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Studies on the Coagulation of Chicken Blood

III. Differentiation of $\varkappa$-, δ- and φ-Factors by Adsorption

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Studies on the adsorption from oxalated plasma of the coagulation factors, measured as $\varkappa$-, δ- and φ-factor activities with thromboplastin and with Russell's viper venom-cephalin, revealed characteristic differences in the adsorbabilities of these factors by a series of crystalline adsorbents. Differences in the coagulation activity of the factors are thus reflected in differences in their physico-chemical properties. A further differentiation of the φ-factor activity can be made by means of the adsorbents. It is shown that the φ-factor activity represents the combined activities of a calcium carbonate adsorbable φ-factor and one or more φ-factors which are not adsorbed from oxalated plasma by this compound.

In an earlier paper [1] a method was described for estimation of the $\varkappa$-, δ- and φ-factor activities of chicken plasma. The method permitted comparison of the $\varkappa$-, δ- and φ-factor activities of different plasmas and was therefore suitable for a study of the effect of adsorbents on the concentration of the coagulation factors measured as $\varkappa$-, δ- and as φ-factors in the assay systems. It had already been found [1] that the three types of factors could be completely adsorbed from oxalated plasma by typical prothrombin adsorbents, such as barium carbonate and strontium carbonate. The motivation for carrying out further adsorption studies was twofold: In the first place, it was thought possible by systematic testing of other well-defined insoluble compounds to find adsorbents with more selective properties, *i.e.* adsorbents which would adsorb one or two of the coagulation factors and leave the other(s) unaffected — or to find adsorbents with at least different adsorption *capacities* for the three types of factors. In the second place, it was of interest to learn if treatment of plasma with increasing amounts of an adsorbent would result in merely partial adsorption of a certain type of activity ($\varkappa$-, δ- or φ-factor). If this should be the case, it would mean that more than one coagulation factor contributed to the measured activity and would permit a distinction between different coagulation factors with similar effects in the test systems described.

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As reported [1] the dilution curves for φ-factor activity obtained with cephalin, Russell's viper venom (RVV)-cephalin and with thromboplastin as assay accelerators, were of different shapes. It was suggested that the activity measured as φ-factor activity, might represent the combined activities of separate entities (φ_1, φ_2, φ_3. . . .), some of which are active only in the thromboplastin assay system, one or more other factors specific for the cephalin assay system, and one or more factors which are measured as φ-factors in both these systems as well as with RVV-cephalin.

There is at present no evidence indicating the existence of more than one coagulation factor with $\varkappa$-factor activity, or more than one factor with δ-factor activity. In the measurements of $\varkappa$-, and of δ-factor activities of plasmas the three assay systems have so far given identical results. The mechanism by which $\varkappa$- and δ-factor influence the coagulation rate thus appears to be equally important with all three coagulation accelerators.

MATERIALS AND METHODS

The materials and methods for estimation of the $\varkappa$-, δ- and φ-factor activities of chicken plasma were described in a previous paper [1]. The detailed procedure for treatment of plasma with adsorbents was also presented.

Adsorbents. Some of the preparations of the adsorbents used in this work were commercially available, but most of the compounds tested as adsorbents were prepared in the laboratory in an attempt to ensure the greatest possible reproducibility in properties. Yet the adsorption properties of different batches of the same compound, although qualitatively similar, were not always quantitatively identical, *i.e.*, their ability to distinguish between different coagulation factors was the same, but their adsorption *capacities* for each of the various factors could vary somewhat. Amorphous preparations usually did not show the marked selective adsorption properties, typical for crystalline preparations. Therefore, great efforts were taken to prepare the adsorbents in a crystalline or at least a micro-crystalline state. The preparation procedures were adapted from methods described in Gmelin-Kraut's Handbuch der Anorganischen Chemie. All preparations were finally thoroughly washed with distilled water, treated with 96 % ethanol and dried overnight at 80°C.

Oxalates of calcium, strontium and barium were prepared from aqueous solutions of the chlorides with excess of an equimolar mixture of oxalic acid and ammonium oxalate. Precipitation was carried out at 80—90°C. The precipitates were washed several times with hot distilled water by stirring, followed by decantation after settling. The washed products were dissolved in boiling dilute hydrochloric acid (8—10 % for calcium, and 4—5 % for strontium or barium salts) until saturation, quickly cooled to 4—5°C and stored at 5°C for 24—48 h. Microcrystalline oxalates were obtained. Final washing was done with hot distilled water.

Carbonates of calcium, strontium, barium, cadmium, and manganese were prepared from solutions of the chlorides with excess of ammonium bicarbonate. Precipitation was carried out very slowly at 80—90°C. Washing of the precipitates was done with cold distilled water. Use of hot water at this point often gave peptization of the crystals, whereby the preparations lost their selective properties.

Lead chromate. Lead acetate was dissolved in a small volume of 6—7 N nitric acid. Potassium bichromate in excess was added very slowly to the hot solution. Washing of the precipitate was done with hot distilled water.

Barium chromate. A solution of barium chloride was precipitated with excess of potassium bichromate. After washing, the product was dissolved in hot 5 % nitric acid and recrystallized by slow neutralization of the solution with sodium acetate. The crystals were washed with hot distilled water.

Calcium molybdate. Six parts of calcium chloride, two parts of sodium molybdate and four parts of sodium chloride were thoroughly mixed by grinding in a mortar and brought

to melting at 800°C. After cooling, the melt was treated with water to remove the soluble material. The insoluble calcium molybdate was ground to a powder and washed with hot distilled water.

Bismuth fluoride. Bismuth nitrate was dissolved in water with a minimum of nitric acid and precipitated by addition of a concentrated solution of potassium fluoride. Washing of the microcrystalline precipitate was done first with cold, then with hot distilled water.

Calcium fluoride. Five parts of calcium chloride, four parts of sodium fluoride and four parts of sodium chloride were thoroughly mixed by grinding in a mortar and brought to melting at 900°C. The melt was treated as for calcium molybdate.

Lead sulfide. Lead acetate was dissolved in 0.5 N nitric acid. The solution was then treated with hydrogen sulfide gas. During the precipitation the solution was gradually diluted with 1 1/2 volumes of distilled water. The precipitate was washed with dilute nitric acid, followed by hot distilled water.

Magnesium arsenate. A solution of 3.5 g $MgSO_4 \cdot 7H_2O$ in 2 000 ml of water was mixed with 3 g $Na_2HAsO_4 \cdot 7H_2O$ and 0.8 g $NaHCO_3$ dissolved in 1 000 ml of water. The mixture was left at room temperature for at least eight months with occasional stirring. The product ($Mg_3(AsO_4)_2.8H_2O$) was finally washed with cold distilled water.

Treatment of plasma with the adsorbents described here, caused no or at most a very slight pH change. During a preliminary testing of an adsorbent the activity of the adsorbed plasma was compared to that of untreated plasma at different dilutions. Corresponding results were taken to indicate that the reduced activity of the treated plasma was caused by adsorption of coagulation factors and not by release of inhibitory material from the adsorbent. The optimal conditions for elution of the adsorbed factors from the various adsorbents have not been determined, but preliminary experiments have indicated that the factors adsorbed by calcium oxalate may be eluted by citrate buffers of suitable pH and molarity.

EXPERIMENTS AND RESULTS

Comparative studies on the adsorption of $\varkappa$-, δ- and φ-factor activities

A series of adsorbents were tested for approximate capacity to adsorb $\varkappa$-, δ- and φ-factor activity from oxalated plasma. This testing was done in different periods in the course of four years. The plasmas were obtained from normal chickens and had different levels of $\varkappa$-, δ- and φ-factors. A different set of substrate plasmas was used in each experiment, and different batches of thromboplastin, Russell's viper venom and cephalin were employed. The activities of the factors were expressed in arbitrary units. The units for $\varkappa$-and δ-factor activities were related to the differences in $\varkappa$-factor and in δ-factor concentrations between the vitamin K-deficient and the coumachlor plasma used as substrates [1], and appeared not to vary significantly from one set of substrate plasmas to another. Different experiments with the same batch of an adsorbent were therefore comparable. Because of lack of a reliable reference, the φ-factor activity of each untreated plasma was arbitrarily set at 100 %, in spite of possible differences between the plasmas employed. Different experiments with the same batch of an adsorbent could therefore scarcely be expected to give identical results, but were regarded mainly as a guide to more detailed studies on the adsorption of φ-factor activity.

A summary of the results of these experiments is given in Table 1. As anticipated the compounds tested as adsorbents showed some pronounced differences in adsorption capacities for different types of factors ($\varkappa$-, δ- or

φ-factor). Selective adsorption of δ-factor was accomplished with two adsorbents (cadmium carbonate and bismuth fluoride), but none of the adsorbents showed an absolute selectivity for $\varkappa$- or φ-factors. All compounds adsorbing φ-factor activity as measured with thromboplastin, also adsorbed the φ-factor activity which was measured with RVV-cephalin. Selective adsorption of a thromboplastin specific φ-factor was thus not achieved with any of the adsorbents. As for the adsorption of $\varkappa$- and of δ-factor the thromboplastin and the RVV-cephalin assay systems gave concordant results. For a comparison of the effects of the different compounds, it seemed convenient to distinguish between three main groups of adsorbents:

The first group of adsorbents comprised the major part of the compounds. These adsorbents had a smaller or greater adsorption capacity for all three types of coagulation factors ($\varkappa$-, δ- and φ-factors). Considerable differences between the individual adsorbents of this group were noted regarding their relative adsorption capacities for the different factors. Thus calcium oxalate and magnesium arsenate had greater adsorption capacities for δ-factor than for $\varkappa$-factor, whereas adsorbents such as calcium carbonate, manganese carbonate, strontium carbonate, strontium oxalate and lead sulfide had about equal adsorption capacities for these two factors, and barium chromate had definitely greater adsorption capacity for $\varkappa$-factor than for δ-factor. The adsorption studies thus provided additional evidence for the non-identity of the $\varkappa$-factor and δ-factor activities.

In the first group of adsorbents calcium carbonate was found to be fundamentally different from the rest. This adsorbent showed a small, but definite adsorption capacity for $\varkappa$- and δ-factor and, in the range studied, these two coagulation factors were adsorbed proportionally to the amount of adsorbent added. Only a part of the φ-factor activity could, however, be adsorbed by calcium carbonate. This was observed both with thromboplastin and with RVV-cephalin as assay accelerators. The partial absorbability of φ-factor activity by calcium carbonate can probably be explained only by assuming that the activity measured as φ-factor activity with thromboplastin and with RVV-cephalin, represents the combined activities of at least two different coagulation entities: one which can be completely adsorbed from oxalated plasma by treatment with less than 100 mg calcium carbonate per ml of plasma, and one or more factors which cannot be adsorbed by this compound. The designation φ_2-factor was chosen for this coagulation entity, which is a part of the φ-factor activity measured with thromboplastin as well as with RVV-cephalin and can be completely adsorbed from oxalated plasma by treatment with calcium carbonate.

The adsorption of φ_2-factor by calcium carbonate was apparently independent of the adsorption of $\varkappa$- and δ-factors, since maximal adsorption of φ-factor activity was accomplished with adsorbent quantities far below those required for complete adsorption of $\varkappa$- and of δ-factor activities. The adsorption of $\varkappa$- and of δ-factor appeared to be mutually independent, since the $\varkappa$-factor activity adsorbed per unit amount of adsorbent was unrelated to the δ-factor activity of the plasma, and *vice versa*.

Bismuth fluoride and cadmium carbonate represent a second group of adsorbents. In the range studied these two compcunds adsorbed δ-factor only,

Table 1. Effect of adsorbents on δ-, $\varkappa$- and φ-factor levels of plasma.

	Plasma No.	Assay system	Adsorbent in mg per ml plasma	Activities of factors after (before) the adsorption δ	$\varkappa$	φ	Plasma coagulation time in min/100 after (before) adsorption
Group 1	4671 a	Thr.pl.	100 CaC_2O_4	25 (70)	35 (60)	40 (100)	
	8706 b	RVV-ceph.	100 »	40 (90)	25 (50)	50 (100)	30 (20.3)
	4671 b	Thr.pl.	100 $CaMoO_4$	45 (75)	60 (80)	45 (100)	
	8706 a	RVV-ceph.	100 »	5 (30)	50 (75)	78 (100)	25.5 (24)
	4671 b	Thr.pl.	100 $MnCO_3$	55 (75)	60 (80)	75 (100)	
	8706 a	RVV-ceph.	150 »	10 (30)	40 (75)	60 (100)	27.5 (24)
	4702	Thr.pl.	10 $SrCO_3$**	20 (80)	10 (70)	15 (100)	
	8834	RVV-ceph.	10 »	25 (100)	5 (70)	30 (100)	41 (20.8)
	4671 b	Thr.pl.	100 SrC_2O_4	35 (75)	40 (80)	50 (100)	
	8826	RVV-ceph.	100 »	55 (100)	0 (15)	42 (100)	30 (22)
	4702	Thr.pl.	100 PbS	10 (80)	2 (70)	5 (100)	
	8826	RVV-ceph.	100 »	30 (100)	0 (15)	20 (100)	35 (22)
	4702	Thr.pl.	100 BaC_2O_4*	30 (80)	10 (70)	10 (100)	
	8826	RVV-ceph.	100 »	15 (100)	0 (15)	10 (100)	75 (22)
	4671 b	Thr.pl.	10 $BaCO_3$**	0 (75)	0 (80)	1 (100)	
	8834	RVV-ceph.	10 »	15 (100)	10 (70)	15 (100)	51 (20.8)
	4671 b	Thr.pl.	100 $BaCrO_4$	40 (75)	25 (80)	28 (100)	
	8838	RVV-ceph.	100 »	20 (60)	20 (80)	45 (100)	35.6 (21)
	4702	Thr.pl.	100 $Mg_3(AsO_4)_2$	30 (80)	50 (70)	80 (100)	
	8838	RVV-ceph.	150 »	0 (60)	50 (80)	60 (100)	33 (21)
	1212	Thr.pl.	100 $CaCO_3$	55	15	72	
			200 »	45 (65)	5 (25)	72 (100)	
	36	Thr.pl.	100 »	90	90	88	
			200 »	80	80	88	
			300 »	70 (100)	70 (100)	88 (100)	
			400 »	60	60	88	
	8834	RVV-ceph.	200 »	80 (100)	50 (70)	90 (100)	
Group 2	4671 a	Thr.pl.	100 $CdCO_3$	60 (70)	60 (60)	100 (100)	
	2410	»	150 »	33 (48)	— —	100 (100)	
	8848	Thr.pl.	80 »	67	75	100	28.3
			160 »	59 (75)	75 (75)	100 (100)	28.3 (28.3)
			240 »	51	75	100	28.3
	8706 b	RVV-ceph.	200 »	70 (90)	50 (50)	100 (100)	20.3 (20.3)
Group 3	4671 b	Thr.pl.	150 BiF_3	64 (75)	80 (80)	100 (100)	
	2410	»	200 »	34 (48)	— —	100 (100)	
	8849	»	300 »	0 (5)	65 (65)	100 (100)	
	8706	RVV-ceph.	200 »	75 (90)	50 (50)	100 (100)	20.3 (20.3)
	4702	Thr.pl.	150 CaF_2	80 (80)	70 (70)	100 (100)	
	8706 a	RVV-ceph.	200 »	30 (30)	75 (75)	100 (100)	24 (24)
	4671 a	Thr.pl.	100 $PbCrO_4$	70 (70)	60 (60)	100 (100)	
	8834	RVV-ceph.	200 »	100 (100)	70 (70)	100 (100)	20.8 (20.8)

* Different batches of barium oxalate were used in these two experiments.
** Commercial preparations.

Table 2. Effect of column adsorption on δ-, $\varkappa$- and φ-factor levels of normal plasma

Column material	Effluent plasma	Activity of factors in effluent (untreated) plasma			Thromboplastin time in min/100 of effluent (untreated) plasma
2 g CaF_2	0–1 ml	45	90	72	26
	1–2 ml	75 (75)	100 (100)	100 (100)	24.5 (24.5)
	2–3 ml	75	100	100	24.5
2 g $PbCrO_4$	0–1 ml	73	100	98	24.7
	1–2 ml	75 (75)	100 (100)	100 (100)	24.5 (24.5)
	2–3 ml	75	100	100	24.5

proportionally to the amount of adsorbent added. The activities of $\varkappa$- and φ-factors remained unchanged, and no prolongation of the coagulation time of normal plasma could be detected. Complete, selective removal of δ-factor from oxalated plasma might therefore be possible with either of these compounds. It was considered unpracticable and was not attempted since the adsorption capacities for δ-factor were so small *.

A third group of adsorbents is represented by calcium fluoride and lead chromate. Treatment with these compounds had no effect on the activities of $\varkappa$-, δ- or φ-factors as measured with thromboplastin and with RVV-cephalin. Plasma coagulation times was essentially unchanged after adsorption. The coagulation time and $\varkappa$-, δ- and φ-factor levels of normal oxalated plasmas were also found unchanged after passage through a non-wetted column of calcium fluoride or lead chromate, except in the first few drops of effluent plasma (Table 2). These two compounds could thus scarcely be regarded as adsorbents of coagulation factors at all. The experiments do, however, demonstrate that the effects of the other adsorbents (of group 1 and 2) are specific and not simply due to contact of plasma with *any* kind of foreign surface.

Adsorption experiments with "hypoprothrombinemic" plasmas

The coagulation time of plasma is insensitive to variations in $\varkappa$- or δ-factor concentrations above a lower optimal level [1]. A lower optimal level for the concentrations of φ-factors probably also exists. Adsorption of small amounts of $\varkappa$-, δ- or φ-factors from normal plasma would consequently not result in prolonged *plasma coagulation times*. However, the coagulation time of vitamin K-deficient plasma is sensitive to small variations in δ- and φ-factor concentration, and the coagulation time of coumachlor plasma is sensitive to small variations in the concentrations of $\varkappa$- and φ-factors. The effect of some of the

* The procedure for preparation of cadmium carbonate has sometimes given preparation which are less specific than that used in this work. Some preparations showed a small adsorption capacity for φ-factor (but not for $\varkappa$-factor) in addition to the δ-factor adsorption capacity.

Table 3. Adsorption of hypoprothrombinemic plasmas.

Adsorbent in mg per ml plasma	Thromboplastin time of plasma in min/100 after (before) absorption			
	Coumachlor		Vitamin K-deficient*	
50 CaF_2	360	(360)	61	(61)
50 $PbCrO_4$	360		61	
50 $CdCO_3$	360		64.8	
50 BiF_3	360		61.8	
10 $BaCrO_4$	2 000		75	
10 $CaCO_3$	500		73.2	

* The relatively short coagulation time of this plasma indicated a moderately severe vitamin K-deficiency with δ-factor level near, but not below the optimal level.

adsorbents on the coagulation time of these types of plasma was therefore investigated to check the results of the experiments on adsorption of the $\varkappa$-, δ-, and φ-factor activities of normal plasma.

Adsorption with small amounts of calcium fluoride or lead chromate had no effect on the coagulation times of vitamin K-deficient or coumachlor plasma, as seen in Table 3. Similar amounts of cadmium carbonate or bismuth fluoride, however, caused a slight prolongation of the coagulation time of the vitamin K-deficient plasma, but had no such effect on the coagulation time of the coumachlor plasma. The results indicated an adsorption of δ-factor by cadmium carbonate and by bismuth fluoride, and no effect of these adsorbents on $\varkappa$- or φ-factor concentrations. Treatment of the two types of hypoprothrombinemic plasmas with small amounts of barium chromate or calcium carbonate, which reduce the activities of all three types of coagulation factors, definitely prolonged the coagulation times of both plasmas.

The results of these experiments are thus in accordance with the observed effect, or lack of effect, of the respective adsorbents on the activities of $\varkappa$-, φ- and δ-factors.

DISCUSSION

Plasmas from humans with coagulation disorders of genetic origin have been a valuable material for the discovery of previously unknown coagulation factors in man. As far as other species are concerned, hemophilia in dogs has been described by Graham *et al.*[2], and a hemophilia-like disease in swine by Hogan *et al.*[3], but reports on genetic coagulation anomalies in small laboratory animals are lacking. It is, therefore, not possible at present to use genetically deficient chicken plasma for detection of chicken coagulation factors.

This purpose may be accomplished by the use of chicken plasmas with *induced* coagulation deficiencies. The number of coagulation factors which may be studied with such artificial substrates, is limited to the factors which by suitable procedures can be reduced *below optimal levels* in the plasma. These factors do not necessarily correspond to the factors lacking in the different

categories of congenitally deficient human plasmas, recognized at present. The interpretation of the results obtained with artificial test substrates is complicated by the possibility that the reduced coagulation rate of such substrates may be due to lowering of more than one coagulation factor. Thus, the coagulation rate of vitamin K-deficient and of coumachlor plasmas are not determined by the concentrations of one factor only. Vitamin K-deficient plasma has a manifest deficiency in a second factor (δ-factor) and coumachlor plasma in a third ($\varkappa$-factor) in addition to the deficiency in a common factor (φ-factor) [4]. With a substrate of pure vitamin K-deficient plasma the combined activities of δ- and φ-factors would thus be measured, and with pure coumachlor plasma as substrate no distinction between the activities of $\varkappa$- and φ-factors could be made. The information obtained with artifical substrates is therefore of limited value, unless combined with techniques permitting a distinction between the different factors which accelerate the coagulation of the test substrate. Coagulation studies in this laboratory have for some time been devoted to a development of such techniques.

It has been shown that the use of *mixtures* of vitamin K-deficient and coumachlor plasma as substrates permits the differentiation and independent assay of the $\varkappa$-, δ- and φ-factor activities of normal plasma [1]. The present study reveals differences in the adsorbabilities of the factors responsible for these activities. The multiple nature and the non-identity of the activities measured with the pure substrates, could thus have been recognized by studies on the adsorption of the two activities. Adsorbability studies may therefore be of value for differentiation of coagulation activities and for physicochemical characterization of the corresponding coagulation factors.

Thus, adsorption experiments appear to offer a clue to a possible further differentiation of the three activities measured as $\varkappa$-factor, δ-factor and as φ-factor activities. This was indicated by the experiments on adsorption of φ-factor activity by calcium carbonate. The merely partial adsorbability of this activity proves that it is due to at least two different coagulation factors. These preliminary results stressed the necessity of more detailed studies on the effect of adsorbents on the $\varkappa$-, δ- and φ-factor activities of plasma. As will be shown in forthcoming papers, such studies have permitted an even further differentiation of the φ-factor activity and given evidence in favour of the homogeneity of the $\varkappa$-factor and δ-factor activities.

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