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A Study of the Separation of Fibrinogen and Antihemophilic Factor (AHF) in Canine, Porcine, and Human Plasmas

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The separation of fibrinogen and plasma antihemophilic factor (AHF)*) is a problem of both practical and theoretical importance. The practical nature of the problem is encountered in attempts to prepare concentrated AHF, free of fibrinogen, for therapy of hemophilia. Such a preparation is desirable if non-human plasma is used as a source of AHF, since it is necessary to remove fibrinogen as well as other non-AHF proteins as potential antigens. From the theoretical aspect, highly purified AHF is needed for physical and chemical characterization. The normal AHF levels in patients with congenital afibrinogenemia (1), and the normal fibrinogen levels in patients with hemophilia (2—4), show that the separation has been accomplished genetically. However, numerous attempts to prepare plasma or plasma fractions free of fibrinogen but high in AHF have failed or resulted in very low yields of AHF (1, 5—9). The apparent close similarity in chemical and physical properties of AHF and fibrinogen has even led to the suggestion (1) that AHF activity of plasma is but another physiologic function of fibrinogen. This paper reports studies of the separation of AHF and fibrinogen in canine, bovine, porcine, and human plasmas by selective adsorption.

Materials and methods

MATERIALS. — (1) The *barium sulfate* was Merck Reagent Grade.

(2) The *fuller's earth* was Florigel (Floridin Co., Warren, Pennsylvania).

(3) *Aluminum hydroxide* was prepared according to the directions of Biggs and Macfarlane (10) except that a large volume of more dilute ammonium hydroxide was added (1400 ml of 1.8 M NH_4OH per 1/4 mole of ammonium alum). The final precipitate was

*) AHF is defined as the plasma protein lacking in classical hemophilia (Factor VIII deficiency, hemophilia A).

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washed with distilled water until pH of the supernatant was less than 6.7. The dry weight of the suspension used was 27 mg per ml.

(4) The *thrombin* preparation was Topical Thrombin (Parke-Davis), 100 u per ml.

(5) *St-3 pads* (Hercules) 6 cm in diameter were used for Seitz filtration. According to the manufacturer's description, the St-3 is composed of 50 per cent asbestos, 6 per cent cotton, and 44 per cent cellulose.

METHODS. — *Collection and Preparation of Plasmas*: Human and canine blood were obtained by the two-syringe technique. Nine volumes of blood were drawn into a syringe containing one volume of 0.1 M sodium oxalate. Bovine and porcine blood were obtained at the abattoir. Nine volumes of blood were mixed with one volume of 0.1 M sodium oxalate in a plastic beaker. The plasmas were obtained by centrifuging the various bloods at about 3,000 g for 20 minutes, and adsorbed two times with $BaSO_4$ (100 mg per ml).

AHF Assays: Canine, bovine, and porcine plasmas and plasma fractions were assayed for AHF content by the partial thromboplastin time technique, using hemophilic dog plasma as the substrate (11). Human plasma and plasma fractions were assayed for AHF content by the prothrombin utilization technique, using whole hemophilic dog blood as the substrate (12).

Fibrinogen Determinations: Fibrinogen content of the adsorbed plasmas was estimated by a modification of the thrombin clotting time method of Shinowara and Rosenfeld (13). A series of plasmas with known fibrinogen concentration was obtained by preparing fresh mixtures of normal plasma with fibrinogen-free plasma of the same species. Fibrinogen-free plasmas were prepared by heat defibrinogenation. Thrombin (0.2 ml) was added to each mixture (0.2 ml) and the clotting time was obtained at 28 ° C. A control calibration curve was prepared for each species. The thrombin clotting times of the adsorbed plasmas were determined and the fibrinogen content was calculated from the appropriate calibration curve. Because of the presence of interfering proteins (14) our modification of this method gives only approximate results. It has the convenience of being rapidly performed, and is applicable to a wide range of fibrinogen levels.

Adsorption Procedures: Plasmas were adsorbed at either 28 ° or 4 ° C in a constant temperature bath. Adsorbents were removed by centrifugation. Fuller's earth was used as a dry powder. Weighed portions were added to plasma and adsorption was continued with slow mechanical stirring. Aliquots of the $Al(OH)_3$ suspension were pipetted into tubes and centrifuged. The supernatant was discarded and the plasma was added to the $Al(OH)_3$ precipitate. The $Al(OH)_3$ was carefully resuspended and mixed occasionally. Seitz pressure filtration was performed at 4 ° C. A large volume of normal saline was run through the filter pad before use.

Results

A number of adsorbents were studied with the aim of obtaining either selective adsorption of AHF or of fibrinogen. Results obtained with fuller's earth and $Al(OH)_3$ adsorption and Seitz filtration are presented.

Fuller's Earth. Table 1 shows the effect of adsorbing various plasmas with different amounts of fuller's earth for 30 minutes at 28° C. The supernatant plasma was assayed for AHF and fibrinogen; results are expressed as per cent of the initial values. With canine plasma, the fibrinogen was removed without loss of AHF activity. With human, bovine, and porcine plasmas, fibrinogen was also adsorbed to a greater degree than AHF activity at all levels of fuller's earth tested. The difference, however, was less striking than with

Table 1: *Adsorption of AHF and Fibrinogen with Fuller's Earth*)*

Type of Plasma	Fuller's Earth mg per ml	Residual Fibrinogen in Supernatant per cent	Residual AHF in Supernatant per cent
Canine	0	100	100
	20	< 8	100
	50	< 5	100
Human	0	100	100
	20	56	100
	50	37	63
	100	2	29
	150	< 2	19
Bovine	0	100	100
	50	100	100
	150	23	52
Porcine	0	100	100
	20	50	65
	100	< 4	33
	150	< 4	11

Table 2: *Effect of Adsorption Time on Fuller's Earth treatment of bovine Plasma**)*

Time of Adsorption min.	Residual Fibrinogen in Supernatant per cent	Residual AHF in Supernatant per cent
30	100	101
60	79	100
120	27	37
240	14	< 5

canine plasma. The upper practical limit of fuller's earth that can be used is 150 mg per ml, as at this level, little of the plasma can be removed without washing the adsorbent.

To test whether longer treatment with smaller amounts of fuller's earth might give more selective adsorption of fibrinogen, bovine plasma was treated at 28° C with 50 mg per ml for varying periods of time. The results (Table 2) show that a longer adsorption time does not increase the selectivity of fibrinogen adsorption and that adsorption equilibrium is reached slowly. In other studies, canine plasma was adsorbed with fuller's earth at 4° C instead of at 28° C,

*) Plasmas were adsorbed for 30 minutes at 28 ° C.

**) Plasmas were adsorbed at 28 ° C.

as in Tables 1 and 2. It was found that at 4° C, adsorption of fibrinogen proceeded much more slowly than at the higher temperature. These data again indicate that adsorption equilibrium is attained slowly.

Table 3: *Adsorption of AHF Fibrinogen with Al(OH)₃*)*

Type of Plasma	Temperature (° C)	Al $(OH)_3$ mg per ml	Residual Fibrinogen in Supernatant per cent	Residual AHF in Supernatant per cent
Bovine	28	0	100	100
		2.7	71	93
		5.4	14	79
		10.8	< 4	52
Bovine	4	2.7	70	99
		5.4	14	71
		10.8	< 4	50
Canine	4	0	100	100
		2.7	75	—
		5.4	19	32
		10.8	< 10	32
Human	4	0	100	100
		2.7	22	61
		5.4	17	33
		10.8	< 4	12

Elution of the fibrinogen from the fuller's earth adsorbate of dog plasma has been attempted with a large variety of eluting agents including strong salt solutions, sodium desoxycholate, and buffers over a wide range of concentration and pH. Proper conditions for elution have not been found.

Aluminium Hydroxide. Another adsorbent studied that shows selective fibrinogen adsorption is $Al(OH)_3$ gel. Assays of plasma after $Al(OH)_3$ adsorption are given in Table 3. The first two sets of data show the adsorption of bovine plasma at 28° and 4° C, with different amounts of $Al(OH)_3$. There is no evidence for a temperature effect on adsorption. This contrasts with the findings for fuller's earth. Fibrinogen was selectively adsorbed at all levels of $Al(OH)_3$ for the three species tested. The greatest differential between fibrinogen adsorption and AHF adsorption was obtained at 5.4 mg per ml for bovine plasma and 2.7 mg per ml for human plasma. The differential was slight for canine plasma.

*) All plasmas were adsorbed for 30 minutes.

Seitz Filtration. This type of adsorption was selective for AHF rather than fibrinogen. Four hundred ml of canine plasma were filtered and collected in a series of tubes. The filtration time for each successive sample as well as the results of the AHF assays are given in Table 4. The first 25 ml of filtrate contained only a trace of AHF and about 60 per cent of the initial fibrinogen. The second 25 ml sample contained 6 per cent AHF. The fibrinogen level was normal for samples 2—6.

Table 4: *AHF Level of Seitz filtered Canine Plasma*)*

Sample Number	Filtration Time min	Volume filtered ml	AHF activity of Filtrate per cent of initial
1	12	25	< 2.5
2	14	25	6
3	13	25	53
4	15	25	94
5	27	50	96
6	220	250	97

Discussion

A reliable assay procedure is needed for any quantitative study of plasma antihemophilic factor. While several types of assays have been described, many appear to be lacking in sensitivity or specificity or both. Two types of tests for AHF were used in these studies. One is based on the correction of the delayed partial thromboplastin time, the other on the correction of the slow prothrombin utilization of hemophilic blood. The substrate used in these tests was plasma or whole blood from canine hemophiliacs. It has been shown earlier that the clotting defect in canine hemophilia is identical with that in human hemophilia (15). Also, the results of the assays correlate well with the *in vivo* correction of the hemostatic defect, both in hemophilic dogs and humans (16). It should be emphasized that in both assay procedures all plasmas tested for AHF are first treated with $BaSO_4$. The necessity for preliminary adsorption of this type to insure specificity of the assays has been described previously (11, 17). In these studies it was pointed out that non-AHF clotting accelerators adsorbable on $BaSO_4$ may give false high AHF values (17).

Preferential adsorption of fibrinogen compared to AHF adsorption is obtained when plasmas are treated with fuller's earth or $Al(OH)_3$. With fuller's earth, the preferential adsorption of fibrinogen from canine plasma is far superior to the results obtained with plasmas of other species. In contrast to the

*) Plasma was filtered at 4 ° C.

fuller's earth experiments, $Al(OH)_3$ adsorption gave similar results for all three species tested.

The *total* amount of fibrinogen adsorbed under standard conditions was found to depend on the species of plasma used. This species dependence of fibrinogen adsorption is greater with fuller's earth than with $Al(OH)_3$. Plasma fibrinogen concentrations differ only moderately among the species studied and probably do not account for the variations in adsorbability observed.

Seitz filtration, unlike the other adsorption procedures tested, removed AHF in preference to fibrinogen (Table 4). Heretofore, we have been dependent upon hemophilic plasma as a source of material for preparation of an AHF-deficient fibrinogen solution (20). With the use of Seitz filtration, it is possible to make a similar preparation from normal plasma.

The logical extension of this work is the use of adsorbed plasmas for the purification of AHF. The use of canine plasma adsorbed with fuller's earth as a starting material for salting out and ethanol fractionation procedures has yielded the first potent fibrinogen-free AHF concentrates. These canine plasma fractions have been concentrated over 200-fold in terms of activity and original plasma protein nitrogen (18, 19). $Al(OH)_3$ adsorption provides a promising step for the preparation of human and bovine AHF fractions poor in fibrinogen.

Summary

1. Studies are reported on attempts to separate antihemophilic factor (AHF) from fibrinogen in plasma by selective adsorption with fuller's earth, aluminum hydroxide gel, and Seitz filtration.

2. Fuller's earth adsorption of canine plasma resulted in almost complete removal of fibrinogen without loss of AHF. By the use of this procedure as part of the fractionation procedure, potent AHF fractions free of fibrinogen have been obtained.

3. $Al(OH)_3$ adsorption of fibrinogen from bovine, canine, and human plasmas is greater than the adsorption of AHF. $Al(OH)_3$ adsorption offers promise for the preparation of fibrinogen-poor AHF fractions of human or bovine plasma.

4. Seitz filtration of plasma causes loss of AHF with little or no loss of fibrinogen.

Résumé

1) Revue des méthodes pour la séparation du facteur antihémophilique (AHF) du fibrinogène plasmatique par adsorption sur du "fuller's earth" de l'hydroxyde aluminique ou par filtration sur des filtres de Seitz.

2) Le traitement du plasma de chien avec du "fuller's earth" résulte en une absorption pratiquement complète de fibrinogène sans perte d'AHF. En

employant ce procédé par la méthode de fractionnement, nous avons obtenu une préparation d'AHF non contaminée de fibrinogène.

3) Le traitement de plasma bovin, canin ou humain avec l'hydroxyde d'Aluminium résulte en une absorption plus importante de fibrinogène que du AHF. L'hydroxyde d'Aluminium permet de préparer à partir de plasma humain ou bovin du fibrinogène contenant peu de AHF.

4) Les filtres de Seitz absorbent partiellement le AHF sans diminution importante du fibrinogène.

Zusammenfassung

1. Es wird über Versuche berichtet, den *antihämophilen Faktor* (AHF, Faktor VIII) im Plasma durch selektive Adsorption an Fuller Erde, Aluminiumhydroxyd-Gel und Seitzfiltration vom Fibrinogen zu trennen.

2. Adsorption von Hundeplasma mit Fullererde entfernt Fibrinogen nahezu vollkommen ohne Verlust an AHF (Faktor VIII). Unter Mitverwendung dieses Verfahrens bei der Plasmafraktionierung konnten AHF-Fraktionen erhalten werden, die frei von Fibrinogen waren.

3. Im Rinder-, Hunde- und Menschenplasma wird Fibrinogen stärker als AHF (Faktor VIII) an Al $(OH)_3$ adsorbiert. Die Adsorption mit Al $(OH)_3$ bietet eine Möglichkeit zur Herstellung Fibrinogen-armer AHF-Fraktionen aus menschlichem und Rinderplasma.

4. Die Seitzfiltration von Plasma verursacht einen Verlust von AHF (Faktor VIII) bei geringem oder keinem Verlust an Fibrinogen.

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