that two forms of acid phosphatase may be present in the lysosomes, the possibility cannot be excluded that the minor component represents merely supernatant enzyme which was absorbed to the lysosomes prior to their disruption.

Present findings confirm and extend an earlier report that acid p-nitrophenylphosphatases in rat liver lysosomal and supernatant fractions can be distinguished in the presence of inhibitors(6). Association of a specific acid phosphatase with the lysosomal fraction is not, however, species specific for the rat. In guinea pig livers a lysosomal p-nitrophenylphosphatase can be distinguished from a microsomal p-nitrophenylphosphatase (5). The microsomal enzyme is more sensitive to heat and alloxin inhibition, and less sensitive to fluoride and tartrate than the lysosomal enzyme. Since microsomes were not separated from the soluble fraction in the present study, it is possible that the thermolabile acid phosphatase in the supernatant fraction is associated with the microsomes. On the other hand, in contrast to the rat, guinea pig liver p-nitrophenylphosphatase is located predominantly in the microsomal fraction(5). This suggests a basic difference between the two species and generalizations on the comparative intracellular distributions of acid phosphatase may not be warranted at this time.

In view of the present findings, it is suggested that further studies on possible separate physiological roles for the two enzymes should be studied, particularly as they are related to the process of tissue autolysis in which acid phosphatase is known to be important(1).

Summary. Two acid phosphatases, a lysosomal and supernatant form, have been demonstrated in rat liver by differences in their heat stabilities at 50°C, pH activity curves and sensitivities to tartrate and fluoride inhibition.

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Separation of the Antihemophilic Factor (F. VIII) from Fibrinogen With Thrombin and Manganese Chloride.* (30948)

EMILY M. BARROW, SOLANGE M. AMOS, CRAIG HEINDEL AND JOHN B. GRAHAM
Department of Pathology and The Genetics Curriculum, University of North Carolina, Chapel Hill

When blood is allowed to clot, prothrombin converts to thrombin and fibrinogen to fibrin, while the antihemophilic factor (AHF, Factor VIII) is "consumed" in some manner. AHF disappears during the clotting process and cannot be measured in serum(1). The addition to plasma of thrombin, a proteolytic enzyme, causes the fibrinogen to clot and AHF activity to disappear(2). The inference has been that the thrombin produced during clotting destroys AHF enzymatically. Trypsin, another proteolytic enzyme, also destroys AHF activity(3) and alters fibrinogen, rendering it nonclottable(4). Since hydrolysis of some proteins by trypsin can be prevented by the chlorides of certain metal ions, notably manganese(5), we have investigated the effect of these cations on the destruction of Factor VIII by thrombin and trypsin. As a result, we have discovered conditions under which

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thrombin converts fibrinogen to fibrin without destroying AHF.

Methods and materials. Citrated plasmas were prepared and *AHF assays* performed as described previously(6). The *unit of AHF* has been defined more recently as 1/100 of the AHF activity in 1 ml of freshly pooled normal human plasma(7). Plasma samples were *dialyzed* at 4°C for 90 minutes in Visking cellulose casing. One hundred ml of dialyzing fluid were used for each ml of plasma, and continuous agitation was maintained with a magnetic stirrer.

Fibrinogen levels were measured as clottable protein nitrogen after conversion of the fibrinogen to fibrin by thrombin(8). *Thrombin* of bovine origin was obtained from Parke, Davis and Co. *Trypsin,* twice crystallized, was purchased from Mann Research Laboratories. *Aluminum hydroxide* was obtained as a moist gel from the British Drug House and was prepared for use by suspending 100 g in 200 ml of distilled water. One-tenth ml of the $Al(OH)_3$ suspension was added to each ml of plasma for adsorption of prothrombin and other clotting factors. The mixture was agitated for 30 minutes at 4°C and the precipitate removed by centrifugation at 3,000 rpm.

Citrated saline was prepared by mixing one volume of 3.2% sodium citrate with 19 volumes of 0.9% sodium chloride. *Human fibrinogen,* Fraction I, was prepared by the ethanol method of Cohn(9).

Results. Dialysis against cation solutions increases AHF activity. Dialysis of plasma against weak solutions of several cations increases AHF activity. For example, the AHF activity of 50 separate plasma samples *invariably* increased after dialysis against 0.02 M $MnCl_2$, the increase averaging 89% and ranging from 7-450%.

AHF destruction by thrombin and trypsin can be prevented by dialysis against $MnCl_2$. Columns 1 and 3 of Table I show that AHF gradually diminishes in plasma at 37°C in the presence of 1 U thrombin/ml or 250 μg trypsin/ml. Columns 2 and 4 of Table I show that prior dialysis of the plasma samples against 0.02 M $MnCl_2$ prevents this loss of AHF activity.

TABLE I. Effect of $MnCl_2$ on Destruction of AHF Activity by Thrombin and Trypsin.

	Residual units AHF/ml of Plasma			
	Thrombin*		Trypsin	
Incubation time	No $MnCl_2$†	$MnCl_2$†	No $MnCl_2$†	$MnCl_2$†
Before enzyme added	100	280	100	250
30 min after enzyme added	23	270	73	240
60 min after enzyme added	11	225	29	156
90 min after enzyme added	<6	325	9	160
120 min after enzyme added	<6	300	<6	165

* Fibrin clots which formed in the samples to which thrombin had been added were removed before testing for AHF.

† Plasma absorbed with $Al(OH)_3$ before dialysis and/or addition of enzyme.

Manganese is the most efficient cation in protecting AHF against the action of thrombin. The chlorides of barium, calcium, magnesium, manganese and sodium were tested over a wide range of concentrations to determine the specific cation most effective in protecting AHF against thrombic destruction and its optimal concentration. Table II contains a sample of the extensive data collected. The AHF activity of all samples increased somewhat after dialysis, but a second dialysis against citrated saline did not revert the AHF activity to the pre-dialysis levels. It is apparent that AHF is protected against thrombic destruction by manganese and by barium to a lesser extent. It is clear from these data and those at other concentrations, that manganese is the most efficient cation tested and that 0.02 M is the most effective concentration for *human* AHF.

The protective effect of $MnCl_2$ is not exerted directly on thrombin. It seemed possible that manganese might have inactivated the thrombin, thereby protecting AHF from destruction. This possibility was tested by dialyzing thrombin against NaCl (0.15 M) or $MnCl_2$ (0.02 M), incubating at 37°C, and testing its fibrinogen clotting ability and AHF destroying property at intervals. It appears that dialysis increases somewhat the fibrinogen-clotting ability of thrombin (Table III) but does not diminish its AHF-destroy-

TABLE II. Effects of Several Cations on Thrombic Destruction of AHF in Plasma. Units of AHF/ml of plasma

Dialysis solution	After dialysis for 90 min	After re-dialysis *vs* citrated saline for 90 min	After subsequent incubation with 1 U thrombin/ml 2 hr at 37°C
$BaCl_2$ 0.02M	135	135	27
$CaCl_2$ "	160	140	6
$MgCl_2$ "	150	120	11
$MnCl_2$ "	220	220	105
NaCl 0.15M	120	110	11
No dialysis	100	100	<6

0.02M is not necessarily optimal for cations other than manganese. No cation tested at any concentration even closely approximated the effectiveness of 0.02M $MnCl_2$.

ing property significantly (Table IV).

$MnCl_2$ dialysis does not potentiate or destroy antithrombin. Table V demonstrates that the thrombin neutralizing ability of plasma is neither increased nor decreased by prior dialysis against $MnCl_2$. Thus the stability of the AHF of dialyzed plasma in the presence of thrombin cannot be attributed to alteration of the thrombin-antithrombin relationship.

Dialysis against $MnCl_2$ does not alter fibrinogen clottability. A human fibrinogen solution containing 1030 mg clottable protein/100 ml was dialyzed against 0.02 M $MnCl_2$. The precipitate which formed was removed by centrifugation. The clear supernatant contained 512 mg% clottable protein and clotted in 9 seconds with 100 U thrombin. Untreated fibrinogen from the same batch diluted to 512 mg/100 ml with buffer also clotted in 9 seconds with 100 U thrombin.

AHF was the only clotting factor observed to be appreciably more active after treatment with $MnCl_2$ and thrombin. Specific clotting factor assays were performed on $Al(OH)_3$ adsorbed normal plasmas which had been dialyzed against $MnCl_2$ then incubated with weak thrombin. Residual concentrations of Factors II, VII, IX and X, which are adsorbed by $Al(OH)_3$, were less than 10%. The concentrations of Factors V and XII ranged from 50-100% in different experiments, and Factor VIII activity ranged from 107-550%. Addition of these manganese-dia-

TABLE III. Effect of $MnCl_2$ on Fibrin Clotting Ability of Thrombin.

Incubation time at 37°C*	Clotting time, seconds: Non-dialyzed thrombin (100 U)	Dialyzed thrombin (100 U): 0.15M NaCl	Dialyzed thrombin (100 U): 0.02M $MnCl_2$
0	12.0	8.6	9.2
1 hr	11.4	9.5	7.8
2 "	10.0	12.6	6.5
3 "	12.5	11.4	7.5

* The thrombin solutions were incubated in Lusteroid tubes for the indicated time periods. 0.1 ml of each thrombin solution was added to 0.4 ml of human fibrinogen and clotting time recorded.

TABLE IV. Effect of $MnCl_2$ on AHF Destroying Property of Thrombin.

Incubation time at 37°C	Residual AHF, units/ml plasma: Non-dialyzed thrombin	Dialyzed thrombin: 0.15M NaCl	Dialyzed thrombin: 0.02M $MnCl_2$
Before thrombin added	100	100	100
30 min after thrombin added	57	45	75
60 min after thrombin added	16	<6	22
120 min after thrombin added	7	<6	<6

TABLE V. Antithrombin Activity of Untreated and $MnCl_2$ Dialyzed Human Plasmas.

Incubation at 37°C	Thrombin clotting time (sec)* Substance incubating with thrombin: Untreated plasma	$MnCl_2$ dialyzed plasma	Saline
0 min	19	18	11
15 "	53	50	10
30 "	81	82	14
45 "	110	114	12
60 "	192	189	14

* The test was performed by adding 1.0 ml of thrombin containing 100 NIH units to 1.0 ml of each type of plasma or saline. The clots were removed and 0.1 ml of each incubating mixture was added to 0.4 ml of human fibrinogen at times indicated.

TABLE VI. Transfusion of Normal Dogs.

Material transfused	Units of AHF/dog blood volume*: Before transfusion	Amount transfused	Total after transfusion*: Expected	Observed
$MnCl_2$-thrombin treated normal canine plasma	51,163	770	51,993	73,416
Idem	63,700	720	64,420	217,140
0.08M $MnCl_2$ alone	61,200	0	61,175	85,000

* The method for estimating this value is found in references 5 and 6.

TABLE VII. Transfusion of Hemophilic Dogs.

Material transfused	Units of AHF/dog blood volume: Total before transfusion	Amount transfused	Total after transfusion: Expected	Observed
Normal plasma, untreated	1,960	35,670	37,360	21,675
" " treated with $MnCl_2$ and thrombin	3,452	27,280	30,732	24,570
Hemophilic plasma treated with $MnCl_2$ & thrombin	3,222	800	4,022	3,222

lyzed, thrombin-activated plasmas to fibrinogen did not result in the formation of clots in 24 hours at 37°C. A terminal re-adsorption with $Al(OH)_3$ to remove traces of residual thrombin reduced the AHF "hyperactivity" only slightly.

AHF activity of plasma dialyzed against $MnCl_2$ and clotted with thrombin can be demonstrated in vivo. A crucial test of identity for AHF is to detect and assay the administered AHF activity in an animal which has been transfused with a sample believed to contain AHF. It is well documented that non-specific accelerators of clotting which mimic AHF in the test tube are usually unmasked when transfused into a hemophilic animal. Because of known toxic effects of $MnCl_2$, the *in vivo* experiments were performed on dogs infused with canine plasma. It was found necessary to modify the dialysis procedure to obtain a fibrinogen-free, AHF-active, preparation of canine plasma. The optimal conditions consisted of a $MnCl_2$ concentration of 0.08 M for dialysis and 6 U thrombin per ml of dialyzed plasma to remove the fibrinogen.

Table VI shows the results when 3 normal dogs were transfused with normal canine plasma which had been adsorbed with $Al(OH)_3$, dialyzed against 0.08 M $MnCl_2$ and incubated with 6 U thrombin/ml. Each dog received 2 ml treated plasma/kilo of body weight. All survived but showed signs typical of manganese poisoning. It should be noted that the AHF observed to be circulating in these non-hemophilic dogs after transfusion was more than expected. The response of an unexpectedly large elevation of plasma AHF in one dog prompted the injection of 0.08 M $MnCl_2$ alone. The last line of Table V suggests that "activation" of AHF by manganese *may* occur to some extent *in vivo*. On the other hand, this phenomenon of AHF elevation may be the one also known to occur after administration of adrenalin (10).

Two hemophilic dogs were transfused with similarly treated normal and hemophilic canine plasmas, at a dosage of 10 ml/kg of body weight. Table VII shows a similar effect of untreated normal plasma and manganese-dialyzed normal plasma which had been incubated with thrombin. That is, the expected and observed amounts of AHF circulating after transfusion were not highly discrepant in either case. It should be noted that the observed level of AHF in hemophilic dogs after transfusion was less than expected while that in normal dogs was greater (see above). The data are not sufficient to test whether

these deviations from expectation are statistically significant. It should be noted that $MnCl_2$ dialysis did not appreciably activate *in vitro* the AHF of hemophilic plasma being prepared for transfusion, and that this mixture containing $MnCl_2$ did not "release" or "activate" AHF within the hemophiliac after transfusion.

Discussion and summary. These experiments show that the AHF activity of plasma is enhanced by dialysis, especially against $MnCl_2$, and that thrombic destruction of AHF can be prevented by prior dialysis of plasma against $MnCl_2$. Manganese chloride is clearly the most effective cation tested with respect to protection of AHF against thrombic destruction and 0.02 M is the optimal concentration. Manganese does not protect AHF, however, by destroying thrombin or by altering the thrombin destroying properties of antithrombin. This specific effect of Mn++ in protecting AHF is not surprising because others have observed that Mn++ both activates and stabilizes AHF in plasma(11,12). Our experiments suggest that the protective action of $MnCl_2$ may be related in some manner to changes induced in the AHF molecule. The nature of any such change remains to be clarified.

Identification of the AHF activity in the treated plasmas was established by injection into both normal and hemophilic dogs. These experiments showed that the AHF activity observed *in vitro* could also be observed *in vivo*. Normal dogs circulated somewhat more and hemophilic dogs somewhat less AHF than expected. It is suggested by these results and a similar result using $MnCl_2$ alone that AHF may become "activated" by Mn++ *in vivo* in normal but not hemophilic animals. This apparent "activation" may, however, be nothing more than an indirect method of producing the well known "adrenaline effect."

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Role of the Thymus in Tolerance. II. Transfer of Specific Unresponsiveness to BSA with Thymus Grafting.* (30949)

STANLEY B. SMITH,† KATARINA ISAKOVIC,‡ AND BYRON H. WAKSMAN
Department of Microbiology, Yale University, New Haven, Conn.

In a previous study(1), we have established that thymectomized, irradiated rats, which receive grafts of thymus from donors made tolerant by neonatal exposure to protein antigen (bovine γ-globulin, BGG), themselves show specific inhibition of certain immune responses, notably delayed sensitization

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† Special Fellow of Nat. Inst. of Allergy & Infect. Dis.

‡ Assistant, Microbiological Institute, Faculty of Pharmacy, and Microbiology Unit, Inst. of Biology, University of Belgrade, Belgrade.