SPECIFIC COAGULATION FACTOR ADSORPTION TO INSOLUBLE HEPARIN.

Patricia W. Gentry and Benjamin Alexander

Coagulation Laboratory, The New York Blood Center, N.Y., N.Y. 10021

Received December 6,1972

Summary: An insoluble derivative of heparin has been prepared by coupling to agarose beads, and its adsorption of certain blood clotting factors has been investigated. Factors IX*, XI, and heparin cofactor (He-Co)** are selectively adsorbed, and can be recovered by elution of the heparin-agarose (H-Ag) with an NaCl gradient. Fibrinogen (FI) adsorption is negligible. The affinity for F IX appears to be lower than for F XI and He-Co. Thrombin, F XI, and He-Co seem to be equally tightly bound to the insoluble H.

Introduction: Heparin (H)* acts predominantly as an antithrombin; moreover, it also inhibits F IX* (1-4). There is considerable recent interest in the antithrombogenicity of H-coated surfaces (5-10), but it is difficult to evaluate the precise role of H in such preparations since many of the surfaces per se resemble H in being highly polar, and may thus affect certain clotting factors (11). Moreover, the H may be leached by blood from some H-surface combinations (7,12).

We report herein studies with an insoluble H derivative, in contrast to an H-coated surface, which contribute to our understanding of the anti-coagulant activity of H, and to assessing its affinity for several coagulation components.

Materials and Methods: Heparin (Sigma, Grade 1, hog intestinal mucosa, lot numbers 57B 1450 and 79B 1610) was coupled to agarose beads (Biorad, 0.5M), according to Cuatrecasas (13). The degree of substitution with H was determined by toluidine blue binding (14). Amongst several preparations the H incorporated was consistently 0.3 μgm/ml packed beads.

The H-Ag was equilibrated with 0.05 M Tris buffer (pH 7.5) containing 0.01 M CaCl, and a column (0.7 cm diameter) of 4 ml bed volume was prepared.

---

*Roman numeral nomenclature as recommended by the International Committee on Haemostasis and Thrombosis. Thromb. Diathes. haemorrh. (Stuttg.). Suppl. 13, 455 (1964).

**Abbreviations used: H, He-Co, heparin cofactor; H-Ag, heparin-agarose; C.T., clotting time; NIH, National Institutes of Health; M.W., molecular weight; SDS, sodium dodecyl sulphate.

Nine ml of venous blood from at least 8 random routine donors were mixed with 1 ml of 3.1% sodium citrate, and platelet poor plasma was obtained by centrifugation at 4° at 1200 g. The plasmas were pooled, and 22 ml were passed through the column at 22° at 0.2 ml/min. The first 4 ml to emerge, having been diluted with the Ca^{++} in the residual equilibrating buffer, were discarded since the Ca^{++} interfered with clotting assays. The rest of the emerging plasma was collected in 3 sequential 6 ml portions. To remove the non-adsorbed material, the column was then washed with 0.05 M Tris buffer until the effluent was essentially protein free.

The adsorbed components were eluted with a stepwise gradient consisting of 25 ml of the buffer containing 0.1 M NaCl; 25 ml buffer, containing 0.2 M NaCl; and finally 25 ml buffer, containing 0.4 M NaCl. The eluates were screened for protein by measuring adsorption at 282 nm in a Zeiss spectrophotometer. The fractions comprising the protein peaks were pooled, concentrated approximately 3 fold in an Amicon ultrafiltration cell with Diaflo membrane PM10, the NaCl concentration reduced to 0.1 M, and their protein content was estimated by the method of Lowry et al (15).

The level of various clotting factors, before and after passage through the column, was measured by assessing the ability of test samples to correct the defective clotting of plasma deficient in the relevant factors. Activity was estimated by interpolation on a standard curve correlating clotting activity with factor concentration, prepared with the precolumn material. F XI and VIII were measured against human plasma hereditarily deficient in these factors. F XI was also measured against plasma rendered F XI deficient by adsorption with celite (15 mg/ml) (Johns Manville) (16); F IX and VII were measured, using canine plasma hereditarily deficient in the respective factors; F X was assayed with F X-deficient bovine plasma (Sigma) (17); F V was measured against plasma artificially depleted in F V (18); F II, by a two-stage procedure (19), modified by using purified F V, VII and X (Sigma) instead of bovine adsorbed plasma, and purified FI (AB Kabi, 93% clottable) in the thrombin clotting mix-

TABLE I

VARIOUS CLOTTING FACTOR ACTIVITIES IN PLASMA

PASSED THROUGH HEPARIN-AGAROSE COLUMN

	Activity (Per Cent)*							
Factor	II	V	VII	X	VIII	IX	XI**	XI#
First 6 ml effluent	93	100	77	73	90	5	12	6
Second 6 ml "	92	---	100	110	--	34	15	9
Third 6 ml "	90	---	100	98	--	61	36	17

* Expressed in % of the factor in control parent plasma, arbitrarily considered to contain 100%.

** Assayed by correction of clotting defect of F XI congenitally deficient plasma.

Assayed with plasma depleted in F XI by celite adsorption (15 mg/ml).

ture. He-Co activity was measured in a one-stage thrombin clotting time test, as follows: 5 μl sample, 5 μl H (31.3 units/ml in H_2O) and 10 μl thrombin (25 NIH units/ml in 0.15 M NaCl) were incubated at 37° for 30 seconds. 0.2 ml of FI solution (4 mg/ml) was then added and the C.T. recorded.

Results and Discussion: Various coagulation factor levels in the first three sequential 6 ml fractions of plasma collected after passage through the H-Ag column are shown in Table I. F IX and F XI appear to be the only factors considerably depleted. Although F XII was not measured, we may assume that it was reduced concomitantly with F XI since the F XI activity, as measured with the celite-treated plasma, was always substantially lower than that obtained with the hereditarily deficient plasma. In this instance the normal plasma was treated with 15 mg celite/ml, an amount known to remove F XI completely, and F XII partially (16).

The fractions eluted from the H-Ag column with the stepwise NaCl concentration gradient showed three distinct and reproducible peaks (Fig. 1), each comprising in their pooled material 60, 24, and 10 mg protein, respectively. Since the plasma effluents revealed predominant removal of F IX and XI, each

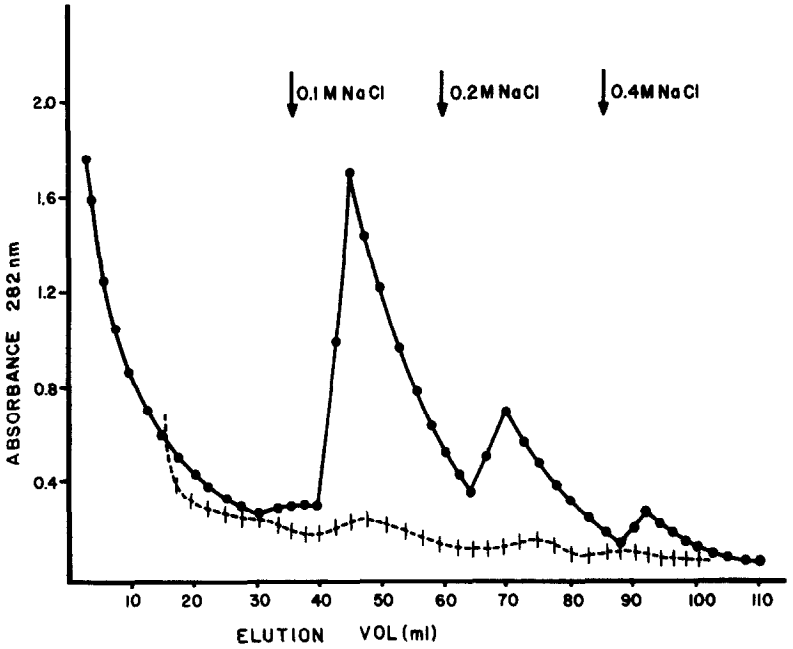

Fig. 1 Protein elution profile following application of plasma to H-Ag (—) or control agarose (1---1) column. Following washing of the columns with 0.05 M Tris buffer, pH 7.5, to remove nonadsorbed proteins, a step-wise increasing NaCl gradient at pH 7.5 was applied as indicated (see Methods). The flow rate was 0.5 ml/min.

eluted pool was assayed for these factors, as well as He-Co. F IX and XI levels were calculated by interpolation on a standard curve prepared with the original plasma on the basis of the amount of protein in the test sample and the total protein recovered in the particular pool. As evident in Table II, F IX is eluted preponderantly in the first and second pools, whereas the major portion of F XI appears in pool 3.

The He-Co in pools 1 and 2 could not be estimated since they contained some FI which interfered with the assay system. Pool 3 contained appreciable He-Co activity (Table II), judged on the basis of the following criteria: (a) the test sample alone retarded F I-thrombin clotting only slightly; (b) a mixture of sample and H markedly retarded clotting, far beyond the effect of each individually; and (c) the degree to which clotting was retarded by a mixture comprising 15 μg protein from pool 3 eluate and 0.62 H units was found to

TABLE II

F IX, XI, AND H-CO IN ELUATES FROM

H-AG COLUMNS EXPOSED TO PLASMA OR SERUM

		F IX Activity		F XI Activity		
Plasma Fraction	Elut. Agent	Units*/mg Protein	%**	Units*/mg Protein	%**	Heparin Cofactor C.T. (sec.)
Pool 1	0.1 M.NaCl	2	58	4	10	--
" 2	0.2 M "	3	37	6	31	--
" 3	0.4 M "	1	4	53	60	144 (7.6)#
Serum						
Fraction	Elut. Agent					
Pool 1	0.1 M NaCl	3	61	2	21	13 (32.4)
" 2	0.2 M "	7	30	5	26	21 (21.4)
" 3	0.4 M "	3	9	27	53	101 (7.4)

* One unit of F IX or XI is arbitrarily defined as that present in 1.0 ml of the original plasma or serum. F XI measured with celite-adsorbed plasma as substrate (16).

** Also expressed in % of the total eluted activity.

Bracketed figures indicate μg protein in 10 μl of sample incubated for 30 sec at 37 ° with 5 μl H (0.16U) and 5 μl thrombin (0.25U), followed by the addition of 0.2 ml FI (0.8 mg). The control C.T. (10 μl buffer instead of sample) was 16 sec.

increase during incubation, as follows: after 15 sec incubation the C.T. was 19.8 sec; after 30 sec incubation, the C.T. was 143 sec; and after 60 sec, the C.T. was 248 sec.

The FI content of the three pools was measured by the procedure of Hawiger et al (20) as modified by Laevelle et al (21). Pool 1 was found to contain 1.1 mg FI; pool 2, 0.3 mg; and pool 3, 0.2 mg - these amounting to 1.8, 1.3, and 2.0 % of the total protein, respectively.

To confirm that the selective removal of F IX and XI from plasma was due to

TABLE III

F IX AND XI ACTIVITY IN THE

FIRST FRACTIONS RECOVERED FROM AGAROSE OR H-AG COLUMNS

		Activity (Per Cent)*		
		F IX	F XI**	F XI#
Control-Agarose:	Plasma	82	--	76
H-Ag:	"	5	12	6
H-Ag:	Serum	13	42	37
Reused H-Ag:	Plasma	24	--	11

* Expressed in % of the factor, the parent material arbitrarily considered to be 100%.

** Assayed against hereditarily deficient plasma.

Assayed against celite-adsorbed plasma.

specific adsorption to the coupled H, and not attributable to the agarose matrix per se, plasma was passed through a column of non-H-treated agarose beads under conditions otherwise identical to those for the H-Ag column except that Ca^{++} was omitted from the equilibrating Tris buffer since with Ca^{++}, a clot forms on the column. Compared with H-Ag, agarose alone reduces F IX and XI only slightly (Table III).

After plasma passage, the agarose column was also eluted with a stepwise gradient of increasing NaCl concentration (Fig. 1). In contrast to 60 mg protein collected in pool 1 from H-Ag, only 2 mg were recovered; of this 0.03 mg was FI. No F IX, F XI or He-Co was detectable in the eluate. The striking differences in the activities eluted from the control compared with the H-Ag columns indicate that F IX, XI, and He-Co are selectively adsorbed by virtue of the coupled heparin.

Except for FI, the factors removed by H-Ag are not consumed during clotting. One would expect, therefore, that the same results should be obtainable with

serum. Non-anticoagulated blood from eight donors was collected into glass tubes, these were kept at 37 ° for 1 hour, and thereafter handled the same as the anticoagulated whole blood. The resulting sera were pooled, and aliquots were similarly applied to an H-Ag column. As with plasma, F IX and F XI were found markedly depleted in the first 6 ml effluent (Table III). Moreover, the profile and total protein eluted by the stepwise NaCl gradient was identical with that for plasma. The distribution of F IX and XI activity were also the same (Table II).

Since the eluates obtained from the serum adsorbed column were devoid of FI, He-Co could be measured in all three pools. No activity was detectable in pool 1, some appeared in pool 2, and the major portion was found in pool 3 (Table II).

To see whether protein had been eluted completely by the NaCl gradient, the columns were washed with 0.15 M NaCl, and samples were examined with the sensitive ninhydrin procedure (22, 23). No reactive material was found. Furthermore, when a column that had already been eluted was re-equilibrated and a second plasma sample applied, F IX and XI were again effectively removed (Table III), and subsequent gradient elution gave a profile and distribution similar to that depicted in Fig. 1. The data thus indicate that F IX, XI, and He-Co are specifically adsorbed by H-Ag, can be quantitatively and readily desorbed, and that the desorbed column is reusable.

Since H acts as an antithrombin, although markedly augmented in this respect by He-Co, the affinity of H-Ag for thrombin was also examined. Two ml of a bovine thrombin (Parke-Davis) solution in 0.01 M NaCl (200 NIH U/ml), purified according to Lundblad (24), were passed through an H-Ag column (1 ml bed volume) equilibrated with the Tris buffer. Virtually all of the thrombin was adsorbed. Following extensive washing with the buffer, the column was eluted with the same gradient system described above. Pool 1 was essentially devoid of thrombin. The fraction corresponding to the pool 2 plasma and serum eluates contained 0.5 thrombin units, and 150 units were found in pool 3. Thrombin adsorption by H-Ag is apparently also specific since with agarose beads

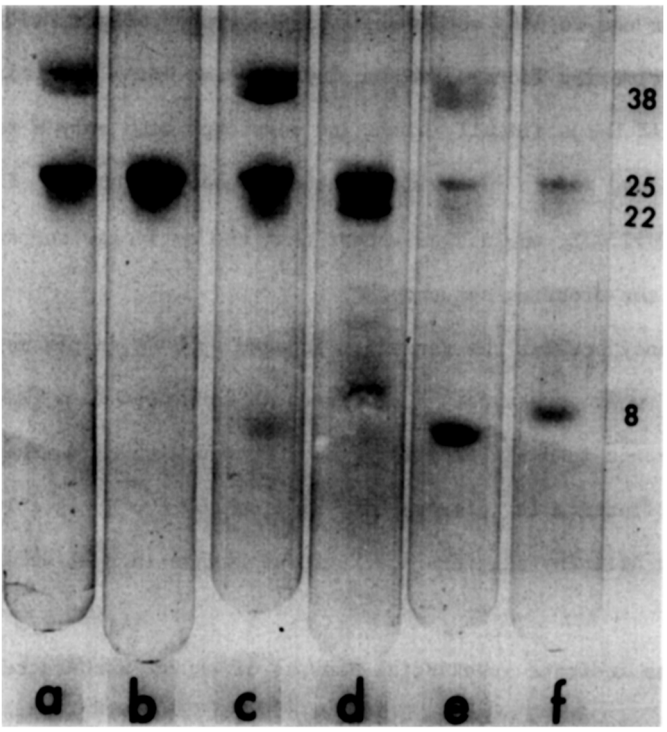

Fig. 2 Electrophoretic patterns of the three pools eluted from the H-Ag plasma and serum columns on 5% polyacrylamide gels in SDS. a and b represent pool 1 from plasma and serum, respectively; c (plasma) and d (serum), pool 2; and e (plasma) and f (serum), pool 3. The numbers on the left indicate M.W. x 10^4. The protein load was 50 μg incubated for 2 hours in 0.01 M sodium phosphate buffer (pH 7.0) containing 1% SDS. The gels were stained with Coomassie blue.

alone, 93 per cent of the thrombin appeared in the wash effluent. Furthermore, H-Ag affinity for thrombin appears similar to that for F XI and He-Co.

The profile of the various eluates was examined by SDS polyacrylamide gel electrophoresis (25), and is shown in Fig. 2. Eight protein markers (Schwartz-Mann) of known M.W. were run as standards. The two bands exhibiting lowest mobility probably represent FI since not only do they conform in M.W. with FI, but also they are absent in the serum profile.

Except for these, the serum and plasma patterns are identical. The band exhibiting the highest mobility is likely He-Co since its M.W.(app) of 8 x 10^4 conforms approximately with 6.5 x 10^4 reported for purified antithrombin III (26).

Assuming that the results obtained with H-Ag truly reflect the interaction of native H with clotting factors, we can for the time being conclude that of many factors tested H specifically binds and complexes with only a few, and, under our conditions, in the following order of binding tightness: F XI = He-Co = thrombin $>$ F IX $\gg$ FI. It would thus appear that the anticoagulant acts at several steps in the clotting sequence.

The discrepancy between the depletion induced by H-Ag in plasma F XI compared with serum, where presumably F XI exists in activated form (XIa), suggests a striking difference in H affinity for F XI compared with XIa. This is also evident in the difference in recovery of F XI from the two He-Ag columns (Table II). The altered affinity suggests a structural change in F XI during its activation.

The data also indicate substantial amounts of as yet uncharacterized protein in the eluates, presently under investigation.

H-Ag is thus a valuable tool, highly useful for studying certain clotting entities, as well as some non-clotting functions of H.

Acknowledgments: This research was supported by USPHS Grants HL 9011 and 11447. Blood samples were voluntarily provided by donors in the Blood Procurement Program of the New York Blood Center. We wish to thank Dr. H. Al-Mondhiry of Memorial Hospital, N.Y. for the samples of human F XI deficient plasma, and Dr. Jean W. Dodds, N.Y. State Department of Health, for F VII and IX deficient canine plasmas.

REFERENCES

1. Shanberge, J. N., Sarelis, A., and Regan, E. E., J. Lab. Clin. Med. 54, 501 (1959).
2. O'Brien, J. R., Nature 181, 1801 (1958).
3. Ratnoff, O. D., and Davie, E. W., Biochemistry 1, 967 (1962).
4. Pitlick, F. A., Lundblad, R. L., and Davie, E. W., J. Biomed. Mater. Res. 3, 95 (1969).
5. Gott, V. L., Whiffen, J. D., and Dutton, R. C., Science 142, 1297 (1963).
6. Leininger, R. I., Cooper, C. W., Falb, R. D., and Grode, G. A., Science 152, 1625 (1966).
7. Usdin, V. R., and Fourt, L., J. Biomed. Mater. Res. 3, 107 (1969).
8. Merrill, E. W., Salzman, E. W., Wong, P. S. L., Ashford, T. P., Brown, A. H., and Austen, W. G., J. Appl. Physiol. 29, 723 (1970).
9. Gott, V. L., and Furuse, A., Fed. Proc. 30, 1679 (1971).
10. Nossel, H. L., Wilner, G. A., and LeRoy, E. C., Nature 221, 75 (1969).
11. Oja, P. D., Holmes, G. W., Perkins, H. A., and Love, J., J. Biomed. Mater. Res. 3, 165 (1969).
12. Falb, R. D., Grode, G. A., Fed. Proc. 30, 1688 (1971).

13. Cuatracasas, P., J. Biol. Chem. 245, 3059 (1970).
14. MacIntosh, F. C., Biochem. J. 35, 776 (1941).
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. 193, 265 (1951).
16. Soulier, J. P., and Prou-Wartelle, O., Brit. J. Haemat. 6, 88 (1960).
17. Bachman, F., Duckert, F., and Koller, F., Thromb. Diath. Haemorrhag. 2, 24 (1958).
18. Owen, C. A., Bowie, E. J. W., Didisheim, P., and Thompson, J. H., in The Diagnosis of Bleeding Disorders 1st Ed., pp. 105-106, Boston: Little, Brown and Company, 1969.
19. Ware, A. G., and Seegers, W. H., Am. J. Clin. Path. 19, 471 (1949).
20. Hawiger, J., Niewiarowski, S., Gurewich, V. and Thomas, D. P., J. Lab. Clin. Med. 75, 93 (1970).
21. Laevelle, D. E., Mertens, B. F., Bowie, E. J. W., and Owen, C. A., Am. J. Clin. Path. 55, 452 (1971).
22. Matheson, A. T., Tigane, E., and Hanes, C. S., Can. J. Biochem. Physiol. 39, 417 (1961).
23. Awad, W. M., and Wilcox, P. E., Biochim. Biophys. Acta. 73, 285 (1963).
24. Lundblad, R. L., Biochemistry 10, 2501 (1971).
25. Weber, K., and Osborn, M., J. Biol. Chem. 244, 4406 (1969).
26. Andersson,L. -O., and Miller-Andersson, M., III Congress, Washington, D.C.: The International Society on Thrombosis and Haemostasis, 1972.