ON THE ASSAY OF THROMBIN PREPARATIONS

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Different techniques of assaying thrombic activity have been compared. The relative strength of three commercial thrombin preparations has been compared with that of an arbitrary standard. Heparinized plasma as used by Burstein and Guinand and TAME, toluene-sulphonyl-arginine methyl ester, as used by Sherry and Troll are both unsuitable as clotting substrates. Only purified fibrinogen can be used. Fibrinogen prepared according to M. and B. Blombäck proved to be suitable, giving only 3–7 per cent error in the thrombin determinations. It is suggested that the strength of the thrombin standard should be expressed by its capacity to split *N*-terminal glutamic acid from fibrinogen with the appearance of *N*-terminal glycine.

Since 1949 a committee has been working on the preparation of a Scandinavian Pharmacopoeia for the three Scandinavian countries and Finland. It is hoped that the first edition will be published within a few years. Simultaneously a subcommittee, Nordisk Biologisk Standardiseringskommitté, NBS, has been engaged in selecting or working out methods for the assay of the activity of biological products, vitamins, hormones, antibiotics, and cardiac drugs, as well as toxicity tests. Also included are assays of heparin and thrombin preparations.

A critical analysis was made in this laboratory of the methods in use for the assay of heparin preparations[1–4] and the results were summarised by one of the present authors[5]. Only whole blood methods using fresh whole blood or blood preserved with sodium sulphate according to the B.P. 1953 method could be recommended, since they gave the same result as *in vivo* tests in sheep. An extended use of the B.P. 1953 method in the routine assay of heparin has since proved it to be comparable to the whole blood method using fresh ox blood. It has therefore been recommended by the subcommittee for the new pharmacopoeia.

This paper is concerned with the determination of thrombin activity. The disagreement in the results obtained with different methods is still more pronounced than with the assay of heparin. The very sensitive enzyme reactions involved in blood coagulation are strongly influenced by the milieu. Hence endeavours should be made to eliminate all external factors and to work with a thrombin-fibrinogen system which is as pure as possible.

Plasma Methods

In the earlier techniques oxalated bovine plasma was commonly used as a clotting medium. Astrup and Darling[6,7], defined as a thrombin unit the amount of thrombin which will clot 1 ml. of oxalated ox plasma in

30 seconds at 37°, the concentration of thrombin in the test being proportional to the reciprocal of the clotting time. Subsequently they found that plasma was not suitable for the purpose. The individual variations were too large and the relation between thrombin concentration and the reciprocal of the clotting time was not always linear. Plasma is, however, still recommended for clinical analyses[8].

For the assay of thrombin preparations of differing strengths and purities, plasma methods cannot be used because of the presence of varying amounts of different less well defined antithrombins in plasma.

Fibrinogen Methods

At an early date Warner, Brinkhous and Smith[9] introduced the use of purified fibrinogen as a clotting medium. The fibrinogen was precipitated from oxalated bovine plasma with 1/3 volume of saturated ammonium sulphate solution, reprecipitated twice and dialysed for 90 minutes against an oxalated saline solution, the final volume being 1/3 of the original plasma volume. They also suggested the use of a clotting interval of 15 seconds as a standard for determining the thrombin unit. The extent to which the unknown solution was to be diluted to give a 15 second clotting time gave a measure of the amount of thrombin present. An interpretation curve was used to correct for deviations of one or two seconds from the 15 second end point. A 15 second coagulation time with a plasma diluted for 1 : 223 corresponded to a concentration of 223 thrombin units/ml. of undiluted plasma.

The technique for the use of purified fibrinogen for the bioassay of thrombin was elaborated by Seegers, Brinkhous, Smith and Warner[10]. The clotting medium contained a stabilising substance, 2 per cent acacia, dissolved in 0·9 per cent physiological saline solution. The pH of the reagent was 7·1 to 7·3 and the temperature 26–29°. Dried thrombin was used as a standard. In studying the details of the method Seegers and Smith[11] demonstrated the difference between plasma and fibrinogen in this respect. Thrombin prepared from bovine plasma did not clot pig plasma as well as it clotted bovine plasma, whereas purified fibrinogen of the two species clotted with equal ease. Some kinds of animal plasmas needed 4 to 5 times more of the bovine thrombin than did the same volume of bovine plasma for coagulation in 15 seconds at 28°.

In 1942 Astrup and Darling[12] used fibrinogen prepared in a number of different ways for the thrombin assay. They found the ammonium sulphate-precipitated bovine fibrinogen to be superior to the Mellanby fibrinogen recovered from the euglobulin fraction. On that occasion they made an extensive study of the different procedures then available for the preparation of fibrinogen. Their technique of assaying thrombin activity was described as follows. To 0·1 ml. of a thrombin solution, 1·0 ml. of ice-cold fibrinogen solution was added and the mixture placed in a water bath at 37°. With the Mellanby fibrinogen no linear relation but a curved line was obtained, whereas the ammonium sulphate-precipitated fibrinogen gave a straight line passing through zero; the thrombin concentration being proportional to the reciprocal of the clotting time.

The Present Work

Before studying the fibrinogen methods we decided to apply one of the plasma methods, that of Burstein and Guinand[13], and the method of Sherry and Troll[14] using toluene-sulphonyl-arginine methyl ester (TAME) as substrate. As expected, neither the plasma method of Burstein and Guinand nor the ester splitting method of Sherry and Troll gave satisfactory responses with the thrombin enzyme. Therefore only fibrinogen methods remained to be tried. For this purpose we selected as substrate the highly purified bovine fibrinogen prepared by Blombäck and Blombäck[15] with their glycine extraction technique. When the clottability with thrombin is 94 to 97 per cent, this fibrinogen may be regarded as free from prothrombin, plasmin and proactivators of plasminogen.

We compared three commercial brands of thrombin: Thrombin Topical (Parke, Davis & Co., Detroit, Mich., U.S.A.) No. 030164–B, Topostasin "Roche" (F. Hoffman-La Roche, Basel) No. B 501087 and Thrombin Upjohn (The Upjohn Company, Kalamazoo, Mich., U.S.A.) No. FA. 620, against a thrombin standard of our own, T 49.

The same number of each brand was used throughout the experiments. In order to prevent inactivation by adsorption to the glass surfaces, all thrombin solutions were stored in siliconised vessels at 0°.

Determination of Thrombin by the Method of Burstein and Guinand

Principle. Thrombin is allowed to act in the presence of heparin upon bovine oxalated plasma whereby the antithrombic activity of the heparin is utilised. To 2 ml. of bovine oxalated plasma is added 1 ml. of a heparinised thrombin solution of varying thrombin concentration. The time necessary for the mixture to reach a certain optical density is noted. A curve is plotted showing the relation between this time and the thrombin concentration of the solution.

Standard preparation. Thrombin T 49. *Material.* Bovine oxalated plasma (1 vol. 0·1M sodium oxalate + 9 vol. blood), Heparin Vitrum 1000 IU/ml., NaCl (0·15M) for dilution of the thrombin and heparin solutions.

Procedure. A Coleman Jr. spectrophotometer is adjusted to zero against a blank consisting of 2 ml. plasma and 1 ml. of 0·15M NaCl at a wavelength of 600 mμ. The optical density of clotted plasma 60 seconds after addition of 1 ml. of T 49 thrombin solution with 60 NIH units/ml. to 2 ml. of oxalated plasma is then determined. The standard preparation, T 49, is then diluted to 60, 57, 54, 51, 48, 45 and 42 NIH units/ml. with 0·15M NaCl, each thrombin dilution containing 1·5 IU heparin/ml.

Determinations are then made of the time necessary for each of the thrombin dilutions to give an optical density of one-third and one-half, respectively, of the known optical density of the plasma clot determined earlier. Each time 1 ml. of thrombin solution is added to 2 ml. of plasma. Similar determination were made with Thrombin Topical, Topostasin Roche, and Thrombin Upjohn, which, on the basis of data on the

ampoules, were diluted with 0·15M NaCl to concentrations of 60, 54, 48 and 42 NIH units/ml., each dilution containing 1·5 IU heparin/ml. All determinations were made at room temperature (20°).

The results are summarised in Figure 1. Since the times were noted for two optical densities, each preparation is represented by two curves (I and II). Figure 1 shows that the method is very sensitive to small differences in thrombin concentration. Evidently the method cannot be used for comparing the activity of different thrombin preparations. One of the preparations, Topostasin "Roche" according to this method was found to be five to ten times weaker than the other two preparations.

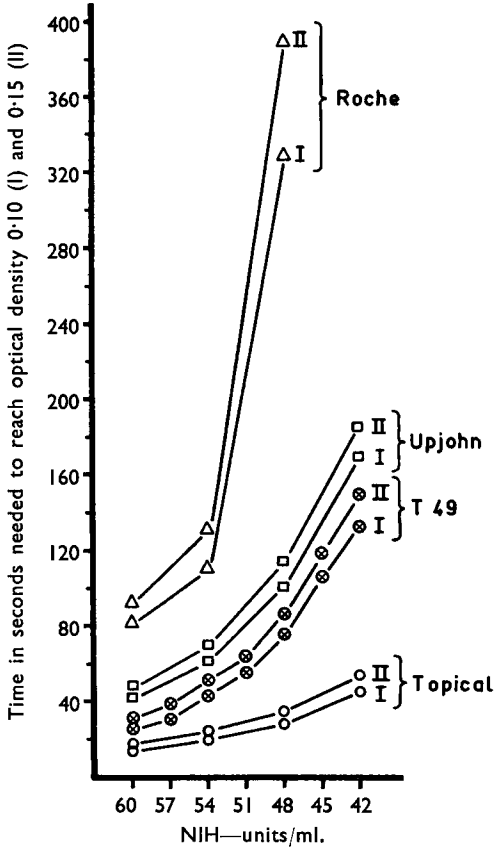

FIG. 1. The assay of the four thrombin preparations with the plasma method of Burstein and Guinand.

Determination of Thrombin According to Sherry and Troll

Principle. Thrombin is allowed to act under definite conditions for a certain time upon TAME (toluene-sulphonyl-arginine-methyl ester). The thrombin activity is directly proportional to the number of released μmols of carboxyl groups. One TAME unit of thrombin is the amount of thrombin which will release one μmol. carboxyl from TAME in 10 minutes under standardised conditions.

Standard preparation. Thrombin T 49. *Material.* TAME (0·2M 6·8 per cent pH 7·0 with NaOH); NaCl (0·15M, 0·88 per cent).

Tris buffer. Tris (hydroxymethyl)aminomethane $(CH_2OH)_3{\cdot}CNH_2$, Sigma 121, Sigma chem. Comp., St. Louis, U.S.A. 1. Tris base (0·3M). 3·64 g. base is dissolved in 100 ml. of 1·75 per cent NaCl. 2. Tris salt (0·3M). 3·64 g. base is dissolved in 30 ml. of N HCl and diluted with water to 100 ml. Tris buffer pH 9 = 90 ml. base + 10 ml. salt.

Procedure

Control of substrate. Eight samples each containing 0·2 ml. of 0·2M TAME + 0·6 ml. of tris buffer (pH 9·0) and 0·2 ml. of Thrombin T 49, diluted to 50 NIH units/ml. with 0·15M NaCl, are incubated at 37°. The reaction is terminated by addition of 1·0 ml. of formalin after 5, 10,

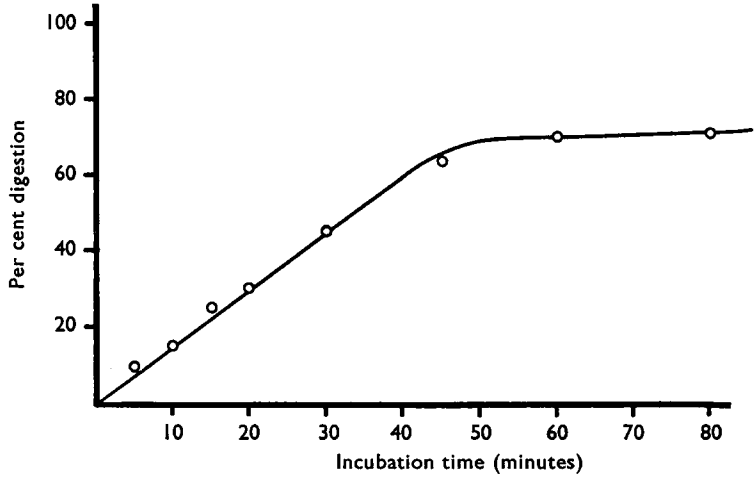

FIG. 2. The relation between the time of incubation and the degree of hydrolysis of the substrate in the TAME method.

15, 20, 30, 45, 60 and 80 minutes, respectively. The number of released μmols carboxyl groups was determined by titration with 0·05N NaOH with 0·01 per cent phenol red as indicator. As a blank, use was made of 0·2 ml. of 0·2M TAME + 0·6 ml. tris buffer + 1·0 ml. of formalin + 0·2 ml. of Thrombin T 49 to 50 NIH units/ml. and titration at time 0.

The digestion of TAME at each interval was calculated in per cent of the amount added (see Fig. 2).

Results. The rate of the reaction is constant up to 70 per cent digestion of the substrate within which region the tests were carried out.

Determination of the Activity of the Different Thrombin Preparations

On the basis of data given on the ampoules the various

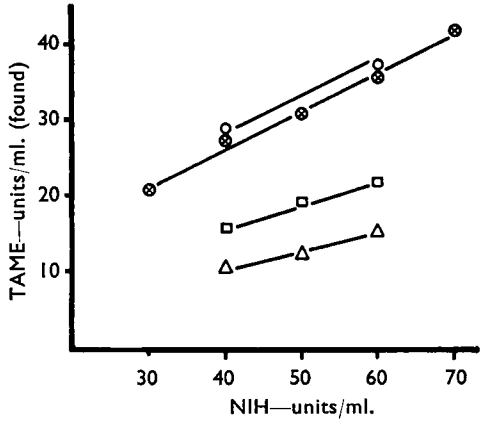

FIG. 3. Assay of the four thrombin preparations with the TAME method of Sherry and Troll.

○ Thrombin Topical ⊗ T 49
□ Upjohn △ Roche

thrombin preparations were diluted to certain definite concentrations in NIH units/ml. A sample of each concentration was incubated for 30 minutes under the same conditions as those described above, the number of released μmols of carboxyl groups was assessed by titration and the number of TAME units/ml. was calculated.

TABLE I

THE TAME METHOD
Incubation time 30 minutes

Date	Preparation	NIH units/ml.	0·05N NaOH consumed ml.		Acid produced μmol.	Digestion per cent	TAME units/ml. found
			Sample	Sample-blank			
15·7	T 49	30	0·650 0·620	0·255	12·75	31·88	21·25
	"	40	0·720 0·710	0·335	16·75	41·87	27·92
	"	50	0·750 0·750	0·370	18·50	46·25	30·83
	"	60	0·825 0·815	0·440	22·00	55·00	36·67
	"	70	0·860 0·910	0·505	25·25	63·12	42·08
	Topostasin "Roche" B 501087	40	0·520 0·530	0·125	6·25	15·63	10·42
	"	50	0·560 0·540	0·150	7·50	18·75	12·50
	"	60	0·600 0·580	0·190	9·50	23·75	15·83
	Thrombin Upjohn FA 620	40	0·600 0·600	0·200	10·00	25·00	16·67
	"	50	0·635 0·635	0·235	11·75	29·38	19·58
	"	60	0·650 0·660	0·255	12·75	31·88	21·25
	Thrombin Topical Parke, Davis & Co. 030174-B	40	0·740 0·740	0·340	17·00	42·50	28·33
	"	60	0·845 0·855	0·450	22·50	56·25	37·50

It is thus apparent that, as judged by this method with T 49 as reference, only Thrombin Topical complies with data given on the ampoules. Topostasin Roche and Thrombin Upjohn give only about 30 per cent and 50 per cent, respectively, of the values given on the ampoules (see Table I and Fig. 3).

Determination of Thrombin using Highly Purified Fibrinogen

Principle. Thrombin is tested for its power to clot a well-defined fibrinogen solution. *Standard preparation.* Thrombin T 49.

Material. Fibrinogen (bovine, prepared according to Blombäck and Blombäck[15]), fraction I–2 with a purity of 94 to 97 per cent with thrombin coagulable fibrinogen.

Method of preparation. Bovine blood was allowed to run into a solution of trisodium citrate (one part 3·8 per cent trisodium citrate ($\times 2H_2O$) to 9 parts of blood). The blood cells were separated by centrifugation at 2000 *g* for about 50 minutes.

Fraction I was precipitated by the method of Cohn and others[16]. The plasma is stirred gently and cooled to 0°. The stirring is continued while sufficient sodium acetate-acetic acid buffer in a 53·5 vol. per cent (at 25°) ethanol-water mixture is added through a capillary jet to bring the pH to 7·2 ± 0·2 and the final ethanol concentration of the system to 8 per cent. During the addition, which takes about 15–25 minutes per litre plasma, the temperature is allowed to fall from 0° to − 3°. This step requires 0·177 l. of 53·3 vol. per cent ethanol (measured at − 5°) per litre of plasma (measured at 0°), and about 1 ml. of 0·8M sodium acetate, adjusted with acetic acid to pH 4·0, is usually sufficient for the pH adjustment. Fraction I consists of between 40 and 50 per cent of thrombin clottable protein. The precipitate is removed by centrifugation at − 3 to − 5° during about 15–20 minutes at 2000 *g*. The precipitate is extracted with an aqueous glycine-citrate-ethanol mixture as described by Blombäck and Blombäck[15].

A 1·6 per cent solution of the fibrinogen in 1·62 per cent (0·055M) trisodium citrate solution (M.V. 294) is freeze-dried. Of the dry powder, which contains 50 per cent trisodium citrate, a 3·2 per cent solution is prepared. This solution has a fibrinogen content of 1·6 per cent and an ionic strength of 0·3.

NaCl (0·30 and 0·15M) 1·75 per cent and 0·88 per cent, respectively.

Tris buffer (0·3M, pH 7·2 = 11 ml. tris base + 89 ml. tris salt).

Siliconised glass vessels, non-siliconised test tubes 60 mm., 9 mm. inner diameter, 10 mm. outer diameter; platinum loops, water bath, stop watches.

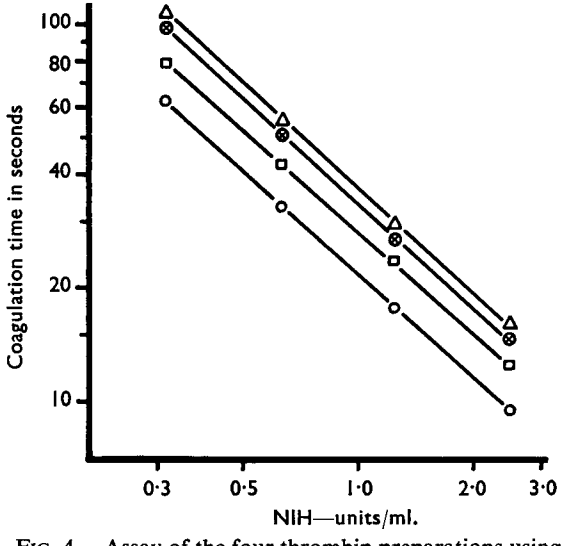

FIG. 4. Assay of the four thrombin preparations using pure fibrinogen.

○ Thrombin Topical ⊗ T 49
□ Upjohn △ Roche

The fibrinogen solution. To 1 part (25 ml.) of the 1·6 per cent fibrinogen solution with the ionic strength of 0·3 is added a mixture of 2 parts (50 ml.) of distilled water and 1 part (25 ml.) of 0·3M tris buffer of pH 7·2. The fibrinogen solution will then contain fibrinogen in a concentration of

0·4 per cent, it will have an ionic strength of 0·15 and a pH of 7·2. The volume is sufficient for testing of 2–4 thrombin preparations against a standard sample.

The thrombin solution. Of the standard preparation, T 49, four dilutions are prepared in siliconised vessels with 0·15M NaCl to concentrations of 25, 12·5, 6·25 and 3·125 NIH units/ml. Corresponding dilutions are made with commercial preparations. Theoretically the clotting time of the strongest solution should not be less than 15 seconds and the corresponding time for the weakest solution should not exceed 110 seconds. In order to avoid inactivation of the thrombin, the dilutions were prepared just before the test and were kept in siliconised vessels at 0°.

Procedure

The determinations were made in a water bath at 37°. The fibrinogen solution and the non-siliconised test tubes were warmed beforehand in the water bath. The various thrombin dilutions were studied separately in series and at least 3 determinations were made of each thrombin dilution.

Exactly 0·1 ml. of diluted thrombin solution was blown into a pre-warmed test tube, 60 × 9 (10) mm., after which 1·0 ml. of pre-warmed fibrinogen solution was added. A platinum loop was slowly moved up and down in the test tube. The interval between the addition of the fibrinogen solution and the visible appearance of fibrin threads caught by the platinum loop was measured with a stopwatch.

Results. The results are summarised in Figure 4. The curves represent the relation between logarithms of the clotting times and the logarithms of the concentrations of the thrombin. In contrast to what was found with the use of the plasma and TAME methods in their relation to T 49 none of the preparations are seen to differ appreciably from what might be expected from the data given on the ampoules.

Discussion

The wide differences in the effect of the different thrombin preparations on heparinised oxalated plasma in Burstein and Guinand's method prohibit the use of the latter.

A comparison between the fibrinogen and TAME methods clearly showed that the activity of the different thrombin preparations in a TAME system is not proportional to their activity in a fibrinogen system. This is due to the presence in varying amounts of esterases in the different thrombin preparations, as pointed out by Landabury and Seegers[17] in 1957. Two preparations, Roche and Upjohn, which in Blombäck and Blombäck's fibrinogen method agree fairly well with the standard preparation T 49, show only 30 per cent and 50 per cent respectively of the activity of T 49, as measured by the TAME method. Consequently, the TAME method is not suitable for the biological standardisation of thrombin preparations.

The only technique that remains is that using fibrinogen. The fibrinogen method works with pure fibrinogen and a system with the ionic

strength, pH and temperature well defined. A statistical analysis of the results shows a quite insignificant margin of error.

An analysis of the data given in Figure 4 showed that the thrombin activity of Topostasin Roche (No. B 501087) corresponded to 60 per cent, that of our standard preparation T 49 to 64 per cent and that of Thrombin

TABLE II

THE FIBRINOGEN METHOD OF BLOMBÄCK AND BLOMBÄCK

Sample	NIH units/ml.	Coagulation time seconds		
T 49	20	16·1	17·2	17·0
	10	27·2	30·0	29·5
	5	52·0	53·0	51·0
"Roche" B 512107	20	15·8	18·0	16·7
	10	29·6	26·5	30·5
	5	50·2	50·6	52·0
Topical Parke Davis 030566 B	20	11·5	11·7	12·6
	10	20·0	18·5	20·1
	5	36·4	36·0	36·8

Upjohn (Fa 620) to 78 per cent of the activity of Thrombin Topical, Parke Davis & Co. (No. 030164 B) all of them within the limits of error of 97 to 103 per cent.

On another occasion, one year later a comparison was made between T 49 and new batches of Topostasin Roche (No. B 512107) and of

TABLE III

STANDARD: THROMBIN TOPICAL (030566 B). TEST: TOPOSTASIN ROCHE (B 512107)

Groups	S_1 5 U/ml.	S_2 10 U/ml.	S_3 20 U/ml.	T_1 5 U/ml.	T_2 10 U/ml.	T_3 20 U/ml.	Sums
1	1·5611	1·3010	1·0607	1·7007	1·4713	1·1987	8·2935
2	1·5563	1·2672	1·0682	1·7042	1·4232	1·2553	8·2744
3	1·5658	1·3032	1·1004	1·7160	1·4843	1·2227	8·3924

Adjustment for mean $= \frac{623{\cdot}01658}{18} = 34{\cdot}612032$

Analysis of variance:

Variation due to	d.f.	Sum of squares	Mean square	Variance ratio
Between doses ..	5	0·80933 (1)	0·161866	381·8
Between groups ..	2	0·00133 (2)	0·000665	1·57
Error	10	0·00424	0·000424 = s^2	
Total ..	17	0·8149011 (3)	s = 0·02059	

(1) $\frac{106{\cdot}26410783}{3} - 34{\cdot}612032 = 35{\cdot}42136 - 34{\cdot}612032 = 0{\cdot}80933$

(2) $\frac{207{\cdot}68021537}{6} - 34{\cdot}612032 = 34{\cdot}61336 - 34{\cdot}612032 = 0{\cdot}00133$

(3) $35{\cdot}42693313 - 34{\cdot}612032 = 0{\cdot}8149011$

Thrombin Topical, Parke Davis & Co. (No. 030566 B). This time the figures found were for Topostasin Roche 64 per cent and for T 49, 64 per cent of that of Thrombin Topical, Parke Davis & Co. The limits of error were 94–107 per cent (P = 0·05). (See Tables II, III and IV.)

TABLE IV

FACTORIAL ANALYSIS (3 × 3)

Variation due to	Factorial coefficients for dose						(1) Divisor $n \cdot S(x^2)$	(2) Sum of products $S(x \cdot T_d)$	(3) Mean square $\frac{2}{1}$ (fg = 1)	(4) Variance ratio
	S_1	S_2	S_3	T_1	T_2	T_3				
1. Differences between preparations..	−1	−1	−1	+1	+1	+1	18	1·3925 = T_a	0·107725 = D^2	254·1
2. Slope	−1	0	+1	−1	0	+1	12	−2·8981 = T_b	0·699915 = B^2	1651
3. Parallellism	+1	0	−1	−1	0	+1	12	0·0097	0·0000078	0·018
4. Curvature	+1	−2	+1	+1	−2	+1	36	0·2097	0·0012215	2·881
5. Departure from curvature	−1	+2	−1	+1	−2	+1	36	−0·1297	0·000467	1·1014
Total effect per dose = T_d	4·6832	3·8714	3·2293	5·1209	4·3788	3·6767				

$n = 3$
$i = 0{\cdot}30103$
$N = 18$
$t = 2{\cdot}228$ (P = 0·05; d.f. = 10) $t^2 = 4{\cdot}9640$

$$M = \frac{4.i.T_a}{3 \cdot T_b} = \frac{4.0{\cdot}30103.1{\cdot}3925}{3\,(-2{\cdot}8981)} = \frac{1{\cdot}676737100}{-8{\cdot}6943} = -0{\cdot}1928547 = 0{\cdot}8071453 - 1$$

Antilog: 0·64141 or 64 per cent

$$b = \frac{T_b}{4.i.n} = \frac{-2{\cdot}8981}{4.0{\cdot}30103.3} = \frac{-2{\cdot}8981}{3{\cdot}61236} = -0{\cdot}8022733$$

$$s_M^2 = \frac{s^2}{b^2}\left[\frac{4}{N}\left(1 + \frac{D^2}{b^2 - s^2.t^2}\right)\right] = \frac{0{\cdot}000424}{0{\cdot}643641966529}\left[\frac{4}{18}\left(1 + \frac{0{\cdot}107725}{0{\cdot}699915 - 0{\cdot}002104736}\right)\right]$$

$$= 0{\cdot}0006588\ [0{\cdot}2222(1{\cdot}15438)] = 0{\cdot}0001464.1{\cdot}15438 = 0{\cdot}0001690$$

$$t.s_M = 2{\cdot}228\ \sqrt{0{\cdot}000169} = \pm \underset{(0{\cdot}971036-1)}{0{\cdot}028964}$$

Antilog: 1·0690 resp. 0·9355 or 94 per cent—107 per cent

ASSAY OF THROMBIN PREPARATIONS

A New Principle for the Determination of the Strength of the Standard Thrombin

Since we now have good knowledge about the chemical processes taking place in the transformation of fibrinogen to fibrin, there seems to be every reason for measuring the thrombin activity by means of a chemical titration. The proteolytic liberation of fibrinopeptides from fibrinogen is accompanied by the appearance in the solution of *N*-terminal glycine. As shown by Bettelheim and Bailey[18], two peptides A and B are liberated from ox fibrinogen, the first one containing the *N*-terminal glutamic acid of fibrinogen. During the initial phase of thrombin action *before any clotting of fibrin takes place*, there is a linear relation between the incubation time and the amount of *N*-terminal glycine appearing in the solution[19] (see Fig. 5).

As shown later by Blombäck and Vestermark[20], only peptide A is liberated during this phase. It could furthermore be demonstrated[21] that the appearance of *N*-terminal glycine in the "soluble fibrin" was followed by a stoichiometric disappearance of glutamyl residues from the fibrinogen (see Fig. 6).

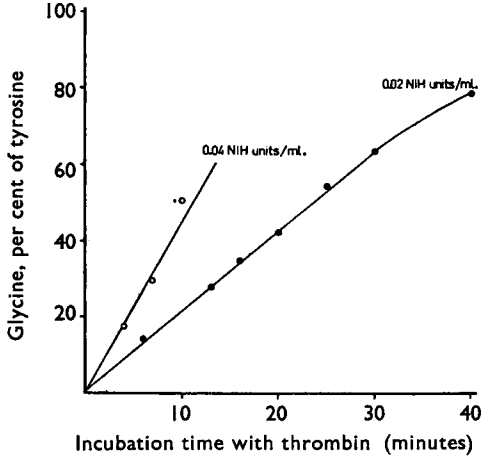

FIG. 5. Appearance of *N*-terminal glycine in the fibrinogen solution at different enzyme concentrations. ●, 0·02 NIH units/ml.; ○, 0·04 NIH units/ml.

There is consequently a possibility of expressing the proteolytic phase of the thrombin action in chemical terms, for example, as μmols of *N*-terminal glycine deriving from pure ox fibrinogen under well defined experimental conditions. Blombäck and Yamashina[19] and Blombäck[21] found that one unit of our thrombin sample T 49 on two different occasions released 0·1 and 0·09 μmols of *N*-terminal glycine from 75 mg. of fibrinogen in 10 minutes as measured with Edman's PTH method.

The fibrinogen used for the assay must be free from prothrombin. In these analyses it should be dissolved in a sodium chloride solution, not in the citrate solution used by Blombäck and Blombäck[15]. The citric acid dissolves in ethyl acetate together with the phenylthiohydantoins of the *N*-terminal amino acids. The experimental conditions governing the substrate and the enzyme concentration, the ionic strength, the pH and the temperature were studied in detail by Blombäck[21].

The Rate of Appearance of N*-terminal Glycine in a Fibrinogen-thrombin System*

Fibrinogen. The preparation of fibrinogen coagulable with thrombin in excess of 98 per cent has been described by Blombäck and Blombäck[15].

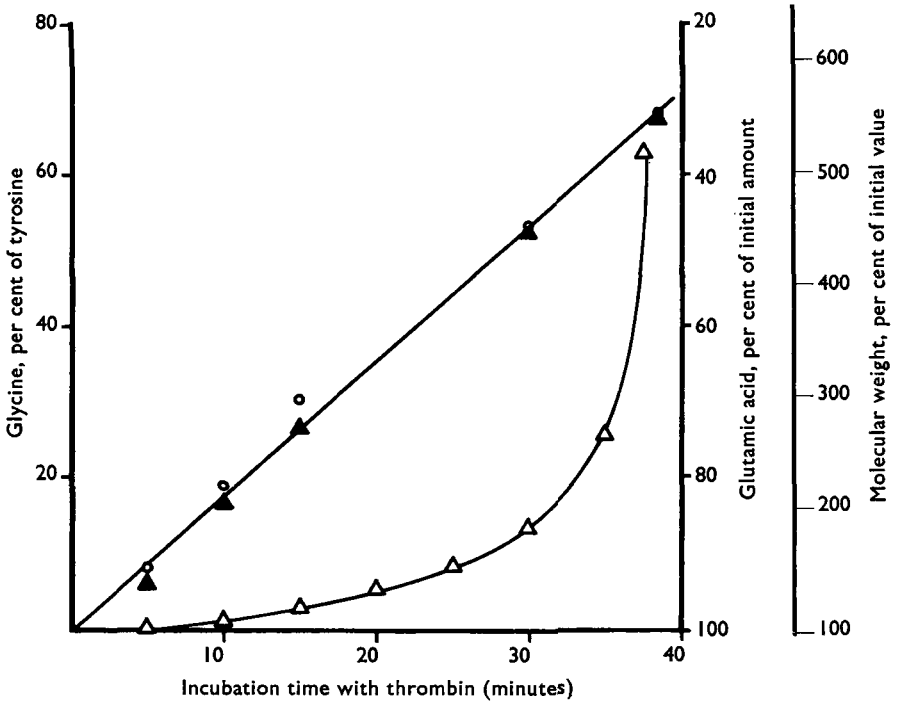

FIG. 6. Changes in *N*-terminal amino acids in the fibrinogen and the increase in light-scattering after addition of thrombin. ○, gly; ▲, glu; △, molecular weight in per cent of initial value.

The fibrinogen made free of glycine through dialysis against three changes of 0·3M sodium chloride at + 5° during 24 hours is diluted with a 0·3M NaCl solution to a protein concentration of 0·5 per cent.

Thrombin. A solution of thrombin containing 1·8 NIH units/ml. is made up in a siliconized glass vessel. The solution is to be used within one hour.

Buffer. A tris (tris(hydroxymethyl)aminomethane)-imidazol buffer is used. The following stock solutions were prepared:

I. A mixture of equal volumes of 0·1M imidazol hydrochloride and 0·1M tris-hydrochloride.

II. A mixture of equal volumes of 0·1M imidazol and 0·1M tris base, both in 0·1M sodium chloride.

These two stock solutions were then mixed in such proportions that a pH of 9·0 is obtained. After dilution of this final buffer with an equal volume of 0·2M sodium chloride, the ionic strength was 0·15.

Procedure. Into several beakers of the same size was put 15 ml. fibrinogen solution (0·5 per cent). Thirty ml. of trisimidazol buffer of pH 9·0 and ionic strength 0·15 were then added. Temperature 20°. The reaction was started by adding thrombin to a concentration between 0·02 and 0·04 NIH units/ml. The reaction was stopped at different intervals (e.g. 5, 10, 15 and 20 minutes) by coupling with phenylisothiocyanate in pyridine (45 ml. pyridine and 2·25 ml. phenylisothiocyanate). The coupling was complete within two hours. The precipitation of the PTC protein as

well as the further treatments including the chromatographic analyses were described in detail by Blombäck and Yamashina[19].

The *N*-terminal glycine is expressed in μmoles. The blank value obtained from the zero time determination is substracted.

Irrespective of the fact that the fibrinogen method described here for the determination of thrombin activity is easy to perform and works with a very small margin of error (3–7 per cent), it may be of value to have a reference standard of thrombin whose activity is checked by chemical means and expressed in chemical terms. A suitable entity would be the equivalent of one or one and a half μmols of *N*-terminal glycine released from pure ox fibrinogen under strictly defined experimental conditions. Of course the same accuracy is not to be expected with this technique as with the coagulation method. The relation between Topostasin Roche, T 49 and Thrombin Topical was namely 68·5; 70:100, whereas the coagulation method gave a relation of 64:64:100. For routine work therefore the coagulation technique is still to be recommended, but the chemical titration may have some value for the expression of the strength of the international thrombin standard.

References

1. Jalling, Jorpes and Lindén, *Quart. J. Pharm. Pharmacol.*, 1946, **19**, 96.
2. Blombäck, Blombäck, Corneliusson and Jorpes, *J. Pharm. Pharmacol.*, 1953, **5**, 1031.
3. Jorpes, Blombäck and Blombäck, *ibid.*, 1954, **6**, 694.
4. Blombäck, Blombäck and Wallén, *Revue d'Hématologie*, 1955, **10**, 45.
5. Jorpes, *Acta pharm. tox., Kbh.*, 1955, **11**, 367.
6. Astrup and Darling, *J. biol. Chem.*, 1940, **133**, 761.
7. Astrup and Darling, *Acta physiol. scand.*, 1941, **2**, 22.
8. Quick, *Hemorrhagic Diseases*, Lea and Fibiger, Philadelphia, 1957, 428.
9. Warner, Brinkhous and Smith, *Amer. J. Physiol.*, 1936, **114**, 667.
10. Seegers, Brinkhous, Smith and Warner, *J. biol. Chem.*, 1938, **126**, 91.
11. Seegers and Smith, *Amer. J. Physiol.*, 1942, **137**, 348.
12. Astrup and Darling, *Acta physiol. scand.*, 1942, **4**, 54.
13. Burstein and Guinand, *Revue d'Hématologie*, 1955, **10**, 571.
14. Sherry and Troll, *J. biol. Chem.*, 1954, **208**, 95.
15. Blombäck and Blombäck, *Arkiv f. Kemi*, 1956, **10**, 415.
16. Cohn, Strong, Hughes, Mulford, Ashworth, Melin and Taylor, *J. Amer. chem. Soc.*, 1946, **68**, 459.
17. Landabury and Seegers, *Proc. Soc. exp. Biol. N.Y.*, 1957, **94**, 708.
18. Bettelheim and Bailey, *Biochem. Biophys. Acta*, 1952, **9**, 578.
19. Blombäck and Yamashina, *Acta chem. scand.*, 1957, **11**, 194.
20. Blombäck and Vestermark, *Arkiv f. Kemi*, 1958, **12**, 173.
21. Blombäck, *ibid.*, 1958, **12**, 321.