FACTORS INFLUENCING THE ACTIVITY OF THROMBIN PREPARATIONS

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Introduction

It has been the practice for some years in this laboratory to assay preparations of thrombin by comparing their activity in plasma with that of a laboratory standard. The purpose of this paper is to record certain of the observations and experiments which have been made from time to time to illuminate the assay.

Thrombin

Thrombin is a comparatively recent addition to the range of animal substances used in medicine and surgery. It is a protein formed in shed blood from an inactive precursor by the action of thromboplastin in the presence of ionic calcium and accelerator globulin (also known as factor V or labile factor) and it brings about the conversion of fibrinogen into insoluble fibrin, the physical basis of the blood clot. The reaction is almost certainly enzymic since it has been demonstrated using highly purified reagents that thrombin is able to convert at least 10,000 times its weight of fibrinogen into fibrin[1] and that the yield is independent of thrombin concentration over a wide range[1,2].

Thrombin of commerce is a partially concentrated serum protein fraction, or it may be obtained *via* prothrombin. It contains buffering salts in varying amount and its activity seldom exceeds 60 National Institute of Health (U.S.A.) Units (originally Iowa Units), per mg. Preparations containing up to 1400 units of activity per mg. dry weight have been prepared[3] and shown to be electrophoretically heterogeneous[4], so it is clear that the actual enzyme is present only in very small amount. The commercial enzyme is stable for many years in the absence of moisture and is comparatively stable in cold aqueous solution provided that micro-organisms are not present in large numbers.

Clotting

When thrombin is added to a fibrinogen-containing solution, fibrin is formed at a rate depending on the concentration of the reactants and the experimental conditions, and the reaction continues until the whole of the fibrinogen has been converted. At a certain point in the course of the reaction the system is observed to clot when the quantity of fibrin formed is sufficient to give rise to a gel capable of supporting its own weight. This is a largely arbitrary point and generally occurs quite early in the process when only a fraction of the fibrinogen has reacted.

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It cannot be uniquely defined since observation of the physical changes in the system merely serves to demonstrate the essential continuity of the process. Further the clot characteristics vary with circumstances, for example, bovine plasma gives an opaque, elastic precipitate, which readily forms a web-like network when agitated, whereas sheep plasma gives rise to a translucent, friable gel. It is therefore essential in carrying out experimental work on clotting systems to define the characteristics of the end-point used at the beginning of the work and to adhere to this.

The Isolated Reaction: Thrombin: Fibrinogen.

A number of workers[2,5,6] have studied the kinetics of the isolated thrombin: fibrinogen reaction in dilute solution, generally by gravimetric determination of the fibrin yield as the reaction proceeds. These studies have shown that in low concentrations the reaction is practically of the first order with respect to both thrombin and fibrinogen, and as would be expected from consideration of the Langmuir equation for heterogeneous catalysis there is a gradual change to zero order for fibrinogen as the concentration increases to an optimal range. Thereafter further substrate actually decreases the rate[5,7]. In solution of ionic strength 0·15 and with 1 unit/ml. of thrombin, the clotting time is almost constant from 0·05 to greater than 0·3 per cent. fibrinogen, whereas with 0·1 units of thrombin per ml., there is a well-marked optimum near 0·1 per cent[5].

Therefore assuming constant experimental conditions and optimal fibrinogen concentration, rate of reaction may be taken as solely dependent on the enzyme concentration. If rate comparisons may be made by comparing clotting times, these should approximate to a linear function of the enzyme dilution. This or the converse that reciprocal clotting time approximates to a linear function of thrombin concentration has been generally confirmed[8,9,10].

This state of affairs may be represented mathematically by the formula,

$$t_c = b\,[\text{thrombin}]^{-1} + c$$

where t_c = clotting time

b = slope of the regression line of clotting time upon thrombin dilution.

c = a constant, and

$[\text{thrombin}]^{-1}$ = reciprocal thrombin concentration.

In all but very fast systems c may be taken as zero.

$$\text{therefore } \log t_c = \log b - \log [\text{thrombin}]$$

and in any particular system b is a constant so that for all practical purposes there should be a straight line relationship between log clotting time and log thrombin concentration and it should be of unit slope. This relationship has the virtue of stabilising the clotting time variance within quite wide limits, and it is also of more general applicability. There are limits to the usefulness of the thrombin concentration: reciprocal clotting time relationship. Astrup and Darling[11] have shown that it does not hold good for impure preparations of fibrinogen, neither does it hold for certain other published data[2] in which the mean slope of the log: log

curve is −0·67. The fibrinogen here used had a mean clottable protein content of 97 per cent. and the thrombin was a physically activated high potency preparation. The physical agency was prolonged exposure to the effect of cold (Seegers *et al.*[4]). We have little experience of the use of solutions of fibrinogen for assay purposes but the above derived relationship fits much published data well. In plasma the reaction is complicated by the presence of other proteins and more especially of thrombin inhibitors. Waugh and Livingstone[6] have shown that non-clottable protein did not influence the course of the reaction, and despite the presence of antithrombin, we find that the log : log relationship holds generally, provided that the clotting times are not less than about 12 seconds. The slope of the regression line of log t_c upon log thrombin concentration is generally about − 0·5 to − 0·7.

The Effect of Temperature on the Reaction in Plasma.

The effect of temperature on the rate of reaction between sheep thrombin and fibrinogen in oxalated bovine plasma, prepared from blood containing 0·16 per cent. of potassium oxalate plus 0·04 per cent. of oxalic acid, was investigated with the aid of a stopwatch, a test-tube, and a water bath.

0·1 ml. of plasma was pipetted directly into the bottom of a scrupulously clean test-tube and after temperature equilibration in the water bath an equal volume of a solution of sheep thrombin in water at the same temperature was blown in and mixed immediately. The system was maintained in the water bath until about one second before it clotted, this having been determined by previous trial, and clotting was detected visually.

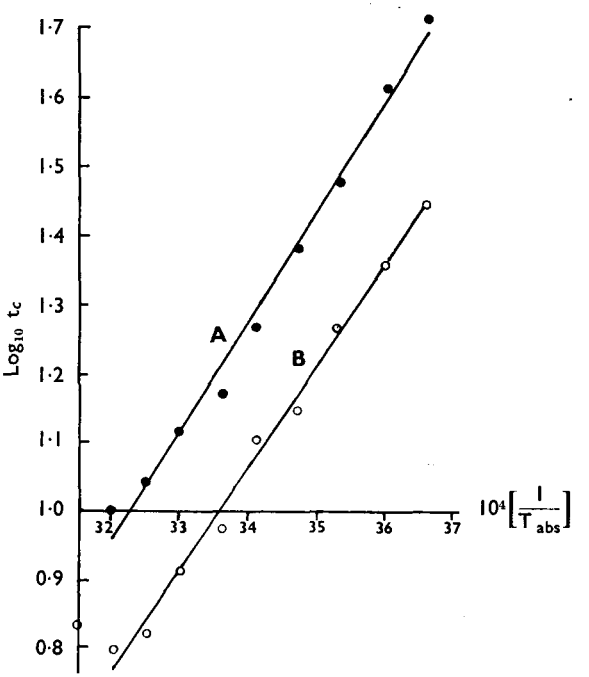

FIG. 1. Effect of temperature on the rate of reaction between thrombin and fibrinogen in oxalated plasma. A, 5 N.I.H. units/ml. B, 10 N.I.H. units/ml. Each point is the mean of not less than 3 determinations.

Two sets of determinations were made, in the one case with a thrombin concentration of 5 and in the other of 10 units per ml. The results are

shown graphically in Figure 1 as a plot of log. clotting time (in seconds) against reciprocal absolute temperature (1/°K). It will be seen that the points are in good agreement with the Arrhenius equation lying very close to a straight line within the range 0° to 40° C. This is by no means always the case with enzyme reactions[12]. Above 40° C., further increase in rate is offset by thermal denaturation of the enzyme. The best fitting straight line has been superposed on the points. The values of E calculated from these slopes are 7·2 and 6·7 kcal., but since the exact nature of the reaction has not yet been established it is not possible to attribute a precise meaning to this parameter.

The Effect of Various Added Substances.

Many substances affect the activity of thrombin preparations, the most important of which have been investigated by various workers[5,13,14,15,16] in buffered solutions of fibrinogen. The effects here considered are those which particularly relate to assay processes and in all cases the substrate employed was citrated or oxalated plasma, and the enzyme concentration was large enough to cause clotting in under half a minute.

Salt Effects.

The most important of these is that due to the presence of sodium salts. Sodium citrate, sulphate and chloride all retard the reaction. In general there was a practically straight line relationship between molar strength and clotting time. The molar concentration of each salt which was the minimum observed to produce marked prolongation of the clotting time and the added maximum salt concentration which produced no evident prolongation in plasma respectively were as follows:

Sodium chloride 0·010 M and 0·005 M
Sodium sulphate 0·008 M and 0·002 M
Sodium citrate 0·005 M and 0·002 M

These figures were determined by consideration of the results obtained in a series of randomised block experiments.

TABLE I

THE EFFECT OF ADDED SODIUM SULPHATE ON THE REACTION BETWEEN BOVINE THROMBIN AND CITRATED BOVINE PLASMA

Molar concentration	Additional ionic strength	Clotting times (seconds)			
0·008	0·024	16	17·5	19	19
0·004	0·012	12	15	16	17
0·002	0·006	14	15	15	14
0·001	0·003	15	14	14	15
0·000	0·000	14	14	15	15

Table I shows a typical set of results; in the majority of instances the clotting time of the system without added salt fell within the range 10 to 20 seconds. The shortest was 9, and the longest 26 seconds.

In addition to the general salt effect shown by the above mentioned salts, certain other salts were found to be capable in low concentration

of exerting an accelerating effect which had previously been observed only in the case of calcium chloride in fibrinogen solutions[2]. These were salts of divalent metals or of transition elements in the divalent state.

The experiment carried out was to mix 0·2 ml. of citrated sheep plasma with 0·1 ml. 0·1M solution of the salt in distilled water. 0·1 ml. of a solution of bovine thrombin in distilled water (of the order of 10 units per ml.) was blown into the mixture from a pipette, and mixing was effected by agitation without the introduction of air bubbles. Table II shows the results obtained.

TABLE II

Added solution	Clotting time (seconds)	Observation
H_2O	16	
$MnSO_4$	10	
$CoCl_2$	10	
$BaCl_2$	8	Very fine precipitate
H_2O	17	
$MnSO_4$	11	
$CoCl_2$	11	
$BaCl_2$	8	Very fine precipitate
$CaCl_2$	8	
H_2O	17	
$CaCl_2$	8	
$CaCl_2$(0·2M)	8	

The precipitate was presumably barium citrate which has a solubility in water of 0·04 per cent. at 18° C.

It is evident that the four cations examined fall into two classes. Cobaltous and manganous cause acceleration to 10 to 11 seconds and barium and calcium cause greater acceleration to 8 seconds. Furthermore this figure was not decreased by doubling the calcium concentration. That this effect is not brought about by initiation of the blood clotting mechanism is shown by the fact that the recalcification time of plasma—0·2 ml. of plasma plus 0·1 ml. of 0·1M calcium chloride—was 8·25 minutes. Barium, cobalt and manganese in corresponding tests were devoid of fibrinogenic action.

Further experiments were carried out in an endeavour to throw some light on the mechanism of the acceleration. Tables III and IV show the relationship between concentration of calcium chloride and clotting time. The maximum rate is attained in the presence of about 0·03 M calcium chloride. In higher concentrations the salt becomes a potent inhibitor. It is interesting to note that Häusler and Schnetz[17] record nickel and cobalt as inhibitors of blood clotting in concentrations greater than 0·003 N.

The smaller variance observed in Table IV is probably associated with formation of calcium oxalate which adsorbed on to the deposited fibrin thus renders the progress of the coagulation particularly lucent.

Various hypotheses would account for this effect but they are of two types only: firstly, those in which there is no change in the mechanism of the reaction, the acceleration being accountable in terms of concentration changes, or, secondly, those in which the course of the reaction is modified with a change associated with the entropy or energy characteristics of

the enzyme-substrate complex. In the former case it could be that either the divalent ions were removing an inhibitor of the forward reaction by combination with it, the segregation being complete before addition of the thrombin, or it could be that calcium is an essential component of the normal mechanism, so that the effective concentration of enzyme is increased by its addition. A further possibility is that removal of the inhibitor occurred at a slower rate so as to complicate the kinetics without actually interfering with the reaction steps. In view of the nature of the results this was not considered likely, and by finding out if there was any marked change in the kinetics it was hoped to throw some light on the mechanism.

TABLE III

THE EFFECT OF CALCIUM CHLORIDE ON THE RATE OF FORMATION OF FIBRIN IN CITRATED SHEEP PLASMA, MEASURED AS CLOTTING TIMES IN SECONDS

Molar concentration of calcium chloride in system					
0·05	0·04	0·03	0·02	0·01	0
9	9	7	8	17	19
8	7	8	11	18	20
9	9	8	12	18	21
10	9	8	11	16	23
36	34	31	42	69	83

TABLE IV

THE EFFECT OF CALCIUM CHLORIDE ON THE RATE OF FORMATION OF FIBRIN IN CITRATED BOVINE PLASMA MEASURED AS CLOTTING TIMES IN SECONDS

Molar concentration of calcium chloride in system					
0·05	0·04	0·03	0·02	0·01	0
14	13	12	12	15	23
15	14	11	11	16	21
14	13	12	12	14	21
43	40	35	35	45	65

Rough determinations of the value of E, the "activation energy," in the presence of 0·03 molar barium and calcium chloride were calculated from experimentally determined temperature coefficients. They were found to be less than the values for thrombin alone and were in good agreement (6·5 and 6·4 kcal.)—but though 0·2 kcal./mol. reduction in "activation energy" would be more than adequate to double the rate of reaction, other factors remaining unchanged, it is not possible to draw any conclusion due to the marked differences between the two values found for thrombin alone. As will be seen from Figure 2, in each case the points in the lower temperature range fell on a straight line, but with calcium chloride the bend towards the optimum commenced at 25° C. It may well be that this premature curvature was an artefact due to the nature of the experimental technique and the shortness of the clotting times. The matter demands fuller investigation.

SPECIFIC EFFECTS

Astrup has presented evidence[18] that the specific effects which undoubtedly occur in the thrombin: fibrinogen reaction[19] are attributable solely to the substrate. But even if this be regarded as established, the possibility also exists that the antithrombins of plasma may be specific in some degree. Several experiments were carried out to determine the magnitude of specific effects in plasma.

In the first two experiments carried out, a statistically confounded 3 factor design was used in order to reduce the block size, with sheep and bovine thrombin each at 4 levels[20] (about 10, 5, 2·5 and 1·25 units/ml. final concentration) and with citrated sheep plasma (0·56 per cent. sodium citrate in blood) and oxalated bovine plasma (0·16 per cent. potassium oxalate plus 0·04 per cent. oxalic acid in blood). The experiments were carried out by two different workers who used the same plasmas but different thrombin solutions and it was evident from consideration of the results represented graphically, that while sheep thrombin had the same activity in sheep and bovine plasma, bovine thrombin was more active than sheep thrombin in bovine plasma when present in higher concentration. The graphs also demonstrate the essential rectilinearity of the log clotting time: log thrombin concentration relationship. Statistical analysis of the results however shows in the one case a quadratic component of the concentration relationship which was just significant at the 5 per cent. level and no other significant effect except the differences due to potency in the thrombin solutions used, and in the other case no quadratic component, a significant plasma effect and a significant interaction between dose and thrombin type. Unfortunately the rigid derivation of the thrombin : plasma : dose interaction was not possible since this was confounded with the sum of squares due to block differences.

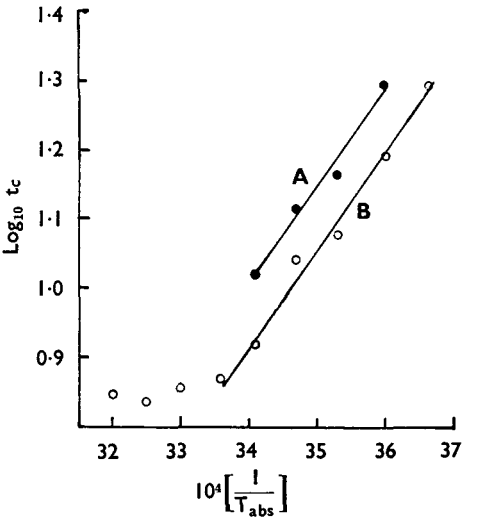

FIG. 2. Effect of temperature on the rate of reaction between thrombin and fibrinogen in oxalated plasma in the presence of calcium and barium. A, 0·05 M barium chloride. B, 0·025 M calcium chloride.

In a further experiment using ox and sheep thrombin each at three levels and oxalated and citrated ox and sheep plasma in a factorial design with three replications, a highly significant plasma effect was found as well as a distinct anticoagulant effect, however there was no significant interaction of any kind, though the random variance was only about 50 per cent. greater than in the confounded block experiment. An identical experiment in the presence of optimal calcium gave significant plasma and anticoagulant effects. In neither of these experiments was the quadratic component of the regression of log clotting time on log concentration significant.

It was evident therefore that interactions between ox and sheep preparations are not generally striking, but can occur. The possibility

of major interspecific effects was however demonstrated quite incidentally in an attempt to relate the "blood clotting dose" of the British Pharmacopœia, or M.R.C. Unit, as we prefer to call it, to the National Institute of Health Unit. The N.I.H. standard was of bovine origin obtained from the National Institute of Health, Bethesda, Maryland, and the M.R.C. preparation was of human thrombin kindly assayed and supplied by Dr. Kekwick of the Lister Institute. Three assays, each satisfying the internal requirements for validity were carried out by three workers, two of whom used bovine plasma as substrate and one a solution of bovine fibrinogen. The two workers who used plasma obtained mutually consistent results which differed markedly from those of the third worker who used fibrinogen. But the fibrinogen data agreed with past experience of the relative magnitudes.

The Effect of Glycerol and Acacia and the Interactions of these with Salts.

These experiments were carried out in randomised blocks generally with each factor at 2 or 3 levels. Glycerol was investigated as it is frequently used as a means of preserving thrombin as a solution in "deep-freeze," and acacia as it is used to increase the stability of dilute solutions of enzymes. Before use the solution of acacia was passed through a column of *biodeminrolit* (mixed bed deionising resin) so that in fact a solution of arabinic acid was used the *p*H of which was found to be 3·0.

The results of the experiments carried out are shown in Tables V, VI, VII and VIII.

TABLE V

THE EFFECT OF SODIUM CHLORIDE, CALCIUM CHLORIDE AND ARABINIC ACID ON THE CLOTTING OF CITRATED SHEEP PLASMA BY THROMBIN

0·1 ml. of each reagent was added to 0·4 ml. of citrated sheep plasma then 0·1 ml. of bovine thrombin (10 μ/ml. in water) was blown in and mixed quickly

	0·019 M sodium chloride			
	0·025 M calcium chloride	Water	0·025 M calcium chloride	Water
	Clotting times in seconds			
0·25 per cent. arabinic acid	20 17	35 29	16 15	28 25
Water	24 20	58 48	22 17	48 50

TABLE VI

ANALYSIS OF VARIANCE OF LOG METAMETERS DERIVED FROM THE RESULTS IN TABLE V

Item	Sum of squares	
Blocks	138·1	
A (arabinic acid) ..	1072·6	
N (NaCl)	138·1	(p 1—0·1 per cent.)
C ($CaCl_2$)	3937·6	(p < 20 per cent.)
A × N	10·6	
A × C	248·1	(p < 0·1 per cent.)
N × C	1·6	
A × N × C	1·6	
Error (7 d.f.)	59·4	∴ mean square 8·5

TABLE VII

THE EFFECT OF SODIUM CHLORIDE, CALCIUM CHLORIDE AND GLYCEROL ON THE CLOTTING OF OXALATED BOVINE PLASMA BY THROMBIN

0·1 ml. of each of the reagents + 0·1 ml. of water was introduced into the bottom of a clean 3 × ½ in. soda glass tube and 0·5 ml. of plasma was blown in and mixed quickly

		Full strength bovine thrombin		2/3 strength bovine thrombin	
		0·04 M sodium chloride	Water	0·04 M sodium chloride	Water
		Clotting times in seconds at 24° C.			
0·025 M calcium chloride	1 per cent. of Glycerol	19	11	22	13
	Water	16	9	21	12
Water	1 per cent. Glycerol	36	19	51	23
	Water	29	18	42	21

TABLE VIII

ANALYSIS OF VARIANCE OF LOG. METAMETERS DERIVED FROM THE RESULTS IN TABLE VI

Item	Sum of squares
N (NaCl)	2730·1
C ($CaCl_2$)	3164·1
G (Glycerol)	138·1
T (Thrombin)	430·6
N × C	18·1
N × G	7·6
N × T	14·1
C × G	0·6
C × T	3·1
G × T	7·6
Residual (5 d.f.)	48·4
	∴ Mean square 9·7

Therefore only the main effects are significant.

From these results the following inferences may be made.

Acacia has a very marked accelerating effect even when present only to the extent of 0·25 per cent., but this is much reduced in the presence of calcium and conversely calcium has a reduced effect in the presence of acacia. It was not at first appreciated that the *p*H of the arabinic acid used was as low as 3 so that a first experiment on the effect of acacia, sodium chloride and calcium chloride served merely to demonstrate the protection of thrombin against hydrogen ions by inorganic salts.

Glycerol was found to have a marked decelerating effect when present to the extent of 1 per cent., but no interaction between glycerol and salts was detected.

SUMMARY

1. The nature of the relationship between thrombin concentration and clotting time is discussed. The rectilinear log:log relationship holds good generally.

2. The effect of temperature on the rate of reaction in dilute plasma is found to be in agreement with the Arrhenius equation and a value for the "activation energy" is calculated.

3. The temperature optimum for thrombin in plasma appears to be between 40° and 45° C.

4. The general retarding effect of salts is noted, and the specific effect of calcium, barium, manganous and nickelous salts is noted and discussed.

5. The activity of thrombin in plasma may show complex dependence on the species of origin.

6. Glycerol retards coagulation, and arabinic acid accelerates it. There is a very marked interaction between the latter and calcium salts.

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