The Control of Prothrombin Conversion. Kinetic Control by Mechanisms Inherent in Two Activation Pathways†

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ABSTRACT: It is known that the activation of prothrombin to thrombin can proceed via two pathways: one initiated by the prothrombin-converting complex (factor X_a, factor V, phospholipid, and Ca^{2+} ions) and the other initiated by the product, thrombin. A kinetic study has shown that the pathways do not proceed with equal ease under all conditions. At high levels of the converting complex, both go to completion: some prothrombin is always cleaved by thrombin, but the resulting intermediate is then activated to give quantitative conversion to thrombin. At slower rates of activation, the product-initiated pathway occurs to a relatively greater extent. Moreover, the intermediate then is not cleaved further but accumulates, so that the generation of thrombin is curtailed. The reason the intermediate is productive only at higher levels of activator may be partly that it is a poorer substrate for the converting complex than prothrombin. More importantly, the activity of the complex is also modulated by thrombin, which rapidly destroys the activity of factor V and factor X_a in a feedback reaction. These concerted controls ensure that prothrombin activation damps itself. Thus thrombin production occurs as a burst, the size of which is regulated by the amounts of factor X_a and factor V initially available.

Blood coagulation is initiated by two pathways, the intrinsic and extrinsic systems, which converge at a reaction in which factor X, a plasma zymogen, is proteolytically converted to a serine protease, factor X_a. Factor X_a catalyzes the conversion of prothrombin to thrombin (Milstone, 1964). Recent experiments in many laboratories have shown that the activation of purified prothrombin involves several steps (Mann et al., 1971; Stenn and Blout, 1972; Owen et al., 1974; Esmon et al., 1974a; Kisiel and Hanahan, 1974). In effect, thrombin is generated by two routes (Figure 1). The more direct route is catalyzed throughout by factor X_a and involves two cleavages (reactions 1 and 2, Figure 1). First, almost half of the prothrombin zymogen is removed from its NH_2-terminus to give a proteolytically inactive, single-chain species (Mann et al., 1971) of molecular weight 39,000, P_3 (Stenn and Blout, 1972; Owen et al., 1974).[1] Cleavage of P_3 then occurs between disulfide bonds, resulting in the formation of active, two-chain thrombin (Heldebrant and Mann, 1973). The alternative pathway (reaction 3 in Figure 1) is initiated by thrombin itself (Stenn and Blout, 1972; Esmon et al., 1974a). Thrombin catalyzes an extra step, the removal of an NH_2-terminal fragment, F_1, from prothrombin to give P_2. Factor X_a then cleaves P_2 to P_3 by removing F_2, the remaining part of the NH_2-terminal peptide, F_{1-2}. P_3 is then cleaved to thrombin (Heldebrant et al., 1973).

Thus thrombin and factor X_a catalyze different proteolytic cleavages of prothrombin, but the alternative products, P_2 and P_3, can both be precursors of thrombin. However, thrombin does not cleave P_2 further to any appreciable extent (Stenn and Blout, 1972). This means that, whichever pathway is followed, the continuing action of factor X_a is essential for the complete conversion of prothrombin to the active protease.

It is well established that the initial rate of thrombin appearance is increased by phospholipid and Ca^{2+} ions (Milstone, 1964) and further increased by a plasma protein, factor V. Factor V is not proteolytic (Barton et al., 1967); we therefore refer to it as a "cofactor" inasmuch as it accelerates the formation of thrombin by factor X_a in the presence of lipid and Ca^{2+} ions (Jobin and Esnouf, 1967). These accelerators in combination with the enzyme factor X_a will here be called the converting complex. However, it must be pointed out that the nature of the interactions between the components of the complex is not completely understood. Therefore the use of this term implies only that all four components are present; no assumptions about the stoichiometry of their interactions can yet be made.

We report here the results of a study of the kinetics of prothrombin activation. When activation is carried out by the complete complex, both factor X_a and thrombin cleave prothrombin to a greater or lesser extent under all the conditions we have examined. In other words, factor X_a and thrombin are always in competition for their common substrate, prothrombin. As a result, the initial activity of the converting complex determines which pathway in Figure 1 predominates. When the activity is lower (because the initial level of factor X_a or factor V is reduced), relatively more P_2 is generated. In addition, at these slower rates of activation P_2 is then converted to thrombin only poorly. Thus as P_2 accumulates, the final yield of thrombin is reduced concomitantly. We show that the persistence of P_2 is mediated by thrombin itself, which regulates the activity of the converting complex. In agreement with others (Rapaport et al., 1963; Colman, 1969b), we find that thrombin potentiates and then destroys the activity of factor V in a

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‡ Part of this work was performed during the tenure of a Postdoctoral Fellowship of the Connecticut Heart Association.

[1] Abbreviations used were: Dip-F, diisopropyl phosphorofluoridate; P_2 and P_3 (Stenn and Blout, 1972), the derivatives of prothrombin formed during activation, which we show are not necessarily intermediates in thrombin formation.

feedback reaction. Although this is the main means of control in our system, we also observe some loss of factor X_a activity. The combined effects of these controls explain the unusual dependence, observed by many investigators, of the final yield of thrombin on the initial level of factor X_a or factor V.

Experimental Section

Materials

Sodium dodecyl sulfate, "Trizma" base, Coomassie Brilliant Blue R, dithiothreitol, 2-mercaptoethanol, diisopropyl phosphorofluoridate, bovine fibrinogen (Cohn fraction I), hen ovalbumin, Taipan snake venom, Tiger snake venom, Russell's viper venom, and bovine plasma deficient in factors II, VII, and X were purchased from Sigma. Benzamidine hydrochloride was obtained from Aldrich. Bovine serum albumin, rabbit muscle phosphorylase A, horse myoglobin, and porcine α-chymotrypsinogen were products of Schwarz/Mann. Acrylamide and *N,N'*-methylenebisacrylamide (Eastman) were recrystallized from chloroform and acetone, respectively. DEAE-Sephadex was purchased from Pharmacia. Other chemicals were reagent-grade products of Baker, Fisher or Mallinckrodt.

Methods

Isolation of Blood Coagulation Factors. PROTHROMBIN AND FACTOR X. A barium citrate eluate was prepared from bovine plasma at the New England Enzyme Center (Tufts University, Boston, Mass.) by the method of Aronson and Menache (1966). The prothrombin and factor X were separated by chromatography on DEAE-Sephadex (Esnouf et al., 1973) in the presence of 25 m*M* benzamidine hydrochloride (Radcliffe and Nemerson, 1975). The leading edge of the prothrombin peak (containing no factor VII or factor IX activity) was pooled, concentrated (Amicon, PM 10 membrane), and dialyzed against 50% glycerol, 0.1 *M* NaCl, and 0.05 *M* Tris-Cl (pH 7.5) containing 2 m*M* benzamidine hydrochloride. It was stored at −20° at a protein concentration of at least 20 mg/ml. The prothrombin concentration was determined from absorbance using a $A_{280}(1\%)$ value of 13.8 (Esnouf et al., 1973). The concentration of factor X_a determined by assay (Esnouf and Jobin, 1967) was less than 0.1 ng/ml in a prothrombin solution of 0.5 mg/ml. The factor X pool was further purified by rechromatography on DEAE-Sephadex using the method of Esnouf et al. (1973). Factor X_a was made using the coagulant protein purified from Russell's viper venom (Jesty et al., 1974), and then chromatographically purified (Jesty and Esnouf, 1973). Factor X_a was stored at a concentration of at least 2 mg/ml in 50% glycerol, 50 m*M* NaCl, and 25 m*M* Tris-Cl (pH 7.5). Its concentration was determined from absorbance at 280 nm, using a value for $A_{280}(1\%)$ of 9.4 (Jesty and Esnouf, 1973).

Factor V was prepared from barium sulfate adsorbed bovine plasma (New England Enzyme Center) by the method of Dombrose et al. (1972), modified by including 0.1 m*M* dithiothreitol and 5 m*M* benzamidine hydrochloride in all the buffers. This gave a product 1300-fold purified, the activity of which could be enhanced tenfold by crude Russell's viper venom (Esmon et al., 1973) and by thrombin (see below). However, in the present experiments the preparation was used without any such initial treatment. The protein was stored at a concentration of 20 absorbance units/ml in 50% glycerol, 10 m*M* $CaCl_2$, 0.1 *M* NaCl, 0.05 *M* Tris-Cl, and 2 m*M* benzamidine hydrochloride (pH 7.5). The preparation was not electrophoretically pure, but gave several bands of molecular weight greater than 100,000 after reduction when electrophoresed in the presence of sodium dodecyl sulfate. However, it contained no prothrombin nor factor X activity and did not cleave prothrombin over a 4-hr period. The activity of the preparation and its ability to be activated further remained constant for some months.

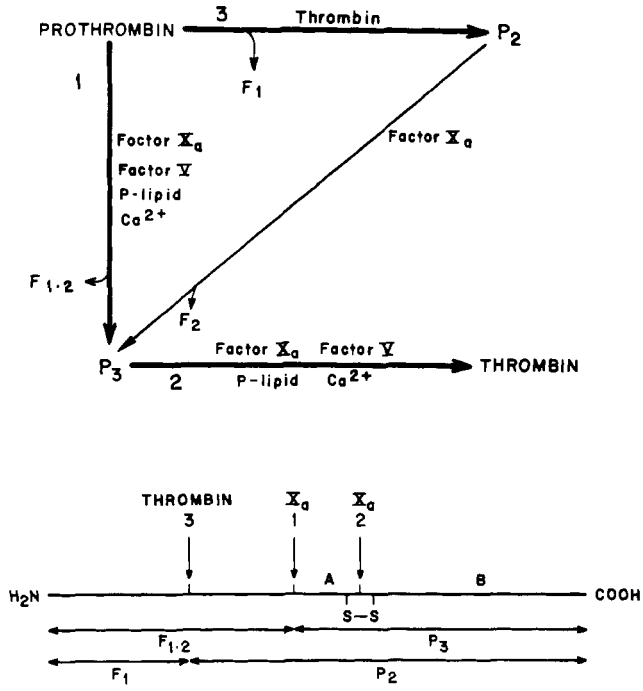

FIGURE 1: The two pathways of prothrombin activation. The findings of Stenn and Blout (1972) and Jackson and coworkers (Owen et al., 1974; Esmon et al., 1974a) are summarized. Reactions 1 and 2 comprise the direct pathway. Reaction 3 is the first step of the alternate route. The corresponding cleavage sites are shown in the lower diagram. The mechanism of activation of P_2, in particular the roles of lipid and factor V, is unclear. These details are therefore omitted.

Thrombin for most of these experiments was a preparation made by the action of Taipan venom coagulant protein (Lanchantin et al., 1973), and purified as described by Radcliffe and Nemerson (1975). The specific activity was 1400 NIH units/mg. In the experiment on the effect of thrombin on factor V, the thrombin used was a highly-purified preparation from human sources, kindly provided by Dr. John Fenton. The specific activity of this material was 2500 NIH units/mg.

P_2 and the corresponding NH_2-terminal peptide of prothrombin, F_1, were made by incubating 200 mg of prothrombin at a concentration of 4 mg/ml in Tris-saline with 10 NIH units/ml of bovine thrombin for 4 hr at 37°. The reaction was stopped by adding 50 μl of 1 *M* Dip-F in anhydrous 2-propanol and stirring at room temperature for 30 min. The mixture was pumped onto a column of DEAE-Sephadex (3 × 30 cm) in Tris-saline at a flow rate of 50 ml/hr. The column was developed with a linear gradient 0.1–0.45 *M* NaCl in 0.05 *M* Tris-Cl (pH 7.5) (2000 ml). The eluted proteins were identified by gel electrophoresis in sodium dodecyl sulfate.

Coagulation Assays. All coagulation assays were done at 37°. Prothrombin was assayed using Tiger snake venom and bovine plasma deficient in factors II, VII, and X (Jobin and Esnouf, 1967). Factor V was assayed by the method of

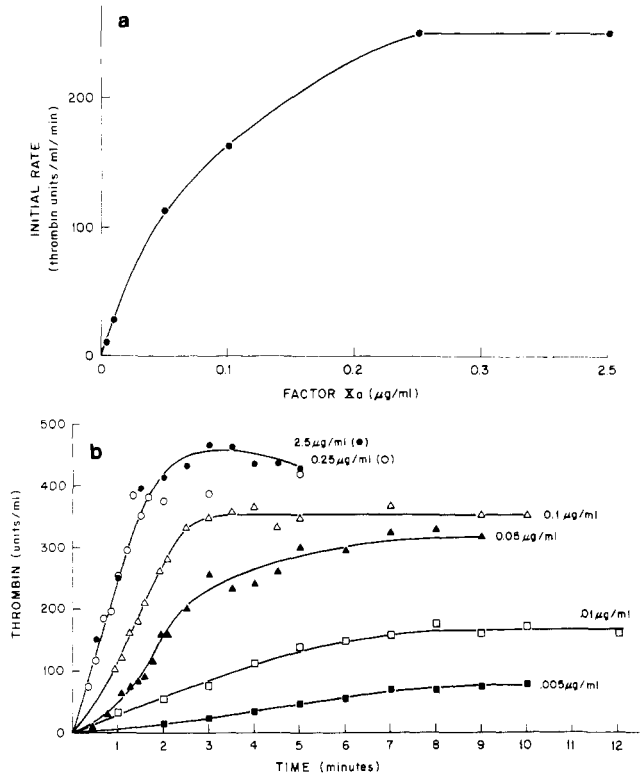

FIGURE 2: Thrombin formation during the activation of prothrombin at various initial concentrations of factor X_a. Prothrombin (0.5 mg/ml) in 0.1 *M* NaCl–0.05 *M* Tris-Cl containing 14 units of factor V, phospholipid, and 10 m*M* $CaCl_2$ was treated with the amount of factor X_a shown to the right of each curve (b). The final volume of each mixture, after addition of factor X_a, was 1.0 ml; 10-μl samples were diluted in 0.02% ovalbumin solution in Tris-saline and assayed for thrombin coagulant activity. (a) Initial rates from these data plotted as a function of initial factor X_a concentration. At a factor X_a concentration of 0.25 μg/ml (○) or more (●), factor V becomes rate limiting.

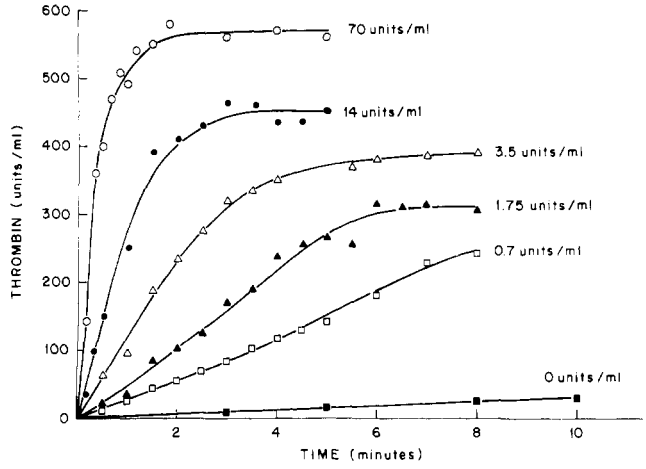

FIGURE 3: Thrombin formation during prothrombin activation at various initial concentrations of factor V. Prothrombin (0.5 mg/ml) was activated with 2.5 μg/ml of factor X_a. The factor V levels were as shown to the right of each curve. The final volume of each mixture, including factor X_a and factor V was 1.0 ml. Other details are in the legend to Figure 2.

Esnouf and Jobin (1967), using Russell's viper venom coagulant protein (Jesty et al., 1974) at a concentration of 2 μg/ml instead of brain thromboplastin. This fraction of the venom lacks the ability to activate factor V present in crude venom (Esmon et al., 1973). Factor V deficient substrate plasma was prepared by incubating human oxalated plasma at 37° for 3 days. Normal citrated bovine plasma is defined as containing 100 units of factor V activity/ml, and results were related to this standard. Thus an activation mixture containing, for example, 35 units/ml has 35% of the factor V activity of bovine plasma. Factor X was assayed using bovine plasma deficient in factors VII and X (Esnouf and Jobin, 1967). Thrombin was assayed by adding 0.2 ml of sample, appropriately diluted in Tris-saline containing 0.02% ovalbumin, to 0.2 ml of fibrinogen solution (10 mg/ml of bovine fraction I, 65% coagulable protein, in 0.1 *M* NaCl–0.05 *M* Tris-Cl (pH 7.5)). The assay was standardized with U.S. Standard Thrombin, Lot B3.

Prothrombin Activations. All activations were done at 37° in Tris-saline buffer (0.1 *M* NaCl–0.05 *M* Tris-Cl (pH 7.5)). The final concentration of $CaCl_2$ in all activation mixtures was 10 m*M*. The source of phospholipids was a crude mixed preparation ("cephalin") made by chloroform extraction of bovine brain (Bell and Alton, 1954). Its concentration was 500 μg of phosphorus/ml, and it was diluted 100-fold in activation mixtures. Factor V and factor X_a were diluted where necessary in 0.02% ovalbumin in Tris-saline. Activations were initiated by the addition of the factor X_a, after preincubation of the other components for 2 min at 37°.

It was particularly important to ensure that proteolysis was completely halted in samples taken during the course of activation. For measurements of thrombin activity, samples were diluted 100-fold or more in ovalbumin solution and kept on ice; 25-μl samples for electrophoresis were added to 25 μl of 10 *M* urea (deionized) and 4% sodium dodecyl sulfate in a boiling water bath and incubated for 2 min. No cleavage of prothrombin by thrombin in urea–sodium dodecyl sulfate could be detected.

Electrophoresis was performed in vertical polyacrylamide slabs 1.5 mm thick (Reid and Bieleski, 1968), containing 7.5% acrylamide, 0.3% bisacrylamide, 5 *M* urea, and 0.1% sodium dodecyl sulfate. The buffer system was that of Fairbanks et al. (1971). Up to 30 samples of 20 μl were electrophoresed per gel. Each sample contained approximately 5 μg of protein. Gels were stained in 0.1% Coomassie Blue in 25% 2-propanol and 10% acetic acid, and destained in 14% methanol and 7% acetic acid at 45°. The intermediates of activation were identified by their molecular weights (Mann et al., 1971). These were obtained by comparison with the electrophoretic mobilities of five reduced standard proteins. The standards were, with their molecular weights: phosphorylase A, 92,000 (Darnall and Klotz, 1972); bovine serum albumin, 68,000; ovalbumin, 43,000; α-chymotrypsinogen, 25,700; myoglobin, 17,200 (Weber et al., 1972).

All samples for electrophoresis were reduced by adding 5% 2-mercaptoethanol after denaturation. This enabled F_3 [the single-chain precursor of thrombin] and the B chain of active thrombin to be separated. However, in this gel system F_1 has an anomalously high molecular weight and runs with or slightly below the B chain. The appearance of thrombin and F_1 therefore could not be estimated reliably by gel scanning. The appearance and disappearance of other bands were estimated as follows. The stained polyacrylamide slab was dried onto filter paper and photographed with an MP 3 Land Camera (Polaroid) using P/N dual film. The negative was cut into strips 0.5 in. wide, and each sample channel (0.25 in. wide) was scanned in a Gilford spectrophotometer (Model 240) coupled with a potentio-

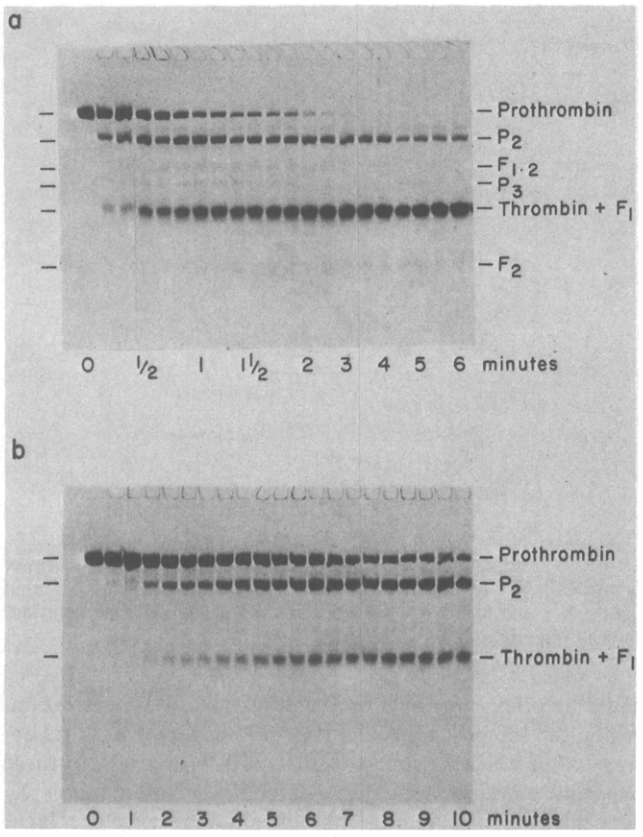

FIGURE 4: Prothrombin conversion at two concentrations of factor X_a: (a) 0.25 μg/ml of factor X_a; (b) 0.005 μg/ml of factor X_a. The factor V concentration was 14 units/ml. Reduced samples were electrophoresed as described under Methods.

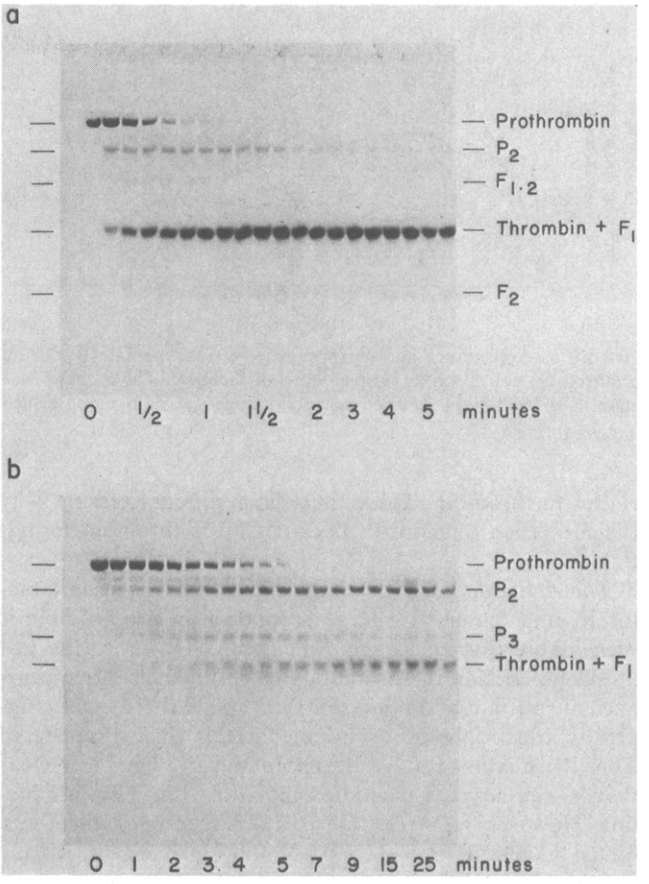

FIGURE 5: Prothrombin conversion at two concentrations of factor V: (a) 70 units/ml of factor V; (b) 0.7 unit/ml of factor V. The factor X_a concentration was 2.5 μg/ml. Other details are described in preceding legends.

metric recorder with the polarity reversed. The scans were quantified by triangulation and the proportion of each protein was expressed as a percentage of total absorbance (i.e., total stained protein) in that sample channel.

Results

In order to determine which components of the activating complex regulate thrombin formation, experiments were performed in which the concentrations of factor X_a and factor V were varied independently. In all these experiments, the prothrombin concentration was 0.5 mg/ml. In the first series, the factor V was at a nonlimiting level (14 units/ml), while the initial factor X_a concentration was varied stepwise from 0.005 to 2.5 μg/ml. The results are shown in Figure 2. In Figure 2a, it is seen that the initial rate of appearance of thrombin is a complex, nonlinear function of the factor X_a concentration. The time course of thrombin formation at several X_a concentrations is shown in full in Figure 2b. This shows the unusual feature on which this work is based, that the *yield* of thrombin, as well as its rate of appearance, depends on the factor X_a concentration.

Figure 3 shows the results obtained when the factor X_a was fixed at a high level (2.5 μg/ml) and the factor V level was varied. Here we see a similar dependence of yield as well as rate of activation on the initial concentration of factor V.

The finding that the final yield of thrombin was dependent on the enzyme and factor V concentrations was unexpected. As prothrombin is activated via two pathways, we investigated the possibility that the yield might depend in some way on which pathway predominates. Samples were therefore withdrawn at intervals from each activation mixture and the products examined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Figures 4 and 5). At a high level of converting complex (70 units of factor V and 2.5 μg of factor X_a per ml), activation was complete, corresponding to a yield of 550 NIH units of thrombin/ml (Figure 5a). [At these highest concentrations, factor X_a could not be detected on the gels, while factor V appears as a single faint band close to the origin.] At lower levels of factor X_a or factor V, the amounts of thrombin formed reached a steady state while P_2 (Figure 4a) and even prothrombin (Figures 4b and 5b) were still present. Subsequently, any remaining prothrombin disappeared to give P_2. At very low levels of factor V (Figure 5b), P_3 also accumulated to a certain extent. Thus at levels of factor X_a or factor V at which the thrombin yield is less than maximal, the intermediates of prothrombin activation accumulate at the expense of thrombin.

When the activating enzyme and the product compete for the same substrate, it is inevitable that the reaction catalyzed by product is favored at lower concentrations of activating enzyme. Hence in slower activations we consistently saw the formation of more P_2 than in more rapid reactions. In addition, we noted that whenever the yield of thrombin was less than quantitative, P_2 reached a steady state and its cleavage products did not appear over several minutes. The formation of P_2 and its failure to be removed can be seen in Figure 6. This shows data obtained by scanning gels of activations at four concentrations of factor V. The persistence of P_2 cannot stem solely from the fact that there are two

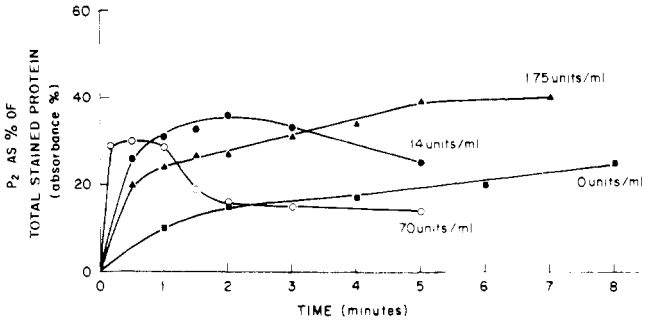

FIGURE 6: Appearance and disappearance of intermediate P_2 during prothrombin activation at various levels of factor V. The P_2 band was quantified from stained, dried polyacrylamide gel slabs as described under Methods.

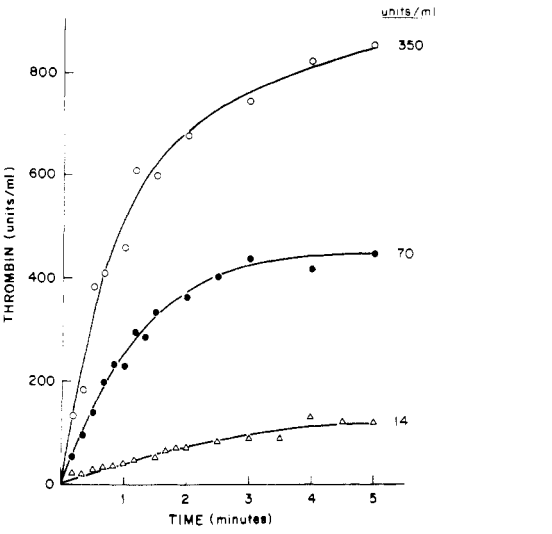

FIGURE 7: Activation of purified P_2 at various initial concentrations of factor V. P_2 (1.2 absorbance units/ml) was activated with 2.5 μg/ml of factor X_a, phospholipid, and Ca^{2+} at the factor V concentrations shown to the right of each curve.

routes to thrombin. There must be additional reasons why the formation of more P_2 does not favor thrombin formation.

The Activation of P_2. At high concentrations of both factor X_a and factor V, a large proportion of prothrombin is presumably cleaved by the direct route. However, the fast initial production of thrombin also resulted in the appearance of substantial amounts of P_2 at the earliest times (Figure 6). Electrophoresis showed that this then disappeared from the mixture to give thrombin and F_2. Thus it is clear that P_2 *can* act as a true intermediate in thrombin production. However, we stress that this was seen only when both factor X_a and factor V were present at high concentration.

It was nevertheless noticeable that P_2 was not consumed as rapidly as prothrombin, even in complete activations. One explanation for this would be that P_2 is less effective as a substrate for the converting complex than prothrombin. The activation of purified P_2 was therefore investigated at various levels of factor X_a and factor V. Activation at different levels of factor V is shown in Figure 7. At a high level of factor X_a, much more factor V was necessary to obtain active thrombin from P_2 than was required for prothrombin activation: indeed, it proved impossible to activate P_2 completely. Electrophoresis showed that this was because some P_2 always remained, the extent of its cleavage being a function of the initial concentration of factor V.

It thus appeared that the activation of P_2 might also be self-damping (as was suggested by Baker and Seegers, 1967). However, these results should be interpreted with caution. For example, it was noted that higher amounts of factor V were required for P_2 activation than were present during quantitative activation of prothrombin (Figure 3). Also, large amounts of P_3 were seen, which does not fit with the fact that P_3 is a transient intermediate in prothrombin activation unless the factor V concentration is very *low*. Thus the activation of purified P_2 may not be a suitable model for the fate of P_2 in the rapid activation of prothrombin. To overcome this criticism, we tried to simulate the activation of P_2 as it would occur during prothrombin activation by adding back F_1, the corresponding peptide removed by thrombin. It was found that this enhanced the rate of thrombin appearance and the yield was increased though not to the maximum level. This result cannot be due to an increase in the phospholipid concentration, since the F_1 was prepared in the absence of lipids. Hence at this time it is uncertain whether P_2 is less readily cleaved by the converting complex or not.

The Effects of Feedback Controls on the Converting Complex. Whether or not P_2 is per se a poor substrate there could be other restraints on its activation. We therefore investigated the possibility that factor V or factor X_a loses activity after an initial burst of thrombin formation. In these experiments, slow activation was initiated at a factor X_a concentration of 0.005 μg/ml, but in the presence of a large excess of factor V (14 units/ml). At a 50-fold greater concentration of factor X_a, this level of factor V would enable almost quantitative activation to occur (Figure 4a). However, the low factor X_a level restricted the yield of thrombin to 15–20% (Figure 8). Electrophoresis showed that the remaining prothrombin continued to be cleaved to P_2, which after 30 min was the main product. This mixture was then used as a source of the products of slow activation, and various additions to it were made.

When the concentration of factor X_a was increased to 0.25 μg/ml and more factor V (14 units/ml) was added at the same time, renewed thrombin formation was seen (Figure 8, curve a). This continued for about 10 min before stopping at a level still below the maximum. By adding further factor X_a (2.5 μg/ml) and still more factor V, the yield of thrombin was raised to the maximum (Figure 8, curve b). Electrophoresis confirmed that at this point, all the P_2 had been converted to thrombin. Thus P_2 in a mixture is capable of being rapidly activated, providing the levels of factor X_a and factor V are both large. However, if factor X_a (2.5 μg/ml) was added *without* fresh factor V, almost no thrombin appeared (Figure 8, curve d). From this we conclude that the factor V originally present had lost its activity, as a result of proteolysis by either factor X_a or thrombin.

It seemed improbable that one component of the converting complex would attack the other proteolytically. We confirmed that the slow activation described above occurred normally if the factor V and factor X_a were preincubated together for 20 min before the addition of the prothrombin. The more likely modulator of factor V activity was thrombin.

The Effect of Thrombin on the Activity of Factor V. The observation that preparations of factor V lose activity when exposed to thrombin was reported by Rapaport and coworkers (1963) and by Colman (1969a,b). These reports also showed that the activity of factor V preparations is enhanced before being lost. We performed an experiment to

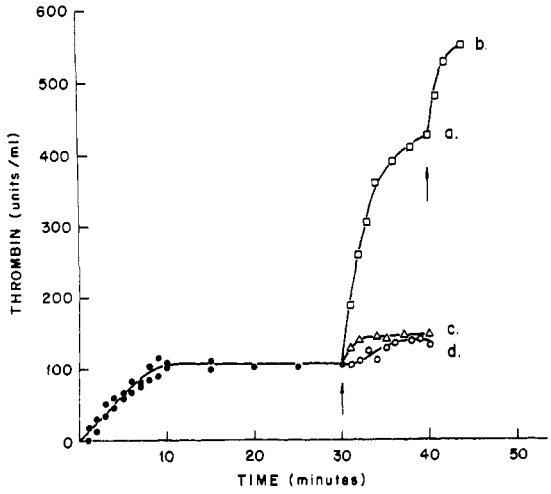

FIGURE 8: Renewal of thrombin formation by increasing the concentrations of components of the activating complex. Slow activation of 0.5 mg/ml of prothrombin was initiated (0.005 μg/ml of factor X_a, 14 units/ml of factor V, phospholipid, and 10 mM $CaCl_2$), and allowed to continue for 30 min (●, two experiments). Further additions to 1-ml samples from this mixture were made at the times shown by arrows, as follows: (a) 0.25 μg (10 μl) of factor X_a and 14 units (10 μl) of factor V; (b) 2.5 μg (10 μl) of factor X_a and 14 units (10 μl) of factor V; (c) 14 units (10 μl) of factor V; (d) 2.5 μg (10 μl) of factor X_a.

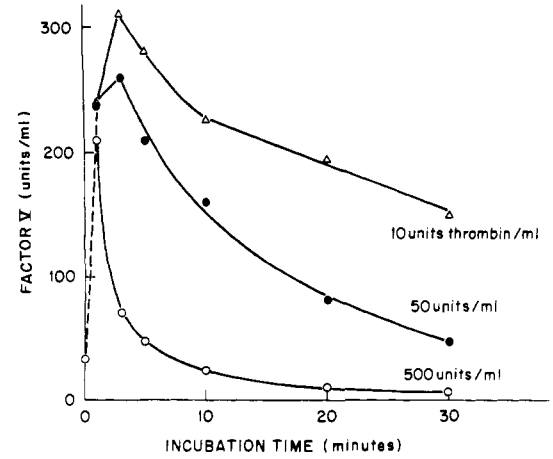

FIGURE 9: Time course of activation and inactivation of factor V by thrombin; a 10-ml factor V solution (35 units/ml in Tris-saline containing 0.02% ovalbumin) was incubated with human thrombin at 500, 50, or 10 units/ml. The mixture also contained phospholipid (5 μg of phosphorus/ml) and 10 mM $CaCl_2$. Activity at zero time was determined on a 1-ml sample to which 1 μl of 5 M Dip-F was added before thrombin. After the addition of thrombin to the remaining mixture, further 1-ml samples were treated with 5 mM Dip-F for 30 min. Factor V activity per milliliter in these samples is plotted as a function of the time of incubation with thrombin. Neither Dip-F treatment nor the 30-min incubation affected the activity of factor V, whereas no detectable thrombin activity remained.

relate the extent and time course of these effects to the conditions used in the activation of prothrombin. Factor V (35 units/ml) was incubated with three different concentrations of thrombin for various times. The reaction was stopped by treating samples from the mixture with 5 mM Dip-F for 30 min. The activity, measured by one-stage assay, is plotted as a function of incubation time in Figure 9. At all three concentrations of thrombin, a dramatic increase in factor V activity occurred first. This was observed whether or not lipid and Ca^{2+} ions were included. At the same level of thrombin as was produced during the present complete activations of prothrombin (500 units/ml), the increase in activity of factor V was too rapid to measure accurately (Figure 9, broken line). Figure 9 also shows the subsequent drop in activity, which was more clearly dependent on the thrombin concentration. This drop was again unaffected by lipid and Ca^{2+} ions. It may be noted that over the entire range of factor V concentrations shown in Figure 3, enough thrombin is formed in only a few minutes to cause appreciable inactivation. Thus the loss of factor V activity is probably a significant control even at the slowest rates of prothrombin activation we have studied.

Activation of Factor X_a Activity. So far we have shown that slow activation has two effects: (i) prothrombin is converted by its product, thrombin, to P_2, which is then activated only slowly at most levels of factor V and factor X_a; (ii) the loss of factor V activity, again through the feedback action of thrombin, helps to prevent the activation of P_2.

The results of other experiments showed that the activity of factor X_a is also curbed during slow activation. In Figure 8 (curve c), it is seen that, when further factor V was added after slow activation had been initiated with a low level of factor X_a, almost no more thrombin was formed. It could be argued that this was because P_2 cannot be activated by such low levels of factor X_a. However, in another experiment (not shown), the extra factor V was added with 0.5 mg/ml of prothrombin, and this additional substrate was also not converted to active thrombin. Therefore the factor X_a originally present, sufficient to catalyze the formation of 100 units/ml of thrombin, had lost its activity.

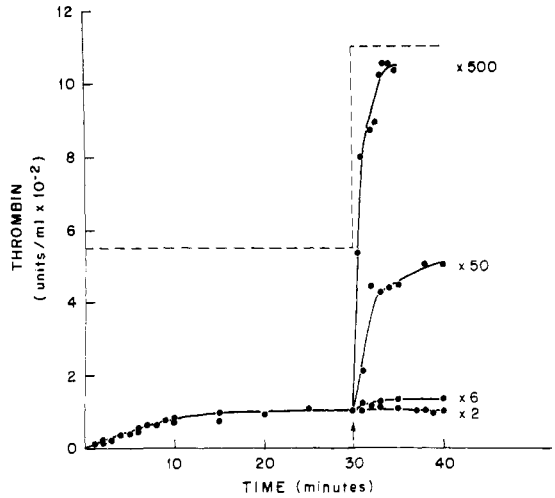

FIGURE 10: Prothrombin conversion at various concentrations of factor X_a in the presence of the products of slow activation; 0.5 mg/ml of prothrombin was activated slowly (see legend to Figure 8). After 30 min, 1-ml samples were withdrawn and a further 0.5 mg of prothrombin (25 μl) and 14 units of factor V (10 μl) added to each, immediately followed by 10 μl of factor X_a. In four experiments, the factor X_a concentration was increased 2-, 6-, 50- and 500-fold as shown, to give a final value of 0.01, 0.03, 0.25, and 2.5 μg/ml, respectively. The broken line shows the maximum yield possible before and after addition of more prothrombin.

We confirmed that factor X_a activity had been lost in further experiments of the same type (Figure 10). Here we tried to initiate the activation of further substrate with additional factor V and different amounts of factor X_a, by adding all these components to the activation mixture. A twofold or sixfold increase over the initial factor X_a concentration gave almost no response. At a 50-fold higher level of factor X_a there was some increase in the yield of thrombin, but for full activation of the new substrate, 2.5 μg/ml of

factor X_a was required. Moreover, as would be predicted from the foregoing results, all the P_2 including that from the initial activation was converted to thrombin at this high level of enzyme. The final yield was therefore 1100 units/ml.

Discussion

Our first indication that prothrombin conversion is under an inherent control was obtained when the purified bovine protein was treated with the coagulant fraction of Taipan snake venom as activator. In this system, the amount of thrombin formed did not always rise to the same final level as predicted for an unregulated enzymic reaction, but depended on the amount of venom used (Nemerson et al., 1974). In the present work, the same phenomenon was seen when prothrombin was activated with combinations of purified factor X_a, factor V, phospholipid, and Ca^{2+} ions. Here, the final yield was a function of the initial concentration of both enzyme and factor V. However, observations of this kind are by no means new. Damped activation of prothrombin was first seen by Mertz et al. (1939a), and since then has been noted by several groups (Shulman and Hearon, 1963; Papahadjopoulos and Hanahan, 1964; Milstone, 1964; Kandall et al., 1972). As the purity of the proteins used in these experiments improved, the possibility that activation was being curtailed by contaminating inhibitors from plasma became less likely. Now that the details of the cleavages occurring during activation are known, we have been able to test the more probable hypothesis that the yield of thrombin depends on the activity of the converting complex because of proteolytic reactions inherent in the activation pathways.

One such control would be the generation of an inhibitor during activation. This was suggested in a general way by Kandall et al. (1972), who observed that, once the plateau of thrombin was reached in an incomplete activation, thrombin generation could not be renewed by the addition of more prothrombin. More specifically, it has been shown that the F_1 [NH_2-terminal] peptide of prothrombin inhibits the assay for factor X_a (Benson et al., 1973), and the generation of thrombin esterolytic activity from prothrombin (Jesty and Esnouf, 1973). In this regard, it should be noted that the F_1 peptide is formed by the action of thrombin on the F_{1-2} peptide (Esmon et al., 1974a) as well as on intact prothrombin. Inhibition by F_1 will therefore be a control in both fast and slow activation, and will be governed by the amount of prothrombin initially present.

In the present work, we considered the ways in which prothrombin conversion, which requires the interaction of three proteins, could in theory be controlled proteolytically. Thus the ability of the substrate to yield product could be altered by proteolysis or, alternatively, the converting complex could lose activity. These possible controls are not mutually exclusive, but could act in concert to limit activation after an initial burst of thrombin formation.

If the appearance of P_2 is used as an indication, prothrombin is readily attacked by thrombin under all the conditions we have been able to study. The results of Jackson and coworkers (Esmon and Jackson, 1974; Esmon et al., 1974a,b) and of Kisiel and Hanahan (1974) show that only the complete, irreversible inhibition of thrombin as it is formed can prevent this feedback and allow activation to occur entirely by the direct route. At the opposite extreme, in very slow activation occurring over several hours the attack on prothrombin by traces of thrombin by far exceeds attack by factor X_a. Therefore the alternative pathway is the only route generating measurable amounts of thrombin because P_2 is the source of P_3 for most of the time (Mann et al., 1971). Between these two extremes, if we consider activation occurring over minutes rather than hours, we find that the product-initiated pathway is effective as an alternative source of thrombin only when the initial concentrations of both factor X_a and factor V exceed certain levels. Above these levels the intermediates of the product-initiated pathway go on to be cleaved completely. However, if the initial concentration of either factor X_a [enzyme] or factor V [cofactor] is below this "critical" level a proportion of the P_2 is not cleaved but accumulates at the expense of product.

Thus under slow activation conditions, the attack of thrombin on prothrombin is effectively nonproductive and can be regarded as a control loop. However, the observation that P_2 accumulates does not tell us whether this is a cause or an effect of control: P_2 would not be activated, regardless of its own properties, if the activity of the converting complex were modulated. Our results show that one major reason why P_2 fails to generate thrombin in slower activations is that the activity of factor V is lost. This inactivation of the cofactor, which we and others have shown can result from thrombin action, is probably the most sensitive control. Significantly, a similar pattern of activation of factor V followed by inactivation is known to occur during the coagulation of plasma (Ware and Seegers, 1948). In the purified system, we also find that some factor X_a activity is lost. However, this loss is small except when the initial X_a concentration is low, and we do not know if it results from proteolysis by thrombin, inhibition by the F_1 peptide, or both.

Whether the alteration of the substrate is a control per se is uncertain at present. In agreement with the results of Marciniak (1970), we find that purified P_2 is never activated as rapidly as prothrombin, even by high levels of factor X_a and factor V. This is probably because P_2 has lost the lipid-binding sites present in the NH_2-terminal region of intact prothrombin (Gitel et al., 1973) and cannot interact normally with the converting complex. However, our data also suggest that the behavior of isolated P_2 does not reflect its behavior during activation of prothrombin. Thus when F_1 is present (as it is when prothrombin is the starting material) the rate and extent of cleavage of P_2 are greater. Jackson and his colleagues have shown that the F_{1-2} peptide is required for the rapid formation of thrombin from P_3 via the direct pathway (Esmon et al., 1974b). They show that P_3 and its corresponding peptide, F_2, remain tightly but noncovalently associated. This is thought to provide a mechanism by which P_3 can remain at the phospholipid surface and be cleaved further by the converting complex. Similarly, we think that, under some conditions, F_1 may accelerate the activation of P_2 even though no longer covalently associated with it. Such a hypothesis would explain why, in rapid activations where both factor X_a and factor V are in excess, P_2 can be a source of thrombin and is in fact quite rapidly activated. Even so, P_2 is probably not activated as efficiently as prothrombin. Hence prothrombin loses its activity in a conventional two-stage assay after incubation with thrombin (Mertz et al., 1939b).

Although cleavage of prothrombin by thrombin may well slow down thrombin production, the modulation of factor V activity is probably the means by which activation is halted entirely. We therefore propose that the destruction of factor V limits activation after a few minutes unless its *initial*

level is high enough to override the effects of thrombin attach and allow the activation of P_2 to proceed to completion. The activity of factor V is so sensitive to modulation by thrombin that its activity will increase in the first seconds according to the amount of thrombin formed via the direct pathway. Our results show that the size and extent of this thrombin burst are also regulated by the factor X_a concentration. Therefore the relevance to coagulation of all the controls mediated by thrombin depends ultimately on whether the factor X_a concentration is variable. Experiments in this laboratory showed that the yield of factor X_a from purified factor X is indeed varied by the amount of factor VII–tissue factor complex used to activate it (Jesty et al., 1974). The mechanisms which control the yield of factor X_a are not strictly analogous to those which limit prothrombin activation. Instead, factor X_a (the product) potentiates and then destroys the activity of the enzyme factor VII, by cleaving it at two sites (Radcliffe and Nemerson, 1975). This has the effect of producing a pulse of factor X-converting activity, the size of which regulates the amount of factor X_a which is finally produced. In prothrombin activation, it is mainly the activity of the nonproteolytic cofactor, factor V, which is modulated; however, the net effects on activation are similar.

We may now consider the consequences of combining two regulated activations, prothrombin and factor X conversion. Since they occur in sequence, these two reactions if unregulated would have the capacity to amplify an initiating stimulus, particularly in view of the relative concentrations of factor VII, factor X, and prothrombin in plasma which are about 20, 160, and 1700 n*M*, respectively. Thus whether a large or a small proportion of plasma factor VII is activated by formation of its complex with tissue factor, tenfold more factor X is available as substrate. Likewise the factor X_a formed is exposed to a tenfold greater amount of *its* substrate, prothrombin. This means that, if the turnover of the two enzymes were unchecked, an initiating stimulus of any size would, in time, produce the maximum 1.7 μmol of thrombin/ml. When postulating his cascade model for coagulation, Macfarlane (1964) pointed out the advantages of this inherent capacity to amplify. However, the advantage to be gained from the explosive local generation of thrombin becomes a liability if the thrombin then endangers the whole circulation. In discussing this difficulty of the model, Macfarlane stated (1972) that macromolecular inhibitors, a variety of which are present in plasma, inactivate the enzymes of coagulation and hence suppress amplification. This possibility was also discussed by Hemker and Hemker (1969). They showed that the kinetics of the extrinsic pathway as a whole fit a model in which each enzyme is formed in its maximum amount but is then inactivated almost instantaneously. Such a model also applies if the concentration of each enzyme never reaches its maximum but is controlled by an inherent, self-damping mechanism as we propose. At present we cannot assess the relative importance of these two types of control, but it is likely that both are necessary for the limitation of minor hemostatic events occurring in vivo.

Acknowledgments

We thank Dr. J. Fenton for his gift of human thrombin, and Dr. C. M. Jackson for showing us several manuscripts prior to publication. We also thank Ms. D. Mohammed for her assistance with electrophoresis.

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Steady-State Analysis of Kinetic Isotope Effects in Enzymic Reactions†

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ABSTRACT: The decrease in the rate of bond cleavage resulting from the presence of heavy isotopes is expressed to a greater or lesser extent as an isotope effect on steady-state kinetic parameters of enzyme-catalyzed reactions, depending upon complex relationships between individual rate constants. This paper describes these relationships and derives general kinetic expressions which allow the determination of the limits of the relative contribution of different reaction components to V_{max} and V_{max}/K_m. The value of the true isotope effect on a carbon–hydrogen bond breaking step, the rate of this step, and its percentage of partial rate limitation of the overall reaction, plus the dissociation constant of the enzyme–substrate complex, can be determined from the derived expressions by comparing deuterium and tritium isotope effects on V_{max}/K_m. In the absence of a measure of the true isotope effect, the lower limit of primary isotope effects of deuterium which may be interpreted as identifying the slowest or "rate-determining" step in an enzyme-catalyzed reaction is found to be $k_H/k_D = 8$, and not $k_H/k_D = 2$ as is currently supposed.

The major reason for determining kinetic isotope effects in enzymology has been to determine whether the maximal velocity is a measure of the rate of the step in which covalent change takes place (Jencks, 1969). Such a step is normally identified as the "rate-determining step" of the enzymatic reaction. The results to date and the steady-state concepts of current kinetic theory suggest, however, that the domination of maximal velocities of enzymatic reactions by a single covalent step is a rare event. It now appears likely that the maximal velocity of most enzymes is dependent upon several "rate-contributing" or "partially rate-limiting" steps. In this latter situation, apparent isotope effects have meaning only in terms of a comparison to the true isotope effects exerted on the catalytic step of covalent change. However, since true isotope effects could not previously be determined, the usual procedure has been to evaluate apparent effects on an absolute scale, based upon an implied comparison with apparent effects for other enzymes and results obtained in chemical reactions. Such an approach ignores the possible wide variation of the true isotope effects in enzyme-catalyzed reactions, which are, in addition, probably much greater than the current standards of comparison.

The primary purpose of the present study is to examine the origins and degrees of expression of isotope effects within the steady-state behavior of enzymes, in order to develop a theoretical basis for evaluating apparent isotope effects and to define the relative contributions of various partially rate-limiting steps to maximal velocities. A method for determining true isotope effects was discovered which clearly obviates the limitations and uncertainties previously encountered in the interpretation of isotopic data. The thermodynamics of bond-breaking processes which give rise to the presence and magnitude of kinetic isotope effects has been reviewed (Jencks, 1969) and is not a subject in this discussion. In addition, the present study is restricted quantitatively to primary isotope effects arising from isotopic substitutions of hydrogen in substrates displaying Michaelis–Menten kinetics, although extensions of the theory to other isotopes and secondary effects are possible in many instances.

Theoretical Analysis

The steady-state kinetic behavior of enzymes as a function of the concentration of one substrate generally obeys the equation

$$v = VS/(K + S) \quad (1)$$

where V is the maximal velocity and K the Michaelis constant. The kinetically independent constants are V and V/K, both complex functions consisting of several rate constants.

† From the School of Pharmacy, University of Wisconsin, Madison, Wisconsin 53706. *Received September 9, 1974.* This investigation was supported by Research Grant GM-18701 of the National Institutes of Health.