ACTA CHEMICA SCANDINAVICA 14 (1960) 2177–2190

Studies on the Coagulation of Chicken Blood

I. Factors which Limit Plasma Coagulation Rate during Vitamin K Deficiency and Treatment with Coumarin Drugs

ØYVIND SØRBYE* and INGER KRUSE

Department of Biochemistry and Nutrition, Polytechnic Institute, Copenhagen, Denmark

It is shown that the coagulation time of vitamin K-deficient and dicumarol or coumachlor plasmas is determined by the concentrations of three different types of factors ($\varkappa$-, δ- and φ-factors).

A method for assay of these factors is described. Coagulation tests with thromboplastin, RVV-cephalin or cephalin alone, are all sensitive to $\varkappa$- and δ-factors. The activity measured as φ-factor activity, in the three different coagulation systems, probably includes both system specific entities and entities, common to all three coagulation systems. The relation of these coagulation factors to well-established human coagulation factors is discussed.

In 1948 Dam and Søndergaard [1] observed that mixtures of plasmas from vitamin K-deficient and dicumarol-treated chickens had shorter thromboplastin coagulation times than either plasma alone. The existence of two coagulation factors, different from prothrombin, was assumed to account for the observation: one, which was especially low in vitamin K-deficient plasma, and another, which was especially low in dicumarol plasma. Prothrombin was assumed to be the third rate-limiting factor, lacking in both plasmas.

The factor in dicumarol plasma which accelerated the coagulation of vitamin K-deficient plasma was named δ (delta)-factor, and the factor in vitamin K-deficient plasma which accelerated the coagulation of dicumarol plasma was named $\varkappa$ (kappa)-factor by Sørbye *et al.*[2,3] The factors were concentrated from dicumarol plasma and vitamin K-deficient plasma, respectively.

A full account of the latter findings and recent progress in the study of coagulation factors, responsible for the phenomena described, is presented in this article. Since the first short communications [2,3] were published, our attention has been drawn to the new experimental possibilities offered by use of mixtures of vitamin K-deficient and anticoagulant drug plasmas as test substrates. It seems that this technique will permit more detailed studies of the

* Fellow of the Royal Norwegian Council for Scientific and Industrial Research.

coagulation entities in normal plasma, which are reduced below optimal levels during vitamin K deficiency and anticoagulant drug treatment. Great interest is attached to the possibility of correlating these coagulation entities with coagulation factors, known from studies of human blood. An established correlation between human and chicken coagulation factors will permit problems of human coagulation to be attacked through work on chicken coagulation factors, and *vice versa*.

MATERIALS AND METHODS

"*Normal plasma*". Blood was obtained from the carotid arteries or jugular veins of chickens, reared on a commercial diet [4] or on special diets (Table 1). The chickens were fasted not less than 4—5 h before blood samples were taken, to avoid turbidity in the centrifuged plasma. Nine parts of blood were drawn into one part of 0.15 M potassium oxalate or 0.10 M trisodium citrate and immediately centrifuged at 1 500 *g* for 20 min. After centrifugation the plasma could be poured off after immersion of the centrifuge tubes in ice-water for 5—10 min. It was completely clear and free from blood cells. In cases where the hematocrit value was abnormally low, the concentration of anticoagulant was adjusted to 18—21 mM oxalate or 12—14 mM citrate. Plasma was used fresh or after storage at —20°C.

Vitamin K-deficient plasma. Chickens were reared on a vitamin K-deficient diet [4] until they showed signs of subcutaneous hemorrhage. Blood was obtained as for "normal plasma". Deficiency in δ-factor was pronounced in plasmas with thromboplastin coagulation times more than 7—8 times the normal value.

Dicumarol plasma. Dicumarol was administered orally to chickens on the commercial diet as single dose (0.7—0.9 mg/g) 48—67 h before blood collection, or in two doses (0.7—0.8 and 0.3 mg/g) 50—58 and 24 h before blood collection. The plasmas obtained had thromboplastin coagulation times of 5—12 times the normal plasma value and showed pronounced κ-factor deficiency.

*Coumachlor * plasma.* Coumachlor was administered orally to chickens on the commercial diet in two doses, 44 and 20 h before blood collection. Usually 0.08 and 0.04 mg/g as first and second dose gave plasmas with thromboplastin times of 7—10 times the normal plasma value. Such plasmas had pronounced κ-factor deficiency. Coumachlor gave more uniform responses than dicumarol and was the preferred anticoagulant in the later part of this work. Coumachlor plasma has been shown to behave identically to dicumarol plasma in coagulation tests [5].

Thromboplastin. Chicken brains were carefully freed from blood and vessels and kept frozen in dry ice. For preparation they were dehydrated and ground with acetone and dried to a light powder in air at + 37°C or under vacuum. The powder was stored at —20°C. Of this powder, 0.15 g was triturated with 3 ml of veronal buffer for 30 min at + 37°C and centrifuged for 10 min at 900 *g* and + 2°C. The supernatant normally had optimal coagulation activity, when diluted with 4—5 ml veronal buffer.

Russel's viper venom (RVV). This product was obtained from the Wellcome Research Laboratories, Beckenham, England; it was dissolved in veronal buffer. Dilution 1:12 000 proved optimal in most cases.

Russel's viper venom-Ca (RVV-Ca). This reagent was prepared by dissolving RVV in 30 mM $CaCl_2$-solution (1:12 000).

$CaCl_2$. A stock solution of 100 mM $CaCl_2$ was prepared. This solution was diluted to optimal concentration (25—30 mM) before use.

Brain "cephalin". This phosphatide fraction was prepared from chicken brains, according to Milstone [6]. A 1 % suspension of "cephalin" in buffer was prepared and stored at —20°C. It was thawed and diluted to optimal concentration (0.06—0.09 %) before use.

Blood cell "cephalin". Packed chicken blood cells, separated by centrifugation of blood, were washed with 3 volumes of oxalated saline by suspension and centrifugation,

* 3-(α-*p*-Chlorphenyl-β-acetylethyl)-4-hydroxycoumarin, supplied by J. R. Geigy, Basle.

treated with 2—3 volumes of 96 % ethanol on a steam bath for one hour and filtered. To the filtrate was added 3 volumes of ether and 3 volumes of distilled water. The mixture was shaken and then allowed to stand for separation of the phases. The ether layer was evaporated to dryness with absolute alcohol under reduced pressure. The residue was washed twice with acetone and finally extracted with ether. The clear ether solution was evaporated, and the lipids were suspended in veronal buffer. The suspension was stored deep frozen. It was thawed and diluted to optimal concentration (0.008—0.06 %) before use.

Buffer. 550 ml of 0.1 M sodium barbiturate, 450 ml of 0.1 M HCl, and 5.8 g sodium chloride were mixed with 1 000 ml of 0.9 % sodium chloride. pH was 7.10—7.14. Usually a trace of potassium oxalate (0.009 %) was added.

Oxalated saline. 0.9 g sodium chloride and 0.009 g potassium oxalate in 1 000 ml distilled water.

Citrate-buffer. One part of 0.1 M trisodium citrate was mixed with seven parts of buffer. This solution had the same concentration of anticoagulant as citrate plasma.

Glassware. Thin-walled conical centrifuge glass tubes (15 ml) were used for coagulation time determinations. All glassware were rinsed in chromic-sulfuric acid, washed with tap water at least 10 times and with distilled water 3 times, finally with ethanol and dried at + 80°C.

Coagulation time determination. All reagents, except the trigger solution ($CaCl_2$ or RVV-Ca), were kept in ice-water during the experiment, mixed in desired proportions and placed in a water bath at + 37°C. Coagulation was started by blowing in prewarmed trigger solution with a syringe. Time for formation of a firm clot was noted by means of a stop watch. The glass was tilted slowly until 3—4 sec. before the expected coagulation time when a rapid, but yet cautious tilting began. With some experience and great accuracy it was possible to duplicate the coagulation times within 1/10 of a second for coagulation times up to 40—50 sec. and within half a second for coagulation times up to 2—3 min. A one-stage coagulation test was normally used:

Thromboplastin coagulation time (tpl.-time).

Coagulation system: 0.2 ml plasma or plasma mixture
0.2 ml thromboplastin solution
0—0.2 ml buffer or test solution
0.2 ml $CaCl_2$ (30 mM).

Thromboplastin time is known to depend on the concentration of prothrombin, proconvertin, Stuart factor and proaccelerin, and is said to be insensitive to variations in the levels of intrinsic factors *.

We have also made use of some modifications of this technique:

RVV-cephalin coagulation time (RVV-ceph.-time). Thromboplastin is replaced by cephalin, and $CaCl_2$ is replaced by RVV-Ca. The RVV-ceph.-time depends on the concentration of prothrombin, Stuart factor and proaccelerin, and is not influenced by the concentration of proconvertin or intrinsic factors.

Cephalin coagulation time (ceph.-time). Thromboplastin is replaced by cephalin. Ceph.-time is insensitive to the concentration of proconvertin, but depends on the concentration of intrinsic factors, Stuart factor, prothrombin and proaccelerin. On determination of ceph.-time, tilting of the glass was avoided. The glass was rotated instead. Time for formation of the first visible fibrin threads was noted.

Serum prothrombin activity.

Coagulation system: 0.2 ml serum or plasma
0.2 ml $SrCO_3$-adsorbed oxalated normal plasma
0.2 ml thromboplastin
0.2 ml $CaCl_2$ (50 mM).

A dilution curve was prepared with normal plasma. Dilutions were made with citrate-buffer. The prothrombin activities of serum, read from this curve, were not expected to

* The intrinsic factors include the antihemophilic factor-A, (AHF, factor VIII), antihemophilic factor-B (PTC, Christmas factor, factor IX), antihemophilic factor-C (PTA) and Hagemann factor.

be correct estimates of the prothrombin concentration, as the concentration of accessory factors (proconvertin, Stuart factor, *etc.*) relative to prothrombin generally is higher in serum than in plasma. However, the test is thought to give an idea of the extent of prothrombin consumption during coagulation.

Serum. Blood samples were collected without anticoagulant and allowed to clot at + 37°C. After 2 1/2—3 h the serum was separated and 1/6 volume of 0.1 M trisodium citrate was added. In this work the serum was examined shortly after citrate addition.

Adsorbed plasma. Plasma samples were stirred with the adsorbent for 15 min. at 0°C, centrifuged for 10 min at 1 500 *g* and + 2°C. The supernatant plasma was poured off and stored at 0°C until used.

Elution of adsorbed factors. The sedimented adsorbent, obtained in the procedure above, was first washed twice by stirring for 10 min. with oxalated saline at 0°C. The volume of washing fluid corresponded to the original volume of plasma. After centrifugation the supernatant was discarded. The adsorbed factors were eluted with citrate or phosphate buffers. The pH and molarity of the eluant will be indicated in the text (p. 000). Elution was done by stirring for 15 min. at 0°C, followed by centrifugation. The supernatant was dialyzed against distilled water, when the preparation subsequently was to be freeze-dried. When the preparation could be used immediately after dialysis, oxalated saline was used as dialysis fluid with buffer as the last change of outer phase.

Dialysis. Dialysis was carried out at + 1 — + 2°C for 40—44 h with 5—6 changes of outer phase.

EXPERIMENTS AND RESULTS

Coagulation times for mixtures of different sets of vitamin K-deficient and dicumarol plasmas are seen in Fig. 1 (A, B, C). It is apparent that replacement of small amounts of one plasma by the other causes some acceleration of the coagulation. This indicates the presence of relatively large amounts of a factor in vitamin K-deficient plasma, which is reduced below its optimal level in dicumarol plasma (*x*-factor), and relatively large amounts of a factor

Table 1. Composition of special diets.

F—6		F—7		F—8	
Ground barley	170	Ground barley	170	Ground barley	170
Ground oats	50	Ground oats	60	Ground oats	60
Wheat bran	55	Wheat bran	50	Wheat bran	50
Ground corn	70	Ground corn	90	Ground corn	100
Corn oil	50	Corn oil	25	Corn oil	20
Dried skimmed milk	50	Dried skimmed milk	55	Dried skimmed milk	65
Pancreas powder [1]	140	Pancreas powder [1]	130	Pancreas powder [1]	100
Casein [2]	220	Casein [2]	225	Casein [2]	240
Dried alfalfa	40	Dried alfalfa	40	Dried alfalfa	40
Salt mixture [1]	50	Salt mixture [1]	50	Salt mixture [1]	50
Vitamin mixture [3]	105	Vitamin mixture [3]	105	Vitamin mixture [3]	105
	1 000		1 000		1 000
Vitamins A and D[1]		Vitamins A and D[1]		Vitamins A and D[1]	

[1] As described by Dam and Søndergaard [4].

[2] From A/S Dansk Mejeri Industri & Export Kompagni, Stege, Møn, Denmark.

[3] This mixture contained: Thiamine hydrochloride, 6 mg; riboflavin, 8 mg; nicotinic acid, 100 mg; calcium pantothenate, 24 mg; pyridoxine, 7 mg; biotin, 0.2 mg; folic acid, 4 mg; choline chloride, 2 000 mg; inositol, 1 000 mg; *p*-aminobenzoic acid, 300 mg; DL-α-tocopheryl acetate (Ephynal, Roche), 100 mg; bicalcium 2-methyl-1,4-naphthoquinone biphosphate (Synkavit, Roche), 10 mg; mixed with sugar to make 105 g.

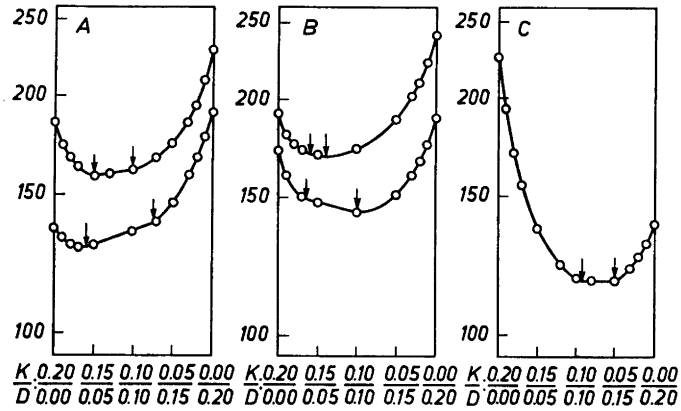

Fig. 1. (*A, B, C*). Coagulation times for plasma mixtures with thromboplastin and 0.2 ml buffer: K, vitamin K-deficient plasma; D, dicumarol plasma; Abscissæ, plasma mixtures in ml; Ordinates, coagulation time in min./100.

in dicumarol plasma, which is below its optimal concentration in vitamin K-deficient plasma (δ-factor). It should be especially noticed, that with increasing concentration of the other plasma in the mixtures a point is reached where the concentration of the factor, supplied by the other plasma, appears to become optimal. These points are indicated by arrows. In the range between the two points the curve appears to be straight-lined when drawn on semi-logarithmic paper.

If the interpretation given above is correct, then plasma mixtures within this range will contain $\varkappa$-factor and δ-factor in optimal amounts. The coagulation time will then depend on the concentration of factors — other than $\varkappa$-factor and δ-factor — which are depressed below optimal levels both in vitamin K-deficient and in dicumarol plasma. In previous reports [1–3] this "common deficiency" has been assumed to be a deficiency in prothrombin. We now prefer to call it a deficiency in φ (phi)-factor(s) until a proper identification with a known coagulation entity becomes possible.

Mixtures of different sets of dicumarol plasmas show no similar depression of the coagulation times (Fig. 2 A), indicating deficiencies in the same rate-limiting factors. The same is true for mixtures of two different vitamin K-deficient plasmas (Fig. 2 B).

These experiments confirm the original observations by Dam and Søndergaard [1].

On treatment of oxalated plasma with $BaCO_3$, all factors, able to shorten the coagulation time of vitamin K-deficient or dicumarol plasma, were removed from normal, vitamin K-deficient and dicumarol plasmas (Fig. 2 C).

It was thus evident that the $\varkappa$, δ- and φ-factors can be adsorbed from oxalated plasma by typical prothrombin adsorbers, such as $BaCO_3$ and $SrCO_3$.

Attempts were made to prepare concentrates of $\varkappa$- and δ-factor from oxalated vitamin K-deficient and dicumarol plasma, respectively, by treatment with

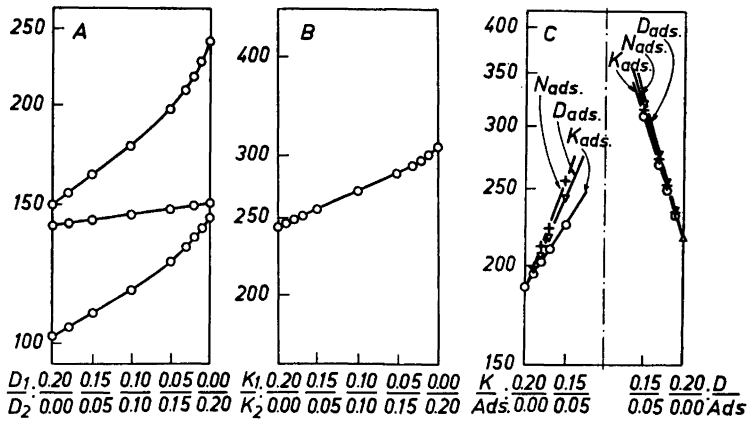

Fig. 2. (*A*, *B*, *C*). Coagulation time for plasma mixtures with thromboplastin and 0.2 ml buffer. *2 A:* D_1 and D_2, dicumarol plasmas; *2 B:* K_1 and K_2, vitamin K-deficient plasmas; *2 C:* K, vitamin K-deficient plasma; D, dicumarol plasma; N, normal plasma; Ads., $BaCO_3$-adsorbed plasma; Abscissæ, plasma mixtures in ml; Ordinates, coagulation time in min./100.

1 % $BaCO_3$, elution twice with 1/3 volume of 0.1 M sodium citrate followed by dialysis and lyophilization. The preparations were redissolved in buffer. Details of the various preparations used in this work are given in Table 2.

The effect of addition of the concentrates of $\varkappa$-factor and δ-factor on the coagulation time of mixtures of vitamin K-deficient and dicumarol plasmas is seen in Figs. 3 and 4.

Figs. 3 A and 4 A show the effect of addition of increasing amounts of δ-concentrates. Coagulation of the plasma mixtures was accelerated only in mixtures with high percentages of vitamin K-deficient plasma. There was no effect on mixtures with high percentages of dicumarol plasma. This proved that the latter mixtures already contained the added factor in optimal concentration. The range for optimal $\varkappa$- and δ-factor concentration (to be referred to

Table 2. Details of $\varkappa$- and δ-factor preparations.

Preparation	Original material			Final volume
	Volume	Plasma	tpl.-time min./100	
$\varkappa$-216	9 ml	vit. K-deficient	275	3 ml
$\varkappa$-420	13 »	vit. K-deficient	300	2 »
$\varkappa$-391	11 »	vit. K-deficient	310	2 »
$\varkappa$-611	10 »	vit. K-deficient	255	2 »
$\varkappa$-615	12 »	vit. K-deficient	435	3 »
δ-436	8 »	dicumarol	360	3 »
δ-427	20 »	dicumarol	470	6 »
δ-228	18 »	dicumarol	375	6 »

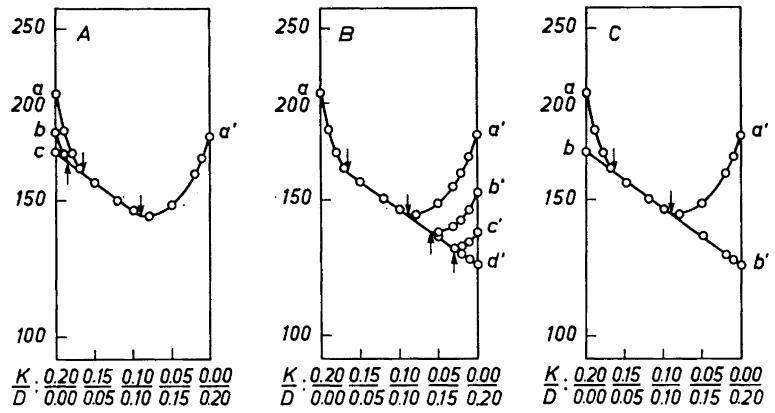

Fig. 3. (*A, B, C*). Coagulation time for plasma mixtures with thromboplastin. Effect of test solutions. Curves a—a', 0.20 ml buffer; *3 A:* Curve b—a', 0.05 ml δ-436 and 0.15 ml buffer; Curve c—a', 0.10 ml δ-436 and 0.10 ml buffer; *3 B:* Curve a—b', 0.10 ml ϰ-216 and 0.10 ml buffer; Curve a—c', 0.20 ml ϰ-216; Curve a—d', 0.10 ml ϰ-420 and 0.10 ml buffer; *3 C:* Curve b—b', 0.10 ml ϰ-420 and 0.10 ml δ-436; K, vitamin K-deficient plasma; D, dicumarol plasma; Abscissæ, plasma mixtures in ml; Ordinates, coagulation time in min./100.

as the optimal range) was extended to the left only, proportionally to the amount of δ-factor added. Full correction of the δ-factor deficiency for all mixtures occurred with 0.1 ml of preparation δ-436 (Fig. 3 A) and with 0.1

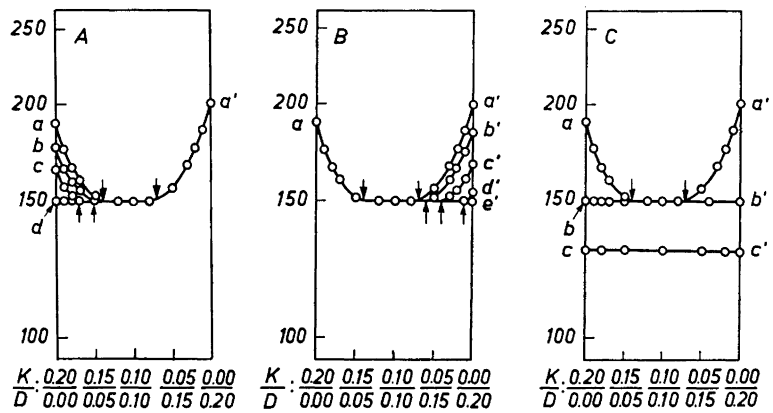

Fig. 4 (*A, B, C*). Coagulation time for plasma mixtures with thromboplastin. Effect of test solutions. Curves a—a': 0.20 ml buffer; *4 A*: Curve b—a', 0.02 ml δ-427 and 0.18 ml buffer; Curve c—a', 0.05 ml δ-427 and 0.15 ml buffer; Curve d—a', 0.10 ml δ-427 and 0.10 ml buffer. *4 B:* Curve a—b', 0.03 ml ϰ-615 and 0.17 ml buffer; Curve a—c', 0.10 ml ϰ-615 and 0.10 ml buffer; Curve a—d', 0.20 ml ϰ-615; Curve a—e', 0.10 ml ϰ-391 and 0.10 ml buffer; *4 C:* Curve b—b', 0.10 ml ϰ-391 and 0.10 ml δ-427; Curve c—c', 0.10 ml ϰ-611 and 0.10 ml δ-228; K, vitamin K-deficient plasma; D, dicumarol plasma; Abscissæ, plasma mixtures in ml; Ordinates, coagulation time in min./100.

ml of preparation δ-427 (Fig. 4 A). No $\varkappa$- or φ-factor activity could thus be demonstrated in these preparations.

Figs. 3 B and 4 B show the effect of addition of increasing amounts of $\varkappa$-concentrates. Coagulation time of the plasma mixtures was shortened only in mixtures with high percentages of dicumarol plasma. The lack of effect on mixtures with high percentages of vitamin K-deficient plasma proved that these mixtures already contained the added factor in optimal amounts. The optimal range was extended to the right only, proportionally to the amount of $\varkappa$-factor added. Full correction of the $\varkappa$-factor deficiency for all mixtures occurred with 0.1 ml of preparation $\varkappa$-420 (Fig. 3 B) and with 0.1 m l of prparation $\varkappa$-391 (Fig. 4 B). No measurable δ- or φ-factor activity was thus present in these preparations.

In Figs. 3 C and 4 C it is seen that simultaneous addition of sufficient amounts of $\varkappa$- and δ-factors completely wiped out every indication of deficiencies in these factors. The coagulation times were shortened only in mixtures with high percentages of either vitamin K-deficient or dicumarol plasma.

The coagulation times for mixtures within the optimal range were not affected. The curves were completely straightened out to give the expected picture of the coagulation time for mixtures of two plasmas with a slight difference in (Fig. 3 C) or identical amounts (Fig. 4 C) of the same rate-limiting factors.

In the lower curve of Fig. 4 C the $\varkappa$- and δ-factor deficiencies had also been corrected, but the entire curve was shifted to slightly shorter coagulation times, indicating the presence of small amounts of φ-factor in the preparations $\varkappa$-611, or δ-228, or both.

The present experiments very strongly support the original interpretation [1] of the "anomalous" coagulation time observed for mixtures of vitamin K-deficient and dicumarol plasmas. They also justify the more detailed interpretation given here (pp. 2080—2081). Furthermore it is evident that the effect of test solutions on the coagulation time of such mixtures can be used to distinguish sharply between $\varkappa$-, δ- and φ- factor type of activities.

Method for the assay of $\varkappa$-, δ- and φ-factors

The results above suggested the possibility that mixtures of vitamin K-deficient and 'dicumarol (or coumachlor) plasmas might be used as test substrates for individual determinations of $\varkappa$-, δ- and φ-factor levels in plasma. It could be expected that addition of a constant, small amount of plasma to all mixtures would shorten their coagulation times, thereby: 1) extending their optimal range to the left, proportionally to the amount of δ-factor, 2) extending their optimal range to the right, proportionally to the amount of $\varkappa$-factor, and 3) shifting the entire curve to shorter coagulation times, depending on the amount of φ-factor(s), in the plasma added.

These expectations were fully met, as seen in Figs. 5 A and 5 B. Strict proportionality was observed between the amount of added plasma and the extension of the optimal range to either side. Fig. 5 A shows the effect of addition of varying amounts of a plasma (N_1), which appeared to have a relatively high δ- and low $\varkappa$-factor level. The plasma was therefore used to

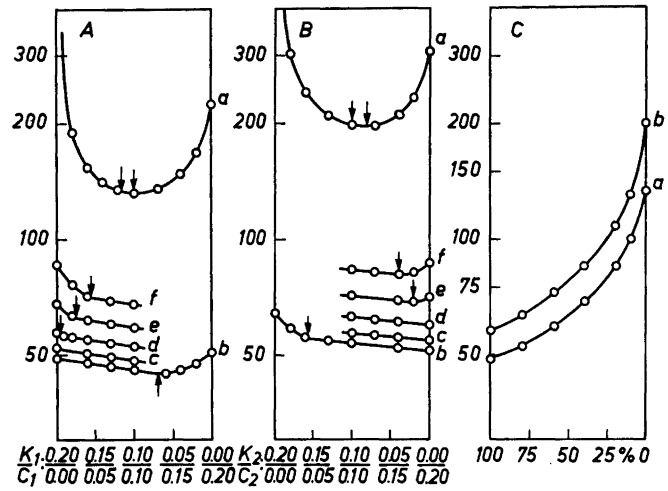

Fig. 5 (*A*, *B*). Coagulation time for plasma mixtures with thromboplastin. Effect of normal plasma.

5 A (*5 B*) Curves a, K_1/C_1 (K_2/C_2) and 0.010 ml buffer.
» b, » » » 0.012 » plasma N_1 (N_2).
» c, » » » 0.010 » »
» d, » » » 0.008 » and 0.002 ml buffer.
» e, » » » 0.006 » » » 0.004 » »
» f, » » » 0.004 » » » 0.006 » »

K_1 and K_2; vitamin K-deficient plasmas; C_1 and C_2, coumachlor plasmas; N_1 and N_2 normal plasmas; Abscissæ, plasma mixtures in ml; Ordinates, coagulation time in min./100.

Fig. 5 C. Dilution curves for φ-factor activity. Diluted normal (N) plasma (0.01 ml) added to a mixture of 0.10 ml vitamin K-deficient (K) and 0.10 ml coumachlor (C) plasma. Curve a, with N_1, K_1 and C_1; Curve b, with N_2, K_2 and C_2; Abscissa, concentration of normal plasma; Ordinate, coagulation time in min./100.

study the relation between amount of added plasma and extension of the optimal range to the left (δ-effect). The plasma, used in Fig. 5 B (N_2), appeared to have a high $\varkappa$- and a relatively low δ-factor level. It was therefore used to bring out the relation between amount of added plasma and extension of the optimal range to the right ($\varkappa$-effect).

The technique makes it possible to compare the $\varkappa$- and δ-factor levels of different plasmas by comparing the extension of the optimal range to either side, which occurs on addition of a constant, small amount of plasma to mixtures of vitamin K-deficient and anticoagulant drug plasmas.

Comparison of the φ-factor levels of different plasmas is also possible from the results of this type of experiments. Estimation of φ-factor(s) alone is, however, most easily done with a substrate of vitamin K-deficient and anticoagulant drug plasmas in proportion 1:1. Such mixtures normally contain optimal amounts of both $\varkappa$- and δ-factors, and the observed coagulation time depends only on the concentration of φ-factor(s). Fig. 5 C shows the rela-

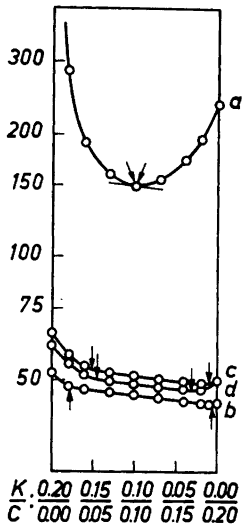

Fig. 6. Coagulation time for plasma mixtures with thromboplastin. Effect of different plasmas. Curve a, 0.010 ml buffer; Curve b, 0.010 ml plasma F-6; Curve c, 0.009 ml plasma F-7 and 0.001 ml buffer; Curve d, 0.010 ml plasma F-8; K, vitamin K-deficient plasma; C, coumachlor plasma; Abscissa, plasma mixtures in ml; Ordinate, coagulation time in min./100.

tion between concentration of added normal plasma (φ-factor(s)) and observed coagulation time with this special mixture of substrate plasmas. The normal plasma and substrate plasmas were the same as those used in Figs. 5 A and 5 B.

Fig. 6 shows the results of an assay of $\varkappa$-, δ- and φ-factor levels in plasmas from 3 different chickens. The chickens were given slightly different diets (Table 1). This experiment may serve as an example of evaluation of the $\varkappa$-, δ- and φ-factor activities of different plasmas. Optimal ranges were read

Table 3. $\varkappa$-, δ- and φ-factor levels in plasma.

Added to substrates		Optimal range $\left(\frac{K}{C}\right)$	Extension of the optimal range		Activity of factors		
Volume (v) ml	Material		to the left(d)	to the right(k)	$\delta\left(\frac{d}{v}\right)$	$\varkappa\left(\frac{k}{v}\right)$	φ*, %
0.010	buffer	$\frac{0.100}{0.100} - \frac{0.100}{0.100}$					
0.010	plasma F-6	$\frac{0.175}{0.025} - \frac{0.005}{0.195}$	0.075	0.095	7 1/2	9 1/2	100
0.009	plasma F-7	$\frac{0.150}{0.050} - \frac{0.010}{0.190}$	0.050	0.090	5 1/2	10	80
0.010	plasma F-8	$\frac{0.145}{0.055} - \frac{0.030}{0.170}$	0.045	0.070	4 1/2	7	84

* Read from a φ-factor dilution curve made with F-6 plasma.

from Fig. 6, and φ-factor activities of the other two plasmas were read from a dilution curve (similar to the curves of Fig. 5 C) made with F-6 plasma. The results are given in Table 3.

The variations in the activities of $\varkappa$-, δ- and φ-factors, observed in this experiment, suggest that the concentration of the factors may vary to some extent with the diet. Thus it seems possible that the formation of $\varkappa$-, δ- and φ-factors depends on dietary factors other than vitamin K. This is, however, an entirely new type of problem, which will receive further treatment in later publications.

Substitution of other Procoagulants for Thromboplastin

In all experiments reported so far, coagulation of the plasma mixtures was accelerated by addition of optimal amounts of chicken brain thromboplastin. It was therefore of interest to see if the deficiency symptoms, observed with thromboplastin as procoagulant, also could be observed in other coagulation systems, where thromboplastin was replaced by RVV-cephalin or by cephalin alone.

The results of two different experiments are seen in Figs. 7 (A, B, C). With all procoagulants the same type of curves was obtained. In all coagulation

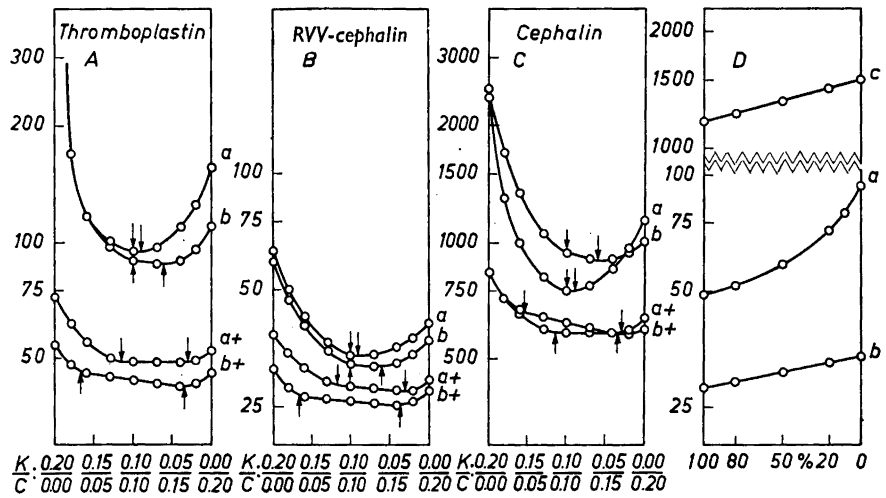

Fig. 7 (A, B, C). Coagulation time for plasma mixtures with thromboplastin (7 A), RVV-cephalin (7 B) and cephalin (7 C). Effect of normal plasma. Curves a, with K_1, C_1 and 0.01 ml buffer; Curves a+, with K_1, C_1 and 0.01 ml plasma N_1; Curves b, with K_2, C_2 and 0.01 ml buffer; Curves b+, with K_2, C_2 and 0.01 ml plasma N_2; K_1 and K_2, vitamin K deficient plasmas; C_1 and C_2, coumachlor plasmas; N_1 and N_2, normal plasmas; Abscissæ, plasma mixtures in ml; Ordinates, coagulation time in min./100.

Fig. 7 D. Dilution curves for φ-factor activity. Diluted normal (N) plasma (0.01 ml) added to a mixture of 0.10 ml vitamin K-deficient (K) and 0.10 ml coumachlor (C) plasma. Curve a, with thromboplastin; Curve b, with RVV-cephalin; Curve c, with cephalin; Abscissa, concentration of normal plasma; Ordinate, coagulation time in min./100.

systems both vitamin K-deficient plasma and coumachlor plasma seemed to contain relatively large amounts of a factor which was reduced below optimal levels in the other plasma. The range, where these factors appeared to be optimal, was, for each set of plasma mixtures, the same in all three coagulation systems (curves a and b). Furthermore, when an assay of the concentration of these factors in normal plasma was performed, according to the method suggested, identical results were obtained with the three different coagulation accelerators (curves a+ and b+).

Thus, it is apparent that $\varkappa$- and δ-factors are important in all three coagulation systems.

It is, however, not immediately clear, whether the activity, measured as φ-factor(s) in the three different coagulation systems, is due to the same factor(s), or if different entities are measured.

The dilution curves for φ-factor(s) appeared to be straight lines with RVV-cephalin and with cephalin, when plotted on semi-logarithmic paper, whereas the curve with thromboplastin was definitely curved (Fig. 7 D).

These findings suggest that the activity, referred to as φ-factor activity, may include one or more factors which are specific for the thromboplastin system. The possibility that the cephalin system may be sensitive to a φ-factor, which is without effect in the two other systems, must also be taken into consideration. The exact number of different entities, measured as φ-factors in the different coagulation systems, is thus still an unsolved problem.

Behavior of $\varkappa$- and δ-factor during coagulation

Comparison of plasma and its corresponding serum, obtained by spontaneous coagulation of blood at + 37°C, showed regularly that δ-factor disappeared during coagulation. The activity of $\varkappa$-factor was, on the contrary, increased. A typical experiment is summarized in Table 4.

The disappearance of δ-factor and activation of $\varkappa$-factor during spontaneous coagulation of blood at + 37°C was measured in all three coagulation systems. Identical results were obtained.

DISCUSSION

The experiments reported demonstrate that the "anomalous" coagulation times, observed for mixtures of vitamin K-deficient and anticoagulant drug plasmas, has been correctly interpreted. Particularly illustrating is the effect

Table 4. Behavior of $\varkappa$- and δ-factors during coagulation.

Activity of	Prothrombin %	δ-factor %	$\varkappa$-factor %
Plasma	100	100	100
Serum	25	0	172

of addition of concentrates, prepared from vitamin K-deficient and anticoagulant drug plasmas, on the coagulation time of such mixtures. The experiments prove that the accelerated coagulation, observed for mixtures of vitamin K-deficient and anticoagulant drug plasmas, is due to great differences in the concentrations of two factors ($\varkappa$- and δ-factors) in these plasmas. However, the prolonged coagulation time of vitamin K-deficient and anticoagulant drug plasmas cannot be completely accounted for by the reduced concentrations of these two factors alone. Three different types of coagulation factors ($\varkappa$-, δ- and φ-factors) are responsible for the prolonged coagulation time of plasma during severe vitamin K-deficiency and after short-time treatment with coumarin drugs.

It is evident that the proposed assay method, based on mixtures of vitamin K-deficient and anticoagulant drug plasmas as substrates, can only be sensitive to the concentration of factors which have been reduced *below optimal levels* in the substrate mixtures. The assay method can therefore not be expected to be sensitive to *all* coagulation factors, affected by vitamin K deficiency and coumarin drug treatment. The reduction of some of the affected factors may not be so extensive that their concentrations drop below optimal levels.

The method should, however, be of value for studies of the factors in normal plasma which are most highly affected by vitamin K deficiency and coumarin drug treatment.

The adsorbability, vitamin K dependence and anticoagulant drug sensitivity of $\varkappa$-, δ- and φ-factors exclude their identity with Hageman factor, PTA, AHF or proaccelerin.

It even seems impossible to identify $\varkappa$- or δ-factor with any of the adsorbable coagulation factors known from studies of human blood. As $\varkappa$-factor and δ-factor are important for the coagulation mechanisms, initiated by addition of thromboplastin, RVV-cephalin and cephalin, neither can be identified with proconvertin or PTC. (RVV-ceph.-time and ceph.-time are not proconvertin sensitive, and tpl.-time and RVV-ceph.-time are insensitive to PTC.) They cannot be identical to prothrombin, as no δ- and increased $\varkappa$-factor activity is found in fresh serum where the prothrombin activity is 25 % of that of plasma.

The possibility discussed by Dam and Søndergaard [7] that differences in ratio of prothrombin to proconvertin might account for the coagulation times of vitamin K-deficient plasma, anticoagulant drug plasma and mixtures of the two, has thus not been supported by the present experiments.

An identification of δ-factor with Stuart factor seems impossible, since this coagulation factor usually is found in serum with unchanged [8] or increased [9] activity. The rate and extent of decrease reported for Stuart factor during anticoagulant drug treatment [9,10], does not conform with the great sensitivity of $\varkappa$-factor to anticoagulant drugs.

Theoretically, it remains a possibility that proconvertin might be registered as a thromboplastin-specific φ-factor, that PTC might behave as a cephalin-specific φ-factor, and that Stuart factor and prothrombin could be measured as φ-factors, common to all three coagulation systems. All these factors are reduced during vitamin K deficiency and coumarin drug treatment, but we do not know if the reduction is so extensive and so rapid that they become

rate-limiting coagulation factors under the conditions described. Further experimentation is necessary to establish the nature and exact number of the different entities in normal plasma, that are measured as φ-factors in the three coagulation systems.

A better knowledge of, and control over, the $\varkappa$-, δ- and φ-factors should be of value for anticoagulant drug therapy, as they seem to control the coagulation rate of plasma during vitamin K deficiency and after treatment with coumarin drugs.

REFERENCES

1. Dam, H. and Søndergaard, E. *Biochim. et Biophys. Acta* **2** (1948) 409.
2. Sørbye, Ø., Kruse, I and Dam, H *Acta Chem Scand* **4** (1950) 549.
3 Sørbye, Ø., Kruse, I. and Dam, H. *Acta Chem. Scand.* **4** (1950) 831.
4. Dam, H. and Søndergaard, E. *Acta Pharmacol. Toxicol.* **9** (1953) 131.
5. Dam, H. and Søndergaard, E. *Acta Pharmacol. Toxicol.* **9** (1953) 137.
6. Milstone, J. H. *Yale J. Biol. and Med.* **22** (1950) 675.
7. Dam, H. and Søndergaard, E. *Acta Physiol. Scand.* **31** (1954) 376.
8. Johnston, Jr., C. L., Ferguson, J. H., O'Hanlon, F. A. and Black, W. L. *Thromb. et Diath. Haemorrhag.* **3** (1959) 367.
9. Bierstedt, P. *Z. ges. inn. Med. u. ihre Grenzgebiete.* **14** (1959) 16.
10. Koller, F. *Thromb. et Diath. Haemorrhag.* **2** (1958) 407.

Received July 22, 1960.