ON THE RELIABILITY OF THE METHODS USED IN THE ASSAY OF HEPARIN

BY BIRGER and MARGARETA BLOMBÄCK, ERNST V. CORNELIUSSON and J. ERIK JORPES

From the Chemistry Department II, Caroline Institute, Stockholm, Sweden

Received August 31, 1953

THE determination of the anticoagulant activity of heparin is an extremely difficult task. The results are influenced by a number of factors, not least by the procedure of assay which is chosen. Identical results are hardly to be anticipated when such different procedures are applied as those listed below (Table I).

TABLE I

METHODS USED IN THE ASSAY OF HEPARIN

A.	Unaltered blood.	
	Howell[9]	Fresh cat blood
	Scott and Charles[10]	Fresh cat blood
	Jaques and Charles[3]	Fresh cat blood.
	Jorpes[11] and Wilander[12]	Fresh ox blood.
	Schütz[13]	Fresh rabbit blood.
B.	Heparinised plasma.	
	Neutralization of heparin with kinase	
	Dam and Glavind[14]	Human plasma.
C.	Oxalated blood + thrombin.	
	Jaques and Charles[3]	Oxalated ox blood.
D.	Oxalated or citrated plasma + thrombin.	
	Kjems and Wagner[4]	Oxalated ox plasma.
	Studer and Winterstein[2]	Citrated ox plasma.
E.	Citrated plasma + calcium salt.	
	Reinert and Winterstein[15]	Citrated ox plasma.
	Foster[16]	Citrated ox plasma.
	Mangieri[8]	Citrated ox plasma.
	Kuizenga, Nelson and Cartland[17] (U.S.P. XIV, 1950)	Citrated sheep plasma.
F.	Oxalated plasma + calcium salt + tissue extract in excess.	
	MacIntosh[18]	Oxalated horse plasma.
G.	Sulphated whole blood + thrombokinase.	
	Adams and Smith[19] (B.P., 1953)	Ox blood "salted" with sodium sulphate.
H.	Bird plasma + tissue extract.	
	Fischer and Schmitz[20]	
	Chargaff, Bancroft and Stanley-Brown[21]	
	Astrup[22]	
	Jaques and Charles[3]	

It is to be expected that the results of the assay will vary in accordance with the method applied. Discrepancies of this kind were experienced in the assay of the different commercial brands of heparin used in anticoagulant therapy.

Our interest in this question arose when several control laboratories claimed that a brand of heparin assayed by the whole blood method (see Jalling, Jorpes and Lindén[1]) had been found with the U.S.P. method of assay to have only 80 per cent. of the declared strength. We were soon able to confirm this finding. We consequently found it necessary to

extend our studies. We were then able to show that the U.S.P. method, using citrated sheep plasma and recalcification with no addition of thrombin or thrombokinase, almost consistently gives a 10 to 15 per cent. lower figure for the anticoagulant activity than the whole blood method[1] and the thrombin method of Studer and Winterstein[2]. This applies to heparin samples varying in strength between 25 and 110 international heparin units per mg. of water-free substance. Since the two last-

TABLE II

THE METHOD USING FRESH OX BLOOD

Sample	Checked against	Date	Number of Stands	Units/mg. or ml.	
				Calculated	Found
International heparin standard	Swedish heparin standard ..	17.6.1952	8	130	130
Provisional international heparin standard, 1942	,,		7 (8)	130	130 (140)
Swedish heparin standard	International heparin standard	18.6.1952	18	80	85
,,	,,	30.7.1952	17	80	82
Commercial sample E 1952 of Heparin Vitrum	,,	25.7.1952	18	5000	4950
Commercial sample of Heparin Novo	Swedish heparin standard	23.7.1952	18	5000	4950
,,	,,	6.5.1952	18	5000	5325
Commercial sample of Liquemin Roche	,,	15.2.1952	11	5000	5000

mentioned methods are those most commonly used in Scandinavia and on the Continent, the discrepancy deserves attention.

Potency of the Original Swedish Heparin Standard. When assayed by the whole blood method against the international heparin standard, both commercial samples of heparin and the original Swedish heparin standard showed a correct potency (Table II).

When the Swedish heparin standard, having a potency of 81 units per mg. as assayed by the whole blood method, was assayed by the thrombin

TABLE III

ASSAY OF THE ORIGINAL SWEDISH HEPARIN STANDARD AGAINST THE INTERNATIONAL HEPARIN STANDARD

Whole blood method	Thrombin method of Studer and Winterstein	Plasma method of the U.S.P. XIV 1950
80	83	65
80	79	68
85	86	70
82	83	70
80* (25.11.51)	83	65
81† (23.7.52)	78	65
76† (6.5.52)	—	68
81	82	67

* Checked against Liquemin Roche
† Checked against Heparin Novo

method of Studer and Winterstein, 82 units/mg. were found. The U.S.P. method, however, gave only 67 units/mg. (Table III). The figures represent means of 7 or 8 assays performed on different days.

Analysis of Heparin Samples of Different Strength. We extended our studies to the analysis of 20 samples of heparin sodium varying in strength between 25 and 130 units per mg. (see Table V). All the samples were subjected to the 4 most commonly used methods of assay, i.e., the thrombin method of Studer and Winterstein[2], the fresh ox blood method of Jalling, Jorpes and Lindén[1], the U.S.P. XIV method using recalcified citrated sheep plasma, and the method of the British Pharmacopœia 1953, using salted ox plasma. As a rule, the assay was made both against the original Swedish heparin standard (I) and against the international heparin standard (II).

METHODS

The Whole Blood Method Using Fresh Ox Blood[7].

The coagulation time is determined in non-paraffined test tubes of pyrex glass holding 2·5 ml. (70 × 8 mm.) with exclusion of air. 10 tubes are placed in an oak rack (30 × 4 × 2 cm.), provided with a cover of the same size. The lower side of the cover is lined with rubber, as is also the upper surface of the rack, so that all the tubes can be tightly closed at the same time. Since the exclusion of air is essential, the hinge and the metal hook closing the stand must be strongly fixed. In order to permit the stand to be closed tightly after filling, it is further equipped with a screwing device in the middle.

Each tube contains 0·2 ml. of a diluted heparin solution and a glass bead, of somewhat smaller size than the bore of the tube. 5 of the tubes contain the standard heparin, 5 the unknown. The diluted solutions of standard heparin contain, in the first tube 10 mg. of water-free substance per 32 ml. of physiological saline solution, in the second 5 mg. and so on; corresponding amounts of the unknown are taken. In summer the heparin is dissolved in 16 ml., during winter the same amount sometimes requires 64 ml.

The blood is taken directly from the vessel into a paraffined dish when the animals are slaughtered at the abattoir. One after the other, the tubes are quickly filled with blood, the cover then being tightened without allowing air to enter the tubes, and the stand is turned over several times. Mixing is obtained by means of the glass bead. Thorough mixing is essential before the stands are left. Only 2 stands can be filled with blood from one dish and in every second stand the tubes with the standard heparin are filled first.

The first reading is made at the laboratory 2 hours later. Further readings are made after 4, 8 and 24 (26) hours. Since the slowing up of the speed of the glass bead indicates the beginning of coagulation, the time for the initial coagulation is noted, as well as that of the final stage. Tubes containing air bubbles are discarded.

The temperature need not be regulated since its influence is the same on the standard and the unknown.

The evaluation of the reading is a simple empirical one described in the original publication. Samples varying 200 per cent. in strength can be compared with each other.

When 16 stands were used at the same time, the method allowed a differentiation of samples varying in strength by 5 per cent. The results were not submitted to any statistical analyses. Since the steps between the different tubes differ in strength by 100 per cent., the method is not particularly well suited for an accurate analysis. Moreover, the accuracy of an assay is largely dependent upon the skill of the person who fills the tubes with fresh blood at the abattoir.

The *Thrombin Methods.*

In discussing the different methods available for the assay of heparin, Jaques and Charles[3] in 1941 gave a definite preference to the whole blood methods, using either freshly drawn ox blood or oxalated whole blood, to which a fixed amount of thrombin had been added. The oxalated whole blood was more stable than the plasma prepared from it. Moreover, the addition of thrombin made the end-point sharper.

The idea of assaying heparin against thrombin was taken up again by Kjems and Wagner[4] in 1948. They added varying quantities of heparin (0·04 to 0·1 ml. of a solution containing 2·5 I.U./ml.) to 1 ml. of oxalated ox plasma and a constant amount of thrombin. The unknown and the standard heparin were run simultaneously and the coagulation times were plotted against concentration of heparin over the range about 20 seconds to about 60 seconds. The readings were made in a water bath at 37° C. The fresh plasma was left standing at 0° C. for at least 24 hours before it was used. Even during the time of the experiment it had to be kept at +0·5° C. The volume of the heparin solution was adjusted to 0·2 ml. Due attention was paid to the salt concentration and to the acidity of the solutions.

In its final form, the thrombin method used by Studer and Winterstein was as follows:—

Reagents.

Citrated Ox Plasma. 19 volumes of fresh ox blood were added to 1 volume of 8 per cent. w/v trisodium citrate solution. 2 hours later, plasma was separated by centrifugation for 30 minutes at 3000 r.p.m. The plasma was left standing at 0° C. for 24 hours before freezing. It could then be stored in 150 ml. portions for several weeks at −20° C. It was thawed in warm water before use, filtered through gauze and kept in ice-water during the time of the experiment.

Heparin. A 0·1 per cent. solution of heparin with 90 to 130 I.U./mg.

Thrombin. An approximately 0·1 per cent. solution of thrombin with about 60 N.I.H. units/mg. A concentration should be chosen which gives a coagulation time of about 60 seconds for the 0·6 point on the curve.

Technique.

For the 0 point of the standard curve without heparin, 0·5 ml. of distilled water is added to 5 ml. of thrombin solution and 0·1 ml. of this

solution is diluted in a 1 ml. tuberculin syringe to 1 ml. with distilled water prewarmed to 20° C. 0·5 ml. of this solution is again diluted in the syringe to 1 ml. with water. The content of the syringe is added to 1 ml. of the thawed plasma, warmed in a water bath at 37° C., a stop-watch being started simultaneously. The test-tube is moved lightly to and fro in the water bath for 5 to 10 seconds. A platinum needle is then moved up and down in the test-tube until the first threads of fibrin are formed, when the clotting time is read off. The process is repeated 3 to 5 times. The clotting time of the 0 point should be about 20 seconds.

For the 0·2 *point of the curve*, 5 ml. of the thrombin solution is pipetted into a mixture of 0·1 ml. of the standard heparin solution and 0·4 ml. of water, after which the above procedure is repeated.

The clotting times of the 0·4 and 0·6 points and, if necessary, of further points in between are determined in the same way.

For each concentration of the heparin standard the same procedure is repeated 3 to 5 times. The clotting time of the 0·6 point should fall between 60 and 70 seconds on the standard curve. Approximately the same dilutions are made of the unknown heparin solution.

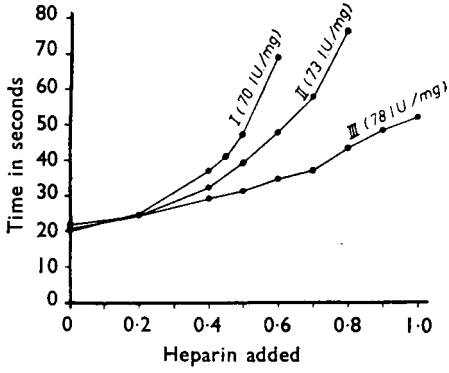

FIG. 1. Influence of storing plasma at 0° C. before freezing on the estimate of the potency of heparin.

I. Plasma frozen immediately after centrifuging.
II. Plasma stored for 24 hours before freezing.
III. Plasma stored for 48 hours before freezing.

The figures found for the anticoagulant activity of the same heparin sample are given in brackets. Concentration of the international heparin standard 0·331 mg./ml.

Only the ascending part of the standard curve can be used.

Comments on the Plasma Thrombin Method.

Kjems and Wagner, as well as Studer and Winterstein, pointed out that the plasma should not be frozen until it had been left for 24 hours at 0° C. In our experience, plasma prepared according to these authors tends to give too high values, as compared with the figures obtained in using plasma frozen immediately after being centrifuged. We have, therefore, preferred to freeze the plasma immediately. A new batch of fresh plasma was used every 3 weeks during 1 year, and always with reproducible figures. When thawed, the plasma can be stored for at least 4 to 5 hours in ice-water with no alteration in the standard curve. The effect of storing the plasma at 0° C. for 24 and 48 hours after centrifugation is shown in Figure 1.

The blood must be collected with the greatest care. It must be filtered through gauze before centrifugation. If small clots have been formed,

the blood is discarded. After thawing, the temperature of the plasma is never allowed to exceed + 5° C.

Our first experience[1] with the Studer and Winterstein method was disappointing. The assay of 16 different heparin preparations on as many days consistently gave about 20 per cent. higher figures than the whole blood method, irrespective of the strength of the samples, which varied between 30 and 130 units/mg. On changing over to a new batch of fresh plasma, lower and more consistent results were obtained. The original Swedish standard heparin was then found to contain 83, 79, 86, 83, 83 and 78 (mean 82) units/mg. of water-free substance as compared with the international heparin standard. The results given in Table III all refer to fresh plasma. We were unable to find any explanation for the

TABLE IV

ASSAY OF THE SWEDISH HEPARIN STANDARD AGAINST THE INTERNATIONAL HEPARIN STANDARD BY THE U.S.P. XIV METHOD
SHEEP PLASMA OF AUGUST 26, 1952

Date: September 14, 1952

Swedish heparin standard

Concentration:	12·095 mg. air-dry substance/10 ml.				Dilution 0·85 : 100				
Volume taken:	0·8	0·76	0·72	0·68	0·65	0·635	0·62	0·59	0·56
Grade of clotting:				>0·5	>0·5	0·5	>1	>1	1

Concentration:	12·095 mg. air-dry substance/10 ml.			Dilution 0·95 : 100			
Volume taken:	0·8	0·76	0·72	0·68	0·65	0·62	0·59	0·56
Grade of clotting:				0·25	>0·5	>0·5	0·5	>0·75

International heparin standard

Concentration:	5·05 mg./10 ml.			Dilution 0·9 : 100							
Volume taken:	0·8	0·77	0·74	0·71	0·68	0·66	0·64	0·62	0·60	0·58	0·56
Grade of clotting:		>0·25	0·25	>0·5	0·5	>0·75	>0·75	0·75	>1	1	1

Concentration:	5·05 mg./10 ml.			Dilution: 1 : 100						
Volume taken:	0·8	0·77	0·74	0·71	0·68	0·66	0·64	0·62	0·60	0·58	0·56
Grade of clotting:						0·25	>0·5	0·5	0·75	1	1

Calculation:

$$\frac{12{\cdot}095 \times 0{\cdot}85 \times 0{\cdot}635}{10 \times 100} \times x_1 = \frac{5{\cdot}05 \times 0{\cdot}9 \times 0{\cdot}68}{10 \times 100} \times 130; \quad \frac{12{\cdot}095 \times 0{\cdot}85 \times 0{\cdot}635}{10 \times 100} \times x_2 = \frac{5{\cdot}05 \times 1 \times 0{\cdot}62}{10 \times 100} \times 130$$

$$\frac{12{\cdot}095 \times 0{\cdot}95 \times 0{\cdot}59}{10 \times 100} \times y_1 = \frac{5{\cdot}05 \times 0{\cdot}9 \times 0{\cdot}68}{10 \times 100} \times 130; \quad \frac{12{\cdot}095 \times 0{\cdot}95 \times 0{\cdot}59}{10 \times 100} \times y_2 = \frac{5{\cdot}05 \times 1 \times 0{\cdot}62}{10 \times 100} \times 130$$

$x_1 = 62$; $x_2 = 62$; $y_1 = 59$; $y_2 = 60$ Mean: 61

difference in behaviour of the two batches of plasma. As a precautionary measure every new plasma sample should therefore be tested with two heparin samples of known strength.

As pointed out by Studer and Winterstein, the amount of thrombin remaining in excess after the addition of heparin strongly influences the shape of the curve. With a too high thrombin activity per ml. the curve will have a flatter appearance, which makes it less suitable. We found 0 points between 18 and 20 seconds most convenient to work with. If weaker thrombin solutions are used the heparin solution must be correspondingly diluted.

Studer and Winterstein recommend a heparin solution containing 100 to 130 I.U./ml. We have consistently used 30 to 40 I.U./ml. probably due to the fact, that we have used immediately frozen plasma samples (see Fig. 1).

We found the purity of the thrombin preparations less essential. We obtained equally good results when using preparations with from 10 to 70 Astrup units[5] per mg. The usual strength was 60 Astrup units per mg. and 40 to 50 mg. of thrombin was dissolved in 100 ml.

The Method of U.S.P. XIV, 1950.

The citrated sheep blood was centrifuged within 1 hour after slaughter and the plasma stored in small paraffined paper boxes at − 20° to − 25° C. After thawing the plasma and filtering through gauze it was left standing at + 2° C. for 1 hour. If a precipitate formed which did not redissolve at room temperature the plasma was discarded. The reading of the tests, a very delicate matter, was made by the same person who, in the course of a year and a half, performed readings on about 400 series of dilutions.

The technique applied is demonstrated in Table IV.

The Sulphated Whole Blood Method

With this method, aqueous heparin solutions are used to dilute whole blood, kept incoagulable by means of a high concentration of salts. The fresh ox blood is "salted" by mixing with one-fifth of its volume of 7 per cent. w/v solution of anhydrous sodium sulphate and then stored at 4° C. It is stable for 3 or 4 weeks.

In order to shorten the coagulation time a water extract of acetone-dried ox brain is added. The assay is conducted at room temperature.

Procedure. Prepare in *water* 3 dilutions of the Standard Preparation containing 1·28, 1·6 and 2·0 units/ml. and three expected equivalent dilutions of the preparation to be tested. Place 1 ml. of each dilution in 6 × $\frac{1}{2}$ in. test-tubes followed by 0·2 ml. of thrombokinase extract; the amount of thrombokinase extract to be added may be varied slightly according to conditions, but should be chosen so that the longest clotting times range between 9 and 12 minutes. Add 1·0 ml. of sulphated whole blood and mix by gentle inversion, avoiding the formation of air bubbles. For each tube, record the time to the nearest 15 seconds from this addition to the formation of a firm clot which remains in the bottom of the tube when it is completely inverted. For a complete assay repeat the comparison 4 times.

The method offers many advantages. The equipment needed is very simple. The method is rapid and accurate. The data can be submitted to analysis of variance, the relationship of the log coagulation time to the log concentration of heparin being linear. Figures for the fiducial limits are easily obtained.

Discussion

As is evident from Table V, the thrombin method of Studer and Winterstein gives very small deviations between the values obtained on different days and in using the two different standard preparations. Along the whole line there is a surprisingly good agreement between the values for the same sample. No such agreement was obtained with the other methods.

The whole blood method, which we used earlier, requires much training in filling the glass tubes with fresh ox blood. The 100 per cent. difference between the concentrations, which is necessary for a clear-cut reading, is also a drawback. We have used the method because it is a whole blood method, without the introduction of any foreign factors into the system, except for the influence of the glass surface of the tubes. The technique also excludes any effect of the air, which is known to give rise to thrombokinase formation. The anticoagulant activity is determined under almost physiological conditions and the figures obtained seem to be

TABLE V

THE POTENCY OF HEPARIN SAMPLES IN I.U. AS ASSAYED ON DIFFERENT DAYS BY DIFFERENT METHODS AGAINST THE ORIGINAL SWEDISH HEPARIN STANDARD (I) AND THE INTERNATIONAL HEPARIN STANDARD (II)

Sample No.	Water free substance per cent.		Plasma + thrombin (Studer and Winterstein)		Fresh ox blood (Jalling, Jorpes and Lindén)		Recalcified plasma (U.S.P. XIV 1950)		Diluted salt plasma (B.P. 1953)	
	S	N	I	II	I	II	I	II	I	I
1	13·07	2·77	126 138	124 136		128	125 135 136	128 140 144		140 135
2	13·41	2·21	130	126 135		126	116 117	110 121	122	126
3	13·20	2·39	124 124	123 124	117	108 102 102	148 139 138	132 133 124 139	122	119 111 111
4	13·00	2·17	113 115 113 110	109 112	108	105 98 102	115 112 124	105 116 116 117	111	106 107 115
5	12·69	2·31	104 110	105 109		104 102	102 110 113	104 116	100	104 106
6	12·96	2·44	114	113		101 104 110	107	110	112	109 107
7	12·93	2·56	122	121		120	117 112	114	128	117
8	12·53	2·12	109	113		107 118	97 99 101	96 104		110
9	12·77	2·51	89 91	90	96	83 78 78	78 78	80 79		76 73 75
10	12·71	2·35	93	93		89	81 87	83	92	82 85
11	11·97	2·46	87 83	81 85		75 73	80 76	82 71	90	80 75
12	12·80	2·49	87 83	85		77 72	77	74	74	78 73 69
13	11·76	2·49	83 79	83		94 82	76 77	79		77 75
14	12·03	2·58	81			78	72 70	66		78
15	11·40	2·47	81			80	69	70		70 72
16	12·68	2·20	78	81 81		79	79		82	
17	10·03	3·21	73 73	71	85		69 66	69	69	
18	10·01	4·64	53 52	53	56		46 46		57	
19	8·39	4·09	30 27 29	29	28	29	15 17 17	15 17	24	
20	6·41	2·99	26 25	26 25		23		32	32	

In the thrombin method two concentrations of the unknown were analysed to be read off on the standard curve. In the whole blood method usually only 9 stands were taken each day. In the U.S.P. method two concentrations of the unknown were compared with two concentrations of the standard.

reliable within 5 to 10 per cent., which is the requirement in anticoagulant therapy.

We do not, however, wish to give any preference to this method, which for various reasons is inferior to the thrombin method of Kjems and Wagner and to that of Studer and Winterstein. As is shown in Table V, every second sample showed about 10 per cent. lower activity with the whole blood method as compared with the figures found with the thrombin method.

The least satisfactory results were obtained with the U.S.P. XIV method. The deviations between the values obtained on different days were quite remarkable. 10 of the 12 samples with an activity below 110 units/mg. showed 10 to 15 per cent. lower figures than with the thrombin method, and out of 8 samples with an activity above 110 units/mg., 2 presented 5 to 10 per cent. too low figures.

Our experience with the method of the British Pharmacopœia has hitherto been limited.

The Thrombin Method Versus the U.S.P. XIV Method. It is evident that recalcified sheep plasma is less suitable for the assay of ordinary heparin samples. Below 110 units/mg., it gave in our series figures 10 to 15 per cent. lower than the thrombin method and above this strength the figures are 5 to 10 per cent. either higher or lower. Different control laboratories also found well-known brands of commercial heparin less satisfactory when this method of assay was applied. We recently (June 1953) analysed a sample of a Danish heparin, which showed an activity of 3335 units/ml., as assayed against the international heparin standard with the U.S.P. method instead of the declared strength of 5000 I.U./ml. There was no reason to assume that the sample was of a low grade quality.

In their studies on treburon, a sulphated polygalacturonic acid methyl ester methyl glycoside, Mangieri, Engelberg and Randall[6] found that the U.S.P. XIV method did not show the true relative activities of treburon and heparin. Assayed by the U.S.P. method, treburon was approximately one-tenth as active as heparin. By Mangieri's recalcification method using frozen beef or sheep plasma, treburon was one-fourth as active as heparin. Also by Quick's[7] antithrombin method of 1936, using fresh rabbit plasma or frozen sheep plasma, treburon was one-fourth as active as heparin. The relative activities 4:1 found with these two methods *in vitro* agreed with the relative activities found *in vivo* in animal experiments. From the results of Mangieri and co-workers, the conclusion must be drawn that the U.S.P. XIV method is less sensitive in respect to the properties by means of which heparin and the heparinoids exert their anticoagulant activity in whole blood and *in vivo*.

From the theoretical point of view, the thrombin methods seem to be the most suitable. Heparin is, after all, an antithrombin more than anything else. In the first place, in the thrombin methods recalcification becomes superfluous. An important source of error is thereby eliminated. The extreme sensitivity of the coagulation mechanism to the concentration of the calcium ions is most clearly stressed by Mangieri[8], who found it necessary to titrate the amount of calcium to be added with concentrations varying in strength by 5 per cent.

Furthermore, in the non-thrombin methods the accelerating factors V and VII, proaccelerin and proconvertin, as well as the antihæmophilic globulin and the thrombokinase system, play their part in the reaction mechanism, in one way or another influencing the results. It is not surprising that the thrombin methods with the direct reaction between heparin and thrombin give the best reproducible results.

SUMMARY

1. The anticoagulant activity of 20 samples of heparin sodium varying in strength between 25 and 130 I.U./mg. has been assayed by 4 different methods: a fresh whole blood method, a thrombin method on plasma, and the methods of the U.S.P. XIV and the B.P. 1953.

2. The U.S.P. XIV method gave 10 to 15 per cent. lower figures than the thrombin method for samples with 25 to 110 I.U./mg.

3. The whole blood method did not give as satisfactorily reproducible results as the thrombin method on plasma.

4. Commercial samples of heparin assayed with the U.S.P. XIV method tended to give lower figures for the anticoagulant activity than when assayed with the thrombin method.

5. Since different factors such as the recalcification, the proaccelerin and the proconvertin, as well as the thrombokinase system, influence the results in the non-thrombin methods, they are considered less suitable for the assay of heparin. Preference is therefore given to the thrombin methods, in which the heparin is neutralised by preformed thrombin, preferably with some whole blood method as an alternative.

REFERENCES

1. Jalling, Jorpes and Lindén, *Quart. J. Pharm. Pharmacol.*, 1946, **19**, 96.
2. Studer and Winterstein, *Helv. Physiol. Pharmacol. Acta*, 1950, **9**, 6.
3. Jaques and Charles, *Quart. J. Pharm. Pharmacol.*, 1941, **14**, 1.
4. Kjems and Wagner, *Acta Pharmacol.*, 1948, **4**, 155.
5. Astrup, *Acta Physiol. scand.*, 1941, **2**, 22.
6. Mangiere, Engelberg and Randall, *J. Pharmacol.*, 1951, **102**, 156.
7. Quick, *Amer. J. Physiol.*, 1936, **115**, 317.
8. Mangieri, *J. Lab. clin. Med.*, 1947, **32**, 901.
9. Howell, *Amer. J. Physiol.*, 1924–5, **71**, 553.
10. Charles and Scott, *J. biol. Chem.*, 1933, **102**, 425, 437.
11. Jorpes, *Biochem. J.*, 1935, **29**, 1817.
12. Wilander, *Skand. Arch. Physiol.*, 1938, **81**, Suppl. XV.
13. Schütz, *Quart. J. Pharm. Pharmacol.*, **14**, 15.
14. Dam and Glavind, *Skand. Arch. Physiol.*, 1939, **82**, 221.
15. Reinert and Winterstein, *Arch. int. Pharmacodyn.*, 1939, **62**, 47.
16. Foster, *J. Lab. clin. Med.*, 1942, **27**, 820.
17. Kuizenga, Nelson and Cartland, *J. biol. Chem.*, 1943, **148**, 641.
18. MacIntosh, *Biochem. J.*, 1941, **35**, 770.
19. Adams and Smith, *J. Pharm. Pharmacol.*, 1950, **2**, 836.
20. Fischer and Schmitz, *Z. physiol. Chem.*, 1932, **210**, 129.
21. Chargaff, Bancroft and Stanley-Brown, *J. biol. Chem.*, 1936, **115**, 149, 155.
22. Astrup, *Enzymologia*, 1938, **5**, 12.