AN ASOLECTIN ADSORBED SUBSTRATE FOR PROACCELERIN ASSAY

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In this report, evidence is presented that asolectin, a commercially available soybean lecithin, is capable under controlled conditions of specifically binding and removing proaccelerin from human plasma. To our knowledge no other selective binding agent or adsorbent of proaccelerin has been reported.

MATERIALS

Asolectin (commercially available from Associated Concentrates, Inc., 57–01 32nd Avenue, Woodside, New York) Lot Number 2085 was used in this investigation. Fifty mg. per ml. was suspended in distilled water with a Potter homogenizer in the amounts needed immediately prior to use.

Barium sulfate (Baker) analytical grade.

Adsorbed ox plasma. Ox blood freshly collected in one-tenth volume of 2.5 per cent potassium oxalate was centrifuged for 45 minutes at 2500 rpm (2° C.). The plasma was siphoned off and passed first through a 20 per cent asbestos, then through a 50 per cent asbestos filter by a technique previously reported (1).

Russell viper venom (Stypven, Burroughs Wellcome and Company, London, England) was diluted 1:40,000 in a 1:30 suspension of cephalin in veronal buffer as described by Hjort, Rapaport, and Owren (2), as was the technique for preparation of:

Prothrombin free ox plasma.

Normal human plasma was prepared from the blood of healthy persons. Following venipuncture with a 16 gauge needle, the first 5 ml. of blood was collected separately and discarded. The desired quantity of blood was then collected by gravity flow in a second glass bottle containing a quantity of 0.1 M sodium citrate solution equal to one-tenth the final volume, or when oxalated blood was necessarily used, into one-tenth volume of 2.5 per cent potassium oxalate solution. In either case, the freshly collected blood was centrifuged at 2500 rpm and 2°C. for 45 minutes. The plasma was transferred to another container with a Pasteur pipette and glass-activated by stirring for 5 minutes with one-eighth volume of fine quartz glass powder (particle size less than 0.07 mm.) by the technique of Rapaport, Aas, and Owren (3). Following centrifugation for 15 minutes at 2500 rpm and 2°C. to remove the glass powder, 20-ml. aliquots of the activated plasma were transferred to glass bottles and stored at minus 20°C. until they were thawed just prior to use.

Citrated, proconvertin deficient plasma was prepared from the blood of a patient with a congenital deficiency of this factor. Studies of the coagulation characteristics of this patient's plasma have been reported previously (4). Collection and processing were as described for citrated normal plasma. Aliquots kept at minus 20°C. were thawed just before use.

Citrated, proaccelerin deficient plasma was prepared in a similar manner from the blood of a patient with parahemophilia. The clotting characteristics of this patient's blood plasma have been reported in detail elsewhere (5).

Diluting fluid I was prepared as follows:

Sodium citrate 0.1 M solution	200 ml.
Sodium chloride 0.9 per cent solution q. s.	1200 ml.

Diluting fluid II was made by mixing:

Sodium citrate 25.66 mM solution	200 ml.
Veronal buffer	200 ml.
Sodium chloride 0.9 per cent solution q. s.	1000 ml.

Veronal buffer (pH 7.35, ionic strength 0.154) was prepared as previously described (2).

Buffer saline was prepared as follows:

Veronal buffer	100 ml.
Sodium chloride 0.9 per cent solution q. s.	1000 ml.

Calcium chloride aqueous solutions were prepared with the molarities indicated in each test system.

Saline extract human brain thromboplastin was prepared as described previously (6).

Thrombin: 1000 units of Hemoclaudan Adrenon Leo (Lövens Kemiske Fabrik, Köbenhavn) were dissolved in 10 ml. veronal buffer. Further dilutions were made with buffer saline to the extent noted in the text.

ASSAY SYSTEMS

All incubations and tests were performed at 37°C. in duplicate.

Fibrinogen reactivity:

Citrated control or test plasma, undiluted	0.2 ml.
Buffer saline	0.2 ml.
Incubate 3 minutes. Time from addition of:	
Thrombin (1:15 in buffer saline)	0.2 ml.

Thromboplastin time [Quick (7)]:

Citrated control or test plasma, undiluted	0.2 ml.
Thromboplastin	0.2 ml.
Incubate 3 minutes. Time from addition of:	
Calcium chloride 25 mM solution	0.2 ml.

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Proaccelerin assay:

Test plasma (1 : 10 in diluting fluid II)	0.2 ml.
Parahemophilia plasma	0.2 ml.
Thromboplastin	0.2 ml.
Incubate 3 minutes. Time from addition of:	
Calcium chloride 30 mM solution	0.2 ml.

Proconvertin assay:

Test plasma (1 : 10 in diluting fluid II)	0.2 ml.
Plasma congenitally deficient in proconvertin	0.2 ml.
Adsorbed ox plasma	0.2 ml.
Thromboplastin	0.2 ml.
Incubate 3 minutes. Time from addition of:	
Calcium chloride 35 mM solution	0.2 ml.

Prothrombin assay: [method of Hjort, Rapaport, and Owren (2)]

Test plasma (diluted 1 : 5 in diluting fluid I; then 1 : 10 in diluting fluid II; final dilution, therefore, 1 : 50)	0.2 ml.
Russell viper venom (1 : 40,000 in suspension of cephalin 1 : 30 in veronal buffer)	0.2 ml.
Prothrombin free ox plasma	0.2 ml.
Incubate 3 minutes. Time from addition of:	
Calcium chloride 30 mM solution	0.2 ml.

Anti-thromboplastin detection:

Test plasma, undiluted	0.2 ml.
Citrated, platelet poor, glass-activated normal plasma, undiluted	0.2 ml.
Adsorbed ox plasma	0.2 ml.
Thromboplastin	0.2 ml.
Incubate 3 minutes. Time from addition of:	
Calcium chloride 30 mM solution	0.2 ml.

PREPARATION OF PROACCELERIN DEFICIENT PLASMA FROM NORMAL PLASMA BY ASOLECTIN BINDING AND SEPARATION

To citrated, platelet poor, glass-activated, normal plasma, asolectin homogenized in distilled water was added in the amount of 25 mg. of asolectin for each ml. of citrated plasma. Sufficient additional distilled water was then added until the original volume of citrated plasma equalled one-sixth the final diluted volume. The glass container was swirled to disperse the asolectin suspension throughout the mixture, covered with Parafilm, and placed in a water bath at 37° C. for 2 hours. After this period, the dilute plasma-asolectin suspension was transferred to lusteroid tubes, capped, and spun in a Spinco preparatory ultracentrifuge at 40,000 rpm for 1 hour (2° C.). The tubes were removed with utmost care to avoid agitation of the contents. Caps were *not* removed since the cuff traps a small quantity of the original suspension. Instead, the central screw plug was removed (again without agitation of the tubes' contents) and a lumbar puncture needle fastened to a 20-ml. Luer-Lok syringe was carefully introduced to a point just above the bottom of the tube on the side opposite the sedimented asolectin. The clear dilute plasma was slowly aspirated until the thin floating surface layer of lipids approached the needle tip. Aspiration was then discontinued and the syringe contents transferred to a graduated cylinder where the clear dilute plasma from all tubes was pooled, measured, and transferred to a glass vacuum flask. The flask's contents were shell-frozen by spinning in a bath of methyl alcohol and carbon dioxide snow. Lyophilization was carried out. When dry, the plasma was reconstituted with distilled water equal to one-sixth the volume of the material transferred to the lyophilizing flask before drying. Correction of the pH to 7.3 was made by adding 0.5 N HCl dropwise with stirring. Multiple lyophilized samples were reconstituted simultaneously, pooled so that a single reference curve would serve for all, frozen and stored in 1-ml. aliquots at minus 20° C.

The assay results in Table I are representative for such reconstituted plasma:

It is seen that proaccelerin was almost completely removed, prothrombin concentration was slightly reduced, proconvertin activity less so. Fibrinogen reactivity was good. Anti-thromboplastic activity was not demonstrated.

The control plasma, without asolectin, was similarly diluted 1 : 6 with distilled water, alkalinized to pH 9.1 with 0.5 N NaOH, and incubated 150 minutes at 37° C. It was then centrifuged at 40,000 rpm for 60 minutes (2° C.), shell frozen, lyophilized, and reconstituted in distilled water equal to one-sixth the total dilute volume. The pH was corrected to 7.3 with 0.5 N HCl added dropwise. No comparable loss of proaccelerin in the reconstituted control plasma occurred as judged by its Quick thromboplastin time of 20.9 and 21.0 seconds in contrast to 75.0, 74.6 seconds for the asolectin adsorbed plasma similarly reconstituted.

It should also be noted that the reconstituted lyophilized asolectin adsorbed plasma was tested undiluted in the anti-thromboplastin detection test. This would actually represent a six-fold increase in concentration of any such substances in the clear supernate since reconstitution was effected with one-sixth the total dilute volume. Actually, no

TABLE I

The following assay results are representative for reconstituted asolectin adsorbed plasma

	Reconstituted asolectin adsorbed plasma *time in seconds*	Per cent	Unadsorbed plasma same source* *time in seconds*	Per cent
Thromboplastin time (Quick)	75.0, 74.6		11.9, 11.6*	
Prothrombin	39.8, 39.2	56	32.8, 32.3	72
Proaccelerin	81.6, 83.3	0	23.7, 23.2	100
Proconvertin	23.6, 23.2	70	22.6, 19.9	100
Fibrinogen reactivity	22.1, 22.9		20.7, 19.9	
Anti-thromboplastin detection	12.1, 11.7		11.9, 12.0	

* Same plasma diluted 1:6 with distilled water, pH adjusted to 9.1 with 0.5 N NaOH, incubated 150 minutes at 37° C., centrifuged 60 minutes at 40,000 rpm, shell frozen and lyophilized gave Quick thromboplastin times after reconstitution with distilled water equal to one-sixth of the total dilute volume and correction of pH to 7.3 with 0.5 N HCl of 20.9 seconds and 21.0 seconds on duplicate samples.

anti-thromboplastins were demonstrable even under these conditions.

Evidence that asolectin specifically binds proaccelerin rather than destroys it is seen in the following experiment: 25 mg. of asolectin per ml. of citrated normal plasma (platelet poor, glass-activated) was suspended directly in the plasma with a Potter homogenizer. This mixture was then transferred to a glass tube, covered with Parafilm and incubated in a water bath at 37° C. Periodically, samples were taken for testing in the following system:

Diluting fluid II	0.2 ml.
Thromboplastin	0.2 ml.
Homogenized asolectin in normal plasma (citrated, platelet poor, glass-activated) 25 mg. per ml.	0.2 ml.
Incubate 3 minutes. Time from addition of:	
Calcium chloride 30 mM solution	0.2 ml.

Incubation time in minutes	Time in seconds of duplicates
0	38.6, 39.2 (same plasma in same system without asolectin 14.2, 14.6 after 0 minutes incubation)
15	38.8, 39.4
30	40.4, 38.9
45	40.8, 39.2
60	40.6, 39.8
75	41.7, 39.1
90	41.2, 39.0
105	42.3, 39.5
120	41.6, 40.7

Plasma incubated with asolectin 25 mg. per ml. in fine suspension for 120 minutes, diluted 1:6 with distilled water, spun 40,000 rpm for 60 minutes to remove asolectin, lyophilized and reconstituted in original volume gave 88.0 and 87.6 seconds in the same system.

We interpret these results to mean that no considerable deterioration of proaccelerin occurs on incubation with 25 mg. per ml. asolectin for two hours at 37° C. We have previously demonstrated that dilution, alkalinization, incubation, centrifugation, lyophilization, neutralization, and reconstitution of normal plasma in the initial volume does not result in comparable loss of proaccelerin activity. The marked depletion of proaccelerin that occurs when asolectin is spun out reflects the specific binding and co-sedimentation of a proaccelerin-asolectin complex, in our opinion. Whether this binding is physical or chemical cannot be stated with certainty at this time. Further investigation along this line is planned as is further consideration of the inhibitory effect of asolectin on the thromboplastin time and whether this is related to the asolectin-proaccelerin complex (*i.e.*, an anti-proaccelerin effect). Reutilizing once used asolectin sediment in equal concentration to adsorb a second equal portion of dilute normal plasma by the identical technique results in marked reduction of its ability to remove proaccelerin. Reconstituted plasma adsorbed with once previously used asolectin gave proaccelerin reduction to only 60 per cent of original activity instead of 0 per cent as in the first adsorption.

The following curves (Figure 1) compare the proaccelerin assay of known dilutions of normal citrated plasma in diluting fluid I when parahemophilia plasma was the substrate (dotted line) and when reconstituted asolectin adsorbed plasma was the substrate (solid line).

Demonstration of the mutual inability of para-

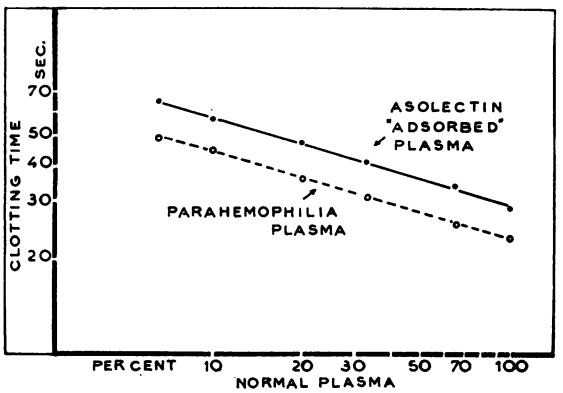

FIG. 1. COMPARISON OF PARAHEMOPHILIA PLASMA AND ASOLECTIN ADSORBED PLASMA AS SUBSTRATE FOR PROACCELERIN ASSAY

hemophilia plasma and reconstituted asolectin adsorbed plasma to correct one another is shown in the following results:

Parahemophilia plasma substrate	0.2 ml.
Reconstituted asolectin adsorbed plasma (1:10 in diluting fluid II)	0.2 ml.
Thromboplastin	0.2 ml.
Calcium chloride 30 mM solution	0.2 ml.
	81.6, 83.3 seconds (equals 0 per cent proaccelerin)

Reconstituted asolectin adsorbed plasma substrate	0.2 ml.
Parahemophilia plasma (1:10 in diluting fluid II)	0.2 ml.
Thromboplastin	0.2 ml.
Calcium chloride 30 mM solution	0.2 ml.
	87.6, 88.4 seconds (equals 0 per cent proaccelerin)

To determine if the results of the assay (Figure 1) were solely a reflection of the proaccelerin content and to exclude prothrombin and proconvertin activity as possible causes of the variations, the following experiment was designed:

Freshly prepared, oxalated, platelet poor, glass-activated, normal plasma was divided into two portions. One was mixed with 75 mg. of barium sulfate per ml. of oxalated plasma for 5 minutes at 20° C., then separated by centrifugation for 25 minutes at 2500 rpm (2° C.). Since some proaccelerin is non-specifically removed during barium sulfate adsorption, the unadsorbed oxalated plasma was diluted with buffer saline solution until its proaccelerin activity in correcting parahemophilia plasma substrate equalled that of the barium sulfate adsorbed plasma against the same substrate. The adjusted unadsorbed plasma mixture that accomplished this was 90 per cent oxalated unadsorbed plasma and 10 per cent buffer saline. Varying mixtures of barium sulfate adsorbed and unadsorbed plasma were tested in the proaccelerin assay system using asolectin adsorbed plasma as substrate. If this substrate is corrected by the proaccelerin content of the mixture and is insensitive to the varying prothrombin and proconvertin content, one would expect the same correction by all the mixtures. In Table II it can be seen that this was substantially the case. Test system is as described in section headed "ASSAY SYSTEMS" for proaccelerin with the difference that asolectin adsorbed reconstituted plasma is substituted as the substrate plasma for parahemophilia plasma.

COMMENT

Asolectin is not completely separable by centrifugation from undiluted plasma and, indeed, rises to the surface. Reduction of specific gravity and ionic strength by dilution with distilled water seems necessary for its complete separation by

TABLE II

Effect of varying prothrombin and proconvertin content on proaccelerin content in mixtures of $BaSO_4$ adsorbed plasma and unadsorbed plasma

Plasma mixtures						
Oxalated $BaSO_4$ adsorbed plasma (%)	100	80	60	40	20	0
Unadsorbed plasma (%)	0	20	40	60	80	100
Prothrombin (%)	0	20	40	60	80	100
Proconvertin (%)	0	20	40	60	80	100
Proaccelerin (%)	Constant in all mixtures					
Time in seconds	33.0	35.0	34.0	33.6	32.2	
Duplicates	32.6	34.8	33.6	33.0	33.6	

sedimentation on centrifugation. The adsorbed dilute plasma can then be lyophilized and reconstituted with an amount of distilled water equal to the original plasma volume.

Twenty-five mg. of asolectin per ml. of citrated plasma was determined to be the optimum amount and 120 minutes at 37° C. the optimum incubation period. Lesser amounts of asolectin or shorter periods of incubation resulted in less complete adsorption of proaccelerin. Greater concentrations of asolectin resulted in progressively larger adsorption of prothrombin in addition to proaccelerin and, with still greater concentrations, in considerable loss of fibrinogen reactivity.

One additional interesting thing becomes apparent from inspection of the systematic assay data (not presented here) when larger quantities of asolectin than recommended in this paper (for adsorption of proaccelerin) are used. Prothrombin adsorption becomes almost complete while proconvertin activity is only slightly diminished. Further examination of this phenomenon is discussed in another communication (8).

Paralleling their remarkable similarity of behavior in some other respects, we have found that cephalin derived from human brain by a modification of the technique of Milstone (9) also behaved as a proaccelerin adsorbent when substituted for asolectin in the technique outlined in this paper. Because asolectin is commercially available, whereas the preparation of cephalin from human brain is tedious, the technique using the former alone is reported in detail here.

It is interesting to speculate that possibly the similar lipid material of thrombocytes may be responsible for their demonstrated behavior as an adsorbent of proaccelerin (10).

SUMMARY

Evidence is presented that asolectin in an amount of 25 mg. per ml. of the original citrated plasma volume, suspended in normal plasma diluted 1 : 6 with distilled water, incubated 120 minutes at 37° C., and removed by centrifugation at 40,000 rpm for 1 hour, is capable of specifically binding and removing the proaccelerin of the normal plasma. The adsorbed dilute plasma was shell frozen, lyophilized, and reconstituted with one-sixth volume of distilled water. Control normal plasma similarly treated with the omission of asolectin exposure showed no comparable loss of proaccelerin.

Such asolectin adsorbed reconstituted plasma was comparable in its behavior as a substrate plasma for a proaccelerin activity assay system to the naturally occurring parahemophilia plasma used as a substrate plasma in the same system.

Evidence of the specificity of the asolectin adsorbed plasma substrate response for proaccelerin activity of the test samples is presented.

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