XLIX. THROMBIN AND CALCIUM CHLORIDE IN RELATION TO COAGULATION.

BY JOHN OGLETHORPE WAKELIN BARRATT,

Beit Memorial Research Fellow¹.

From the Lister Institute.

(Received September 24th, 1915.)

The investigation which forms the subject of this article was undertaken with the object of studying more fully than has hitherto been done the quantitative relation between the time of coagulation of fibrinogen, on the one hand, and the amount of thrombokinas taking part in the process of coagulation, in presence of calcium chloride, on the other hand.

This aspect of blood coagulation has received the attention of several investigators during the last two decades. Thus E. Fuld [1902], using the plasma of geese and turkeys as source of fibrinogen, and as ferment solution the freshly prepared extract, in 0·8 % NaCl solution, of muscle, found that the equation

$$\log \frac{x}{x_0} = 0\cdot585 \log \frac{y}{y_0}$$

is valid when y = the amount of ferment corresponding to coagulation time x and y_0 similarly corresponds to x_0 .

C. J. Martin [1905], employing oxalated plasma of the sheep and dog, found that the coagulation time multiplied by the quantity of ferment (snake venom) was constant, but this law failed when a relatively large amount of venom was added, the observed times being then longer than the calculated periods. L. J. Rettger [1909] observed that the product of the coagulation time and the amount of thrombin employed was constant if the amount of fibrinogen used was fixed, but that if excess of thrombin was used a maximum amount was reached beyond which further increase no longer diminished the time of coagulation; the latter represented the time at which the contents

¹ Towards the expenses of this research a grant was made by the British Medical Association.

of the tube were sufficiently coagulated for the tube to be inverted, without the contents being spilt.

It early became obvious in the course of my investigation that the fowl's plasma employed in the experiments contained some thrombokinase, for although, when oxalated, it remained free from coagulation (a varying amount of white precipitate made its appearance on keeping) during the period it was kept under observation (four weeks at 32° C.), showing that it did not contain thrombin in appreciable quantity, nevertheless on the addition of calcium chloride coagulation always occurred after a shorter or longer period of time. It was also clear that the amount of thrombokinase present in different samples of fowl's plasma exhibited great variation. Thus coagulation at 35° C. sometimes occurred at the end of thirty minutes after the addition of calcium chloride, while when separation of plasma had been more successful its occurrence under the same conditions was not observed until the expiration of several hours. This separation was accomplished by collecting blood from the femoral artery in paraffined tubes by a paraffined cannula and obtaining plasma therefrom by centrifugalisation. Similar variations in respect of spontaneous coagulability were observed in fibrinogen solutions prepared from such samples of plasma by dilution with twenty parts of distilled water through which a stream of CO₂ had been passed. The resulting precipitate of fibrinogen was collected by centrifugalisation, dissolved in 0.85 % NaCl solution and again centrifuged, the bulk of the resulting solution being made equal to that of the plasma, from which the fibrinogen was obtained. It was therefore clear that in estimating the coagulative effect of a given amount of thrombokinase it would be necessary to ascertain, if possible, the quantity already present in the fibrinogen solution or plasma employed. The mode in which this is effected will now be described.

In Table I a series of experiments is shown in which varying amounts of a solution of thrombokinase prepared from the testis of the rabbit were added to 0.325 cc. of fibrinogen solution in the presence of 0.05 cc. of N/10 CaCl₂ solution, the total volume of fluid being in each case made up to 0.5 cc. and the time of coagulation noted. The solution of thrombokinase was prepared by grinding (in a mortar) the body of the testis with sand in presence of distilled water, the resulting emulsion being made up to the original volume of the testis or to any desired multiple of the same. The extract so obtained was centrifuged in order to remove all coarse suspended particles.

In attempting to discover if any relation exists between the time of coagulation, x (i.e. the period required for *complete* separation of fibrin),

and the amount of thrombokinase, y , added it will be observed that the product xy does not vary greatly for the last four experiments. If this relation is assumed to hold good throughout the series, then the amount of thrombokinase present in the 0.325 cc. of fibrinogen solution employed in each experiment can be shown by ordinary algebraical methods, as will be indicated for the experiments given on p. 517, to be equivalent to about 0.0068 cc. of the thrombokinase solution employed; the quantities given in the third column of Table I must therefore be increased by this amount in order that the total quantity of thrombokinase present in each experiment

TABLE I.

Determination of the coagulation time of a series of mixtures of thrombokinase and fibrinogen solutions in presence of CaCl_2 , a solution of NaCl (0.85 %) being added in amount sufficient to make a total volume of 0.5 cc. in each experiment.

No. of experiment	Fibrinogen solution (cc.)	Thrombokinase solution (testis of rabbit) diluted 1 in 1000* (cc.)	N/10 CaCl_2 solution (cc.)	Time of coagulation (35°) (minutes)
1	0.325	—	0.050	408
2	"	0.097	"	88
3	"	0.292	"	30
4	"	0.877	"	11
5	"	2.633	"	3

* In Experiments 3, 4 and 5 more concentrated solutions of thrombokinase were employed, in order that the total volume of fluid in each tube might not exceed 0.5 cc.

may be exhibited. This correction is made in Table II and the corrected product xy determined. For this the value 9.22 was ultimately selected and it then became possible to recalculate the times of coagulation corresponding to different amounts of thrombokinase from the equation

$$xy = \text{const.} \dots\dots\dots(1),$$

the value of the constant being taken at 9.22. This has been done in the last column of Table II and it will be seen that the observed and calculated coagulation times, except in the case of the first experiment, are in good accord; the calculated curve (the one farthest from the axes of coordinates) is given at *A*, Fig. 1. The variation observed in the first experiment will come under consideration later, when some of the factors concerned in producing such variations are taken into account.

A further series of experiments was performed, using 0.160 cc. of the same fibrinogen solution, instead of 0.325 cc. These experiments are recorded in

Table III, the product xy being given in the fifth column, after correction for the amount of thrombokinase present in the smaller amount of fibrinogen solution employed, the latter being equivalent to about 0.00335 cc. of the thrombokinase solution used. Here again the product xy does not differ very greatly in the different experiments. The value selected to represent this product is slightly greater than the mean of the observed values. By the aid of this constant the values of x corresponding to varying amounts of thrombokinase (y) are calculated in the last column of the table (curve B, Fig. 1).

TABLE II.

The data in the second and third columns are taken from Table I.

No. of experiment	^y Thrombokinase solution (testis of rabbit) diluted 1 in 1000 (cc.)	^x Time of coagulation (minutes)	$y_{\text{corrected}}$ (cc.)	$x \times y_{\text{corrected}}$	Value of xy selected	Calculated values of x , corresponding to $y_{\text{corrected}}$ (minutes)
1	—	408	0.0068	2.77	9.22	1355
2	0.097	88	0.1038	9.23	„	89
3	0.292	30	0.2988	8.96	„	31
4	0.877	11	0.8858	9.74	„	10.5
5	2.633	3	2.6398	7.92	„	3.5
				mean 7.92		

TABLE III.

Determination of the coagulation time of a series of mixtures of thrombokinase and fibrinogen (0.160 cc.) solutions in presence of CaCl_2 (0.050 cc. of N/10 solution), a solution of NaCl (0.85 %) being added in amount sufficient to make a total volume of 0.5 cc. in each experiment.

No. of experiment	^y Thrombokinase solution (testis of rabbit) diluted 1 in 1000* (cc.)	^x Time of coagulation (minutes)	$y_{\text{corrected}}$ (cc.)	$x \times y_{\text{corrected}}$	Value of xy selected	Calculated values of x , corresponding to $y_{\text{corrected}}$ (minutes)
6	—	1050	0.00335	3.52	4.54	1355
7	0.048	88	0.05135	4.52	„	89
8	0.144	30	0.14735	4.42	„	31
9	0.432	11	0.43535	4.79	„	10.5
10	1.296	3	1.29935	3.90	„	3.5
				mean 4.23		

* In Experiments 9 and 10 more concentrated solutions of thrombokinase were employed, in order that the total volume of fluid in each tube might not exceed 0.5 cc.

Yet another series of experiments was made, a still smaller quantity of the fibrinogen solution, namely 0.050 cc., being used for each experiment (Table IV). As before a correction is made for the amount of thrombokinase

which is present in the fibrinogen solution employed. The resulting values of xy are given in the fifth column of the table. The values of x in the last column are calculated from the constant 1.42, which is somewhat higher than the mean observed value of xy . With the aid of this constant the corresponding curve C shown in Fig. 1 is constructed. It will be noted that

TABLE IV.

Determination of the coagulation time of a series of mixtures of thrombokinasase and fibrinogen (0.050 cc.) solutions in presence of CaCl_2 (0.050 cc. of $N/10$ solution), a solution of NaCl (0.85 %) being added in amount sufficient to make a total volume of 0.5 cc. in each experiment.

No. of experiment	y Thrombokinasase solution (testis of rabbit) diluted 1 in 1000 (cc.)	x Time of coagulation (minutes)	$y_{\text{corrected}}$ (cc.)	$x \times y_{\text{corrected}}$	Value of xy selected	Calculated values of x , corresponding to $y_{\text{corrected}}$ (minutes)
11	—	1050	0.00105	1.10	1.42	1355
12	0.015	41	0.01605	0.66	„	89
13	0.045	22	0.04605	1.01	„	31
14	0.135	11	0.13605	1.50	„	10.5
15	0.410	3	0.41105	1.23	„	3.5
				mean 1.10

Fig. 1. Curves for the experiments given in Tables I–IV. Abscissae, time in minutes; ordinates, cc. of thrombokinasase solution. C corresponds to experiments in which 0.05 cc. of fibrinogen solution was employed; B to 0.160 cc. and A to 0.325 cc.

in Exps. 11, 12 and 13 the observed are lower than the calculated values; the significance of these variations will be considered later.

All the above series of experiments were made in immediate succession, the same solutions of fibrinogen and thrombokinase being used throughout.

When the mean observed values of $x \times y$ (the latter being corrected for the amount of thrombokinase present in the fibrinogen solution used) were compared, it was found that they were approximately in the ratio of the amounts of fibrinogen solution employed, namely 0.325 cc., 0.160 cc. and 0.050 cc. The values of xy chosen in the above tables are accurately in this ratio and, as the values of x calculated therefrom are in fair agreement with the observed values, it follows that the relation between these quantities is very closely expressed by the equation

$$xy = nz \dots\dots\dots(2),$$

where x represents the time of coagulation, y and z showing the respective amounts of the solutions of thrombokinase and of fibrinogen present in the total volume of 0.5 cc. of fluid employed in each experiment, and n being a constant. To each tube 0.05 cc. of N/10 CaCl_2 solution was added; the temperature of experiment was 35° ; the value of n is 28.4.

TABLE V.

Determination of the coagulation time of a series of mixtures of thrombokinase and fibrinogen (0.050 cc.) solutions in presence of CaCl_2 , a solution of NaCl (0.85 %) being added in amount sufficient to make a total volume of 0.5 cc. in each experiment.

No. of experiment	Fibrinogen solution (cc.)	^y Thrombokinase solution (testis of rabbit) diluted 1 in 1000* (cc.)	N/10 CaCl_2 solution (cc.)	^x Time of coagulation (35°) (minutes)
11	0.050	—	0.050	46
12	"	0.040	"	33
13	"	0.100	"	25
14	"	0.300	"	13
15	"	1.000	"	8

* In Exp. 15 a more concentrated solution of thrombokinase was employed, in order that the total volume of fluid in each tube might not exceed 0.5 cc.

In the above experiments a solution of fowl's fibrinogen was employed, which contained exceedingly little thrombokinase. In the experiments recorded in Table V a relatively large amount of thrombokinase was already present in the fibrinogen solution used, coagulation (at 35°) occurring, after

the addition of calcium chloride alone, within a period of one hour. In these experiments if y (amount of thrombokinase added) is uncorrected, the product xy increases with each member of the series. The amount, e , of thrombokinase originally present in the quantity (0.050 cc.) of fibrinogen solution introduced into each tube (the total volume of fluid present being as before in each case 0.5 cc.) is determined, in terms of the equivalent amount of the thrombokinase solution employed, from the data given by the first two experiments, by putting the product xe in the former equal to the product $x'(y' + e)$ in the latter. Thus

$$xe = x'(y' + e) \dots\dots\dots(3),$$

whence

$$e = \frac{x'y'}{x - x'} \dots\dots\dots(4).$$

In this way the following series of values of e is obtained:

Exps. 11 and 12 (Table V)	$e = 0.10$,
„ „ 13 „	$e = 0.12$,
„ „ 14 „	$e = 0.12$,
„ „ 15 „	$e = 0.19$,
	mean 0.13.

The value of e finally chosen was 0.136.

If now to the amounts of thrombokinase introduced into each tube the equivalent (in terms of the thrombokinase solution employed) amount of thrombokinase originally present in the quantity (0.050 cc.) of fibrinogen

TABLE VI.

Recalculation of values of y (thrombokinase) and x (time of coagulation) from Table V.

No. of experi- ment	$y_{\text{corrected}}$ (cc.)	$x \times y_{\text{corrected}}$	Value of xy selected	Corresponding value of	
				$x_{\text{calculated}}$ (minutes)	x_{found} (minutes)
11	0.136	6.25	6.0	44	46
12	0.176	5.80	„	34	33
13	0.236	5.90	„	25	25
14	0.436	5.66	„	14	13
15	1.136	9.09	„	5	8
		mean 6.54			

solution used is added, the corrected values of y are obtained and from these the true values of the product xy are found. These are set forth in Table VI. The observed values of x , corresponding to the corrected values of y , are shown in Fig. 2, curve A being calculated for $xy = 6$, this being the value

ultimately selected. It will be noted that the observed and calculated values agree fairly closely. This series of experiments illustrates how a relatively heavily contaminated solution of fibrinogen—and the same applies to the plasma from which it is obtained—may be employed for the purpose of investigation, provided the amount of admixture of thrombokinase is determined. The disadvantage of such solutions, however, lies in the circumstance that the range of investigation is limited because the time of coagulation for very small concentrations of thrombokinase, corresponding to the lower

TABLE VII.

Determination of the coagulation time of a series of mixtures of thrombokinase and fibrinogen (0.160 cc. for Exps. 6 to 10 ; 0.325 cc. for Exps. 1 to 5) solutions in presence of CaCl_2 (0.050 cc. of N/10 solution), a solution of NaCl (0.85 %) being added in amount sufficient to make a total volume of 0.5 cc. in each experiment. These experiments complete the series, of which those recorded in Table V form a part.

No. of experiment	Thrombokinase solution (testis of rabbit) diluted 1 in 1000* (cc.)	x Time of coagulation (minutes)	$y_{\text{corrected}}$ (cc.)	$x \times y_{\text{corrected}}$	Value of xy selected	Calculated values of x , corresponding to $y_{\text{corrected}}$ (minutes)
1	—	52	0.884	45.90	39.0	44
2	0.030	40	0.914	36.56	„	43
3	0.100	31	0.984	30.52	„	40
4	0.300	28	1.184	23.14	„	33
5	1.000	20	1.884	37.68	„	21
				mean 34.76		
6	—	54	0.436	23.52	19.2	44
7	0.035	38	0.471	17.90	„	41
8	0.100	30	0.536	16.08	„	36
9	0.300	27	0.736	19.87	„	26
10	1.000	14	1.436	20.08	„	13
				mean 19.49		

* In Exps. 4, 5, 9 and 10 more concentrated solutions of thrombokinase were employed.

parts of the curves shown in the figures (e.g. to the right of the vertical lines at 44 and 27 in Figs. 2 and 3 respectively on the axis of abscissae), cannot be determined, since these represent amounts of thrombokinase which are less than that already present in the plasma or fibrinogen solution employed.

For the sake of comparison with Table VI the remaining experiments of the group from which Table V is constructed are given in Table VII, the corresponding curves (*B* and *C*) being shown in Fig. 2. As in the preceding series the observed values of x do not differ greatly from those

calculated with the aid of the values of xy chosen (39.0, 19.2 and 6.0), which it will be noted are proportional to the amounts of fibrinogen solution (0.325 cc., 0.160 cc. and 0.050 cc. respectively) employed; thus conforming to equation (2).

The enquiry now presents itself, Does the time of coagulation represent the time required to convert prothrombin into thrombin or does it correspond to the period during which thrombin acts upon fibrinogen, giving rise thereby to the appearance of fibrin or does each of these processes occupy an

Fig. 2. Curves for the experiments given in Tables V-VII. Abscissae, time in minutes; ordinates, cc. of thrombokinase solution. The vertical line at 44 on the axis of abscissae represents the outer limit of investigation of the curves. Curve *A* corresponds to experiments in which 0.050 cc. of fibrinogen solution was employed; *B* to 0.160 cc. and *C* to 0.325 cc.

appreciable fraction of the coagulation time? To determine this point thrombin in the form of venom from *Echis carinatus* [Barratt 1913, 1] was added to fibrinogen in varying amounts, and the period of coagulation (at 35°) noted. These experiments, which are given in Table VIII, fall into two groups: in the first (Exps. 1 to 14) plasma to which calcium chloride had been added, as in the preceding series, was employed; in the second group (Exps. 15 to 19) oxalated plasma was used. It will be observed that the course of the first group is similar to that of the experiments already recorded, in which thrombokinase was used and, as in the latter, the times

of coagulation have been calculated on the assumption that the product xy is constant for each of the three amounts of fibrinogen employed, the observed and calculated values being in fair agreement, except for the control experiments 1, 6 and 11, in which the observed periods were longer than the

TABLE VIII.

Determination of the coagulation time of a series of mixtures of venom of Echis carinatus and calcified fowl's plasma (0.325 cc. for Exps. 1 to 5; 0.160 cc. for Exps. 6 to 10 and 0.050 cc. for Exps. 11 to 14, in presence of 0.050 cc. of N/10 CaCl₂ solution) or oxalated fowl's plasma (0.325 cc. for Exps. 15 to 19), a solution of NaCl (0.85 %) being added in amount sufficient to make a total volume of 0.5 cc. in each experiment.

No. of experiment	y Venom solution, 1 in 300,000* (cc.)	x Time of coagulation (minutes)	$y_{\text{corrected}}$ (cc.)	$x \times y_{\text{corrected}}$	Value of xy selected	Calculated values of x (minutes)	Plasma (cc.)
1	—	52	0.11	5.7	3.00	27	0.325 calcified
2	0.030	20	0.14	2.8	"	21	" "
3	0.100	14	0.21	3.2	"	14	" "
4	0.300	4	0.41	1.6	"	7	" "
5	1.000	2	1.11	2.2	"	3	" "
6	—	54	0.054	2.9	1.476	27	0.160 "
7	0.035	14	0.089	1.2	"	17	" "
8	0.100	10	0.154	1.5	"	10	" "
9	0.300	4	0.354	1.4	"	4	" "
10	1.000	2	0.737†	1.5	"	2	" "
11	—	44	0.0169	0.74	0.461	27	0.050 "
12	0.040	10	0.0469	0.47	"	10	" "
13	0.100	4	0.1169	0.47	"	4	" "
14	0.300	2	0.2310†	0.46	"	2	" "
15‡	—	∞	—	—	3.44	∞	0.325 oxalated
16	0.030	129	—	3.87	"	115	" "
17	0.100	33	—	3.30	"	34	" "
18	0.300	9	—	2.70	"	11	" "
19	0.590	6	—	3.54	"	6	" "
				mean 3.97			

* In Exps. 4, 5, 9, 10, 14, 18 and 19 more concentrated solutions of venom were employed.

† Effective values of y (cp. p. 526).

‡ In Exps. 15 to 19 the plasma employed was not the same as that used for Exps. 1 to 14.

calculated. In control experiments of this type irregular variation from the calculated periods is met with more frequently than in experiments in which thrombokinase or thrombin has been added. If the experiments are repeated such differences do not appear to be constant, as may be seen by comparing the corresponding experiments in the preceding tables. The degree of

contamination of the plasma used can be determined as in the previous experiments, except that the estimation is now made in terms of thrombin instead of thrombokinase. Finally it will be noted that the values of xy selected (3.00, 1.476 and 0.461 respectively) are in the same ratio as the amounts of fibrinogen solution added (0.325 cc. in Exps. 1 to 5; 0.160 cc. in Exps. 6 to 10; 0.050 cc. in Exps. 11 to 14), equation (2) being valid, within the limits of experimental error, for these experiments with thrombin. In the second series of experiments shown in Table VIII (Exps. 15 to 19), in which the action of thrombin takes place in presence of potassium oxalate (a solution of fibrinogen different from that employed in the preceding experiments being used), the product xy is, as in the previous experiments, constant and the calculated values of x exhibit a good agreement with the observed values; in these experiments no correction for contamination of plasma is required, the plasma remaining uncoagulated when kept in presence of potassium oxalate. The observed coagulation times are reproduced in Fig. 3; the curves are calculated, *A* representing Exps. 1-5; *B* 6-10 and *C* 11-14.

The above experiments, which, like the preceding experiments made with thrombokinase, take place according to equation (2), indicate that the time of coagulation is essentially the period required for the conversion of fibrinogen into fibrin.

Since the experiments with thrombin in Table VIII indicate therefore that the influence of thrombin (y) upon the coagulation time (x) in presence of a given amount of fibrinogen (z) takes place according to equation (2)

$$xy = nz$$

(n in this particular series of experiments being 10.6), and since, in addition, this equation also expresses the action of thrombokinase in causing coagulation

Fig. 3. Curves for the experiments given in Table VIII. Abscissae, time in minutes; ordinates, cc. of 1 in 300,000 venom solution. The vertical line at 27 on the axis of abscissae represents the outer limit of investigation of the curves. Curve *C* corresponds to experiments in which 0.050 cc. of fibrinogen solution was employed, curve *B* to 0.160 cc. and curve *A* to 0.325 cc.

(Tables I to VII), it follows that thrombokinase and thrombin are interchangeable terms, in respect of fibrin production, in other words that a given amount of thrombokinase, in presence of calcium chloride, will produce a fixed amount of thrombin, whatever may be the concentration of prothrombin, provided of course that the latter is present in excess. As soon as this amount of thrombin has been produced the thrombokinase added has either disappeared or has become so far modified as to be incapable of further action in the direction of giving rise to the production of thrombin in presence of prothrombin and calcium chloride. In no other way can the similarity of the results obtained with thrombin (Table VIII) to those obtained with thrombokinase (Tables I to IV and V to VII) be explained.

The validity of equation (2) may be tested in a manner which in some respects offers an advantage as regards the ease with which simultaneous observations may be made. It is obvious that if, in the equation

$$xy = nz,$$

y is made proportional to z , i.e.

$$y = dz,$$

where d is a constant, then the equation becomes

$$xdz = nz,$$

or

$$x = \frac{n}{d} = \text{const.} \dots\dots\dots(5),$$

that is to say the coagulation time is independent of the concentration of fibrinogen present in solution. This condition is realised if thrombokinase is first added to fibrinogen solution and the mixture thus obtained introduced in varying amounts into tubes, each containing the same quantity of decinormal calcium chloride solution, the total volume of fluid in each case being made up to 0.5 cc. This was in fact done in the series of experiments recorded in Tables I to IV. Thus in the three experiments numbered 2, 7 and 12 a suitable admixture of thrombokinase and fibrinogen solutions was first made and from this amounts of 0.422 cc., 0.208 cc. and 0.065 cc. (containing respectively 0.325 cc., 0.160 cc. and 0.050 cc. of fibrinogen solution) were transferred to tubes into which the requisite amounts of N/10 CaCl_2 solution and 0.85 % NaCl solution had already been introduced; and so on with the remaining tubes with the exception of those numbered 1, 6 and 11, to which no thrombokinase was added.

It will be observed that corresponding tubes exhibit equi-coagulative periods, except in Exps. 12 and 13, Table IV; some of the causes of such variations are considered on p. 540.

THE INFLUENCE OF CALCIUM CHLORIDE UPON THE ACTION OF
THROMBOKINASE AND THROMBIN IN EFFECTING COAGULATION
OF A FIBRINOGEN SOLUTION.

The constant n in equation (2) (p. 516) is, in the mathematical sense, a function of all those potential variables which for these experiments are kept constant. Of the latter two are more immediately important, namely the concentration of CaCl_2 and the temperature, the former of which alone will be considered here.

To this end a series of forty experiments with thrombokinase, similar in type to those shown in Tables I and II, was carried out in the numerical order given in Tables IX and X. These experiments fall into two groups: in the first (Table IX) 0.325 cc. of fibrinogen solution was employed in each experiment; in the second (Table X) 0.160 cc. of fibrinogen solution was used.

TABLE IX.

Determination of the coagulation time of a series of mixtures of thrombokinase and fibrinogen (0.325 cc.) solutions in presence of CaCl_2 , a solution of NaCl (0.85 %) being added in amount sufficient to make a total volume of 0.5 cc. in each experiment.

No. of experiment	N/10 CaCl_2 solution (cc.)	^y Thrombokinase solution (testis of rabbit) diluted 1 in 1000 (cc.)	^x Time of coagulation (minutes)	$y_{\text{corrected}}$ (cc.)	Value of xy selected	$x_{\text{calculated}}$ (minutes)
1	0.075	0.1	44	0.148	7.98	54.0
3	"	0.2	31	0.248	"	32.2
5	"	0.3	23	0.348	"	22.9
7	"	0.6	13	0.648	"	12.2
9	"	1.0	10	1.048 [0.897]	"	7.6 [8.9]
11	0.125	0.1	50	0.148	9.42	63.6
13	"	0.2	39	0.248	"	38.0
15	"	0.3	23	0.348	"	27.1
17	"	0.6	13	0.648	"	14.5
19	"	1.0	10	1.048 [0.897]	"	9.0 [10.5]
21	0.5	0.1	300	0.148	42.8	289.5
23	"	0.2	129	0.248	"	173.0
25	"	0.3	100	0.348	"	123.2
27	"	0.6	59	0.648	"	66.0
29	"	1.0	46	1.048 [0.897]	"	40.8 [47.8]
31	1.5	0.1	> 6500	0.148	327.5	2218
33	"	0.2	> 5000	0.248	"	1323
35	"	0.3	> 4000	0.348	"	944
37	"	0.6	1200	0.648	"	505
39	"	1.0	780	1.048 [0.897]	"	313 [365]

In these two tables the experiments are seen to be arranged in four groups, each of five experiments, the amounts of decinormal CaCl_2 solution employed in each group being respectively 0.075 cc., 0.125 cc., 0.5 cc. and 1.5 cc. The experiments in each individual group are arranged, as in the preceding tables, in the order of the amount of thrombokinase introduced into each tube. As in the preceding experiments each tube contained 0.5 cc. of fluid. When the amount of thrombokinase or calcium chloride solution was so great that this amount would have been exceeded, more concentrated solutions were employed, but in the tables, for the sake of uniformity and consequent ease of calculation the amount used is always referred to the same standard of concentration.

As before the first step in dealing with these experiments was to determine, by means of the method given on p. 517, the amount of thrombokinase contained in the fibrinogen solution used and, at the same time, to ascertain if for each group values of xy could be obtained which would satisfy equation (1) (p. 513), namely

$$xy = \text{const.}$$

The amount of thrombokinase present in 0.325 cc. and 0.160 cc. of the fibrinogen solution employed was estimated to be equal to 0.048 cc. and 0.024 cc. respectively of the thrombokinase solution used. When these quantities were added to the thrombokinase employed, the total amount of thrombokinase taking part in each experiment became known and it was possible to select values representing the product of x (coagulation time) and y (amount of thrombokinase present) for each group. The values of xy so chosen yield, it will be seen, calculated values of x differing (except in cases to which reference will shortly be made) from the observed values only within the limits of experimental error.

It will further be seen that the values of xy (7.98 and 3.61 respectively) selected for the first group in Tables IX and X, in which the amount of CaCl_2 solution employed is lowest, are approximately in the ratio of the amounts of fibrinogen solution added (0.325 cc. and 0.160 cc. respectively), but that with increasing amounts of CaCl_2 a departure from this ratio is observed.

It has already been pointed out that certain calculated values of x differ from those actually observed, to an extent which lies beyond the range of experimental error. These fall into two groups: (1) those in which a very large amount of thrombokinase has been employed (Exps. 9, 19, 29 and 39, Table IX, and 8, 10, 18, 20, 28, 30, 38 and 40, Table X); and (2) those in

which a very large amount of CaCl_2 has been used (Exps. 31 to 40). The latter group will be dealt with later but the former may now conveniently be considered. For a long time this class of variations from the calculated value of the coagulation time (x) presented a difficulty, until it was observed that the coagulation time became stationary as soon as the amount of

TABLE X.

Determination of the coagulation time of a series of mixtures of thrombokinase and fibrinogen (0.160 cc.) solutions in presence of CaCl_2 , a solution of NaCl (0.85 %) being added in amount sufficient to make a total volume of 0.5 cc. in each experiment.

No. of experiment	N/10 CaCl_2 solution (cc.)	^y Thrombokinase solution (testis of rabbit) diluted 1 in 1000 (cc.)	^x Time of coagulation (minutes)	$y_{\text{corrected}}$ (cc.)	Value of xy selected	$x_{\text{calculated}}$ (minutes)
2	0.075	0.1	30	0.124	3.61	29.1
4	"	0.2	20	0.224	"	16.1
6	"	0.3	13	0.324	"	11.1
8	"	0.6	10	0.624 [0.442]	"	5.8 [8.2]
10	"	1.0	10	1.024 [0.442]	"	3.5 [8.2]
12	0.125	0.1	39	0.124	3.73	30.1
14	"	0.2	17	0.224	"	16.7
16	"	0.3	11	0.324	"	11.5
18	"	0.6	7	0.624 [0.442]	"	6.0 [8.5]
20	"	1.0	7	1.024 [0.442]	"	3.6 [8.5]
22	0.5	0.1	56	0.124	6.59	53.2
24	"	0.2	26	0.224	"	29.4
26	"	0.3	20	0.324	"	20.4
28	"	0.6	15	0.624 [0.442]	"	10.6 [14.9]
30	"	1.0	12	1.024 [0.442]	"	6.4 [14.9]
32	1.5	0.1	>6500	0.124	31.03	250.0
34	"	0.2	1440	0.224	"	138.7
36	"	0.3	780	0.324	"	95.7
38	"	0.6	780	0.624 [0.442]	"	49.7 [70.2]
40	"	1.0	780	1.024 [0.442]	"	30.3 [70.2]

thrombokinase (y) reached a certain value, beyond which further increase caused no appreciable shortening of the time of coagulation. Whatever may be the mode, physical or physico-chemical, in which this event takes place, its occurrence may be conveniently expressed by saying that in practice the effective maximum value of thrombokinase is fixed and cannot be exceeded. This value was determined approximately by substituting varying lower values of y for the highest amounts actually employed and selecting those

which gave the best concordance between the calculated and observed values of x^1 . Since the maximum effective values of y which were at first selected for the two series of experiments (Tables IX and X) were found to be approximately in the ratio of the amounts of fibrinogen solution employed, it was assumed that this relation actually existed and on this assumption the values finally adopted were chosen (0.897 cc. of thrombokinase solution when

TABLE XI.

The experiments recorded in Table IX, rearranged in a form suitable for calculating the values of a and b in the equation $w^2 = ax - b$. The observed coagulation times are given within brackets in the fourth column.

No. of experiment	w N/10 CaCl_2 solution (cc.)	y Thrombokinase solution (testis of rabbit) diluted 1 in 1000 (cc.)	$x_{\text{calculated}}$ Time of coagulation (minutes)	$a = \frac{w_1^2 - w^2}{x_1 - x}$	$b = ax - w^2$
9	0.075	0.897	[10] 8.9		0.0504
19	0.125	"	[10] 10.5	0.0062	"
29	0.5	"	[46] 47.8	0.0063	"
39	1.5	"	[780] 365.0	"	"
7	0.075	0.648	[13] 12.2		"
17	0.125	"	[13] 14.5	0.00455	"
27	0.5	"	[59] 66.0	"	"
37	1.5	"	[1200] 505.0	"	"
5	0.075	0.348	[23] 22.9		"
15	0.125	"	[23] 27.1	0.00246	"
25	0.5	"	[100] 123.2	"	"
35	1.5	"	[>4000] 944.0	"	"
3	0.075	0.248	[31] 32.2		"
13	0.125	"	[39] 38.0	0.00174	"
23	0.5	"	[129] 173.0	"	"
33	1.5	"	[>5000] 1323.0	"	"
1	0.075	0.148	[44] 54.0		"
11	0.125	"	[50] 63.6	0.00103	"
21	0.5	"	[300] 289.5	"	"
31	1.5	"	[>6500] 2218.0	"	"

0.325 cc. of fibrinogen solution was employed and 0.442 cc. of the former for 0.160 cc. of the latter). With the aid of these values of y and of the values of the product xy already selected the divergent values of x under consideration were recalculated; these are given within brackets in the last column of the tables and are seen to approach more nearly to the observed coagulation times than do the lower values obtained when the full amounts of thrombokinase present are taken.

¹ In this way the effective values of y in Table VIII were obtained.

In order to study the effect of CaCl_2 upon the time of coagulation the experiments given in Tables IX and X were rearranged in Tables XI and XII respectively according to the effective amounts of thrombokinase present. This course was adopted because it was found, when searching for a relationship between the amounts of CaCl_2 (w) present and the calculated coagulation times (x), in presence of a fixed amount of thrombokinase (y), that the coagulation times, when plotted as abscissae with the corresponding amounts

Fig. 4. Hyperbolic (x, y) and parabolic (x, w) curves for the experiments given in Tables IX and XI. Abscissae (x), time in minutes; ordinates, cc. of solution of thrombokinase (y) for hyperbolic curves ($\cdot$) or of N/10 calcium chloride (w) for parabolic curves ($\bullet$). The amount of fibrinogen solution employed in each experiment is 0.325 cc. The hyperbolic curve nearest to the axes corresponds to those experiments in which 0.075 cc. of decinormal calcium chloride solution was employed; the remaining hyperbolic curves to 0.125 cc., 0.5 cc. and 1.5 cc. respectively. The parabolic curve nearest to the axis of ordinates corresponds to those experiments in which 0.897 cc. of thrombokinase solution was employed; the remaining parabolic curves to 0.648 cc., 0.348 cc., 0.248 cc. and 0.148 cc. respectively.

of decinormal CaCl_2 solution as ordinates, lay on a curve which appeared to be parabolic (Fig. 4 for Tables IX and XI; Fig. 5 for Tables X and XII). If parabolic the values of x must satisfy the equation

$$w^2 = ax - b \quad \dots\dots\dots(6),$$

where a and b are constants. It will be observed that in each table five curves are investigated, corresponding to five different concentrations of

thrombokinase and that four points on each curve are determined. If only a single curve had been studied a larger number of points could have been determined, but the advantage of investigating simultaneously a series of values of w would have been lost.

The manner in which a and b were determined is sufficiently indicated in the 5th and 6th columns of Tables XI and XII. Guidance in selecting

TABLE XII.

The experiments recorded in Table X, rearranged in a form suitable for calculating the values of a and b in the equation $w^2 = ax - b$. The observed coagulation times are given within brackets in the fourth column.

No. of experiment	w N/10 CaCl ₂ solution (cc.)	y Thrombokinase solution (testis of rabbit) diluted 1 in 1000 (cc.)	$x_{\text{calculated}}$ Time of coagulation (minutes)	$a = \frac{w_1^2 - w^2}{x_1 - x}$	$b = ax - w^2$
10	0.075	0.442	[10] 8.2		0.290
20	0.125	"	[7] 8.5	0.0362	"
30	0.5	"	[12] 14.9	"	0.291
40	1.5	"	[780] 70.2	"	0.290
8	0.075	"	[10] 8.2		
18	0.125	"	[7] 8.5	0.0362	"
28	0.5	"	[15] 14.9	"	"
38	1.5	"	[780] 70.3	"	"
6	0.075	0.324	[13] 11.1		
16	0.125	"	[11] 11.5	0.0265	"
26	0.5	"	[20] 20.3	"	"
36	1.5	"	[780] 95.7	"	"
4	0.075	0.224	[20] 16.1		
14	0.125	"	[17] 16.7	0.01835	"
24	0.5	"	[26] 29.4	"	"
34	1.5	"	[1440] 138.7	"	"
2	0.075	0.124	[30] 29.1		
12	0.125	"	[39] 30.1	0.01015	"
22	0.5	"	[56] 53.2	"	"
32	1.5	"	[>6500] 250.0	"	"

final values for these constants is afforded by studying the effect, upon the recalculated values of x , produced by slightly varying the values first selected. When preliminary determinations of a and b had been made it was found that the values of a for each curve were roughly proportional to the amounts of thrombokinase employed, while the values of b were not far different for all the curves. It was therefore concluded that these indications were valid and values of a and b were accordingly selected which appeared best to fulfil the two conditions thus foreshadowed. It will be noted that, with the

values selected, the calculated coagulation times agree with those actually found, within the limits of experimental error for the first three experiments of each group, but that a divergence between the two occurs when the equivalent of 1.5 cc. of decinormal CaCl_2 solution is employed (Exps. 39, 37, 35, 33 and 31, Table XI; Exps. 40, 38, 36, 34 and 32, Table XII) and that the divergence is more marked when 0.160 cc. of fibrinogen solution is employed (Table XII) than when 0.325 cc. is used (Table XI). In other words equation (6) fails when the concentration of CaCl_2 is increased relatively to the fibrinogen solution employed to such an extent that the ratio of the

Fig. 5. Hyperbolic (x, y) and parabolic (x, w) curves for the experiments given in Tables X and XII. Abscissae (x), time in minutes; ordinates, cc. of solution of thrombokinase (y) for hyperbolic curves ($\bullet$) and of N/10 calcium chloride (w) for parabolic curves ($\bullet$). The amount of fibrinogen solution employed in each experiment is 0.160 cc. The hyperbolic curve nearest to the axes represents two curves, corresponding to those experiments in which 0.075 cc. and 0.125 cc. respectively of N/10 CaCl_2 were employed, the remaining hyperbolic curves to 0.5 cc. and 1.5 cc. respectively. The parabolic curve nearest to the axis of ordinates corresponds to those experiments in which 0.442 cc. of thrombokinase solution was employed; the remaining parabolic curves to 0.324 cc., 0.224 cc. and 0.124 cc. respectively.

two reaches 4.6 (Table XI). With this ratio the observed values of x are two to four times as great as the calculated; when the ratio becomes 9.4 (Table XII) the former are roughly ten times as great as the latter.

Since a has been found to be proportional to y , in other words since

$$a = ky,$$

where k is a constant, equation (6) may be written

$$w^2 = kxy - b \dots\dots\dots(7).$$

It was further observed that the values of xy when the amount of CaCl_2 (w) was small were approximately proportional to the amount of fibrinogen

solution (z) employed. It was therefore determined to introduce this relationship into the above equation by putting $k = p$, where p is a constant, and at the same time to express the constant b in the form of a multiple of p , namely pn , n of course being itself a constant. The equation thus becomes

$$w^2 = \frac{p}{z} xy - pn,$$

or
$$w^2 = p \left(\frac{xy}{z} - n \right) \dots\dots\dots(8).$$

If in the forty experiments at present under consideration approximate values of xy are first determined, in the manner indicated in Tables IX and X, and if from these the values of the constants p and n are next determined, it will be found that the estimated values of n lie close together for all the groups in both series of experiments, so that it seems legitimate to infer that n is independent of variations of z . This inference was adopted in calculating p and n in the experiments recorded in Tables IX to XII, the values selected being

$$\begin{aligned} n &= 22.12, \\ p_{0.325} &= 0.00228, \\ p_{0.160} &= 0.0131. \end{aligned}$$

The mode of determining the two constants is illustrated in the series of experiments recorded in Tables XIII to XVI.

Having determined the values of the constants p and n from the approximate values of xy first reached according to the method indicated in Tables IX and X, the values of the product xy for varying concentrations of CaCl_2 may now be recalculated by means of equation (8). It thus becomes possible to determine the slight variations in the value of xy corresponding to the lower values of w , variations which may be too small to be determined satisfactorily by direct observation of coagulation times. It was in this way that the final values of xy , given in Tables IX and X were chosen, the effect of slight variations of p and n upon the calculated values of xy having been carefully studied before the values of these constants ultimately selected were adopted.

It may here be pointed out as a matter of practical convenience that the determination of p and n [equation (8)] is much simpler than the estimation of a and b [equation (6)] or of k and b [equation (7)]. As already mentioned n is independent of w , xy and z ; p remains a function of z , but is independent of xy and w . If $w = 0$ in equation (8), a condition of experiment which

cannot be realised experimentally however, xy becomes proportional to z and equation (2) (p. 516) is obtained, thus

$$\frac{xy}{z} - n = 0,$$

or

$$xy = nz.$$

It is thus seen that equation (2) is theoretically admissible only if w is relatively very small. If w is not sufficiently small to be negligible, then the complete formula

$$xy = \left(\frac{w^2}{p} + n \right) z$$

must be used.

Equation (6) is investigated in Tables XI and XII with a minimal addition of 0.075 cc. of decinormal calcium chloride solution (w) in each 0.5 cc. of fluid. The equation is valid until w falls to 0.03 cc.; below this amount a gradual lengthening of the coagulation time occurs (the amount of thrombokinase added remaining, as before, constant), the observed values of x being greater than the calculated values, so that equations (6) to (8) are no longer applicable. The complete w, x curve which is of the type shown in 2, Fig. 8 (p. 539) appears to be made up of two curves: (1) the parabolic curve already described; and (2) a flat curve, lying below 0.03 on the axis of ordinates, which as it proceeds to the right approaches the axis of abscissae. The latter curve, the accurate investigation of which is difficult, will not be further considered here; it appears to represent the extent to which thrombin has been produced from prothrombin in presence of relatively low percentages of calcium chloride. The form of the complete curve shows that it is not a hyperbola, such as is obtained when the inhibitory action of amboceptor upon complement is represented [Barratt 1913, 2 and 3]; the application of the equation for a hyperbola to the complete w, x curve is impossible.

The enquiry now presents itself, whether the influence of calcium chloride upon coagulation is concerned with the activity of thrombokinase or with that of thrombin. To this end the series of experiments recorded in Tables IX to XII were repeated, with thrombin, in the form of snake venom, in place of thrombokinase. In these experiments (Tables XIII and XIV) the values of xy were first determined as in the preceding series and it is seen that equation (1)

$$xy = \text{const.}$$

is applicable just as when thrombokinase is employed, allowance being made as before for the amount of thrombokinase already present in the fibrinogen

solution employed, this being determined in terms of the thrombin solution employed. It will be observed, however, that in addition to the four groups given in each of the preceding Tables (IX and X) an additional group has been added in which no calcium chloride has been used, venom thrombin (and the same is true of thrombin produced from prothrombin by the action of thrombokinase) being capable of causing coagulation of fibrinogen when no calcium chloride has been added or when, by adding potassium oxalate,

TABLE XIII.

Determination of the coagulation time of a series of mixtures of venom and fibrinogen (0.325 cc.) solutions in presence of CaCl_2 , a solution of NaCl (0.85 %) being added in amount sufficient to make a total volume of 0.5 cc. in each experiment.

No. of experiment	N/10 CaCl_2 solution (cc.)	Venom (<i>Echis carinatus</i>) solution, 1 in 900,000 (cc.)	Time of coagulation (minutes)	$y_{\text{corrected}}$ (cc.)	Value of xy selected	$x_{\text{calculated}}$ (minutes)
I 1	—	4.5	6	4.650	24.30	5.2
11	—	0.6	35	0.750	"	32.4
21	—	0.115	102	0.265	"	91.5
31	—	0.045	120	0.195	"	124.0
41	—	0.015	152	0.165	"	147.0
51	—	—	510	0.150	"	162.0
II 3	0.075	4.5	4	2.94 [4.604]	11.12	3.8
13	"	0.6	12	0.704	"	15.8
23	"	0.035	50	0.209	"	53.2
33	"	0.015	76	0.149	"	74.7
43	"	0.005	122	0.119	"	93.5
53	"	—	160	0.104	"	107.0
III 5	0.125	4.5	4	2.94 [4.604]	11.25	3.8
15	"	0.6	12	0.704	"	16.6
25	"	0.115	42	0.209	"	53.9
35	"	0.045	76	0.149	"	75.5
45	"	0.015	102	0.119	"	94.5
55	"	—	160	0.104	"	108.0
IV 7	0.5	4.5	5	2.94 [4.604]	14.19	4.8
17	"	0.6	18	0.704	"	20.1
27	"	0.115	62	0.209	"	67.9
37	"	0.045	100	0.149	"	95.1
47	"	0.015	137	0.119	"	119.2
57	"	—	182	0.104	"	136.2
V 9	1.5	4.5	44	2.94 [4.604]	39.10	13.3
19	"	0.6	94	0.704	"	55.5
29	"	0.115	330	0.209	"	187.3
39	"	0.045	413	0.149	"	262.3
49	"	0.015	640	0.119	"	319.5
59	"	—	640	0.104	"	376.0

the calcium salt present in solution has been reduced to a point determined by the extremely low solubility of calcium oxalate. It is further to be noted that when no calcium chloride has been added the time of coagulation, after the addition of varying amounts of venom, is longer than is the case in presence of a small quantity of calcium chloride. For the present, however, attention will be given only to the IIInd to Vth groups of Tables XIII and XIV, which alone can be compared with the preceding experiments with thrombokinase in Tables IX and X.

TABLE XIV.

Determination of the coagulation time of a series of mixtures of venom and fibrinogen (0.050 cc.) solutions in presence of CaCl_2 , a solution of NaCl (0.85 %) being added in amount sufficient to make a total volume of 0.5 cc. in each experiment.

No. of experiment	N/10 CaCl_2 solution (cc.)	Venom (<i>Echis carinatus</i>) solution, 1 in 900,000 (cc.)	x , Time of coagulation (minutes)	$y_{\text{corrected}}$ (cc.)	Value of xy selected	$x_{\text{calculated}}$ (minutes)
I						
2	—	4.5	4	4.560	7.30	1.6
12	—	0.6	11	0.660	"	11.0
22	—	0.115	35	0.175	"	41.7
32	—	0.045	76	0.105	"	69.6
42	—	0.015	102	0.075	"	97.2
52	—	—	640	0.060	"	122.0
II						
4	0.075	4.5	4	0.453 [4.516]	1.73	3.8
14	"	0.6	5	0.453 [0.616]	"	3.8
24	"	0.115	16	0.121	"	14.3
34	"	0.045	25	0.061	"	28.4
44	"	0.015	49	0.031	"	35.8
54	"	—	103	0.016	"	108.1
III						
6	0.125	4.5	4	0.453 [4.516]	1.80	4.0
16	"	0.6	5	0.453 [0.616]	"	4.0
26	"	0.115	16	0.121	"	14.9
36	"	0.045	25	0.061	"	29.5
46	"	0.015	55	0.031	"	58.0
56	"	—	102	0.016	"	112.4
IV						
8	0.5	4.5	6	0.453 [4.516]	3.26	7.2
18	"	0.6	8	0.453 [0.616]	"	7.2
28	"	0.115	27	0.121	"	27.0
38	"	0.045	48	0.061	"	53.5
48	"	0.015	93	0.031	"	105.0
58	"	—	180	0.016	"	204.0
V						
10	1.5	4.5	31	0.453 [4.516]	15.75	34.8
20	"	0.6	40	0.453 [0.616]	"	34.8
30	"	0.115	159	0.121	"	130.1
40	"	0.045	248	0.061	"	258.0
50	"	0.015	510	0.031	"	507.4
60	"	—	> 2500	0.016	"	985.0
Bioch. rx						35

When calcified venom was employed the lowest values of xy first selected in the two series (11.12 and 1.73 were ultimately chosen) were found to be very nearly in the ratio of the amounts of fibrinogen solution added (0.325 cc. and 0.050 cc. respectively), indicating that these groups conformed approximately to equation (2)

$$xy = nz.$$

It was also found that the thrombin of venom was effective until an amount equivalent to 2.94 cc. of the venom solution for the first series (Table XIII) and 0.453 cc. for the second series (Table XIV) was used; increase beyond this amount did not cause a corresponding diminution of the coagulation

TABLE XV.

The experiments recorded in Table XIII rearranged in a form suitable for calculating the values of p and n in the equation $w^2 = p \left(\frac{xy}{z} - n \right)$. The observed coagulation times are given within brackets in the fourth column.

No. of experiment	w N/10 CaCl_2 solution (cc.)	y Venom solution (<i>Echis carinatus</i>), 1 in 300,000 (cc.)	$x_{\text{calculated}}$ Time of coagulation (minutes)	xy	$p = \frac{w_1^2 - w^2}{x_1 y_1 - xy}$	$n = \frac{xy}{z} - \frac{w^2}{p}$
3	0.075	2.94	[4] 3.8	11.12		34.01
5	0.125	"	[4] 3.8	11.25	0.026	34.04
7	0.5	"	[5] 4.8	14.19	"	34.07
9	1.5	"	[44] 13.3	39.10	"	33.90
13	0.075	0.704	[12] 15.8	11.12		34
15	0.125	"	[12] 16.6	11.25	"	"
17	0.5	"	[18] 20.1	14.19	"	"
19	1.5	"	[94] 55.5	39.10	"	"
23	0.075	0.209	[50] 53.2	11.12		"
25	0.125	"	[42] 53.9	11.25	"	"
27	0.5	"	[62] 67.9	14.19	"	"
29	1.5	"	[330] 187.3	39.10	"	"
33	0.075	0.149	[76] 74.7	11.12		"
35	0.125	"	[76] 75.5	11.25	"	"
37	0.5	"	[100] 95.1	14.19	"	"
39	1.5	"	[413] 262.3	39.10	"	"
43	0.075	0.119	[122] 93.5	11.12		"
45	0.125	"	[102] 103.2	11.25	"	"
47	0.5	"	[137] 119.2	14.19	"	"
49	1.5	"	[640] 319.5	39.10	"	"
53	0.075	0.104	[160] 107.0	11.12		"
55	0.125	"	[160] 108.0	11.25	"	"
57	0.5	"	[182] 136.2	14.19	"	"
59	1.5	"	[640] 376.0	39.10	"	"

time. In these respects the experiments with venom resemble those already made with thrombokinase and are in agreement with the series shown in Table VIII.

The experiments with calcified venom were now, like the series given in Tables IX and X, rearranged (Tables XV and XVI) for convenience of ascertaining whether or no equation (8)

$$w^2 = p \left(\frac{xy}{z} - n \right)$$

was applicable. It was found that (except in the case of the last group of experiments shown in Tables XIII and XIV) with very slight changes in the values of xy first chosen, values of p and n (given in Tables XV and XVI) could be selected such that the coagulation times recalculated therefrom, given in the tables, were in agreement with the observed periods, within the limits of experimental error. The curves for Tables XIII and XV are given in Fig. 6; those for Tables XIV and XVI in Fig. 7.

It thus appears that in presence of calcium chloride both series (Tables IX to XII and XIII to XVI respectively) behave alike, the same equation being applicable equally to the experiments made with thrombokinase and thrombin. The inhibitory effect of this salt upon fibrin production is therefore manifested in respect of the interaction of thrombin and fibrinogen, the action of thrombokinase upon prothrombin being unaffected. Calcium chloride moreover in the concentrations employed produces from the first retardation of the process of coagulation of fibrinogen by means of thrombin.

If the constants p and n for the experiments with thrombokinase and snake venom are compared as shown below, it will be observed that a striking

		n	p			
			Fibrinogen solution employed in each experiment	0.325 cc.	0.160 cc.	0.050 cc.
Thrombokinase (testis of rabbit) ...	...	22.12	0.00228	0.0131	—	
Thrombin (venom of <i>Echis carinatus</i>) ...	...	34.00	0.026	—	0.0080	

difference occurs between the two in respect of the constant p . In the former experiments p increases with diminution of the amount of fibrinogen solution employed; in the latter the reverse occurs. This is readily explicable on the assumption that different kinds of thrombin are present in the two series of experiments. In reality the conditions of experiment are unavoidably complex, for the thrombin present comes from four sources: (1) that produced

by the action of thrombokinase, contained in relatively small amount in the blood plasma of the fowl, upon fowl's prothrombin in presence of calcium chloride; (2) that produced by the action of thrombokinase from the testis of the rabbit upon fowl's prothrombin; (3) that produced by the action of thrombokinase, contained in relatively small amount in snake venom (cf. p. 540), upon fowl's prothrombin; and (4) that contained in snake venom.

Fig. 6. Hyperbolic (x, y) and parabolic (x, w) curves for the experiments given in Tables XIII and XV. Abscissae (x), time in minutes; ordinates, cc. of 1 in 300,000 solution of venom (y) for hyperbolic curves (•) or of N/10 CaCl_2 (w) for parabolic curves (●). The amount of fibrinogen solution employed in each experiment is 0.325 cc. The hyperbolic curve nearest to the axes represents two curves, corresponding to those experiments in which 0.075 cc. and 0.125 cc. respectively of N/10 CaCl_2 solution were employed, the remaining hyperbolic curves to 0.5 cc. and 1.5 cc. respectively. The parabolic curve nearest to the axis of ordinates corresponds to those experiments in which 2.94 cc. of venom solution was employed; the remaining parabolic curves to 0.704 cc., 0.209 cc., 0.149 cc., 0.119 cc. and 0.104 cc. respectively.

The chief sources of thrombin are (2) and (4). The enquiry naturally arises, Is the thrombin produced from fowl's prothrombin independent of the source of the thrombokinase employed? The difference in the values of p in the two series of experiments appears to indicate that the thrombin of snake venom is different from that produced by the action of the thrombokinase of the rabbit's testis upon fowl's prothrombin; thrombin produced by the action of the thrombokinase of snake venom upon fowl's prothrombin is

present in too small an amount to enable a safe comparison with the other thrombins under consideration to be made. That the thrombin derived from fowl's prothrombin by the action of fowl's thrombokinase (both contained in the fibrinogen solution employed)—the only auto-thrombin present in these experiments—is not similar to snake venom thrombin is suggested by the circumstance that the more marked divergences between observed and calculated values of x are seen in those experiments in Tables XIII and XIV in which the amount of thrombokinase present is low and the amount of the former variety of thrombin is relatively high, e.g. Exps. 53 and 43 of the second group in Table XIII and similar experiments in adjoining groups.

TABLE XVI.

The experiments recorded in Table XIV rearranged in a form suitable for calculating the values of p and n in the equation $w^2 = p\left(\frac{xy}{z} - n\right)$. The observed coagulation times are given within brackets in the fourth column.

No. of experiment	w N/10 CaCl_2 solution (cc.)	y Venom solution (<i>Echis carinatus</i>), 1 in 300,000 (cc.)	$x_{\text{calculated}}$ Time of coagulation (minutes)	xy	$p = \frac{w_1^2 - w^2}{\frac{x_1 y_1}{z} - \frac{xy}{z}}$	$n = \frac{xy}{z} - \frac{w^2}{p}$
2	—	0.453	[4] 3.7	1.70		34.00
4	0.075	"	[4] 3.8	1.73	0.0080	34.00
6	0.125	"	[4] 4.0	1.80	"	33.99
8	0.5	"	[6] 7.2	3.26	"	33.95
10	1.5	"	[31] 34.8	15.75	"	33.85
14	0.075	"	[5] 3.8	1.73	"	34
16	0.125	"	[5] 4.0	1.80	"	"
18	0.5	"	[8] 7.2	3.26	"	"
20	1.5	"	[40] 34.8	15.75	"	"
24	0.075	0.121	[16] 14.3	1.73	"	"
26	0.125	"	[16] 14.9	1.80	"	"
28	0.5	"	[27] 27.0	3.26	"	"
30	1.5	"	[159] 157.4	15.75	"	"
34	0.075	0.061	[25] 28.4	1.73	"	"
36	0.125	"	[25] 29.5	1.80	"	"
38	0.5	"	[48] 53.5	3.26	"	"
40	1.5	"	[248] 258.0	15.75	"	"
44	0.075	0.031	[49] 35.8	1.73	"	"
46	0.125	"	[55] 58.0	1.80	"	"
48	0.5	"	[93] 105.0	3.26	"	"
50	1.5	"	[510] 507.4	15.75	"	"
54	0.075	0.016	[103] 108.1	1.73	"	"
56	0.125	"	[102] 112.4	1.80	"	"
58	0.5	"	[180] 204.0	3.26	"	"
60	1.5	"	[> 2500] 985.0	15.75	"	"

The same process of reasoning applies when, for the latter variety of thrombin, that produced from fowl's prothrombin by the action of rabbit's thrombokinasase (testis) is substituted (Tables IX and X). It is interesting to observe that in the former case the observed coagulation times for low amounts of thrombokinasase are longer than the calculated, while in the latter case the reverse occurs.

Fig. 7. Hyperbolic (x, y) and parabolic (x, w) curves for the experiments given in Tables XIV and XVI. Abscissae (x), time in minutes; ordinates, cc. of 1 in 300,000 solution of venom (y) for hyperbolic curves ($\bullet$) or of N/10 CaCl_2 (w) for parabolic curves ($\bullet$). The amount of fibrinogen solution employed in each experiment is 0.050 cc. The hyperbolic curve nearest to the axes represents two curves, corresponding to those experiments in which 0.075 cc. and 0.125 cc. respectively of N/10 CaCl_2 were employed, the remaining hyperbolic curves to 0.5 cc. and 1.5 cc. respectively. The parabolic curve nearest to the axis of ordinates corresponds to those experiments in which 0.453 cc. of venom solution was employed; the remaining parabolic curves to 0.121 cc., 0.061 cc., 0.031 cc. and 0.016 cc. respectively.

It has already been pointed out that equation (8)

$$w^2 = p \left(\frac{xy}{z} - n \right)$$

only applies so long as the concentration of calcium chloride does not exceed a limit, which is dependent upon the concentration of fibrinogen. When this limit has been passed, the retardation of coagulation becomes greater than

that calculated from equation (8): in other words, the calculated values of xy are lower than the observed values (cp. the last group of experiments in Tables IX, X, XIII and XIV). In the experiments made with thrombokinase (testis of rabbit) the observed retardation was greater when the concentration of fibrinogen was diminished (0.325 cc. was used in each experiment in Table IX; 0.160 cc. in Table X); with thrombin obtained from venom the observed retardation was more marked when the concentration of fibrinogen was increased (0.325 cc. in Table XIII; 0.050 cc. in Table XIV). This circumstance, which is indicated by the increase of p with diminution of z (fibrinogen) in the former experiments and its decrease with increase of z in the latter, is in its ultimate aspect an index of the physical conditions of experiment. Presumably with an auto-thrombin derived from the same source as the fibrinogen employed, p would increase with decrease of z , while with a hetero-thrombin a smaller increase or an actual decrease would occur.

So far we have only considered those experiments in which calcified venom has been used. If no calcium chloride is employed a retardation of the time of coagulation occurs as is exhibited in the first group of experiments in Tables XIII and XIV; with amounts of calcium chloride considerably below 0.075 cc. in each experiment a less marked retardation occurs. The complete w, x curve for snake venom thrombin is therefore of the type shown in 1, Fig. 8. Any attempt to apply the equation for a hyperbola to this curve is unsuccessful, as is obvious from the aspect of the curve. It appears to consist of two parts: (1) the parabolic curve just described; and (2) a low descending curve. The complete curve thus presents a general resemblance to the former curve furnished by thrombokinase (2, Fig. 8). In both an upper parabolic curve is joined below by a lower flat curve. But here the resemblance ceases, the lower curve obtained with thrombokinase approaching but not touching the axis of abscissae, while that obtained with venom thrombin cuts this axis; the former as already mentioned is due to the action of thrombokinase upon prothrombin in presence of minimal amounts of calcium chloride, the latter is due to an accelerator action of calcium chloride upon venom thrombin, an effect which is produced with very low concentrations of the calcium salt and is speedily followed by the inhibitory

Fig. 8. Type curves. Abscissae, time in minutes; ordinates, calcium chloride. (cc.)

action investigated above. It is important to distinguish this action of calcium chloride in low concentration upon venom thrombin from the action exerted upon thrombokinase in presence of prothrombin contained in the fibrinogen solution employed. In point of fact the venom solution contained thrombokinase, for upon heating to 100° for five minutes a solution was obtained free from thrombin, but containing enough thrombokinase to yield in presence of prothrombin and calcium chloride an amount of thrombin equal to about one-twentieth of that originally present, a quantity which if applied as a correction in Tables XIII and XIV would cause a very slight decrease of some of the calculated coagulation periods. Evidence is thus afforded of the increased coagulative activity of venom thrombin in presence of minimal amounts of calcium chloride. Presumably the same effect under similar conditions of experiment is also produced with thrombin derived from the action of thrombokinase (testis of rabbit) upon fowl's prothrombin.

Before concluding this article further reference may be made to the differences between observed and calculated times of experiment exhibited in the tables. Two sources of these differences, namely (1) that due to the circumstance that thrombin has a maximum effective value and (2) that caused by the concentration of calcium chloride exceeding a limiting value, have already been considered. In addition a certain amount of irregularity is observable, which appears to be at present unavoidable in experiments upon coagulation. Although it is not possible to deal with all the sources of experimental error, some of which appear still to elude detection, while others, e.g. decomposition and evaporation, are variable and especially likely to become effective when the coagulation period is long, nevertheless two sources of irregularity of result to which special attention has been given may be referred to here, namely that due to error of observation of the completion of coagulation and that caused by imperfect measurement of the amounts of the various solutions employed.

The former is a serious difficulty when quantitative work upon coagulation is first undertaken. The problem is to judge when coagulation is complete, that is to say when the whole of the fibrin has separated out. The indications that this has occurred are that the clot has reached (1) its maximum consistence and (2) its maximum opacity; the latter, however, does not appear to be so valuable an indication as the former. Since there is no sharp end point, precise measurement of coagulation time is not possible. Even with the training of observation resulting from the repeated performance of serial experiments, in which the degrees of coagulation in mixtures of slightly

different composition are constantly being compared, the measurements made must be regarded as only approximate. Difficulty is sometimes caused by coagulation being irregular, fibrin separating out to form a framework of strands of varying length and thickness, the fibrils being no longer distributed uniformly throughout the clot; this condition which is occasionally troublesome has been met with chiefly when the amount of fibrinogen has been small, but it sometimes appears when relatively large amounts of fibrinogen have been employed. To judge of the consistence of a clot it is necessary to agitate or shake the tube in which the clot is contained. If this is not done gently a soft clot may be detached before complete separation of fibrin has occurred; contraction of the clot may then take place followed by the appearance of a second coagulum, usually of soft consistence, in which case the determination of the coagulation time cannot be made with much approach to precision.

A second source of error arises from the difficulty of delivering accurately the small amounts of fluid which must necessarily be dealt with when large numbers of experiments have to be made with limited quantities of fibrinogen solution. It must be borne in mind that it is not the amount of fluid which is *contained* in the measuring pipette, but the quantity which is actually *delivered*, which requires accurate measurement. Consequently the amounts actually delivered must be determined and also the extent of the variations occurring between successive deliveries from the same scale mark. When this has been done the degree in which the time of coagulation is likely to be affected by unavoidable error of delivery can be estimated with the aid of the data given in the tables. The tendency of fluid to adhere to glass in the neighbourhood of the mouth of a pipette may be overcome by coating the outside of the end of the pipette with hard paraffin. Any greasy material present on the inner surface of the pipette interfering with filling and delivery is best removed by heating to a temperature just sufficient to cause any organic matter present to become charred; the inner surface of the pipette is then readily wetted by water. In performing a series of experiments each pipette should be used exclusively for one constituent of the mixture of liquids employed; if used for several constituents in succession, very careful cleaning may not suffice to avoid contamination. In practice, when performing serial experiments similar to those recorded in the tables, it will be found convenient to prepare a series of dilutions of calcium chloride such that an equal amount of fluid is delivered in each experiment, the same pipette, filled to the same scale mark, being used for each tube, proceeding from lower

to higher concentrations, and being washed out several times with each liquid of higher concentration before commencing to deliver. The solutions of thrombokinase were similarly prepared and dealt with. In order to destroy traces of thrombokinase remaining adherent to the inner surface of the tubes after coagulation has been completed, the latter were, after being previously cleaned, heated just below redness before being again employed.

In conclusion I desire to express my indebtedness to Dr C. J. Martin for the valuable assistance he has given me in connection with this research.

SUMMARY.

(1) A given amount of thrombokinase, in presence of calcium chloride and of a sufficient amount of prothrombin, will produce a definite quantity of thrombin, which is independent of the actual concentration of prothrombin.

(2) In experiments with thrombokinase the coagulation time is concerned with the action of thrombin upon fibrinogen.

(3) The action of thrombin (y) upon fibrinogen (z) when a decinormal calcium chloride solution is present to the extent of 0.05 cc. in a total volume of 0.5 cc. proceeds approximately according to the equation

$$xy = nz,$$

where x = coagulation time and n is a constant.

(4) The effect of adding decinormal calcium chloride solution, in amounts exceeding 0.05 cc. per 0.5 cc. of fluid, to (1) snake venom thrombin or (2) a mixture of thrombokinase and prothrombin is to cause retardation of coagulation of fibrinogen. If the coagulation times (x) are plotted against the amounts of calcium chloride employed, the points so obtained are found to lie on a parabola given by the equation

$$w^2 = ax - b,$$

so long as the amount of calcium chloride lies below a critical limiting value, beyond which this equation is no longer valid. Of the two constants in the equation a is proportional to the amount of thrombokinase present; b is a function of z , whose form has not been determined.

(5) The above action of thrombin upon fibrinogen takes place according to the general equation

$$w^2 = p \left(\frac{xy}{z} - n \right),$$

where n is a constant, independent of w , xy or z , and p is a function of z , not yet determined.

(6) Minimal quantities of calcium chloride (i.e. amounts not exceeding 0.05 cc. of a decinormal solution, contained in a total volume of fluid amounting to 0.5 cc.) accelerate the coagulant activity of thrombin furnished by the venom of *Echis carinatus*.

REFERENCES.

- Barratt, J. O. W. (1913, 1). *Proc. Roy. Soc. B*, **87**, 177.
— (1913, 2). *J. Path. Bacteriol.*, **18**, 270.
— (1913, 3). *Zeitsch. Immun. exp. Therap.*, **18**, 384.
Fuld, E. (1902). *Beiträge*, **2**, 514.
Martin, C. J. (1905). *J. Physiol.*, **32**, 207.
Rettger, L. J. (1909). *Amer. J. Physiol.*, **24**, 406.