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IN VIVO AND *IN VITRO* COMPARATIVE ASSAYS OF THE ANTICOAGULANT ACTIVITY OF DEXTRAN SULPHATE AND HEPARIN

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The recent demonstration by Ricketts (1952), Walton (1952) and Ricketts and Walton (1952), that dextran sulphate preparations of low molecular weight are both anticoagulant and non-toxic, raises the problem of the measurement of potency of semi-synthetic anticoagulants and of the choice of a suitable standard for assay.

Several attempts have been made to assay other semi-synthetic anticoagulants in terms of heparin (Schutz, 1941; Reinert and Winterstein, 1939; Foster, 1942; Kuizenga, Nelson and Cartland, 1943). We have also used heparin, and made some observations on the mode of action of heparin and dextran sulphate. In our hands the *in vitro* methods depending upon the inhibition of blood coagulation proved to be impracticable because the dose-response curves of heparin and dextran sulphate were not parallel. We accordingly devised an *in vivo* method for the comparison of different anticoagulants. This method has the advantage that, compared with *in vitro* tests, the conditions in which potency is measured are closer to those of clinical use. Moreover, since the dose-response curves are steep, high ratios of minimal anticoagulant doses give a useful approximate estimate of comparative potency, irrespective of differences of dose-response of the substances compared.

METHODS

International standard heparin was used throughout. The proposed international standard for dextran sulphate was obtained from the Department of Biological Standards, National Institute for Medical Research, London. The Russell viper venom was reconstituted from desiccated venom obtained from the Central Research Institute, Kasauli, India.

By paper electrophoresis we separated four fractions of Russell viper venom. One of these—the "coagulant fraction"—contained 80% of the total coagulant activity of the crude venom, behaved like a thrombokinase, and had no thrombin activity.

In vitro *Assays.*—*In vitro* assays were made by the prothrombin-time method of Quick, as modified by Meyer, Piroue, and Odier (1952) using oxalated human plasma; 9 ml. venous blood, collected in a silicone-coated syringe, was immediately mixed with 1 ml. 0.1N-sodium oxalate and centrifuged for 10 min. at 2,800 r.p.m. at 15° C. The plasma was kept at room temperature and used within 3 hr. In one series of tests, coagulation was induced by thromboplastin (Difco) and $CaCl_2$; in another series by Russell viper venom and $CaCl_2$. In a few tests, in which guinea-pig plasma was used, the results were similar to those with human plasma, but were less consistent.

The standard heparin was dissolved in 0.85% saline at a concentration of 10 μg./ml.; the dextran sulphate in saline at a concentration of 81.5 μg./ml. Bacto-thromboplastin was added to saline at 50–55° C. to make a 1.875% (w/v) concentration and insoluble material removed by centrifugation; it was kept at 37° C. until used. The dried Russell viper venom was dissolved in saline, and a crystal of thymol added. About 7% of the dried material was insoluble and was removed by centrifugation; the remainder made up in saline, with thymol, to contain 0.1 mg. venom/ml.

Two assay techniques were used: (*a*) *With thromboplastin.* Plasma (0.1 ml.) was added to 0.2 ml. of graded concentrations of the dextran sulphate or of the heparin. The mixtures were kept at 20° C. for 10 min. and thereafter at 37° C. After 1–2 min., first 0.1 ml. thromboplastin and then 0.1 ml. 0.025N-$CaCl_2$—both at 37° C.—were added and mixed by inversion. The formation of a clot was detected by slowly moving a glass hook up and down the column of fluid. (*b*) *With viper venom.* The procedure differed from that with thromboplastin only in that the venom and the $CaCl_2$ were added simultaneously in a volume of 0.2 ml. This step was necessary because the venom initiated coagulation even in the absence of the calcium salt.

In vivo *Assays.*—As in the *in vitro* tests, Russell viper venom solutions were freshly prepared. The electrophoretically purified venom, in thymolized saline, was tested immediately *in vitro* for thrombokinase activity against a standard solution of total

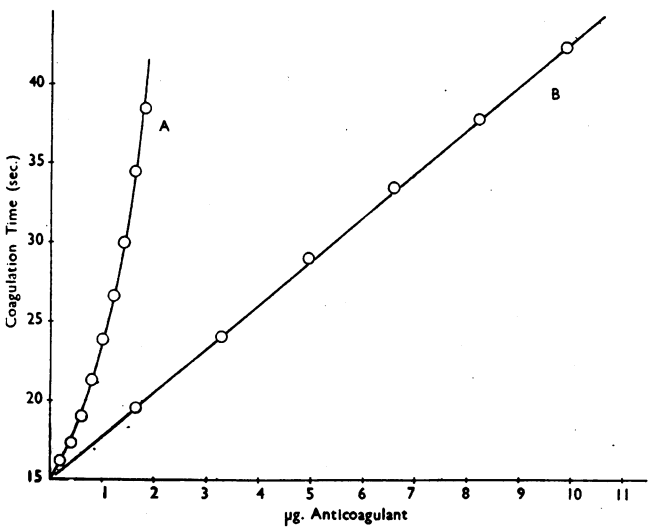

FIG. 1

FIG. 1.—Anticoagulant activity of heparin (A) and dextran sulphate (B). Coagulation time with thromboplastin-treated plasma plotted against dose of anticoagulant. Graded concn. of anticoagulant in 0.2 ml. volumes added to 0.1 ml. fresh oxalated human plasma, held for 10 min. at 20° and 2 min. at 37°; 0.1 ml. 1.875% thromboplastin and 0.1 ml. 0.025N-$CaCl_2$ successively added, and the mixtures agitated with a glass hook at 37° C. until clotting occurred.

FIG. 2.—Anticoagulant activity of heparin (A) and dextran sulphate (B). Experiment as in Fig. 1, except that log. coagulation time is plotted against dose.

FIG. 3.—Anticoagulant activity of heparin (A) and dextran sulphate (B). Coagulation time with Russell-viper-venom-treated plasma, plotted against dose of anticoagulant. Graded concn. of anticoagulant in 0.2 ml. volumes added to 0.1 ml. fresh oxalated human plasma, held 10 min. at 20° C. and 2 min. at 37° C.; and a mixture of 0.1 ml. 0.025N-$CaCl_2$ and 0.1 ml. of 0.1 mg./ml. venom added, and the final mixtures agitated with a glass hook at 37° until clotting occurred.

FIG. 4.—Anticoagulant activity of heparin (A) and dextran sulphate (B). Experiment as in Fig. 3, except that log. coagulation time is plotted against dose.

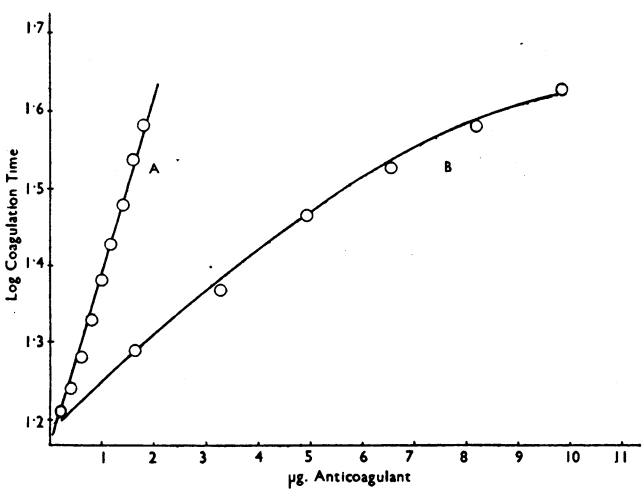

FIG. 2

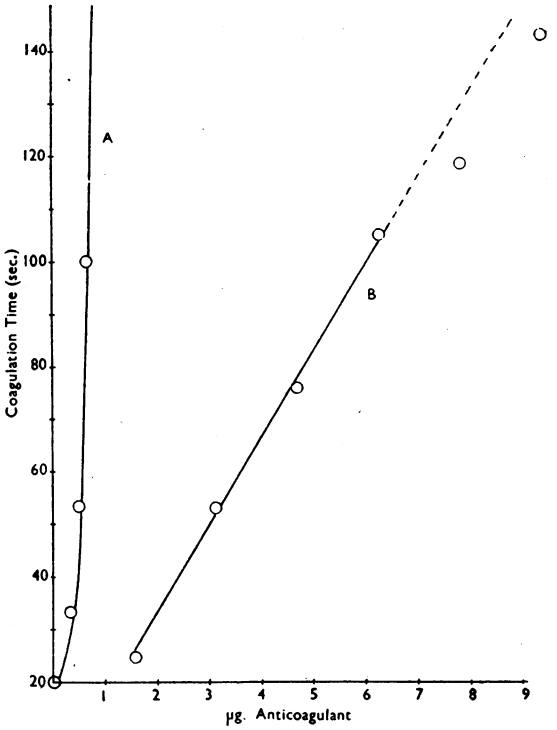

FIG. 3

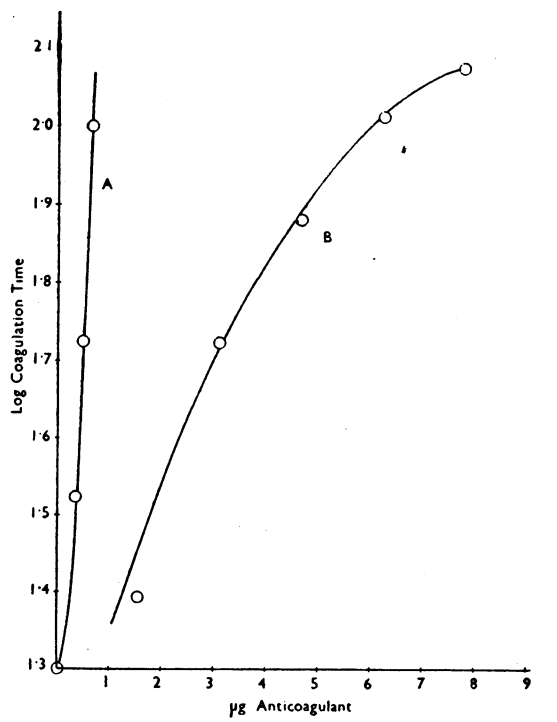

FIG. 4

Russell viper venom, and immediately used for injections.

The mixture of anticoagulant (heparin or dextran sulphate) with crude or purified Russell viper venom was made up to 5 ml. with physiological saline, allowed to stand for approximately 10 min. at room temperature, and slowly injected into an ear vein of a conscious rabbit over 2 to 3 min., carefully avoiding any subcutaneous leak. The animals were observed closely during the subsequent hours. Autopsies were usually carried out immediately after respiration had stopped. Often the heart was still beating or fibrillating. The cavities of the heart and the lumen in large vessels, particularly the pulmonary vessels, were searched for the presence of clots.

RESULTS

In vitro *Assays*

With either bacto-thromboplastin or Russell viper venom, the relation of dose of heparin to coagulation time was curvilinear (Fig. 1A); with respect to the logarithm of the coagulation time it was linear (Fig. 2A). The dose of dextran sulphate on the other hand was linearly related to the coagulation time; its relationship to the logarithm of the coagulation time was curvilinear (Figs. 1B and 2B). For a comparison of activity it would be possible to select an arbitrary coagulation time—for example 30 sec.—and compare the doses of the two substances which correspond to this response. On this basis, we found that dextran sulphate had about one-third the activity of heparin. The invalidity of this method is evident when another period is chosen as a response level. Thus at 40 sec. the ratio of activities is 1:9. Similar discrepancies were found when the logarithm of the dose of heparin was used as abscissa. Similar results were obtained with Russell viper venom (Figs. 3 and 4).

In other words, since the dose-response curves are not linear or parallel, neither the orthodox assay nor the slope-ratio assay (Finney, 1952) are applicable. The discrepancies in the dose-response curves of the two substances indicate that we are dealing with fundamentally different substances, thus violating the hypothesis of similarity (Finney, 1952) upon which valid assay is based.

In vivo *Assays*

The results are summarized in Table I. Rabbits receiving approximately 0.2 mg. of Russell viper venom alone, or its coagulant fraction, died within ½ to 3 min.

Post mortem there was a massive clot in the right heart cavities and thrombosis in large pulmonary vessels; the lungs were pale yellow and ischaemic.

Crude Venom (Table I, A).—In the test rabbits there was a certain critical weight ratio of anticoagulant to venom below which both heparin and dextran sulphate failed to inhibit the rapid coagulation of blood by Russell viper venom. The test animals died almost as quickly as the controls and the post-mortem picture was similar. Above this critical ratio, the anticoagulant inhibited the coagulant action of Russell viper venom and thus prevented immediate death. The animals survived the injection without apparent discomfort. Later, however, they developed progressive signs of general intoxication together with, after 3 to 4 hr., intense oedematous infiltration of the eyelids. They died within 3 to 20 hr.

Post mortem, these animals had lung oedema with extensive zones of hyperaemia surrounding haemorrhagic foci and, usually, congestion of most other organs. However, there were no clots in the chambers of the heart or in the large vessels. The delayed death appeared to be due to toxic components other than the coagulant fraction which are not inhibited by heparin or dextran sulphate. This view is consistent with the fact that repeated injections of heparin up to a total of 5 times the anticoagulant dose fail to prevent the later death.

When the minimum amount either of dextran sulphate or of heparin was used to neutralize the

TABLE I

THE ANTICOAGULANT ACTIVITY OF HEPARIN AND DEXTRAN SULPHATE IN RABBITS TREATED WITH RUSSELL VIPER VENOM

	Anti-coagulant	Rabbit Wt. (g.)	Dose of Venom (mg.) *a*	Dose of Anti-coagulant (mg.) *b*	Venom: Anti-coagulant Ratio (*a* : *b*)	Survival Time
A Crude venom	None	920	0·2			2 min.
		1,515	0·22			1·5 ,,
		1,320	0·2			3 ,,
	Standard heparin	980	3·0	6·0	1 : 2	2½–3½ hr.
		1,150	1·5	3·0	1 : 2	2½–3½ ,,
		2,250	0·6	1·2	1 : 2	12–20 ,,
		920	0·6	0·6	1 : 1	6 ,,
		1,070	0·6	0·6	1 : 1	10 ,,
		980	0·6	0·3	1 : ½	2½–3½ ,,
		1,050	0·6	0·3	1 : ½	1½ ,,
		1,200	0·6	0·3	1 : ½	3 min.
		1,290	0·3	2·8	1 : 9	12–24 hr.
	Standard dextran sulphate	1,200	0·3	81·5	1 : 272	2¼ hr.
		1,300	0·6	12·0	1 : 20	2 ,,
		1,090	0·6	9·0	1 : 15	2–3 min.
		2,460	0·6	6·0	1 : 10	2 ,,
		2,720	0·6	2·4	1 : 4	3 ,,
B Purified venom	None	1,480	0·15			>14 days
		1,440	0·2			3 min.
		1,140	0·35			2 ,,
	Standard heparin	1,470	0·8	1·0	1 : 1·25	>14 days
		1,140	0·35	0·35	1 : 1	>14 ,,
	Standard dextran sulphate	1,120	0·8	35·4	1 : 44·3	>14 days
		960	0·35	7·0	1 : 20	>14 ,,
		1,300	0·35	3·5	1 : 10	3 min.

immediate coagulant effect of the venom (venom: heparin = 1 : 0.5 ; venom : dextran sulphate = 1 : 20), death sometimes resulted from a late and progressive thrombus formation. In each of three rabbits so dying, a thrombus was found in the portal vein.

Our results agree with those of Ahuja, Veeraraghavan, and Menon (1946, 1947) as regards the weight ratio of heparin necessary to prevent death, and show clearly that the semi-synthetic anticoagulant dextran sulphate, if used in sufficient amount, has a total *in vivo* effect similar to that of heparin.

Purified Venom : Coagulant Fraction Isolated by Paper Electrophoresis (Table I, B).—As with crude venom, there was a certain critical ratio below which both heparin and dextran sulphate failed to prevent immediate death. Test rabbits died with the same signs and lesions as the controls. Above this ratio (for heparin 1 : 1 ; for dextran sulphate 1 : 20) they survived the immediate " coagulation " death ; indeed they survived for more than two weeks. Unlike those tested with crude venom, they did not show any signs of subsequent delayed intoxication, including the oedema of the eyelids —a useful criterion of the purity of the coagulant fraction of the viper venom.

Discussion

The data in Table I provide no basis for a comparison of the dose-response in heparin-treated and dextran-sulphate-treated animals. Nevertheless, it is clear that both with heparin, where the doses increase by twofold steps, and with dextran sulphate, where the dose increments are arithmetic, in steps of 3 mg., the response changes sharply from the acute to the delayed death.

With such steep dose-response curves, we may therefore take acute death as an index and directly compare minimal protective doses of the two anticoagulants. Potency ratios so determined have an added advantage over those obtained by *in vitro* methods, in that the conditions of the *in vivo* test are closer to those in clinical use of the substances, and so give a better indication of clinical efficacy.

From the results of these *in vivo* experiments, it is clear that, like heparin, dextran sulphate inhibits the coagulant action of either crude or purified Russell viper venom, whether it is used as a crude venom or as a purified fraction ; and that dextran sulphate of a potency equal to that of the proposed standard has, weight for weight, one-twentieth to one-fortieth of the anticoagulant efficacy of the international standard heparin.

Summary

1. The *in vitro* test for anticoagulant activity, based on Quick's method of measuring prothrombin time, does not provide a biometrically valid measure of the anticoagulant potency of dextran sulphate in terms of heparin.

2. Dextran sulphate and heparin both inhibit the coagulant action of Russell viper venom thrombokinase, *in vitro* and *in vivo*.

3. *In vivo*, intravenous Russell viper venom kills rabbits within a few minutes. When this acute death is prevented by previous mixing of the venom with either heparin or dextran sulphate, the animals die within 3 to 20 hr., owing to toxic substances other than the coagulant. These other toxins can be removed by paper electrophoretic fractionation of the crude venom.

4. An *in vivo* test, using Russell viper venom or the purified coagulant fraction, is described for the comparison of anticoagulant activity of heparin and anticoagulants of the dextran sulphate type. According to this test, the dextran sulphate constituting the proposed international standard is 20 to 40 times less potent than the international standard heparin.

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