 4 represents a typical reaction of α-chymotrypsin with *N*-acetyl-DL-tryptophan *p*-nitrophenyl ester at *p*H 2.7, using a stock enzyme solution at *p*H 4.03. Unfortunately the y value in this experiment is too small to be measured with great accuracy, but it is possible to determine an approximate value of k_{-d}. This value and the other constants of this reaction, calculated in the manner described, are listed in Table II. These constants form a reasonably self-consistent set, although the accuracy is not as high as that found in the β-chymotrypsin experiments. Here again, the initial burst is quasi-instantaneous and the turnover reaction is absolutely linear. Table II indicates that $k_3 < k_{-d}$ for α-chymotrypsin while the opposite is true for β-chymotrypsin. Significantly, k_3 is identical for both α- and β-chymotrypsin reactions. These data indicate that equation (3) is a correct description of the α-chymotrypsin reaction.

Rate of Conversion of the β-Chymotrypsin Dimer to Monomer. Since the dimer-monomer conversion with β-chymotrypsin was particularly amenable to experiment, this reaction was investigated in some detail. Table III gives values of k_3 and k_{-d} for this reaction under a number of different conditions. The experiments at *p*H 2.7 show that k_3 as well as k_{-d} are independent of initial enzyme and substrate concentrations over the concentration range studied. In Figure 5, the *p*H dependencies of the rate constants k_3 and k_{-d} for β-chymotrypsin are compared to that for k_3 of α-chymotrypsin. This comparison shows the identity of k_3 values for α- and β-chymotrypsin as well as the similarity of the *p*H dependencies of k_3 and k_{-d}. Above *p*H 4, k_{-d} is not accessible to experiment because of its speed.

TABLE II: Dimerization of α- and β-Chymotrypsin.[a]

	β-Chymotrypsin	α-Chymotrypsin
$E_A \times 10^6$ M	5.03	22.89
$E_0 \times 10^6$ M	2.14	10.38
$2D_0 \times 10^6$ M	2.90	12.51
$k_{-d} \times 10^3$ sec^{-1}	6.42	23
$k_3 \times 10^3$ sec^{-1}	10.3	11.3

[a] *p*H 2.7, 0.05 M citrate buffer, 1.6% acetonitrile-water, 25.0 ± 0.5°.

TABLE III: Hydrolysis of *N*-Acetyl-DL-tryptophan *p*-Nitrophenyl Ester by β-Chymotrypsin.[a]

*p*H	$E_A \times 10^6$ M	$S_0^L \times 10^5$ M	$k_3 \times 10^3$ sec^{-1}	$k_{-d} \times 10^3$ sec^{-1}
2.22	2.54	7.86	3.3	2.9
2.22	2.50	15.8	3.1	3.1
2.25	1.92	15.8	3.4	3.0
2.60	5.32	6.35	8.6	5.9
2.70	5.03	8.07	10.4	7.2
2.70	2.54	8.04	10.2	6.7
2.70	5.03	8.04	10.3	6.4
2.70	2.03	8.04	10.4	7.4
2.70	2.03	8.04	9.9	5.8
2.70	1.02	8.04	10.4	5.6
2.70	1.02	8.04	10.2	5.6
2.70	19.5	15.8	10.2	6.3
2.70	2.54	7.9	10.3	7.2
3.46	1.02	7.9	40.9	22
4.04	1.02	7.9	170	100
2.700	2.54	0[b]	10.3	7.19
2.690	2.54	4.88[b]	19.0	8.18
2.702	1.02	9.80[b]	27.1	7.11
2.692	1.02	14.70[b]	(30.8)[c]	8.58
2.709	1.02	19.6[b]		11.6

[a] 25°, 1.6% acetonitrile-water, μ = 0.05 M. [b] These numbers correspond to % methanol in the solution and not to the concentration of substrate which was 7.9×10^{-5} M. [c] Approximate value since the condition $S_0 \gg K_m$(app) was not completely satisfied.

TABLE IV: Monomer-Dimer Equilibrium of β-Chymotrypsin.

$E_A \times 10^6$ M	$D_0 \times 10^6$ M	E_A/D_0
	*p*H = 2.70[a]	
1.02	0.155	6.58
2.04	0.445	4.57
5.03	1.435	3.51
19.5	6.79	2.88
48.4	19.2	2.53
	*p*H = 6.02[b]	
2858	791	3.61
2858	858	3.33
2858	769	3.72
1429	372	3.84
953	250	3.81
286	61.3	4.66
143	30.2	4.72

[a] Equilibrium experiments (see Experimental), 1.6% acetonitrile-water, 0.05 M citrate buffer, 25 ± 0.5°. [b] Stock experiments (see Experimental), 0.067 M phosphate buffer, 25 ± 0.5°.

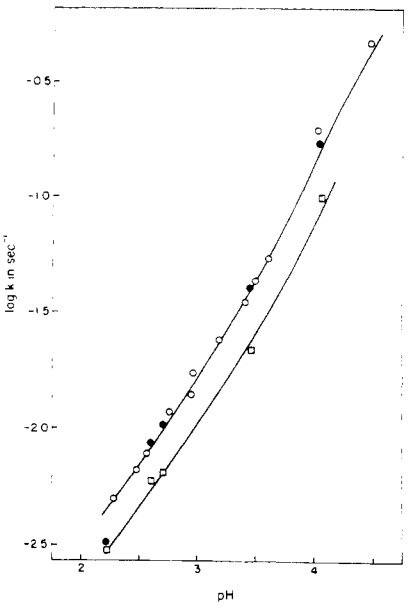


FIGURE 5: The hydrolysis of *N*-acetyl-DL-tryptophan *p*-nitrophenyl ester by chymotrypsins. O, k_3 for α-chymotrypsin (Kézdy *et al.*, 1964); ●, k_3 for β-chymotrypsin; □, k_{-d} for β-chymotrypsin. μ = 0.05.

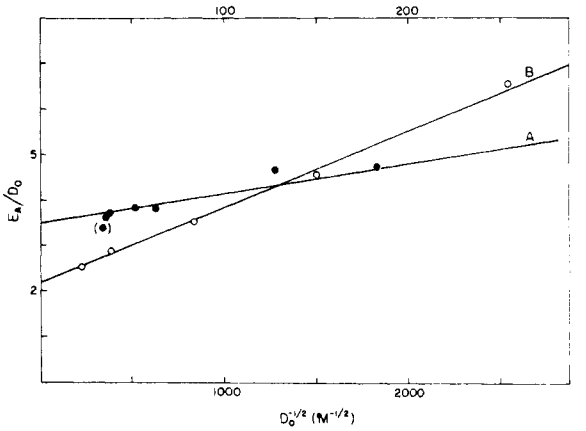


FIGURE 6: The monomer-dimer equilibrium of β-chymotrypsin plotted according to equation (6). A, *p*H 6.02 (upper scale); B, *p*H 2.70 (lower scale).

Table III also records the results of experiments with β-chymotrypsin carried out in methanol-water mixtures. With increasing methanol concentration, k_3 increases whereas k_{-d} remains constant. From these data, one may calculate that $k_3 = 1.85 \times 10^{-4}$ M^{-1} sec^{-1} and k_4 (methanolysis) $= 7.14 \times 10^{-3}$ M^{-1} sec^{-1}, yielding a ratio $k_4/k_3 = 38.6$. This value is essentially identical to the one obtained at *p*H 7.65 ($k_4/k_3 = 39$) (Bender *et al.*, 1964a), demonstrating again the identity of the mechanisms of hydrolysis and alcoholysis, and also the identity of the chymotrypsin mechanism over a wide *p*H range.

Monomer-Dimer Equilibrium of β-Chymotrypsin. Table IV and Figure 6 show results concerning the monomer-dimer equilibrium of β-chymotrypsin, treated according to equation (6). Fairly good straight lines are obtained, indicating that the assumptions underlying equation (6) are satisfied. Moreover, considering the probable error of the individual points, the intercepts of these plots are significantly different from 2, requiring that the hypothesis of a one-step adsorptive dimerization (equation 8) must be discarded and that the hypothesis of a two-step dimerization involving the formation of an acyl-enzyme compound is indicated.

Assuming an acyl-enzyme dimer, a reasonable combination involves a carboxylic acid group of one enzyme molecule and the serine hydroxyl group of a second enzyme molecule. At *p*H 2.7, the *p*H of one experiment described, the carboxylic acid would be completely protonated. Under these circumstances, the intercept and slope of Figure 6 will yield K_d and K_s^E, respectively, according to equation (6). The values for these constants are $K_d = 0.1$ and $K_s^E = 2.69 \times 10^{-5}$ M. The inverse of K_d, $k_d/k_{-d} = 10$, compares favorably with the corresponding constant for the acylation of α-chymotrypsin with the carboxylic acid, *N*-acetyl-L-tryptophan, which varies from 3 to 7 (Kézdy and Bender, 1964).

At *p*H 6.02, the *p*H of the second experiment described above, the carboxylic acid forming the acyl-enzyme would be largely ionized. Since only the protonated carboxylic acid may form an acyl-enzyme (Kézdy and Bender, 1964) while the carboxylate anion may form an adsorptive complex but may not acylate the enzyme, equation (6) must be modified to give (9):

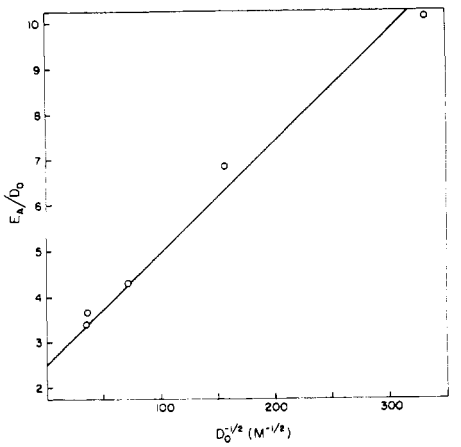


FIGURE 7: The monomer-dimer equilibrium of α-chymotrypsin plotted according to equation (6). *p*H 4.02, 0.2 M acetate.

$$E_A/D = 2\{1 + K_d[1 + (K_i'/H)]\} + \{K_dK_s^E[1 + (K_i/H)]/D\}^{1/2} \quad (9)$$

where K_i is the ionization constant of the carboxylic acid group of the enzyme involved in acyl-enzyme formation and K_i' is the ionization constant of this group in the adsorptive enzyme-enzyme complex. If the carboxylic acid involved in the dimerization is completely ionized at *p*H 6.02 [$(K_i/H) \gg 1$], then the intercept and slope of Figure 6 (data of Table IV) permits the calculation of K_i and K_i', using the values of K_d and K_s^E obtained at *p*H 2.7. These calculations yield pK_i = 4.80 and pK_i' = 5.14, in reasonable agreement with that found previously for *N*-acetyl-L-tryptophan (Kézdy and Bender, 1964).

A number of other experiments were carried out with β-chymotrypsin to elucidate the mechanism of inactivation of this enzyme, principally to test the hypothesis that the inactive enzyme is an acyl-enzyme dimer. (1) Extensive dialysis of a 1.6×10^{-3} M β-chymotrypsin solution at *p*H 4.0 versus *p*H 4 acetate buffer did not change the ratio E_0/E_A; the only change observed was a slight decrease in enzyme concentration, owing to the dilution of the dialyzed sample. Thus the hypothesis that the acylating agent may be a small peptide rather than a second enzyme molecule appears to be improbable, although this possibility cannot be excluded. (2) Ionic strength changes do not affect the position of the equilibrium of dimerization: changing the ionic strength from 0.05 to 0.4 does not produce any detectable change in E_0/E_A. (3) The influence of ionic strength on k_3 and k_{-d} is appreciable. For example, at *p*H 2.85, $k_3 = 1.33 \times 10^{-2}$ sec^{-1} at $\mu = 0.05$, and 5.29×10^{-3} sec^{-1} at $\mu = 0.70$; likewise, $k_{-d} = 0.98 \times 10^{-2}$ sec^{-1} at $\mu = 0.05$, and 3.83×10^{-3} sec^{-1} at $\mu = 0.70$. Interestingly, the effect of ionic strength on k_3 and k_{-d} is identical, as can be seen by the ratios of k_3/k_{-d} at the two ionic strengths, 1.36 and 1.38, respectively, confirming the similarity of k_{-d} to a known deacylation reaction. (4) The titration of a highly dimerized β-chymotrypsin solution (2.9×10^{-4} M) at *p*H 4 by *N-trans*-cinnamoylimidazole proceeds approximately twice as slowly as the titration of a purely monomeric solution (defined as the same solution maintained at *p*H 7.5), although the amount of acyl-enzyme formed in the two experiments is identical. (5) Inhibitors such as *N*-acetyl-L-tryptophan decrease the amount of D_0, thus showing that the active site is involved in the dimerization. (6) Dimerization prevents denaturation by 15% acetonitrile at *p*H 2.7, similar to the protection against denaturation afforded by simple acyl-enzyme formation. In this instance, denaturation was measured by the decrease in absorbance at 290 mμ (Chervenka, 1962). (7) Dimerization is accompanied by apparent spectral changes at 290 and 293 mμ, but the effect is complicated by the increased light scattering of the solution, which leads to an apparent increase in absorbance. (8) Finally, the dimerization is a completely reversible phenomenon, and a true equilibrium: the same amount of inactive enzyme is obtained in a given experimental condition when the equilibrium is approached from either side (by dilution or changing the *p*H).

Monomer-Dimer Equilibrium of α-Chymotrypsin. Table V and Figure 7 show the results of one series of experiments on the dimerization of α-chymotrypsin at *p*H 4.02. Figure 7, showing a plot of equation (6) (or 9), demonstrates a fairly good straight line, with an intercept significantly different from 2, indicating again that an acyl-enzyme must be postulated in addition to an enzyme-enzyme complex. If K_i and K_i' are similar to those obtained for β-chymotrypsin (*vide supra*), we may safely assume that $H \gg K_i$ and K_i'. Then according to equation (6), the slope and intercept allow the calculations, $K_d = 0.26$ and $K_s^E = 2.2 \times 10^{-3}$ M. These values are very similar to the equilibrium constant and the K_s^{AH}, respectively, of *N*-acetyl-L-tryptophan (Kézdy and Bender, 1964).

TABLE V: Monomer-Dimer Equilibrium of α-Chymotrypsin.[a]

$E_A \times 10^6$ M	$D_0 \times 10^6$ M	E_A/D_0
2816	770	3.66
2745	812	3.38
686.3	160	4.30
686.3	166	4.13
274.5	40.1	6.85
91.5	9.04	10.10

[a] *p*H 4.02; 0.2 M acetate.

Instead of measuring the amount of chemically inactive enzyme, D, one may use light-scattering measurements (Steiner, 1954). The amount of dimer from light-scattering measurements will, however, be different

from the above measurements since light-scattering measurements determine enzyme-enzyme complexes as well as acyl-enzyme dimers, whereas the present chemical measurements determine only the latter. However, it is possible to use the present data to determine an apparent equilibrium constant of dimerization that will simulate the light-scattering (and other physicochemical) measurements. This value for α-chymotrypsin is 4.6×10^{-4} M at *p*H 4.02. Interpolation of Steiner's (1954) light-scattering results at this *p*H yields 2.5×10^{-4} M, in good agreement. These experimental results seem, however, to contradict the dimerization constants obtained by sedimentation and gel filtration (Winzor and Scheraga, 1964) (1.05×10^{-5} M) obtained in approximately identical conditions. No detectable dimerization of α-chymotrypsin was observed in our chemical method at *p*H 2.6 and 7, in qualitative agreement with Egan *et al.* (1957).

Discussion

The evidence presented here strongly indicates that the inactive chymotrypsins at low *p*H are dimeric molecules, that the point of dimerization involves the active site, and finally that these active-site dimers are acyl-enzyme dimers.

The effect of the protein concentration on the active-inactive enzyme equilibrium indicates that a dimer is formed. Inactivation by some small degradation product is not very probable on the basis of the dialysis experiments discussed earlier. Large fragments are not possible, because the ratio (normality from titration)/(normality from weight) is too high to allow such an explanation. Therefore a dimeric enzyme molecule must account for the inactive enzyme.

Several indications point to the fact that the dimer is an active site–to–active site interaction. The most powerful evidence is that both enzyme molecules of the dimer are inactive toward specific substrates. Another is that dimerization may be inhibited by specific compounds such as *N*-acetyl-L-tryptophan. A third is that the X-ray diffraction analysis of α-chymotrypsin crystals indicates two molecules of enzyme occupying one unit cell in a configuration in which one of the enzyme molecules is turned 180° with respect to the other (Blow *et al.*, 1964). A final argument is that such an asymmetric active site–to–active site arrangement could lead to a reasonably nonrepulsive form of the dimer at low *p*H values if the multiple positive charges of the enzyme were situated principally on the side of the enzyme away from the active sites.

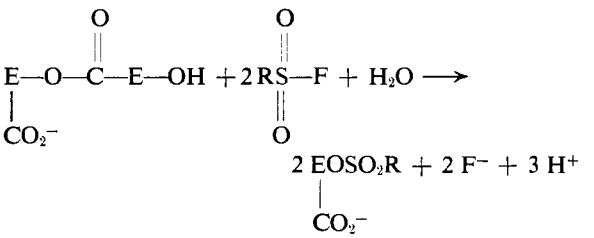
The adherence of the monomer-dimer equilibrium to equation (6) and not to equation (8) indicates that the initial (rapid) absorption process is followed by a (slower) second step in order to form the dimer. This sequence of events can be explained either in terms of a secondary time-dependent acyl-enzyme dimer formation or in a secondary time-dependent conformational change. Rate studies with polyamino acids and proteins indicate that conformational changes are much more rapid than the process observed here (Eigen and Hammes, 1963). Thus the simplest explanation of the phenomenon observed here is the formation of an acyl-enzyme dimer. The effect of *p*H on the monomer-dimer equilibrium shows a maximum amount of dimer at *p*H 4 and minimal amounts of dimer at *p*H 2 and 7. While it is conceivable that such a *p*H dependence could reflect a conformational change, the simplest explanation is that this *p*H dependence is a product of electrostatic repulsion between two positively charged enzyme molecules preventing acyl-enzyme formation (which increases with decreasing *p*H) and of the protonation of a carboxylic acid group necessary for acyl-enzyme formation (which also increases with decreasing *p*H). A number of kinetic results are difficult to explain on the grounds of a conformational change, but are explained immediately on the basis that the active site catalyzes the formation of an acyl-enzyme dimer: (1) the effect of *p*H on k_{-d} parallels the effect of *p*H on a typical deacylation process, k_3; (2) the effect of ionic strength on k_{-d} parallels its effect on a typical deacylation process, k_3; (3) the dimer protects the enzyme against denaturation by acetonitrile in exactly the same way that the acyl-enzyme protects the enzyme against this denaturation; and (4) chymotrypsinogen does not exhibit such a dimerization. These equilibrium, kinetic, and *p*H arguments provide a strong case to postulate that the dimer is an acyl-enzyme.

The fact that methanol increases the rate constant of the deacylation process, k_3, but does not affect k_{-d} is the only piece of evidence which is in apparent contrast with the acyl-enzyme hypothesis. A possible explanation of this anomaly is that methanol cannot come into contact with the enzyme-enzyme dimer, or that the enzyme requires a free acidic group for its activity. This latter explanation seems more probable, if one considers that in the acyl-enzyme dimer *both* the enzyme molecules lose their activity toward specific substrates.

It is possible only to make a tentative identification of the carboxylic acid group involved in the acylation of one enzyme molecule by another. Chymotrypsinogen and δ-chymotrypsin show little tendency to dimerize (δ-chymotrypsin at high enzyme concentration can be shown to undergo dimerization to a small extent) while α- and β-chymotrypsin (as well as γ-chymotrypsin) have a high tendency to acylate and dimerize. The only known difference between these two classes of enzymes is that in α- and β-chymotrypsin the carboxylic acid group of tyrosine 146 (Hartley, 1964) is liberated (by chymotrypsin). Thus, the tyrosine 146 carboxylic acid group has certainly transiently formed an acyl-enzyme with another enzyme molecule, so that reacylation of the enzyme by this carboxylic acid group is at least possible. However, the absolute magnitude of the rate constant k_{-d} indicates a kinetic specificity somewhat poorer than that of *N*-acetyl-L-tryptophanyl-chymotrypsin, whereas it would be expected that that of *N*-acetyl-L-tyrosyl-chymotrypsin would be somewhat greater. On the basis of the magnitude of k_{-d}, the carboxylic acid group might be identified as that of the C terminus of the A chain, leucine

13, which is present in all chymotrypsins. It is hoped in the future to distinguish between these two possible carboxylic acid groups as participants in acyl-enzyme dimer formation.

Several phenomena recorded in the literature may be related to the dimerization described here. (1) The deviation of the hydrolysis rate constant of the methyl cinnamate from the theoretical curve at high enzyme concentration (Bender and Zerner, 1962) may be qualitatively explained by inhibition of the reaction by enzyme: the results indicate a K_I^E of $1\text{–}2 \times 10^{-3}$ M. (2) At low *p*H values the rate of acylation of α-chymotrypsin by *p*-nitrophenyl acetate is accelerated by increasing enzyme concentration (Kézdy and Bender, 1962). In this reaction, the enzyme may behave similarly to indole (Foster, 1961) in the deacylation of acetyl-α-chymotrypsin. (3) The maximum stability of α-chymotrypsin of *p*H 4–6 toward denaturing agents such as urea, heat, and protons (Chervenka, 1960, 1962; Aldrich and Balls, 1958) may be qualitatively explained by the increased stability of the dimer toward denaturing agents. (4) The fluorescence changes observed by the addition of substrate to α-chymotrypsin (Sturtevant, 1962) may possibly be related to a displacement of the dimerization equilibrium of the enzyme. (5) Acylations of α-chymotrypsin at low *p*H (5–6) have invariably produced more protons/mole than acylations at intermediate *p*H (7–8) (Bernhard, 1962; Gutfreund and Sturtevant, 1956a,b; Erlanger *et al.*, 1963; Fahrney and Gold, 1963). A particularly clear-cut example is seen in the acylation of α-chymotrypsin by phenylmethanesulfonyl fluoride, which produces 1.5 protons/mole at *p*H 5 and 1 proton/mole at *p*H 7 (Fahrney and Gold, 1963). These observations may be explained on the basis that the enzyme being acylated at *p*H 7 is the monomer (which liberates 1 proton/mole) but the enzyme acylated at *p*H 5 is the dimer (which liberates 1.5 protons/mole).



(6) Since chymotrypsinogen is able to adsorb compounds related to specific substrates (Vaslow and Doherty, 1953), it certainly must be able to form protein-protein complexes, but of course no acyl-enzyme formation is possible. (7) The time-dependent change in the deacylation of acetyl-α-chymotrypsin at *p*H 7 (Marini and Hess, 1960) is a slower phenomenon than that observed here. (8) The sedimentation constants of an α-chymotrypsin solution at *p*H 7.7 are much higher than that expected for a dimer (Tinoco, 1957). The polymeric aggregates appear to be completely active, in contrast to the results found here. (9) The displacement of the monomer-dimer equilibrium could suffice to explain the many times postulated "induced fit" phenomenon, at least in the case of chymotrypsin (Koshland *et al.*, 1962).

An enzyme-substrate complex has been suggested as the mode of dimerization to explain the increased sedimentation of trypsin in the presence of its degradation products (Desnuelle, 1960). The hypothesis that the dimerization of chymotrypsin at low *p*H values involves the formation of an enzyme-enzyme complex, followed by the formation of an acyl-enzyme dimer, appears to be able to correlate most of the pertinent phenomena for this enzyme. This hypothesis provides the ultimate example of a natural acyl-enzyme consisting of two protein components.

References

Aldrich, F. L., Jr., and Balls, A. K. (1958), *J. Biol. Chem. 233*, 1355.

Bender, M. L., Clement, G. E., Gunter, C. R., and Kézdy, F. J. (1964a), *J. Am. Chem. Soc. 86*, 3697.

Bender, M. L., Kézdy, F. J., and Gunter, C. R. (1964b), *J. Am. Chem. Soc. 86*, 3714

Bender, M. L., and Zerner, B. (1962), *J. Am. Chem. Soc. 84*, 2550.

Bernhard, S. A. (1962), *Brookhaven Symp. Biol. 15* (*BNL738* (*C-34*)), 129.

Bethune, J. L., and Kegeles, G. (1961), *J. Phys. Chem. 65*, 1761.

Blow, D. M., Rossmann, M. G., and Jeffery, B. A. (1964), *J. Mol. Biol. 8*, 65.

Chervenka, C. H. (1960), *J. Am. Chem. Soc. 82*, 582.

Chervenka, C. H. (1962), *J. Biol. Chem. 237*, 2105.

Desnuelle, P. (1960), *Enzymes 4*, 125.

Egan, R., Michel, H. O., Schlueter, R., and Jandorf, B. J. (1957), *Arch. Biochem. Biophys. 66*, 366.

Eigen, M., and Hammes, G. G. (1963), *Advan. Enzymol. 25*, 1.

Erlanger, B. F., Castleman, H., and Cooper, A. G. (1963), *J. Am. Chem. Soc. 85*, 1872.

Fahrney, D. E., and Gold, A. M. (1963), *J. Am. Chem. Soc. 85*, 349.

Foster, R. J. (1961), *J. Biol. Chem. 236*, 2461.

Frenkel, S. Y. (1952), *Biokhimiya 17*, 535; (1953), *Chem. Abstr. 47*, 3660.

Gutfreund, H., and Sturtevant, J. M. (1956a), *Biochem. J. 63*, 656.

Gutfreund, H., and Sturtevant, J. M. (1956b), *Proc. Natl. Acad. Sci. U.S. 42*, 719.

Hartley, B. S. (1964), *Nature 201*, 1284.

Kézdy, F. J., and Bender, M. L. (1962), *Biochemistry 1*, 1097.

Kézdy, F. J., and Bender, M. L. (1964), *J. Am. Chem. Soc. 86*, 938.

Kézdy, F. J., Clement, G. E., and Bender, M. L. (1964), *J. Am. Chem. Soc. 86*, 3690.

Koshland, D. E., Jr., Yankeelov, J. A., and Thoma, J. A. (1962), *Federation Proc. 21*, 1031.

Marini, M. A., and Hess, G. P. (1960), *J. Am. Chem. Soc. 82*, 5160.

Martin, R. B., and Niemann, C. (1958), *J. Am. Chem. Soc. 80*, 1473.

Massey, V., Harrington, W. F., and Hartley, B. S. (1955), *Discussions Faraday Soc. 20*, 24.
Rao, M. S., and Kegeles, G. (1958), *J. Am. Chem. Soc. 80*, 5724.
Schonbaum, G. R., Zerner, B., and Bender, M. L. (1961), *J. Biol. Chem. 236*, 2930.
Schwert, G. W. (1949), *J. Biol. Chem. 179*, 655.
Schwert, G. W. (1951), *J. Biol. Chem. 190*, 799.
Schwert, G. W., and Kaufman, S. (1949), *J. Biol. Chem. 180*, 517.
Schwert, G. W., and Kaufman, S. (1951), *J. Biol. Chem. 190*, 807.
Smith, E. L., and Brown, D. M. (1952), *J. Biol. Chem. 195*, 525.
Steiner, R. F. (1954), *Arch. Biochem. Biophys. 53*, 457.
Sturtevant, J. M. (1962), *Biochem. Biophys. Res. Commun. 8*, 321.
Tinoco, I., Jr. (1957), *Arch. Biochem. Biophys. 68*, 367.
Vaslow, F., and Doherty, D. G. (1953), *J. Am. Chem. Soc. 75*, 928.
Winzor, D. J., and Scheraga, H. A. (1964), *J. Phys. Chem. 68*, 338.

The Activation of Stuart Factor (Factor X) by Activated Antihemophilic Factor (Activated Factor VIII)*

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ABSTRACT: The reaction of activated antihemophilic factor with Stuart factor has been investigated. It was found that activated antihemophilic factor is an enzyme which reacts with Stuart factor converting the latter to an activated product which accelerates clotting. The reaction occurs only in the presence of calcium ions, the optimal concentration being 5 mM. The *p*H optimum was found to be about 7.5. The activation of Stuart factor was not affected by 1×10^{-4} M *p*-mercuribenzoate or 1.0 unit of heparin per ml. Esterase activity toward benzoylarginyl ethyl ester or tosylarginymethyl ester was not detected for either activated antil hemophilic factor or activated Stuart factor. In addition, neither enzyme was inhibited by preincubation with 5×10^{-3} M diisopropylphosphorofluoridate. Soybean trypsin inhibitor was found to be a potent inhibitor of activated Stuart factor. Phospholipid and calcium ions were required for coagulation at some point(s) following the activation of Stuart factor in the intrinsic system.

Stuart factor[1] (factor X) is a plasma protein which is required for normal blood coagulation (Telfer *et al.*, 1956; Hougie *et al.*, 1957). In the intrinsic clotting system, it participates during the middle phase of blood coagulation following the interaction of Hageman factor (factor XII), plasma thromboplastin antecedent (factor XI), and Christmas factor (factor IX) (see Davie and Ratnoff, 1964, and Macfarlane, 1964, for recent reviews). It has been shown that Stuart factor is converted to an activated form (product I) in the presence of activated Christmas factor, antihemophilic factor (factor VIII), and calcium ions (Bergsagel and Hougie, 1956; Hougie *et al.*, 1957; Spaet and Cintron, 1963). Lundblad and Davie (1964) have shown that the initial reaction involving the last three clotting factors is the activation of antihemophilic factor by activated Christmas factor. This reaction requires the presence of phospholipid and calcium ions. The present communication characterizes the second reaction in which activated antihemophilic factor in turn converts Stuart factor to an activated form in a calcium-dependent reaction.

Materials and Methods

Standard grade *ecteola cellulose* with a capacity of 0.3 meq of base/g was purchased from Carl Schleicher and Schuell Co., Keene, N.H. *G-25 Sephadex* (coarse grade) was purchased from Pharmacia Laboratories, Inc., Piscataway, New Market, N.J. Crystalline *soybean trypsin inhibitor* (STI)[2] was purchased from

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† Predoctoral trainee of the National Institutes of Health.

[1] Stuart factor was named after one of the earliest patients in which a deficiency of this clotting factor was observed (Hougie *et al.*, 1957).

[2] Abbreviations used in this work: STI, soybean trypsin inhibitor; DFP, diisopropylphosphorofluoridate; TAME, *p*-toluenesulfonyl-L-arginine methyl ester; BAEE, benzoyl-L-arginine ethyl ester; AHF, antihemophilic factor; PTA, plasma thromboplastin antecedent.