Anti-Heparin Activity of Erythrocyte Hemolysate.* (23152)

SAMUEL I. RAPAPORT AND SARA BETH AMES
Department of Medicine, University of California Medical Center, Los Angeles, and the Medical Service, Vet. Adm. Hospital, Long Beach, Calif.

Quick and his co-workers(1) discovered clotting activity resembling platelet thromboplastic activity in erythrocyte hemolysate. DeVries and his associates(2) suspended in saline the residue of a sedimented erythrocyte hemolysate previously extracted with water. This material contained platelet thromboplastic but not anti-heparin activity. In contrast, we have found that Quick's whole hemolysate preparation possesses anti-heparin activity comparable to that of concentrated platelet suspensions.

Reagents. *Erythrocyte hemolysate* was made by Quick's technic(3) of freezing and thawing washed, platelet-free erythrocytes in physiological saline. Undiluted hemolysate consisted of hemolyzed cells suspended in a volume of saline equal to the original plasma volume. *Intact red blood cells* were the same cells not disrupted by freezing and thawing. *Platelets* were obtained in a concentration of about 11,000,000/cu mm by differential centrifugation and suspension after washing, in a diluting fluid consisting of 3 parts of physiological saline, 1 part of 0.025 M sodium citrate and 1 part of sodium diethyl barbiturate (veronal) buffer. *Cephalin,* an acetone insoluble, ether soluble fraction of human brain, was made as previously described(4). *Saline brain extract thromboplastin* and *veronal buffer* were prepared by Owren's technic(5), and *oxalated, platelet-poor plasma* by high-speed centrifugation of fresh plasma handled with "silicone technic." A bovine *thrombin solution* of 100 units/ml, was made by dissolving the contents of one vial of Parke-Davis Topical Thrombin in a mixture of 25 ml of saline and 25 ml of glycerine. This stock preparation retained full activity for weeks at 4°C. Dilute solutions were prepared in buffer with "silicone technic" and used within 30 minutes. Heparin (Abbott, Heparin Injection 1%) was diluted in saline and added *in vitro* to plasma. Plasma from patients receiving heparin (Upjohn, Heparin sodium, 100 mg/ml) was also examined. The reagents and technic of the prothrombin-proconvertin (P. and P.) assay(6) are described elsewhere.

Results. 1. *Evidence that hemolysate can abolish the anti-thrombic activity of heparin.* Table I summarizes results obtained by mixing 0.4 ml of platelet-poor plasma containing 0.044 unit of heparin/ml with 0.2 ml of material being examined for anti-heparin activity. After 3 minutes at 37°C, 0.2 ml of dilute (5 units/ml), warmed thrombin solution was added and the clotting time recorded. The thrombin potency was checked at the end of the experiment by clotting plasma containing saline instead of heparin. This Table shows that erythrocyte hemolysate, platelets and saline brain extract effectively neutralize heparin. Intact red cells have no, and "cephalin" only weak, anti-heparin activity. Wolf had previously noted(7) that lipid brain extracts could not inactivate heparin.

Table II lists clotting times obtained when 0.2 ml of different concentrations of hemolysate, platelets and "cephalin" were mixed with 0.4 ml of platelet-poor plasma containing 0.037 unit of heparin/ml, and the mixture clotted with 0.2 ml of thrombin as described above. The final concentration of test material in the clotting mixture, therefore, was one-fourth of the concentration given in the Table. As Table II illustrates, an initial hemolysate concentration of 1/25 (final concentration of 1/100) exhibited some anti-heparin activity. Intravascular hemolysis of sufficient intensity to raise the plasma hemoglobin level to 150 mg% would produce approximately this concentration of red cell products *in vivo*. Table II also shows that platelets possess definite anti-heparin activity at their normal circulating concentra-

* This study was supported by grant from the Los Angeles County Heart Assn.

TABLE I. Effect of Various Materials upon Anti-Thrombic Activity of Heparin.

	Test material	Thrombin time (sec.)
Normal plasma containing heparin &	Dil. fluid	No clot
	Intact RBC	Sl. clot 720 sec.
	Cephalin (1/50)	87, 243
	Hemolysate	30, 28
	Platelets	38, 40
	Thrombopl.	42, 42
Saline	Dil. fluid	29, 28

tion (150-300,000/cu mm of blood by the Rees-Ecker method used). "Cephalin" was ineffective at any concentration studied.

Note that thrombin time of mixture of heparinized plasma and undiluted hemolysate, 23 seconds, was shorter than that of the control without heparin or hemolysate, 33 seconds. Therefore, the thrombin time of a mixture of hemolysate and plasma was compared with that of a mixture of saline and plasma without added heparin.

Table III summarizes the results obtained when first 0.2 ml of hemolysate, and then saline, were added to 0.4 ml of oxalated and

TABLE II. Effect of Various Concentrations of Hemolysate, Platelets and "Cephalin" on Thrombin Time of Heparinized Plasma.

Material	Initial conc.	Thrombin time (sec.)
Hemolysate	Undil.	23
	1/ 5	38
	1/10	55
	1/25	135
	1/50	no clot
	x1000	
Platelets	10,860/mm³	34
	5,430	29
	2,715	29
	1,358	33
	679	31
	340	36
	170	60
	85	188
	42	499
"Cephalin"	Undil. (1.1 g %)	no clot
	1/ 10	535
	1/ 25	227
	1/ 50	no clot
	1/100	" "

Thrombin time of control mixture of 0.4 ml of "non-heparinized" plasma and 0.2 ml of diluting fluid was 33 sec.

TABLE III. Effect of Hemolysate upon Thrombin Time in the Absence of Heparin.

Type of plasma	Added material	Thrombin time (sec.)
Oxalated	Saline	64, 68
	Hemolysate	46, 46
Citrated	Saline	53, 52
	Hemolysate	43, 44

citrated plasma. The mixture was clotted with 0.2 ml of a weaker thrombin solution (1 unit/ml) to magnify differences. As this Table reveals, hemolysate was capable of shortening the thrombin time in the absence of added heparin. This implies that while hemolysate shortens the thrombin time of heparinized plasma primarily because of its anti-heparin activity, it also exerts a secondary, more direct effect upon the rate of the thrombin-fibrinogen reaction. A similar property of platelet extracts has been reported(8).

2. *Evidence that hemolysate abolishes heparin interference with prothrombin consumption.* Heparin not only inactivates preformed thrombin but interferes with conversion of prothrombin to thrombin. Therefore, if hemolysate contains anti-heparin activity, it should increase the rate of prothrombin consumption in clotted blood containing heparin. To test this, 2 ml samples of blood from patients receiving heparin were added to each of 4 small tubes. One tube contained no added material, the other 3 contained, respectively, 0.2 ml of buffer, of "cephalin," and of hemolysate. Each tube stood for 1 hour at 37°C after clotting before 0.2 ml of 0.1 M sodium citrate was added to halt prothrombin conversion. The residual serum prothrombin was then estimated by the P. and P. method of Owren and Aas(6).

These data are given in Table IV. The prolonged whole blood clotting times confirm the presence of heparin in the blood at the time of the test. The normal plasma P. and P. values illustrate the reliability of this assay in the presence of heparin. The failure of either buffer or "cephalin" to improve the impaired prothrombin consumption of the heparinized blood again illustrates their lack of anti-heparin activity. The very low P.

TABLE IV. Residual P. and P. Levels in Sera from Heparinized Blood Clotted with Buffer, "Cephalin" and Hemolysate.

Clotting time (13 min. normal), min.	P. and P. levels (%)				
	Plasma	Serum			
		Material added			
		Nothing	Buffer	"Cephalin"	Hemolysate
20	100	39	51	55	<4
21	86	33	33	35	"
20	74	44	31	36	"
17	82	27	15	28	<2

and P. levels obtained with hemolysate demonstrate strikingly its anti-heparin activity.

Discussion. Quick's erythrocyte hemolysate resembles platelet suspensions in possessing both platelet thromboplastic like activity and anti-heparin activity, and in directly shortening the time of the thrombin-fibrinogen reaction. Thus, hemolysate differs from commonly used lipid extract platelet substitutes, such as "cephalin," which possess only platelet thromboplastic like activity. DeVries (2) work indicates that thromboplastic activity of hemolysate can be separated from its other clotting properties.

Hemolysate can be made easily and stored frozen indefinitely. Therefore it is a convenient reagent to test for heparinemia. Our procedure is to add 0.2 ml of a standardized dilute thrombin solution that will clot normal plasma in about 20 seconds to a mixture of 0.4 ml of the plasma to be tested and 0.2 ml of saline. A normal thrombin time rules out heparinemia. If a long thrombin time is obtained, or if no clot forms, the test is repeated substituting 0.2 ml of hemolysate for the saline. If this corrects the long thrombin time, heparinemia should be strongly suspected.

If hemolysate fails to shorten a prolonged thrombin time, heparinemia is not its cause. However, if clotting fails to occur either with saline or with hemolysate, a further step is necessary. Failure to clot could mean that so much heparin is present that enough remains after hemolysate neutralization to inactivate the dilute thrombin used. Therefore, the test must be repeated using a thrombin solution strong enough to clot the mixture containing saline. Then, failure to obtain a much shorter time with hemolysate will definitely eliminate heparinemia.

The use of hemolysate also permits an evaluation of the plasma thromboplastic factors in heparinized blood. This becomes necessary, for example, when bleeding occurs in a patient receiving heparin in amounts that should not be excessive. If, in this circumstance, hemolysate corrects abnormal prothrombin consumption of the heparinized blood (see again Table IV) a co-existing deficiency of anti-hemophilic globulin (AHG), plasma thromboplastic component (PTC) or plasma thromboplastin antecedent (PTA) would be most unlikely.

Summary. 1. Erythrocyte hemolysate possesses anti-heparin activity that can correct the prolonged thrombin time and impaired prothrombin consumption of heparinized blood. This makes erythrocyte hemolysate a useful reagent to detect heparinemia and to permit the evaluation of plasma clotting factor activities in heparinized blood. 2. Erythrocyte hemolysate also produces some shortening of the thrombin time of plasma not containing added heparin.

The authors are grateful to Mrs. Maria Friedland for her technical assistance.

1. Quick, A. J., Georgatsos, J. G., and Hussey, C. V., *Am. J. Med. Sci.*, 1954, v228, 207.

2. DeVries, S. I., Kettenborg, H. K., and Van der Pol, E. T., *Nederl. tijdscher. geneesk.*, 1955, v99, 2967.

3. Georgatsos, J. G., Hussey, C. V., and Quick, A. J., *Am. J. Physiol.*, 1955, v181, 30.

4. Rapaport, S. I., Aas, K., and Owren, P. A., *J. Lab. and Clin. Med.*, 1954, v44, 364.

5. Owren, P. A., *Scandinav. J. Clin. and Lab. Invest.*, 1949, v1, 81.

6. Owren, P. A., and Aas, K., *ibid.*, 1951, v3, 201.

7. Wolf, P., *Brit. J. Hematol.*, 1956, v2, 375.

8. Ware, A. G., Fahey, J. L., and Seegers, W. H., *Am. J. Physiol.*, 1948, v154, 140.

Received March 26, 1957. P.S.E.B.M., 1957, v95.