Some Properties of a Reaction Mechanism Analogous to Thrombin Formation

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Investigation of the early stages of blood coagulation has proceeded rapidly during the past few years (6, 7). At least three plasma factors, V, VIII and IX, together with calcium and platelets, are now recognized as essential for the formation of blood prothrombin activator (3, 11). However, because quantitative experimental study of activator formation can only be made by coupling the reaction to thrombin formation and measuring a clotting time, definitive experiments to test hypotheses are difficult to devise. The evidence now available favors a sequence of reactions each of which would involve two or at the most, three reactants (4). The alternative of a smaller number of reactions involving a large number of reactants is less attractive, if only because of the diminished probability of reactions requiring the simultaneous collision of three, four or five reactants. Nevertheless, the literature on blood coagulation reflects these uncertainties, and occasionally reference is still made to autocatalytic mechanisms (1). The latter term stems from the similarity of the time course of the clotting reactions to known autocatalytic reactions, rather than from direct experimental evidence. Of the various mechanisms suggested, nature has most frequently adopted the device of a consecutive series of reactions, as seen in the transfer of electrons to oxygen by the cytochrome system.

Recent data from this laboratory (10, 11), obtained from a kinetic study of highly purified clotting factors, revealed that rather specific kinetic patterns were produced by variation of concentrations of individual factors. Antihemo-

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philic factor (VIII), for example, affects the rate at which thrombin is formed, but does not alter the length of the induction period which precedes the appearance of thrombin. With this degree of specificity to work with, it appeared worthwhile to examine the kinetic behavior of some hypothetical models of reaction mechanisms, making use of the analog computor. With this instrument, the behavior of the model is formulated in mathematical terms (9). Then an analogous electrical circuit is made up, using electrical functions, which can be varied at will, in place of the mathematical functions. The behavior of any one or more components of the model with respect to time can be observed by scanning the circuit with a cathode ray oscilloscope. In this way, "impossible" models can be quickly eliminated, and, depending upon how characteristic and specific the known behavior of the natural system, more and more "reasonable" analogs can be selected.

A GAP/R Model K-3 Electronic Analog Computor manufactured by G. A. Philbrick Researches, Inc., Boston, was used. The author is grateful to Dr. Peter Curran of the Biophysical Laboratory, Harvard Medical School, for help and advice in setting up the circuits employed.

Choice of a Model

Since it was desired to select a model by comparison with a natural system which yielded thrombin as a product, the model was set up to form an analogous product (cf. E below). One model pattern offered the best approach to a satisfactory solution of the problem, yet was not so complex as to be impossible to study with the available computor components. This model is a follows:

$$A \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} B \qquad (1)$$

$$B \underset{k'_{-2}}{\overset{k'_2}{\rightleftharpoons}} C \qquad (2)$$

$$D \underset{k_{-3}}{\overset{k_3 C}{\rightleftharpoons}} E \qquad (3)$$

The transformations A $\longrightarrow$ B $\longrightarrow$ C represent stages in the formation of C which acts enzymatically on the reaction D $\longrightarrow$ E. The latter represents the conversion of prothrombin to thrombin. For the moment we need not attempt to identify A and B, although C obviously corresponds to the hypothetical prothrombin activator. The constants k'_1, k'_{-1}, k'_2 . . . etc. are rate constants for

the respective reactions.*) The rate equations describing the kinetic behavior of this model were then set up in the standard fashion. Thus, for example, the rate of change of A with time, dA/dt depends upon the rate at which A is utilized to form B ($-k'_1$ A) and the rate at which B is transformed back to A by the reverse reaction ($+ k'_{-1}$ B).

$$\frac{dA}{dt} = - k'_1 A + k'_{-1} B \tag{4}$$

By making the quite reasonable assumption that there is no appreciable back reaction, the term k'_{-1} B drops out and equation (4) then is simplified to:

$$\frac{dA}{dt} = - k'_1 A \tag{5}$$

By similar reasoning and assumption of no back reaction the transformation B $\longrightarrow$ C is described by two equations which deal with the rate of change of B and C respectively:

$$\frac{dB}{dt} = - k'_2 B + k'_1 A \tag{6}$$

$$\frac{dC}{dt} = k'_2 B \tag{7}$$

For the final reaction, the experimental data indicate that the rate of disappearance of D is dependent upon the initial concentration of D, the amount of enzymatically reactive activator C, and the rate constant k_3. Experimentally there is no evidence of significant back reaction from E to D so the disregarding of k_{-3} seems justified. Also, the rate of *appearance* of E is equal to the rate of disappearance of D; the former being the experimentally measured quantity.

$$\frac{dD}{dt} = - k_3 DC = - \frac{dE}{dt} \tag{8}$$

In the analog computor, an analogous electrical circuit is set up by which these equations are integrated simultaneously with respect to time, thus yielding a solution to equation (8) in terms of the functions of A, k'_1, k'_2, k_3 and D. This

*) The rate constants designated by a prime, e.g., k'_1, may actually be *apparent* rate constants. They thus may include a true rate constant and a modifier J_1 so that $k'_1 = k_1 J_1$. This is a convenient simplification which permits us to consider hypothetical enzymes J_1 and J_2 without unnecessarily complicating the electronic analog. The straightforward usage of a true constant k_3 and enzyme C for the third reaction was adopted to permit independent evaluation of reaction 3.

solution is seen on the oscilloscope as a plot of E as a function of time t.

By changing values of electrical functions in the computor components it was then possible to study the effect of variations in k'_1, k'_2 k_3 and A upon the appearance of E. It was assumed that the initial concentrations of B and C were zero, i.e., B and C originate exclusively from A. The initial value of D was kept constant in all tests in keeping with the practice during experiments on the chemical system (11).

Results

As a test of the validity of the electronic analog, its behavior was checked under conditions where the outcome could be predicted or was known. Thus, when any one variable, — A, k'_1, k'_2 or k_3 — was set at zero, regardless of the values of the others, no E should form. This proved to be the case. At the start the behavior of the third reaction was examined. The results are shown in Figure 1, and are in good agreement with the known behavior of such a system. To do this, k'_1 and k'_2 were set at zero and values of C were inserted independent of reactions 2 and 3 as desired.

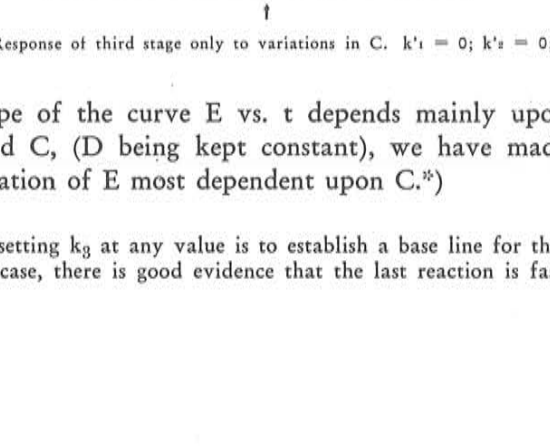

Fig. 1: Response of third stage only to variations in C. $k'_1 = 0$; $k'_2 = 0$; $k_3 = 4.0$.

Since the slope of the curve E vs. t depends mainly upon the choice of values for k_3 and C, (D being kept constant), we have made k_3 high, thus making the formation of E most dependent upon C.*)

*) The effect of setting k_3 at any value is to establish a base line for the relative values of k'_1 and k'_2. In this case, there is good evidence that the last reaction is fast in comparison to earlier ones (4).

The behavior of the electronic analog was observed under a range of conditions of imposed values of A, k'_1 and k'_2. A typical curve is reproduced in Figure 2 in order to illustrate the striking resemblance of the model to the natural system in this respect. In Figure 3 is shown a family of curves obtained

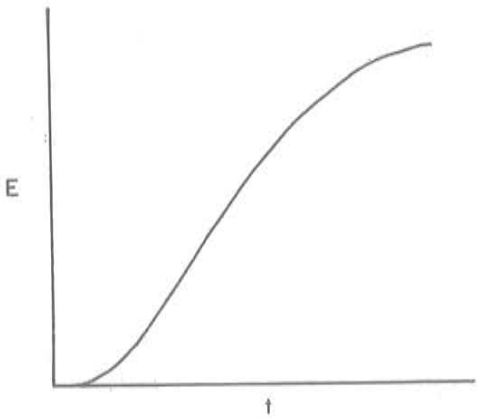

Fig. 2: Example of formation of E by complete model. $k'_1 = 0.5$; $k'_2 = 0.4$; $k_3 = 4.0$; $A = 0.5$.

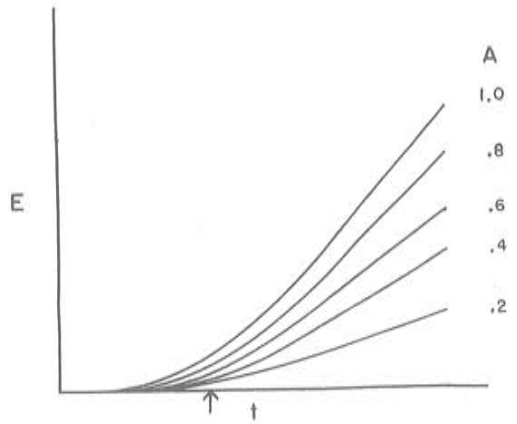

Fig. 3: Effect of variation in initial input of A upon formation of E. $k'_1 = 0.10$; $k'_2 = 0.16$. The arrow designates the common (extrapolated) origin of each curve in the series, reflecting a constant induction period.

by changing the initial value of A under conditions where k'_1 and k'_2 are nearly equal and small with respect to k_3. In this case there is a long time delay before E increases and this delay (induction period) is not influenced by large

imposed changes in the value of A. In contrast, variations in k'_1 (Figure 4) result in displacement of the curve toward lower values on the time axis. At low values of k'_1 there is a simultaneous change in the slope and displacement. Variations in k'_2, shown in Figure 5, result in a similar response, except that

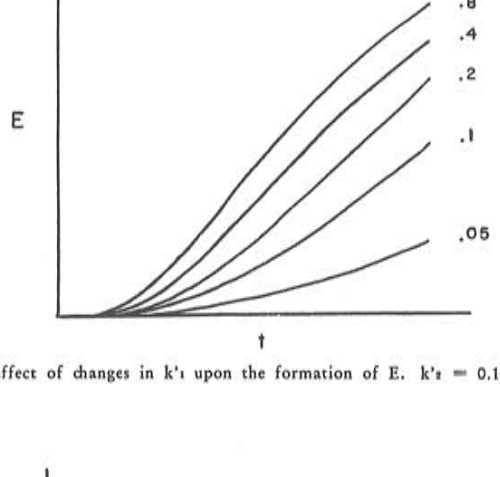

Fig. 4: Effect of changes in k'_1 upon the formation of E. $k'_2 = 0.16$; A = 0.6.

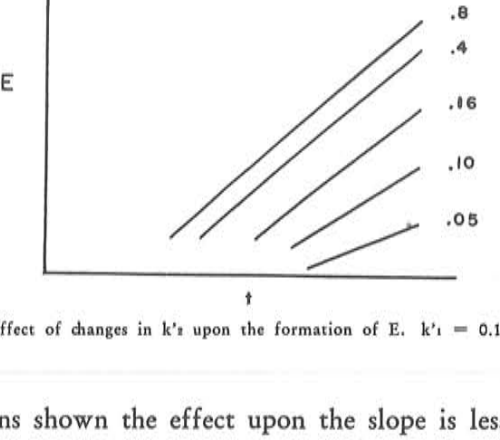

Fig. 5: Effect of changes in k'_2 upon the formation of E. $k'_1 = 0.10$; A = 0.6.

under the conditions shown the effect upon the slope is less pronounced. The characteristic response to changes in A was observed even at quite large values of k'_1 (up to 0.8) and k'_2 (up to 0.8) except that the relative displacement of the curves on the time axis was determined by the values of k'_1 and k'_2.

Further distinctive characteristics of the behavior of the model were found by examining the fate of components A, B and C during the process. These are exemplified by the results shown in Figure 6. When k'_1 is moderately

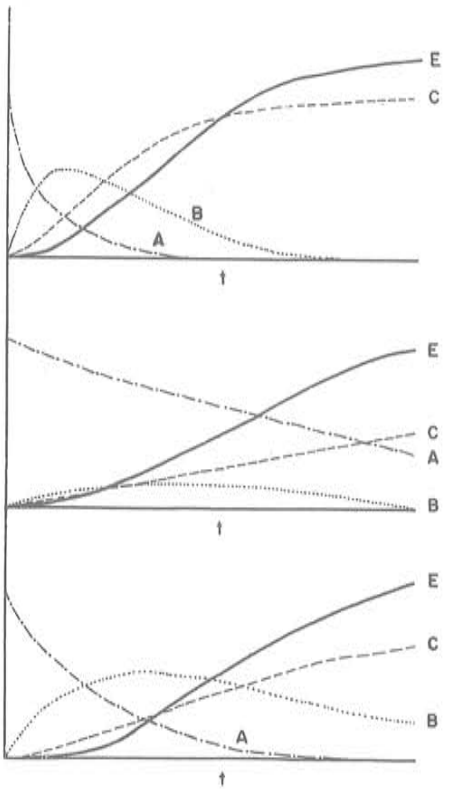

Fig. 6: Simultaneous plots of A, B, C and E as a function of time under various conditions.
Fig. 6a: (uppermost section) $k'_1 = 1.00$; $k'_2 = 0.40$.
Fig. 6b: (middle section) $k'_1 = 0.10$; $k'_2 = 0.40$.
Fig. 6c: (lowest section) $k'_1 = 0.40$; $k'_2 = 0.16$. Initial input of A = 0.6 in each case.

large and k'_2 smaller, A disappears rapidly as it is utilized in forming B (Figure 6a). Simultaneously B is being utilized in the formation of C, so the amount of B first increases and then slowly decreases to zero as C is formed. At the end, there is no A or B left in the system and C is thus formed in 100 per cent yield. If now k'_1 is made one-tenth its previous value, k'_1 becomes the limiting rate. Under these circumstances (Figure 6b). A is only partially utilized and C is produced in less than 50 per cent yield. In consequence, E is formed less rapidly and less completely. It is of interest that the amount of B present

in the system is never very large; it is utilized about as rapidly as it is formed. Similarly the pattern shown in Figure 6c is obtained when k'_1 is small and k'_2 is even smaller, although the ratio k'_1/k'_2 is the same as in Figure 6a. The result is intermediate, as A is completely utilized and more B and less C are formed during the reaction.

From these and other observations of the behavior of the model, certain additional patterns could be discerned, among which the following seem noteworthy. When k'_2 is small, wide variations in the values of k'_1 have practically no effect on the rate of appearance of E. At a low initial level of A, E forms most rapidly when k'_1 and k'_2 are large and least rapidly when k'_2 is small. At a low value of k'_1 and a given value of A, the slope of the linear portion of the appearance of E is the same over a wide range of value of k'_2; only the displacement on the time axis is affected by k_2.

Discussion

Even with the relatively simple mechanism of consecutive reactions represented by the model, there is a striking resemblance between the model and the natural system. In view of this accord, it is worthwhile to consider possible identifications. In the model, A is consumed during the reaction and it affects the rate of formation of E, but not the induction period. In the natural system, these same attributes are characteristic of antihemophilic factor (VIII). Platelets also behave more like A than anything else, especially in the range where they alter the rate but not the induction period. This apparent shortcoming of the model in having only one function where two components operate in the natural system can be overcome by modifying the mechanism to include a stoichiometric reaction between two functions A_1 and A_2 in the first stage.

$$A_1 + A_2 \xrightarrow{k_1 J_1} B \qquad (9)$$

There is support for such a mechanism in the experiments of Shinowara (8). The single function A in the analog does not, however; need to be modified, since it can equally well represent variation of A_1 at constant A_2, or variation of A_2 at constant A_1.

The apparent rate constants k'_1 and k'_2 affect the model in much the same manner that Ac globulin (V) and PTC (IX) moderate thrombin formation in the biochemical system. In this instance, however, the kinetic response of the natural system to factor V and factor IX has not been sufficiently differentiated to suggest assignment of an identity to either k'_1 or k'_2. Assuming validity

of other hypotheses which impute to factor V a later role than that of factor IX (6), we shall tentatively associate factor V with k'_2. The main weakness of so doing lies in the fact that factor V is currently considered to be consumed during coagulation (5). The analogy between k'_1 (or k'_2) and PTC is better because PTC is not consumed during coagulation.

In the natural system, PTC was observed to be activated to a product which reduced the induction period in thrombin formation. Earlier, B e r g s a g e l had shown that PTC in serum is activated by formation of a calcium complex; this product also tended to reduce the induction period under the conditions then used (2). Here the model can be modified by assuming that the factor J (footnote p. 3) is an enzyme which must be activated from a precursor state. This also carries the attractive connotation that it be a "triggering" mechanism for the whole process, a role already assigned to PTC (6).

The quantitative variations in A, B and C with respect to time (Figure 6), lead to interesting teleological suggestions in favor of a mechanism that produces C most efficiently. They may be most useful, however, in designing experiments to produce the intermediate B for example, in optimal yield. No new suggestions derive from the model regarding the role of calcium, which has been assumed to act as a typical metal activator of one or more enzymes in the mechanism.

Summary

Using an analog computor, the behavior with respect to time of a model chemical reaction mechanism has been studied. This mechanism consists of a consecutive series of reactions, the product of which corresponds to a prothrombin activator that acts enzymatically upon prothrombin. The striking resemblance in behavior between the model mechanism and the natural system strengthens current concepts of prothrombin activator formation.

Résumé

L'auteur admet que la conversion de la prothrombine en thrombine représente une réaction enzymatique et que la formation de l'activateur de la prothrombine (thromboplastine sanguine) est également due à une série de réactions enzymatiques succèssives. A l'aide d'une machine à calculer électronique il a examiné l'influence de variations de certains substrate et enzymes sur la formation du produit final (la thrombine). De cette façon il a trouvé des relations mathématiques qui paraissent en accord avec les donnés experimentales.

Zusammenfassung

Der Autor geht von der Voraussetzung aus, daß die Umwandlung des Prothrombins in Thrombin einen enzymatischen Vorgang darstellt und daß auch die Bildung des „Prothrombinactivators" (Blutthrombokinase) eine Reihe von enzymatischen Reaktionen in sich schließt. Mit Hilfe einer Elektronenrechenmaschine hat er die Wirkung von Variationen der einzelnen Größen auf die Bildung des Endproduktes (Thrombin) etc. untersucht. Dabei konnten gewisse Gesetzmäßigkeiten gefunden werden, die sich mit den spärlichen bisher vorliegenden experimentellen Daten in Einklang bringen lassen.

References

(1) Astrup, T.: The autocatalytic reaction in blood coagulation. Danish med. Bull. 4: 160 (1957).
(2) Bergsagel, D. E.: The Role of Calcium in the Activation of the Christmas Factor. Brit. J. Hemat. 1: 199 (1955).
(3) Bergsagel, D. E. and Hougie, C.: Intermediate stages in the formation of blood thromboplastin. Brit. J. Haematol. 2: 113 (1956).
(4) Biggs, R., Douglas, A. S. and Macfarlane, R. G.: The initial stages of coagulation. J. Physiol. (Lond.) 122: 538 (1953).
(5) Douglas, A. S.: Factor V consumption during blood coagulation. Brit. J. Haematol. 2: 153 (1956).
(6) Macfarlane, R. G.: Blood coagulation. Physiol. Rev. 36: 479 (1956).
(7) Seegers, W. H. and Johnson, S. A.: Conversion of prothrombin to autoprothrombin II (Platelet Cofactor II) and its relation to the blood clotting mechanism. Amer. J. Physiol. 184: 259 (1956).
(8) Shinowara, G. Y.: The isolation and characterization of thromboplastic cell and plasma components. J. Lab. clin. Med. 38: 11 (1951).
(9) Soroka: Analog methods in computation and simulation. McGraw Hill, New York (1954).
(10) Surgenor, D. M. and Steele, B. B.: Platelet antihemophilic factor interactions. Fed. Proc. 17: 1262 (1958).
(11) Surgenor, D. M., Steele, B. B. and Wallach, D. F. H.: Thrombin formation in purified systems. Thrombosis et Diathesis Haemorrhagica 3: 214 (1959).