peripheral effect on mammalian striated muscle.

1. Langley, J. N., and Dickinson, W. L., *J. Physiol.*, 1809, v11, 265.
2. Amantea, C., *Arch. farm. sper. e Sc. aff.*, 1920, v30, 3.
3. Galamini, A., *Boll. Soc. ital. biol. sper.*, 1928, v3, 1206.
4. Longo, V. C., and Bovet, D., *Boll. Soc. ital. biol. sper.*, 1952, v28, 612.
5. Langley, J. N., *J. Physiol.*, 1905, v33, 374.
6. Bacq, Z. M., and Brown, G., *J. Physiol.*, 1937, v89, 45.
7. Brown, G. L., *J. Physiol.*, 1937, v89, 438.
8. Cahen, R. L., and Lynes, T., *J. Pharm. and Exp. Therap.*, 1951, v103, 44.
9. Cahen, R. L., Thomas, J., and Tvede, K., *J. Pharm. and Exp. Therap.*, 1953, v107, 424.
10. Longo, V. C., and Bovet, D., *Farmaco.*, 1949, v4, 515.
11. Auer, J., and Meltzer, S., *J. Pharm. and Exp. Therap.*, 1913-1914, v5, 521.
12. Gross, E., and Cullen, J., *J. Pharm. and Exp. Therap.*, 1943, v78, 358.
13. Comroe, J. H., and Dripps, R., *Anesthesiol.*, 1946, v7, 260.
14. Pick, E., and Richards, C., *J. Pharm. and Exp. Therap.*, 1947, v90, 1.
15. Lang, D. A., Kimura, K., and Unna, K., *Arch. internat. pharmacodyn.*, 1951, v85, 257.
16. Shallek, W., PROC. SOC. EXP. BIOL. AND MED., 1946, v63, 79.
17. Artusio, J. F., Riker, W., and Wescoe, W. C., *J. Pharm. and Exp. Therap.*, 1950, v100, 227.

Received July 30, 1953. P.S.E.B.M., 1953, v84.

Relative Inability of Heparin to Prolong Clotting Time of Oxalated and Resin-Treated Blood and Plasma.*† (20683)

LUCY SALOMON AND MARIO STEFANINI.

From the Ziskind Laboratories, New England Center Hospital, and J. H. Pratt Diagnostic Clinic, and the Department of Medicine, Tufts College Medical School, Boston.

In the course of studies on the anticoagulant activity of heparin it was noticed that this drug failed to prolong the clotting time of recalcified, oxalated or resin-treated blood and plasma. In view of the possible importance of this phenomenon as a source of error in the evaluation of the anticoagulant activity of heparin, it was decided to investigate the matter further.

Materials and methods. A) *Collection of blood and plasma in various anticoagulants.* Blood was collected from healthy donors in Silicone-coated glass syringes through Arquad 2-C‡ coated needles. It was transferred to Silicone-coated test tubes and made incoagulable by the addition of one of the following reagents: (a) sodium or potassium oxalate, 0.1 M; (b) sodium citrate, 0.2 M; (c) Sequestrene-Na_2§ 1% in 0.7% NaCl, all in the volume of one ml to 9 ml of blood. In other experiments, whole blood was decalcified by passage through a column of cation-exchange resin (2.5 g of resin to 10 ml of blood) with the technic described by one of us(1). Dowex-50‖ and IR-100¶ in the sodium cycle were used in this work. In all cases plasma was separated by centrifugation of the whole blood at 2000 rpm for 10 minutes. B) *Addition of heparin.* Saline solution containing various amounts of heparin was added, in the

* Supported by grant-in-aid from the American Heart Assn.

† The authors wish to express their appreciation to Dr. Peter Bernfeld and to Mr. Mordecai Berkowitz, Cancer Research Unit, Tufts College Medical School, for repeating and confirming some of the results reported.

‡ Dicoco-dimethyl-ammonium chloride; available from Armour Co., Chicago, Ill.

§ Disodium ethylendiamine-tetracetate-dihydrate; available from Alrose Chemical Co., Providence, R. I.

‖ Available from Dow Chemical Co., Midland, Mich.

¶ Available from Rohm and Haas Co., Philadelphia, Pa.,

TABLE I. Clotting Time of Recalcified Plasma, in Presence of Various Amounts of Heparin Sodium (Average Values of Several Experiments). [Plasma was obtained by centrifugation from blood made incoagulable by various technics.]

Anticoagulant originally used (final conc.)	Sodium oxalate, .01 M	Sodium citrate, .02 M	Sequestrene-Na_2, 0.1%	Dowex-50	IR-100	Native plasma*
Heparin, γ/ml	Clotting times (min.)					
0	3.5	4	4.5	3	3.5	4
1	3.5	14	12.5	3.5	4.5	15.5
2	4	26	29	3.5	4	26
3	4	62	58	4	5.5	56
4	4.5	∞	∞	7	6	∞
5	5.5	∞	∞	8.5	8.5	∞
10	7	∞	∞	12	11.5	—
20	12.5	—	—	17	19	—
30	∞	—	—	∞	∞	

* No recalcifying agent added.

volume of 0.1 ml for every 0.9 ml of blood or plasma. C) *Determination of clotting time of recalcified blood and plasma.* Blood or plasma made incoagulable by the technics described under A), with or without the addition of heparin, were transferred to glass test tubes in water bath at 37°C and recalcified with 1/10 volume 0.2 M $CaCl_2$. The test tubes were then tilted every 30 seconds for 10 minutes and every minute thereafter, until they could be inverted without escape of content. D) *Determination of prothrombin time* was carried out by the one-stage method of Quick, using dehydrated human brain thromboplastin.

Results. Heparin and clotting time of recalcified blood and plasma collected in various anticoagulants. Table I shows that as much as 5 γ of heparin sodium per ml failed to prolong very significantly the clotting time after recalcification of plasma from blood made incoagulable by addition of sodium or potassium oxalate or by passage through cation-exchange resins. This result was in sharp contrast with that obtained with native, citrate or Sequestrene-Na_2 treated plasma. In the latter case, the clotting time after recalcification was normally retarded by the addition of heparin. The effect of oxalate and resins was not modified by changing the concentration of $CaCl_2$ used to recalcify the mixture, although all clotting times were uniformly delayed when the concentration of $CaCl_2$ was over 0.02 M in the final mixture. Essentially similar results were obtained when whole blood instead of plasma, dog and rabbit blood or plasma were used in similar experiments.

Clotting time of recalcified plasma collected by various technics from patients receiving heparin. The phenomenon described was studied in plasma and blood collected from patients receiving intravenous heparin. Doses of one mg/kilo weight were being administered for therapeutic purposes. Blood was collected 10 minutes after the injection of the full dose of the drug (to allow maximum anticoagulant effect) and made incoagulable by any of the technics described in A). It was again noticed that, on recalcification, samples containing sodium oxalate or passed through cation-exchange resins would clot

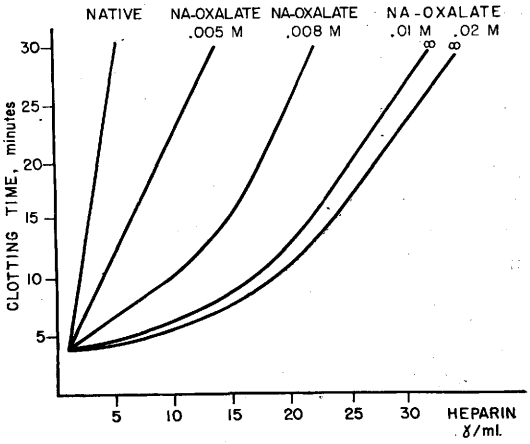

FIG. 1. Relationship of heparin concentration to clotting time of native or recalcified plasma (made incoagulable by addition of various concentrations of calcium oxalate). Plasma was recalcified with concentrations of $CaCl_2$ equimolecular to that of the sodium oxalate used as anticoagulant.

TABLE II. Prothrombin Times (One Stage) of Samples of Oxalated and Citrated Plasma in Presence of Heparin (Avg 2 Exp.).

Heparin sodium	γ/ml	0	1	5	10
Anticoagulant in plasma		Prothrombin time (sec.)			
Sodium oxalate	.01 M	13.0	14.5	19.0	25.4
" citrate	.02 M	12.5	13.5	15.0	19.5

much faster than samples collected in sodium citrate or Sequestrene-Na_2.

Quantitative aspects of the sodium oxalate-heparin antagonism. As indicated in Fig. 1, the anticoagulant activity of heparin became more pronounced as the concentration of sodium oxalate was decreased below the optimum level.

Prothrombin time of plasma from blood made incoagulable with various technics, in the presence of heparin. Blood was collected from healthy donors as described and made incoagulable by the addition of sodium citrate 0.02 M or sodium oxalate 0.01 M. Various amounts of heparin were added and the prothrombin time determined by the one-stage method. A similar trend was noticed for both citrated and oxalated plasmas (a behavior completely opposed to the conduct observed for whole blood and plasma clotting time), although the prothrombin time of the citrated sample was uniformly shorter (Table II).

Discussion. The most significant finding of this work is that the anticoagulant activity of heparin was limited when the drug was added to blood or plasma which had been made incoagulable by decalcification with addition of sodium oxalate or passage through cation-exchange resins. The phenomenon was consistently observed and clearly reproducible when sodium oxalate was the anticoagulant used. When cation-exchange resins were used, however, we often observed that excellency of technic in venipuncture and the speed with which the blood was passed through the resin would change somewhat the values obtained; the anticoagulant effect of heparin becoming more pronounced if blood was collected with trauma to the tissue and was not promptly passed through the resin. The phenomenon was not related to changes in the pH of the plasma due to the various technics of decalcification, since direct measurements of the pH showed that this remained relatively constant.

There is obviously a relationship between mechanism of decalcification and anticoagulant activity of heparin. The mechanism through which sodium oxalate causes incoagulability of blood is apparently different from that of sodium citrate (and according to our experience, Sequestrene-Na_2) (2,3). The heparin-resistance of oxalated blood (directly proportional to the concentration of oxalate used) when compared to the heparin-sensitivity of citrated and Sequestrene-Na_2 treated blood could be related to the different mechanism of decalcification of these anticoagulants. One wonders whether the presence of calcium, combined or free, is necessary to the anticoagulant activity of heparin, or whether oxalate can directly inhibit the activity of the anticoagulant.

Whatever the explanation of these findings, it should be emphasized that not cation-exchange resin or sodium oxalate treated but only native, sodium citrate or Sequestrene-Na_2 treated blood and plasma are suitable for studies of the anticoagulant activity of heparin *in vitro*.

Summary. Plasma decalcified with sodium oxalate or passage through cation-exchange resins is relatively resistant to the anticoagulant effect of heparin on recalcification. Native plasma, and sodium citrate or Sequestrene-Na_2 tested plasma react normally to heparin. The latter plasmas only should be used when testing the anticoagulant effect of heparin *in vitro*.

1. Stefanini, M., PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 22.

2. Quick, A. J., and Stefanini, M., *J. Gen. Physiol.*, 1948, v32, 190.

3. Stefanini, M., Salomon, L., Chatterjea, J. B., and Silverberg, J. H., *Am. J. Clin. Path.*, 1952, v22, 146.

Received August 3, 1953. P.S.E.B.M., 1953, v84.