STUDIES ON THROMBOPLASTIN GENERATION TEST

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ABSTRACT

In this paper, an attempt to devise a most satisfactory method of thrombo-plastin generation test (TGT) was made and the results were as follows:

1) As anticoagulant for the preparation of plasma, sodium citrate is superior to potassium oxalate, because the various factors in the former are more stable than those in oxalated plasma.

2) In adsorption of plasma, $BaSO_4$ and $Al(OH)_3$-gel are both useful. In citrated plasma, the optimum dose was 200 mg/ml and preferable adsorption time at 37°C ranged from 5 to 20 minutes with $BaSO_4$, and with $Al(OH)_3$ 0.03 ml/ml, 1–3 minutes. However, there is need to use $BaSO_4$ of purity above 99% and with grain size mostly of 1–2 μ.

3) Serum should be separated 1 hour after initiation of the incubation in case of normal whole blood clotting time, and 3 hours after in hemophiliacs. There is no need to activate the serum with glass beads.

4) The optimum concentration of platelet suspension was found to be 200,000–300,000/cmm and washing of platelets should be made for at least 4 times. But in general, platelet substitutes should be used, and the potency of such substitutes should preferably possess minimal substrate clotting time of about 10 seconds.

5) The normal value of TGT was 9.97±0.938 seconds for the minimal substrate clotting time, or 100±23.3% in thromboplastic activity.

6) The standard dilution curve of TGT can be plotted linearly on a bilogari-thmic scale and they should be prepared for each time of measurement, and the results of TGT indicated by percentages of the normal value.

I. INTRODUCTION

Since the original publication of the thromboplastin generation test (TGT) by Biggs and her coworker[1], various modifications have been reported by many investigators[2]−[13], so that today, methods termed to be TGT are diverse as regards the details.

In this paper, a comparative study was made of these modifications of TGT, in an attempt to devise a most satisfactory method.

II. MATERIALS AND METHODS

1) Collection of blood: The two-syringe technique was used.

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As anticoagulant, 3.8% sodium citrate, 0.1 M potassium oxalate and 1% EDTA-Na₂ solutions were used, in the proportion of 9 parts of blood to 1 part of each of these anticoagulant solutions. All apparatuses used for collection of plasma and platelets were siliconized with Dow Corning 1107.

2) *Preparation of plasma*: Whole blood was centrifuged at 3,000 rpm for 30 minutes at 4°C to separate the plasma, and the upper 2/3 portion of this plasma layer was collected and examined.

3) *Platelet suspension*: Whole blood was centrifuged 2 times at 800-1,000 rpm for 10 minutes to prepare a platelet rich plasma, which was centrifuged further at 3,000 rpm for 30 minutes to separate the platelets. The platelets were washed 4 times with physiologic saline solution, and a final suspension of platelet with a concentration of 300,000/cmm was prepared.

4) Barium sulfate (a special grade product of DAIICHI PURE CHEMICALS CO., LTD.) was mixed in a proportion of 200 mg per 1 ml plasma, and the mixture was agitated at 37°C for 15 minutes for adsorption.

5) Aluminium hydroxide gel was prepared according to the method of Miale and Wilson[14], and was mixed in a proportion of 0.03 ml per 1 ml of plasma and the mixture was agitated at 37°C for 3 minutes for adsorption.

6) *Platelet substiutes*: Trostin (CHUGAI PHARMACEUTICAL CO., LTD.), Platelin (WARNER-CHILCOTT), Cephaloplastin (DADE REAGENTS, INC.) and Bell-Alton's partial thromboplastin prepared according to the modification of the method of Langdell, Wagner and Brinkhous[15], were used.

7) The methods of various tests are listed as follows.

Whole blood clotting time: Method of Lee and White[16].
Prothrombin time: Quick's method[17].
Prothrombin in plasma: Method of Rosenfield and Tuft[18].
Prothrombin in serum: Method of Stefanini and Crosby[19].
Factor VII + X: Method of deVries and Alexander[20].
Factor VIII: Pitney's method[21].
Factor IX: Method of Fukui and Umegaki[22].

Thromboplastin generation test was performed based on the original method of Biggs and Douglas, employing adsorbed plasma diluted 1:5, serum diluted 1:10, Trostin diluted 1:200 and 0.025 M CaCl₂ solution.

III. PREPARATION OF ADSORBED PLASMA

I) Selection of anticoagulant.

In studying the technique of TGT, the selection of anticoagulant used in the preparation of plasma was examined.

A comparison of stability between citrated and oxalated plasmas at 4°C was made (Fig. 1). In oxalated plasma the activity of Factor V and Factor VIII
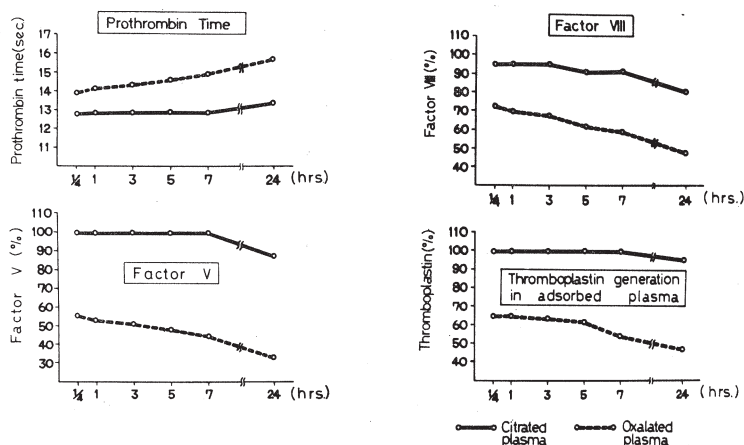
FIG. 1. Stability of citrated plasma and oxalated plasma.
—stored at 4°C—

showed remarkable reduction as compared with citrated plasma.   This indicates
that oxalated plasma is less adequate than citrated plasma in stability.

2) Selection of adsorbent.

A comparative study was carried out of the grade of adsorption in citrated
plasma between alminium hydroxide gel (Al(OH)$_3$) and barium sulfate (BaSO$_4$).
The incubation time for adsorption was 3 minutes in the former and 15 minutes
in the latter (Table 1).

TABLE 1.   Adsorptive effects of BaSO$_4$ and Al(OH)$_3$

Adsorbent (/ml)		Prothrombin time (sec.)	Prothrombin (%)	Factor V (%)	Factor VIII (%)	Thromboplastin generation (%)
Al(OH)$_3$	0.02 ml 0.03 0.04	66.6 94.7 102.2	0 0 0	70 70 57	98 100 97	78 76 73
BaSO$_4$	100 mg 200 300	18.0 $>$240 $>$240	4 0 0	80 80 80	102 100 86	100 100 93

In case of Al(OH)$_3$ a concentration of 0.03-0.04 ml per 1 ml plasma was
found to be appropriate for adsorption in prothrombin, but when used in a con-
centration of 0.04 ml, a reduction in activity of Factor V was noted.   Therefore
the optimum concentration of Al(OH)$_3$ was found to be 0.03 ml.

In case of 100 mg/ml of BaSO$_4$ the prothrombin time was extremely short,
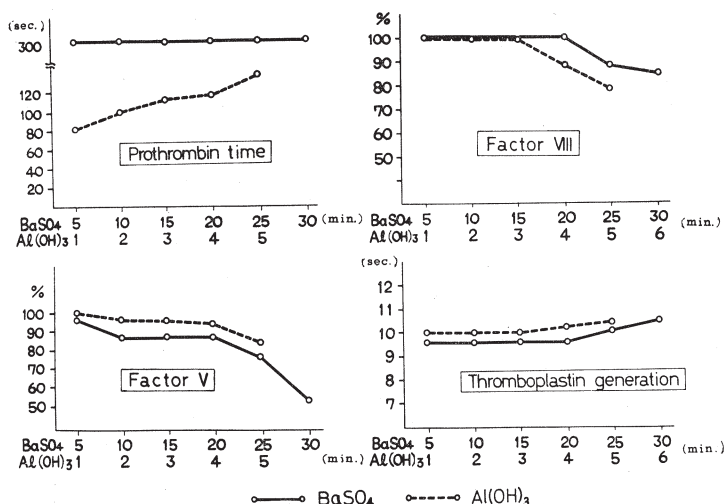being 18 seconds and adsorption was found to be poor.   But with 200 mg/ml

FIG. 2.   Adsorption time.   —comparison of BaSO₄ and Al(OH)₃—

the prothrombin time exceeded 240 seconds and adsorption became extremely satisfactory, and the activities of Factor V and Factor VIII were well preserved when compared with the case of Al(OH)₃, whereas with 300 mg/ml the activity of Factor VIII fell slightly.   Thus the optimum dose of BaSO₄ was found to be 200 mg/ml for citrated plasma.

Figure 2 shows the results of a study on the adsorption time of Al(OH)₃ and BaSO₄ for citrated plasma.   Adsorption was conducted with Al(OH)₃ (0.03 ml/ml) for times ranging from 1 to 5 minutes and with BaSO₄ (200 mg/ml) from 5 to 30 minutes at 37°C respectively.

In experiments with Al(OH)₃ the activity of Factor VIII and Factor V fell with the adsorption time of 4–5 minutes, while with BaSO₄ a similar fall in activity of Factor VIII and Factor V occurred when adsorption was conducted for more than 25 minutes.   Prothrombin failed to be demonstrated when adsorption was conducted with Al(OH)₃ for 1 minute and with BaSO₄ for 5 minutes. These findings indicate extremely high adsorption in both adsorbents.

The optimum adsorption time of Al(OH)₃ was 1–3 minutes and of BaSO₄ 5–20 minutes.

For citrated plasma, both BaSO₄ and Al(OH)₃ were equally useful as adsorption agents, but as Al(OH)₃ has the disadvantage of poor preservation, BaSO₄ is more preferable for general use.

3) *Comparison of various brands of barium sulfate*

A study was made of six lots of barium sulfate (No. 1–6) from various firms, and the results are shown in Table 2.   The purity of these 6 products ranged

TABLE 2. Various samples of BaSO₄ preparations

	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
Purity (%)	99.1	99.0	99.4	98.5	99.5	97.6
Specific gravity (v/w)	0.720	0.676	0.670	0.610	0.810	1.433
$<1\mu$ (%)	33.9	71.2	55.8	73.7	21.8	7.2
$1-2\mu$ (%)	53.6	21.5	25.6	19.0	28.9	10.4
$2-5\mu$ (%)	10.6	5.9	15.0	5.8	46.5	80.9
$>5\mu$ (%)	1.9	1.4	3.6	1.5	2.8	1.5

(Grain size)

TABLE 3. Comparison of various BaSO₄ preparations as adsorbent for citrated plasma

	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
Prothrombin time (sec.)	not clot.	not clot.	not clot.	154	11.5	14
Prothrombin (%)	0	0	0	1	2	34
Factor V (%)	75	56	58	62	70	94
Factor VII+ X (%)	0	2	1	0	2	2
Factor VIII (%)	100	85	78	81	84	106
TGT BaSO₄ (%)	100	77	64	70	80	109
TGT serum (%)	5	4	4	5	3	12

from 97.6% to 99.5% and was approximately equal, but the specific gravity varied greatly, ranging from 0.610 to 1.433, and the distribution of size of the grains in case of No. 1 was mostly $1-2\mu$, but was less than 1μ in products No. 2, 3 and 4, and mostly $2-5\mu$ in No. 5 and 6, which indicated that their sizes were proportional to the specific gravities.

When the adsorption of citrated plasma was compared using these barium sulfate products (Table 3), No. 1, 2 and 3 exhibited perfect adsorption for pro-thrombin, but No. 2, 3 and 4 products with grain size mostly less than 1μ showed over-adsorption of Factor V and Factor VIII, while No. 4, 5 and 6 of purity under 99% and/or with grain size mostly $2-5\mu$ showed poor adsorption of prothrombin. Namely, in the preparation of adsorbed plasma for TGT, product No. 1 with grain size mostly $1-2\mu$ was found to be most suited.

IV. PREPARATION OF SERUM
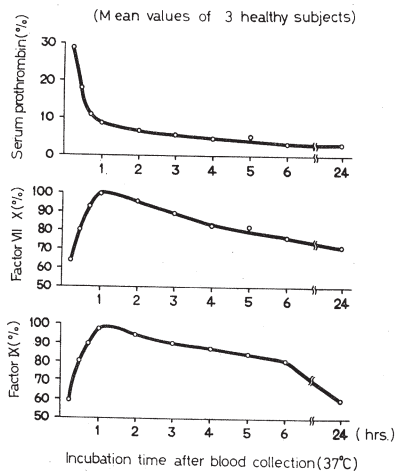
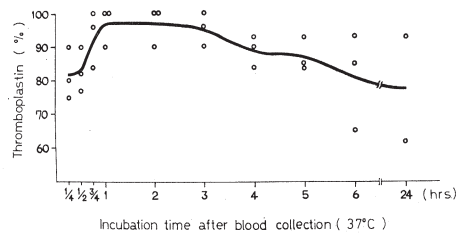1) *In cases of normal whole blood clotting time*

FIG. 3.

FIG. 4.

FIG. 3. Incubation time of whole blood and Factor II, VII+X and IX in serum

FIG. 4. Effect of whole blood incubation befor serum separation on thromboplastin generation (Healthy cases).

Blood collected from healthy humans was kept at 37°C and the serum was separated at various times after the initiation of incubation. The separated serum was frozen immediately at − 40°C, and 3 days later was tested after thawing.

In Figures 3 and 4 are shown the results of 3 cases examined.

It will be seen that prothrombin decreased rapidly after blood collection, namely, to less than 10% after one hour, while Factor VII + X and Factor IX showed gradual rise in activities after blood collection, reaching a maximum at the end of one hour.   Thereafter, the activity gradually fell, to about 60-70% at the end of 24 hours.   However, the activity of serum component in thromboplastin generation was maintained at a maximum during 1-3 hours after blood collection (Fig. 4).

2) *In cases of prolonged whole blood clotting time*

Two cases of hemophilia B were subjected to this study (Table 4).

In the case of severe hemophiliac the clotting time showed 4 hours and 30 minutes and Factor IX 0%, while in the moderate hemophiliac the clotting time was 25 minutes and Factor IX 1.7%. The changes in activity of Factor VII + X and Factor IX showed a tendency similar to that seen in healthy cases, but maximal activity was seen at 3-4 hours (Fig. 5).   The prothrombin in serum was as high as 66% in the severe homophiliac and 16% in the moderate case even at the end of 24 hours, indicating the existence of high to medium grade prothrombin consumption impairment.

In order to stimulate this prothrombin consumtion glass beads of 3 mm in diameter were added to blood in a ratio of 5 beads to 2 ml blood and gently
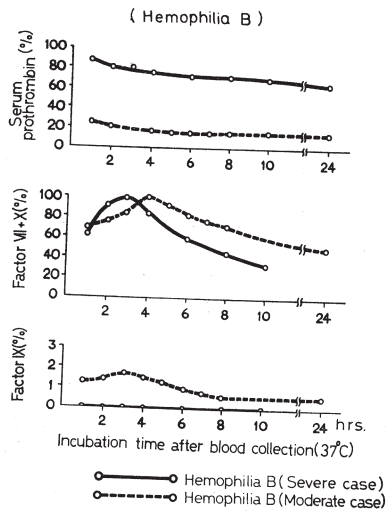
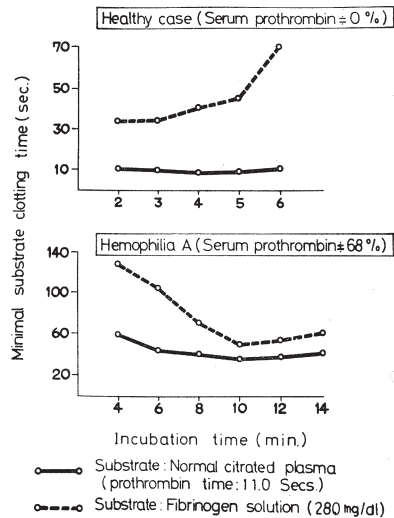FIG. 5. Incubation time of whole blood and Factor II, VII+X and IX in serum.

FIG. 6. Serum prothrombin and thromboplastin generation.

TABLE 4. Effects of activation of serum factors by glass beads in hemophilia A

		Moderate case of hemophilia B			Severe case of hemophilia B		
Incubation time after blood collection(37°C)		1	3	6 (hrs)	1	3	6 (hrs)
Serum prothrombin (%)	*I	25	19	16	88	80	72
	**II	0	0		64	48	44
Factor VII+X (%)	*I	69	85	83	63	100	46
	**II	104	96		76	88	83
Factor IX (%)	*I	1.3	1.7	1.0	0	0	0
	**II	1.0	1.7		0	0	0
TGT serum activity (%)	*I	5.4	5.3	5.3	10.6	7.5	7.3
	**II	5.8	6.0		7.4	7.3	7.3

*I: Whole blood clotting in glass tube
**II: Acceleration of whole blood clotting with glass beads

shaken, when the whole blood clotting time of the moderate case was shortened from 25 minutes to 10 minutes and the prothrombin content became 0, and the activation of Factor VII + X was also promoted. In case of the severe hemo-

philiac whole blood clotting time was shortened from 4 hours and 30 minutes to 1 hour and 26 minutes, but serum prothrombin remained in large amounts. However, the serum activity of thromboplastin generation remained unchanged regardless of activation of serum by the glass beads, as shown in Table 4.

Next, a study was made of the effects of prothrombin caused by impaired consumption on thrombin generation in the incubation mixture (Fig. 6). In both serum of normal and of hemophilia A with conspicuous prothrombin consumption impairment the thrombin activity in the incubation mixture was far weaker than thromboplastin activity, so that even when high impairment of intrinsic thromboplastin formation exists the serum prothrombin could produce practically no influence on the minimal substrate clotting time of TGT.

3) *Relation of time elapsing after diluion of serum to activity of serum component in thromboplastin generation*

Diluted serum was left to stand at room temperature (20°C), and the relation between time elapsing after dilution and activity of serum component in thromboplastin generation was studied (Table 5), and cotrary to the reports of Biggs[13], Nour-Eldin[23] and Seaman[24], no gradual increase in activity of serum was recognized, and there was seen practically no change in the activity at 10 minutes and up to 80-180 minutes.

From the above it is desirable that serum be separated 1 hour after blood collection when the clotting time is normal, and 3-4 hours after, when the clotting time is prolonged, and there is no need to activate Factor VII, IX and X with glass beads, or to allow serum after dilution to stand at room temperature.

TABLE 5. Relation between time elapsing after dilution
of serum and thromboplastin generation

Elapsing time after dilution of serum (min.)	Serum I	Serum II	Serum III*
	Thromboplastin generation —minimal substrate clotting time— (sec.)		
0	9.5	7.7	7.7
10	10.1	8.2	8.3
20	10.0	8.3	8.5
30	10.2	8.5	8.7
40	10.1	8.2	8.2
50	10.4	8.3	8.3
60	10.0	8.4	8.6
70	10.3	8.3	
80	10.2	8.6	
180	10.1		

* Serum III was agitated with glass stick.

V. PLATELET SUSPENSION AND PLATELET SUBSTITUTE

1) *Concentration of platelet suspension*

Platelet suspension prepared from blood added with sodium citrate, potassium oxalate and EDTA showed shortest minimal substrate clotting time at the platelet concentration of 750,000/cmm, and with decrease in platelet number the clotting time gradually lengthened (Fig. 7). Moreover, there was seen a fairly marked difference in minimal substrate clotting time, depending upon the various kinds of anticoagulant as indicated in the figure.

However, when the platelet suspension was subjected to repeated freezing at – 40°C and thawing in order to destroy the platelets the above mentioned difference practically disappeared (Fig. 8).

These findings suggested that potassium oxalate and EDTA act on the surface of the platelets to inhibit the release of factor 3 from the platelets; therefore in the preparation of platelet suspension the use of citrated blood is recommended[25].
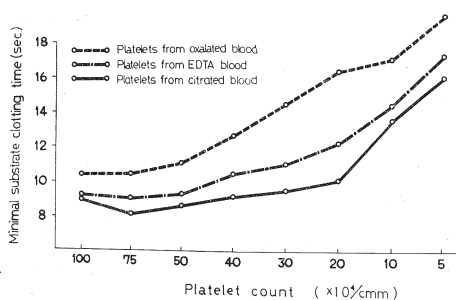
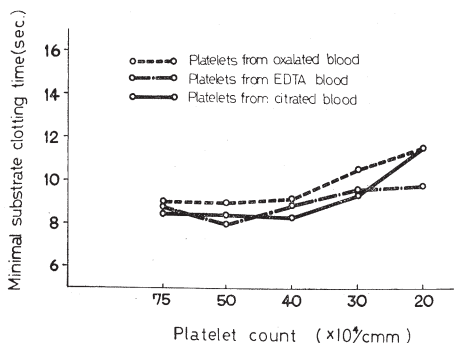FIG. 7. Platelet concentration and thromboplastin generation. —without freezing and thawing—

FIG. 9. Washing of platelets and thromboplastin generation.

FIG. 8. Platelet concentration and thromboplastin generation. —with repeated (twice) freezing (−40°C) and thawing (37°C)—
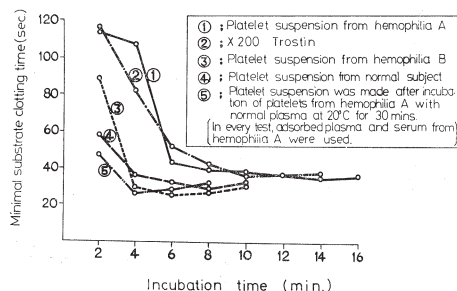
FIG. 10. Various platelet suspensions and platelet substitutes in TGT of hemophilia A.

The platelet concentration recommended is a range of 200,000-300,000/cmm. by which the minimal substrate clotting time becomes about 10 seconds, for the clotting time in TGT should not be too short nor too long in order to avoid large errors in measurement, and a time of about 10 seconds is accompanied by minimal measurement errors.

2) *Frequency of washing in preparation of platelet suspension*

In conducting TGT with an incubation mixture composed of adsorbed plasma and serum of hemophilia A and platelets of normal humans, thromboplastin generation gradually fell with increase in frequency of washing of platelets, and after more than 4 washings an approximately constant activity of thromboplastin generation resulted (Fig. 9).

However, when the adsorbed plasma and serum of the above mentioned incubation mixture were kept the same and only the platelet suspension changed in various samples there was seen a clear difference in thromboplastin generation (Fig. 10). For example, when the platelets of hemophilia A were used the minimal substrate clotting time was 36 seconds at the end of 14 minutes after incubation, but with platelets of hemophilia B and normal humans the times were respectively shortend to 26 seconds at the end of 6 minutes and to 29 seconds at the end of 8 minutes. Again, when platelets of hemophilia A were incubated with normal plasma at room temperature for 30 minutes and then were washed 4 times, there was seen a shortening of clotting time as shown in the figure. This indicates the failure of complete removal of Factor VIII which is absorbed to the surface of the platelets even after washing for 4 times.

On the other hand, as shown in Figure 10, practically similar results were obtained in the experiment using a solution of Trostin diluted 1 : 200 and in that using platelets of hemophilia A as well. From this finding, coupled with the fact that preparation of a constant concentration of platelet suspension is
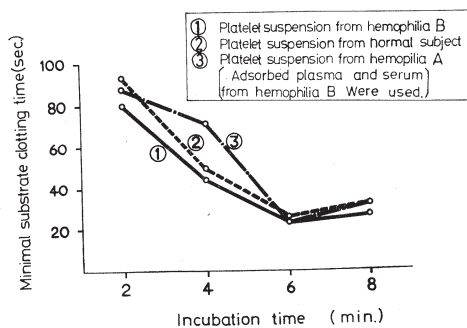

FIG. 11. Various platelet suspensions in TGT of hemophilia B.

TABLE 6. Effect of CaCl₂ concentration on incubation mixture of TGT

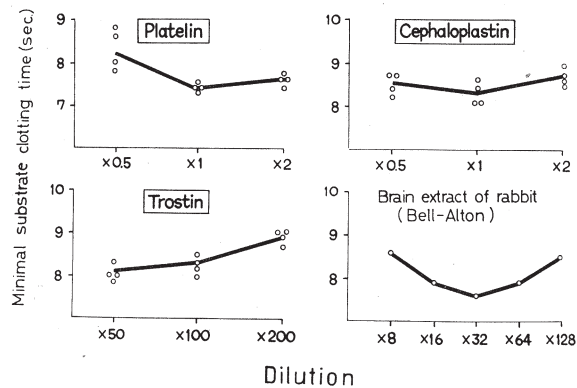| CaCl₂ concentration (M)	TGT minimal substrate clotting time (sec.)
0.01	12.7
0.02	12.1
0.025	11.7
0.03	11.8
0.04	12.2
0.05	12.4

FIG. 12.   Comparison of thromboplastic activity of various platelet substitutes.
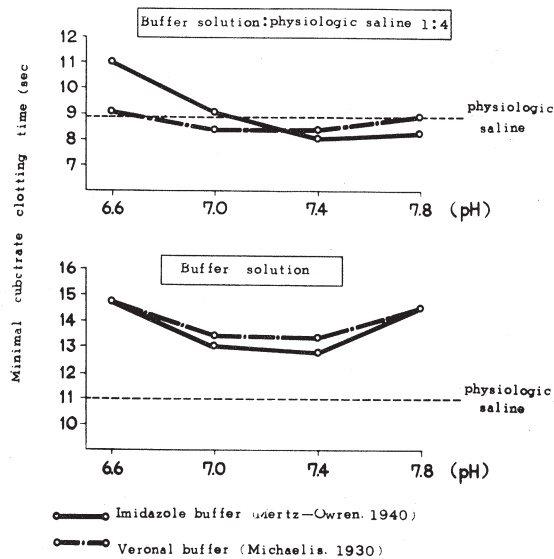

FIG. 13.   Buffer solution and pH in incubation mixture of TGT.

rather difficult, the use of platelet suspensions may in general be avoided, and platelet substitutes described below should be used instead in TGT.

In similar experiments using adsorbed plasma and serum of hemophilia B such results could not be obtained, (Fig. 11) therefore it may be considered that Factor IX fails to be adsorbed by the platelets.

3) *Platelet substitutes*

At present various platelet substitutes are available.   The results with the use of Plateline, Cephaloplastin, Trostin and brain extract of rabbit prepared by

us are shown in Figure 12. It will be seen that the results with these platelet substitutes are quite reproducible and so they are useful.

VI. CONCENTRATION OF CaCl₂

As was described in the original method of Biggs *et al.* the concentration of $CaCl_2$ used in TGT was optimum at 0.025 M which was confirmed by this study, and with concentrations above or below this there resulted a prolongation of the minimal substrate clotting time (Table 6).

VII. BUFFER SOLUTION

The pH of physiologic saline solution was 6.2, but as blood possesses a powerful buffer action, the pH of the incubation mixture becomes 6.8. Hence, when a 1:5 dilution of adsorbed plasma and 1:10 dilution of serum are used in TGT, there need no use of a buffer solution (Fig. 13).

VIII. PREINCUBATION TIME OF INCUBATION MIXTURE AND SUBSTRATE PLASMA

The preincubation time of the incubation mixture, namely, the time from the initiation of incubation at 37°C. of the mixture to addition of $CaCl_2$ solution was checked and there was found no change in the intrinsic thromboplastic activity of the incubation mixture at the preincubation time ranging from 1 to 7 and 1/2 minutes (Fig. 14), while the appropriate preincubation time of citrated plasma as substrate was 1-5 minutes (Fig. 15).
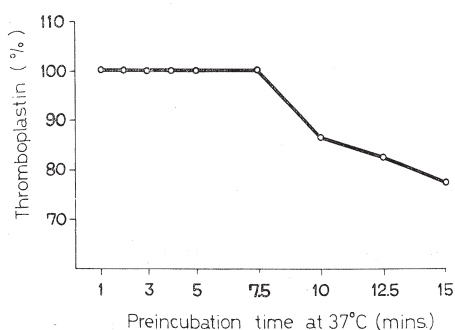

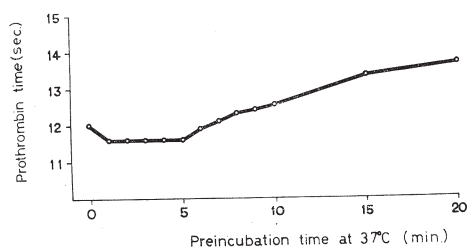FIG. 14. Preincubation time of incubation mixture on thromboplastin generation.

FIG. 15. Preincubation time of substrate plasma in TGT.

IX. NORMAL RANGE OF TGT

The range of error of TGT conducted by the procedure described above was 0.2-0.4 seconds as shown in Table 7, so that when differences exceeding 0.5 seconds occured in experiments they were taken to be significant. Further, our range of error of prothrombin time was found to be 0.2 seconds.

TABLE 7. Range of error in TGT

| | | No. of incubate mixture | | | | |
		1	2	3	4	5
	1	7.5	8.6	9.0	8.9	11.6
Minimal	2	7.6	8.5	8.6	9.1	11.5
substrate	3	7.5	8.8	8.6	9.0	11.6
clotting	4	7.6	8.5	8.6	9.1	11.3
time	5	7.4	8.5	8.8	8.9	11.4
(sec.)	6	7.5	8.9	9.0	9.2	11.5
Mean (sec.)		7.52	8.63	8.77	9.03	11.50
Range of error (sec.)		0.2	0.4	0.4	0.3	0.3

Employing our TGT method the activity of thromboplastin generation in 73 normal humans was estimated and it was found that the minimal substrate clotting time was 9.97 ± 0.938 seconds or 100 ± 23.3 in per cent thromboplastin, while 66 out of the 73 cases (90%) fell within the range of 9.03–10.91 seconds (70–130%).

X. RELATION OF LEVELS OF FACTOR VIII OR IX TO TGT

Employing mixtures of various proportions of normal plasma with hemophilia A plasma, or normal serum with hemophilia B serum, a study was made of the relation between the levels of Factor VIII in adsorbed plasma or Factor IX in serum with activity of thromboplastin generation, and it was found that with decrease in Factor VIII or IX a gradual fall in thromboplastin generation occurred.   And when the levels of Factor VIII or IX fell to 20–30% the thromboplastin generation clearly showed abnormal values, as compared with the above mentioned normal range (Fig. 16, 17).
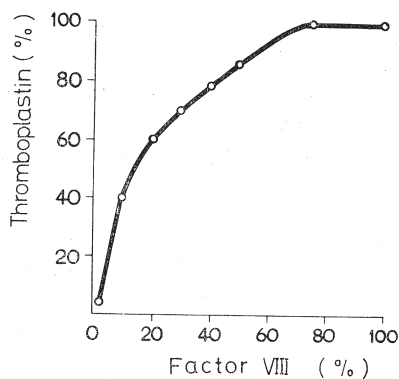

FIG. 16. Relation between Factor VIII levels in adsorbed plasma and thromboplastin generation.
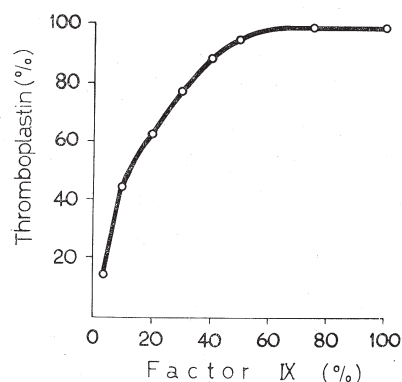
FIG. 17. Relation between Factor IX levels in serum and thromboplastin generation.

XI. STANDARD DILUTION CURVE OF TGT

Employing adsorbed plasma, and serum obtained from normal human blood and phospho-lipid the TGT was conducted according to the method above de-scribed, and when the thromboplastic ac-tivity of the incubation mixture reached a maximum the mixture was immediately cooled in melting ice and diluted with physi-ologic saline in ascending dilutions to ob-tain the standard dilution curve of TGT. In this curve the thromboplastin concen-tration of the mother solution is repre-sented by 100% and the values are lineally plotted on a bilogarithmic scale (Fig. 18). However, when the time of measurement differ the curves did not necessarily coin-cide, so that there is need to prepare stand-ard dilution curves for each measurement.
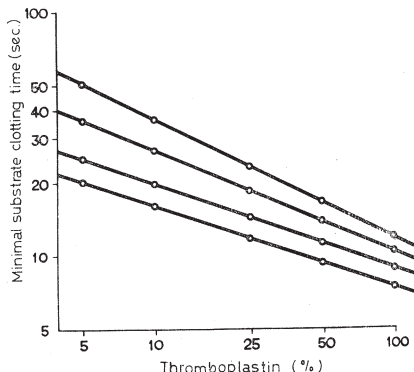
FIG. 18. Standard dilution curve of TGT.

Hence, the results obtained for differ-ent times of measurement should be compared not by the minimal substrate clotting time but by the percentages obtained from the standard dilution curve for each time of measurement, as such will be more rational.

XII. DISCUSSION AND SUMMARY

Based on the experimental results described above the most satisfactory procedure of TGT will be as follows.

Citrated plasma is superior to oxalated plasma, as the stability of the various factors was greater, which support the findings in the reports of Mustard[25] and others[26]-[28].

In preparing adsorbed plasma with citrated plasma, 200 mg/ml for 5-20 minutes in barium sulfate or 0.03 ml/ml for 1-3 minutes in alminium hydroxide gel are both useful, but from the standpoint of preservation, $BaSO_4$ is believed to be more convenient. However, there is need to use $BaSO_4$ of purity above 99% and with grain size mostly of 1-2 μ.

Serum should be separated within 1-3 hours after blood collection, in case with normal whole blood clotting time at the end of 1 hour, and in hemophiliacs at the end of 3 hours.

Prothrombin in serum influences the reaction speed of TGT but not the minimal substrate clotting time[29]. Hence, there is no need in hemophilia to activate the serum with glass beads.

No theoretical grounds were obtained for the need to leave the serum after

dilution at room temperature with the object of gradually raising the activity of the serum component in thromboplastin generation, contrary to the reports of Biggs[30], Pitney[31], and Pool and Robinson[32].

To prepare platelet suspension, citrated plasma should be used, and the washings should be made for at least 4 times. The optimum concentration of platelet suspension was found to be 200,000-300,000 per cmm, and corresponded with the reports of Biggs and Klein[7], but differed from those of Miale and Garrett[6] and of Umegaki and Fukui[3].

In general platelet substitutes should be used, but in this case the potency of such substitutes should preferably possess minimal substrate clotting time of about 10 seconds, as has been pointed out by many investigators[3,4,13,14,33]. We employed Trostin diluted 1 : 200. There was found no need to use buffer solutions for diluting each component of TGT.

The appropriate preincubation time of the incubation mixture was 1-7 minutes, while the appropriate preincubation time of citrated plasma as substrate was 1-5 minutes.

The appropriate concentration of $CaCl_2$ was 0.025 M[1,2,30].

The normal value of TGT estimated by the method described above was 9.97 ± 0.938 seconds for the minimal substrate clotting time, or $100 \pm 23.3\%$ in thromboplastic activity.

Regarding the relation of Factor VIII and IX with TGT, there was seen a clear deviation of thromboplastin generation from the above mentioned normal range, evidenced by the appearance of abnormal values when the levels of Factor VIII and IX fell to less than 20-30%.

The standard dilution curve of TGT can be plotted linearly on a bilogarithmic scale, and they should be prepared for each time of measurement[8]. And the results of measurements made at different times should be obtained as percentages from the standard dilution curve.

REFERENCES

1) Biggs, R. and Douglas, A. S., The thromboplastin generation test, *J. Clin. Path.*, **6**, 23, 1953.

2) Duckert, F., Flückiger, P., Isenschmid, H., Matter, M., Vogel-Meng, J., and Koller, F., A modification of the thromboplastin generation test, *Acta Hamat.*, **12**, 197, 1954.

3) Bell, W. N. and Alton, H. G., A brain extract as a substitute for platelet suspension in the thromboplastin generation test, *Nature*, **174**, 880, 1954.

4) Newlands, M. J. and Wild, F., Sources of platelet factor for the thromboplastin generation test, *Nature*, **176**, 885, 1955.

5) Verstraete, M., The thromboplastin generation test. Some practical modifications and study of the role of factors V and VII on the formation of plasma thromboplastin, *Acta Med. Scand.*, **155**, 37, 1956.

6) Miale, J. B. and Garrett, V. R., Sudies on the thromboplastin generation test, III. The effects of dilution, storage and concentration of platelets, *Amer. J. Clin. Path.*, **27**, 701,

1957.

7) Klein, E. and Fiorentino, R., Effects of varying concentrations of platelets and their lyophilized derivatives on generation of thromboplastin, *Proc. Soc. Exp. Biol. Med.*, **94**, 357, 1957.

8) Umegaki, K. and Fukui, H., A modification of thromboplastin generation test, *J. Nara Med. Ass.*, **8**, 34, 1957. (in Japanese.)

9) Connor, W. E. and Carter, J. R., Influence of soybean phosphatide on blood coagulation and its use in the thromboplastin generation test, *Proc. Soc. Exp. Biol. Med.*, **97**, 38, 1958.

10) MacMillan, R. L. and Ritter, H., A modification of the thromboplastin generation test, *Amer. J. Med. Sci.*, **235**, 437, 1958.

11) Davidson, E. and Tomlin, S., Observation thromboplastin generation using platelet suspensions prepared by a simplified method, *Thromb. Diath. Haem.*, **5**, 74, 1960.

12) Thompson, J. H., Owen, C. B., Spittell, J. A. and Pascuzzi, C. A., The "retarded" thromboplastin-generation test. Its use in the study of accelerated blood coagulation, *Amer. J. Clin. Path.*, **37**, 63, 1962.

13) Biggs, R. and Macfarlane, R. G., Human blood coagulation and its disorders. Blackwell, Oxford, 1962. p. 372.

14) Miale, J. B. and Wilson, M. P., Studies on the thromboplastin generation test., I. Method and clinical applications, *Amer. J. Clin. Path.*, **26**, 969, 1956.

15) Langdell, R. D., Wagner, R. H. and Brinkhous, K. M., Determinations of the partial thromboplastin time (PTT). In Blood coagulation, hemorrhage and thrombosis, Edited by L. M. Tocantins, and L. A. Kazal, Grune and Stratton, Inc., New York, 1964. p. 103.

16) Lee, R. I. and White, P. D., A clinical study of the coagulation time of blood, *Amer. J. Med. Sci.*, **145**, 495, 1913.

17) Quick, A. J., Tne clinical application of the hippuric acid and the prothrombin test, *Amer. J. Clin. Path.*, **10**, 222, 1940.

18) Rosenfield, R. E. and Tuft, H. S., Estimation of prothrombin level from prothrombin time, *Amer. J. Clin. Path.*, **17**, 405, 1947.

19) Stefanini, M. and Crosby, W. H., The one stage prothrombin consumtion test, Blood, **5**, 965, 1950.

20) deVries, A., Alexander, B. and Goldstein, R., A factor in serum which accelerates the conversion of prothrombin to thrombin I. Its determination and some physiolgic and biochemical properties, *Blood*, **4**, 247, 1949.

21) Pitney, W. R., The assay of antihemophilic globulin (AHG) in plasma, *Brit. J. Haemat.*, **2**, 250, 1956.

22) Fukui, H. and Umegaki, K., Plasma thromboplastin component assay in the serum, *J. Nara Med. Ass.*, **8**, 40, 1957 (in Japanese).

23) Nour-Eldin, F., The action of serum on plasma thromboplastin, *Acta haemat.*, **28**, 265, 1962.

24) Seaman, A. J. and Karlsen, K., Influence of serum incubation condition on asolectin thromboplastin generation results, *Thromb. Diath. Haemorrh.*, **4**, 83, 1959.

25) Mustard, F. J., Some *in vitro* effects of various concentration of disodium ethylenediamine tetracetate, potassium oxalate and sodium citrate on coagulation of blood, *Amer. J. Clin. Path.*, **30**, 498, 1958.

26) Penick, G. D. and Brinkhous, K. M., Relative stability of plasma antihemophilic factor (AHF) under different condition of storage, *Amer. J. med. Sci.*, **232**, 434, 1956.

27) Fahey, J. L., Ware, A. G. and Seegers, W. H., Stability of prothrombin and Ac-globulin in stored human plasma as influenced by conditions of storage, *Amer. J. Physiol.*, **154**, 122, 1948.

28) Spaet, T. H. and Garner, E. S., Studies on the storage lability of human antihemophilic

factor., *J. Lab. Clin. Med.*, **46**, 111, 1955.

29) Umegaki, K., Thromboplastin generation test, *Jap. J. Clin. Med.*, **22**, 161, 1964. (in Japanese).

30) Biggs, R., Eveling, J. and Richards, G., The assay of antihemophilic globulin activity, Brit. *J. Haemat.*, **1**, 20, 1955.

31) Pitney, W. R., The assay of antihaemophilic globulin (AHG) in plasma, *Brit. J. Haemat.*, **2**, 250, 1956.

32) Pool, J. G. and Robinson, J., Observation on plasma banking and transfusion procedures for haemophilic patients using a quantitative assay for antihaemophilic globulin (AHG)., *Brit. J. Haemat.*, **5**, 24, 1959.

33) MacMillan, R. L., Thromboplastin generation test of Biggs and Douglas, *In* blood coagulation, hemonrhage and thrombosis. Edited by L. M. Tocantins and L. A. Kazal, Grune and Stratton, Inc., New York, 1964. p. 89.