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Studies on the Coagulation of Chicken Blood

IX. Labile Factor Activity of Fresh Oxalated Plasma: the Combined Activity of Sixteen Discrete Labile Coagulation Factors

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The labile factor activity of fresh oxalated chicken plasma is fully accounted for by the combined activity of sixteen discrete labile coagulation factors which are active with chicken brain thromboplastin. With Russell's viper venom (RVV)-cephalin only eight of these factors are active, and the combined activity of the eight factors accounts for all labile factor activity measured by this agent. The labile factor activity measured with RVV, unsupplemented by cephalin, can be accounted for by the combined activity of seven of the eight factors measured by RVV-cephalin. The eight factors specific for the thromboplastin assay system, may be regarded as co-factors for generation of hypothetical intermediate activation products of thromboplastin. The remaining eight factors are probably involved in the transformation of thromboplastin activation products and of RVV-cephalin into prothrombin converting principles. It is suggested that the various labile factors of chicken plasma may be involved in alternative mechanisms for formation of such principles.

The idea that the labile factor activity of fresh oxalated chicken plasma is of complex nature and due to the combined activity of discrete labile coagulation factors, originated in the observations that only part of the total activity was adsorbed by treatment of the plasma with crystalline adsorbents [1–5]. The selective adsorption thus obtained, was interpreted as adsorption of one (or a few) labile coagulation factor(s) which, in this respect, differed from the factors responsible for the non-adsorbable part of the activity. As a result of continued testing of insoluble crystalline compounds, more than one hundred adsorbents with selective properties are known at present [2–5]. The effects obtained by use of different adsorbents were compared by matching experiments. Thus, all selective adsorbents were in turn classified according to the identity or non-identity of the activities adsorbed. These experiments gradually led to the recognition of sixteen different categories of selective

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adsorbents and accordingly to the existence of sixteen discernible labile coagulation factors in fresh, oxalated chicken plasma [2-5].

The present experiments were carried out to examine to what extent the labile factor activity of fresh oxalated chicken plasma could be accounted for by the combined activity of these sixteen factors.

MATERIALS AND METHODS

The materials and methods used in this study were as described previously [2-5]. Labile factor activity [2] was determined using stored, oxalated normal chicken plasma as substrate and the assay accelerators chicken brain thromboplastin, Russell's viper venom (RVV)-cephalin, and RVV alone. The coagulation time of the substrates was usually 3 – 6 times the value for fresh normal plasmas. Substrates with longer coagulation times were generally adjusted to coagulation times of 4 – 5 times the value for normal plasma by addition of traces of fresh plasma.

In this work the quantities of the adsorbents used for preparation of the adsorbed plasma samples were always adequate for *complete* adsorption of the adsorbable factor(s).

EXPERIMENTS AND RESULTS

Strontium carbonate adsorbable labile factor activity. It has been shown already [2] that barium oxalate and calcium carbonate adsorb two different parts (σ-factor and ψ-factor) of the labile factor activity adsorbed by strontium carbonate, and that the combined activity of these two factors is equal to the activity adsorbed by strontium carbonate. Some additional experiments are shown in Table 1, confirming that the activity adsorbed by strontium carbonate is fully accounted for by the combined activity of the σ- and ψ-factors, regardless of large variations in the relative activities of the two factors in different experiments.

Strontium carbonate non-adsorbable labile factor activity. By means of the fourteen different categories of selective adsorbents described in three preceding papers [3-5], fourteen different components of the labile factor activity of fresh, oxalated, strontium carbonate preadsorbed plasma could be distin-

Table 1. Combined activity of the ψ- and σ-factors.

Adsorbent in mg/ml	Activity of adsorbed factor(s) in % of total activity of plasmas Nos.:										Labile factor(s) adsorbed
	5053	3441		4397	4413	4418	4681	5052*	5230	5348	
Ca-carbonate 5	10	15	12.5	3.5	14	16	5	4	11	4	ψ
Ba-oxalate 5	12	12	11	12.5	15	5	4	4	11	4	σ
Sr-carbonate 25	22	27	23.5	16	29	21	9	8	22	8	$\psi + \sigma$
Assay accelerator **:	V	Vc	Chicken brain thromboplastin								

* Coumachlor plasma, thromboplastin coagulation time > 20 min.

** V: Russell's viper venom; Vc: Russell's viper venom-cephalin.

Table 2. Effect of adsorption of single factors on the specific activity of the others. 0 indicates no change, *i.e.* the factors act independently (additively); + indicates reduced specific activity, *i.e.* the factors have synergistic effects.

Adsorption of factor	Effect on the specific activity of factors													
	ϱ	ν	μ	π	γ	β	ε	λ	α	η	ζ	ϑ	ξ	ω
ϱ		+	0	0	0	0	0	0	0	0	0	0	0	0
ν	+		+	0	0	0	0	0	0	0	0	0	0	0
μ	0	+		+	0	0	0	0	0	0	0	0	0	0
π	0	0	+		+	0	0	0	0	0	0	0	0	0
γ	0	0	0	+		+	0	0	0	0	0	0	0	0
β	0	0	0	0	+		+	0	0	0	0	0	0	0
ε	0	0	0	0	0	+		+	0	0	0	0	0	0
λ	0	0	0	0	0	0	+		+	0	0	0	0	0
α	0	0	0	0	0	0	0	+		0	0	0	0	0
η	0	0	0	0	0	0	0	0	0		+	0	0	0
ζ	0	0	0	0	0	0	0	0	0	+		0	0	0
ϑ	0	0	0	0	0	0	0	0	0	0	0		0	0
ξ	0	0	0	0	0	0	0	0	0	0	0	0		0
ω	0	0	0	0	0	0	0	0	0	0	0	0	0	

guished. Of these factors, only six were components of the labile factor activity measured by RVV-cephalin. The activity of one of the latter six factors was shown to depend on the presence of added cephalin in the test mixture.

For the development of a satisfactory procedure for determination of the combined activity of the fourteen labile factors active with thromboplastin, the following facts had to be considered: With the exception of three factors the specific activity of each of the fourteen factors depends on the concentrations of the others in a complex and characteristic manner [3–5]. In Table 2 is presented a summary of previous experiments showing how the specific activity of each of the fourteen factors is related to the concentration of the other thirteen. Nine of the factors are interlinked synergistically (ϱ—ν—μ—π—γ—β—ε—λ—α), whereas the specific activity of each of these factors is independent of the concentrations of the ϑ-, ξ-, ω-, η-, and ζ-factors, and *vice versa*. Of the latter five factors, only the η- and ζ-factors are interdependent. The ϑ-, ξ-, and ω-factors are thus the only labile factors, the specific activity of each of which is independent of the concentrations of all the others. The combined activity of these three factors could consequently be determined as the sum of activities adsorbed by the three corresponding selective adsorbents in parallel experiments. The η- and ξ-factors are synergists, but the specific activity of each of these two factors is independent of the concentrations of the other twelve. The combined activity of the η- and ζ-factors would thus be equal to the activity of η-factor, determined in the presence of ζ-factor, plus the activity of ζ-factor, determined in the absence of η-factor, or *vice versa*. A two-step adsorption procedure with the respective selective adsorbents would then be required for determination of the combined activity of the η- and ζ-factors.

Table 3. Adsorption of labile factors by mixtures of selective adsorbents (thromboplastin assay).

Chick No.	Pretreatment(s) of plasma	Adsorbent(s) in mg/ml	Activity of adsorbed factor(s) in % of $SrCO_3$ plasma	Labile factor(s) adsorbed
2885	$SrCO_3$, 25 mg/ml (A)	Bi-phosphate 15 (B)	11	ϱ
		Bi-oxalate 15 (C)	11	μ
		Mixture of (B) and (C)	22	$\varrho + \mu$
2887	(A)	Pb-carbonate 35 (D)	9	π
		Pb-phosphate 70 (E)	7	β
		Mixture of (D) and (E)	16	$\pi + \beta$
		Ni-oxalate 15 (F)	6	ε
		Mg-borate 30 (G)	7	α
		Mixture of (F) and (G)	13	$\varepsilon + \alpha$
2896	(A)	Cd-phosphate 70 (H)	11	ν
	(A) followed by (H)	Bi-phosphate 15 (B)	3	ϱ
		Bi-oxalate 15 (C)	3	μ
		Mixture of (B) and (C)	6	$\varrho + \mu$
	(A)	Mixture of (B), (H) and (C)	17	$\varrho + \nu + \mu$
2896	(A)	Ba-phosphate 80 (I)	6.5	γ
	(A) followed by (I)	Pb-carbonate 35 (D)	5.5	π
		Pb-phosphate 70 (E)	5.5	β
		Mixture of (D) and (E)	11	$\pi + \beta$
	(A)	Mixture of (D), (I) and (E)	17.5	$\pi + \gamma + \beta$
2885	(A)	Co-carbonate 120 (K)	18	λ
	(A) followed by (K)	Ni-oxalate 15 (F)	3	ε
		Mg-borate 30 (G)	3	α
		Mixture of (F) and (G)	6	$\varepsilon + \alpha$
	(A)	Mixture of (F), (K) and (G)	24	$\varepsilon + \lambda + \alpha$
3858	(A)	Al-oxide $IIIb_6$ 110 (L)	20	η
	(A) followed by (L)	Al-oxide II 90 (M)	8	ζ
	(A)	Mixture of (L) and (M)	28	$\eta + \zeta$

The synergistic interrelationships between the nine remaining factors (ϱ—ν—μ—π—γ—β—ε—λ—α) indicate that the sum of activities adsorbed from strontium carbonate plasma by the respective selective adsorbents, would give erroneously high results for the combined activity of these nine factors. A correct estimate of this activity would require a nine-step adsorption procedure, starting with selective adsorption of ϱ-factor, followed by successive adsorption of the ν-, μ-, π-, γ-, β-, ε-, λ-, and α-factors, or by successive adsorption of these factors in the reverse order. The number of adsorption steps could be reduced to five, starting with selective adsorption of γ-factor, followed by successive adsorption of the π-, μ-, ν-, and ϱ-factors and of the β-, ε-, λ-, and α-factors in two parallel series of adsorption experiments. However, the limited stability of the labile factor activity of adsorbed plasmas [5] did not leave sufficient time for such multi-step adsorption procedures.

Another approach to this problem was suggested by the observation that adsorption of a labile factor by a selective adsorbent was unaffected by a

Table 4. Combined activity of the strontium carbonate non-adsorbable, labile factors; thromboplastin assay.

Adsorbent(s) in mg/ml *		Activity of adsorbed factor(s) in % of strontium carbonate adsorbed plasmas, Nos.:										Labile factor(s) adsorbed
		5898	5895	6190	6201	6324	6321	6329	6208[a]	6316[b]	3856	4155	
Mixture of (B), (H) and (C)		5	6	12.5	11	7	7	5	10	5	—	—	ϱ, ν, μ
Mixture of (D), (I) and (E)		28	18	14	13.5	20	14	17	15	19	—	—	π, γ, β
Mixture of (F), (K) and (G)		6	13	11.5	11.5	7	7	7	12	8	—	—	ε, λ, α
Mixture of (L) and (M)		38	37	35.5	36	45	43	41	34	51	40	31	η, ζ
Zn-tungstate	10	5	10	11.5	11	6	7	10	10	5	6.5	8	ϑ
Fe-oxide	20	12	12	11.5	11	6	14	11	12	6	6.5	8	ξ
Bi-sulfide	50	12	12	12.5	15	15	14	16	15	13	6.5	8	ω
Mg-borate	30	—	—	—	—	—	—	—	—	—	6.5	8	α
	Sum:	106	108	109	109	106	106	107	108	107	66	63	
	Max. expected [c]	104	106	108	108	105	105	104	107	104			
Al-oxide I	350	—	—	—	—	—	—	—	—	—	65.5	63	α,ϑ,ξ,ω,η,ζ

* Symbols (B)–(M) as in Table 3.
[a] Coumachlor plasma, thromboplastin coagulation time 175 min/100 (normal plasma 24–26).
[b] Vitamin K-deficient plasma, thromboplastin coagulation time > 10 min.
[c] 100 + 1/3 of the activities adsorbed by the [(B), (H), (C)] and [(F), (K), (G)] mixtures.

simultaneous adsorption of one or two other labile factors by selective adsorbents of other categories. Table 3 shows that the activity adsorbed by certain mixtures of selective adsorbents was equivalent to the sum of activities adsorbed in parallel or successive adsorptions using the individual components of these mixtures. The combined activity of the η- and ζ-factors could thus be determined by a one-step adsorption using a mixture of the respective selective adsorbents (see Table 3). Similarly, the activities of the ($\varrho-\nu-\mu$), ($\pi-\gamma-\beta$), and ($\varepsilon-\lambda-\alpha$) groups of factors could be determined separately by parallel one-step adsorptions using three adsorbent mixtures, each composed of selective adsorbents from three different categories of such compounds. The sum of the activities adsorbed by the latter three adsorbent mixtures will, however, not represent the correct value for the combined activity of the nine factors, since the synergistic effects of the μ- and π-factors and of ε- and β-factors are counted twice. Correct values might be obtained by successive adsorption of the three groups of factors, or by simultaneous adsorption of all nine factors by use of a single mixture containing all the corresponding nine selective adsorbents. However, neither of these two alternative procedures has been tried out experimentally. For routine work parallel adsorptions were preferred to more time-consuming procedures involving two or three successive adsorptions; and the chances of undesirable interactions between the adsorbents is presumably reduced by use of three mixtures of three adsorbents each, instead of the complex and rather voluminous mixture of nine adsorbents. The activities of the ($\varrho-\nu-\mu$) ($\pi-\gamma-\beta$), and ($\varepsilon-\lambda-\alpha$) groups of factors were consequently determined separately in parallel experiments. The combined activity

Table 5. Combined activity of the strontium carbonate non-adsorbable labile factors; Russell's viper venom (RVV)-cephalin assay.

Adsorbent(s) in mg/ml*		Activity of adsorbed factor(s) in % of strontium carbonate adsorbed plasmas, Nos.:										Active labile factor(s) adsorbed
		5906	5925	6183	6187	6201	6322	6330	6204[a]	4156	4155	
Pb-phosphate	70	9	9	8	20	15	12	10	11	7	7	β
Co-oxalate	100	20	18	11.5	10	15	12	10	12.5	37	31	λ
Mixture of (L) and (M)		29	38	50	52	42	44	54	39	32	32	η, ζ
Fe-oxide	20	21	19	11.5	10	10	10	8	14	11	15	ξ
Bi-sulfide	50	22	17	20	8	18	22	19	24	13	15	ω
	Sum:	101	101	101	100	100	100	101	100.5	100	100	
Al-oxide I	350	—	—	—	—	—	—	—	—	56	62	ξ, ω, η, ζ

* Symbols (L) and (M) as in Table 3.
[a] Vitamin K-deficient plasma, RVV-cephalin coagulation time 90 min/100 (normal plasma: 19—21).

of the η- and ζ-factors, and the activity of each of the ϑ-, ξ-, and ω-factors were determined as outlined above by simultaneous adsorption of four additional aliquots of the plasma. The sum of the adsorbed activities determined by this procedure will then exceed the correct values by the synergistic effects of the μ- and ε-factors on the π- and β-factor activities, respectively. This "excess activity" will be somewhat less than the combined activity of the μ- and ε-factors and, therefore, probably no more than one third of the combined activity of the $(\varrho-\nu-\mu)$ and $(\varepsilon-\lambda-\alpha)$ groups of factors.

Table 4 is a summary of representative experiments using chicken brain thromboplastin as assay accelerator and plasmas from chickens fed on a vitamin K-deficient diet or on various normal diets [6,7]. The experiments showed that the combined activity of the fourteen factors added up to 106—109 %. The results were thus slightly (1—3 %) above the expected maximum values, which in these experiments were estimated to be in the range of 104—108 %.

It is concluded, therefore, that the labile factor activity of strontium carbonate plasmas is adequately accounted for by the combined activity of the fourteen labile factors reported in previous papers. Table 4 also shows that the activity adsorbed by Al-oxide I is equivalent to the combined activity of the α-, ϑ-, ξ-, ω-, η-, and ζ-factors, in agreement with previous experiments [5].

Table 5 is a summary of experiments with RVV-cephalin as assay accelerator. The ϱ-, ν-, μ-, π-, γ-, ε-, α-, and ϑ-factors are inactive in this assay system [3-5], and the combined activity of the β-, λ-, η-, ζ-, ξ-, and ω-factors accounts for all labile factor activity of strontium carbonate plasmas. The activity adsorbed by Al-oxide I is equivalent to the combined activity of the ξ-, ω-, η-, and ζ-factors, as predicted by previous experiments [5].

Table 6. Combined activity of the strontium carbonate non-adsorbable labile factors; Russell's viper venom (RVV) assay.

Adsorbent(s) in mg/ml*		Activity of adsorbed factor(s) in % of strontium carbonate adsorbed plasmas, Nos.:										Active labile factor(s) adsorbed
		5927	6183	6190	6321	6329	6187	5895	6202[a]	3856	4152	
Co-oxalate	100	12	9	14	14	17	17	18	15	29	26	λ
Mixture of (L) and (M)		62	58	62	50	62	50	42	52	55	52	η, ζ
Fe-oxide	20	14	14	9	14	9	17	22	15	6	11	ξ
Bi-sulfide	50	12	19	15	22	13	17	18	18	10	11	ω
	Sum:	100	100	100	100	101	101	100	100	100	100	
Al-oxide I	350	—	—	—	—	—	—	—	—	71	74	ξ, ω, η, ζ

* Symbols (L) and (M) as in Table 3.

[a] Vitamin K-deficient plasma, RVV-buffer coagulation time 155 min/100 (normal plasma: 35—40).

Table 6 shows the results obtained with RVV, unsupplemented by cephalin. In the absence of added cephalin β-factor is inactive [3], and the combined activity of the λ-, ξ-, ω-, η-, and ζ-factors accounts for all labile factor activity of strontium carbonate plasmas *. The activity adsorbed by Al-oxide was equivalent to the combined activity of the ξ-, ω-, η-, and ζ-factors (*cf.* Ref.[5]).

DISCUSSION

The results presented here indicate that the labile factor activity of fresh oxalated chicken plasma is adequately accounted for by the combined activity of sixteen discrete labile factors, measured by chicken brain thromboplastin. Similarly, the labile factor activities measured with RVV-cephalin and RVV as assay accelerators, are accounted for by the combined activity of eight and of seven, respectively, of these sixteen factors. The experimental possibilities of a differentiation of the labile factor activity of chicken plasma by adsorption with selective adsorbents [2–5], thus seem to be exhausted.

Labile factor or proaccelerin activity of plasmas is generally recognized as the activity of a single entity: the labile factor or proaccelerin. The very complex nature of the labile factor activity of chicken plasma detected by adsorption analysis, may therefore appear surprising. However, chicken plasma differs from a number of mammalian plasmas in certain respects, *e.g.* by the absence of "contact activation" [10], and by low yield and slow formation of intrinsic plasma thromboplastin [11], due to deficiencies in prothromboplastic factors [10,11] (Hageman factor, PTA, and PTC). It is conceivable, therefore, that the hemostatic mechanism of chickens may depend on plasmatic coagulation factors (possibly labile) which are not present in mammalian plasma. They very rapid deterioration of chicken labile factor actitity (and of hemophilic factor A) during storage [2,11] indicate the further possibility that some of the factors which are labile in chicken plasma may correspond to factors which are stable in mammalian plasma, or at least not reduced below optimal levels during storage or incubation. A relation between mammalian labile factor activity and the chicken labile factors may probably be established through studies on the adsorption of mammalian labile factor activity by the selective adsorbents used for characterization of the chicken labile factors.

The individual labile factors in chicken plasma do not appear to be functionally identical, as judged by their complex interrelationships. These facts indicate that none of the labile factors can be replaced completely by any of the others. We are thus presented with the problem of assigning a specific function to each of sixteen labile coagulation factors. A possible clue to a future solution of this problem is suggested by the observed inactivity of the ϱ-, ν-, μ-, π-, γ-, ε-, α-, and ϑ-factors in the RVV-cephalin assay system [3–5]. These eight factors are accordingly thromboplastin specific and may be considered as cofactors for generation of possible intermediate activation products of thrombo-

* When the RVV assay is used, attention should be paid to the fact that the RVV coagulation time is sensitive to variations in the plasma levels of certain lipids [8,9]. Therefore, occasional failures to reach 100 % by the combined activity of the five labile factors, do not necessarily prove the existence of additional labile factors. Such results may be due to a very low concentration of active lipids in the stored plasma substrate compared to that of the fresh plasma employed.

plastin, one or more of which may have coagulant properties qualitatively similar to that of RVV-cephalin. The thromboplastin specific φ-factors [12] are probably also involved in these reactions, which may be analogous with the formation of convertin from proconvertin and thromboplastin in humans [13,14]. The λ-, ξ-, ω-, η-, ζ-, ν-, ψ- and σ-factors could then be regarded as co-factors for formation of prothrombin converting principle(s) (prothrombinase(s) [14]) from RVV-cephalin and from the above hypothetical intermediate activation product(s) of thromboplastin. Some of the latter group of factors might also be considered as possible accelerators of the fibrinogen-fibrin conversion. The inactivity of the β-factor in the RVV assay system [3] indicates that this factor is involved in reactions which only operate in the presence of added cephalin (as such, or in the form of thromboplastin).

The sixteen labile factors may not all be regarded as coagulation factors in the sense that a deficiency in one factor will cause a hemorrhagic diathesis or grossly abnormal plasma coagulation times. Reduction (by adsorption) of the ψ- and σ-factor levels of plasma to about 15 % of normal values had no effect on the thromboplastin coagulation time [2]. Complete adsorption of either of the two factors was associated with a minor prolongation of the thromboplastin coagulation time only. It appears unlikely, therefore, that the thromboplastin specific factors should all be essential for generation of RVV-cephalin equivalent activation products of thromboplastin, or that the β-, λ-, ξ-, ω-, η-, ζ-, ψ-, and σ-factors should all be indispensable for the transformation of these agents into prothrombin converting principles. It seems more likely that the formation of activation products of thromboplastin and of RVV may occur by more than one mechanism in chicken plasma. The labile factors could then be considered as parts of *alternative mechanisms* for formation of such activation products. Some of these mechanisms may not be expected to contribute significantly to observed coagulation rates of plasmas except under special experimental conditions. The accelerating effect of, *e.g.* β-factor is thus manifest only in plasma coagulation systems containing added cephalin.

So far no systematic approach has been made to the problem of elution of the adsorbed factors from the respective selective adsorbents, but unpublished data indicate that elution may be accomplished by use of phosphate buffers. However, the yields were low, presumably due to the labile nature of the factors, stressing the importance of using plasmas with high levels of labile factors for such studies. Great variations in the relative activities of the various factors were observed in different experiments [2–5]. These results diverted our immediate attention away from the elution problems and towards development of methods for quantitative determination of each of the sixteen labile factors, with the purpose of studying the causes of such variations. A series of forthcoming papers will be devoted to this subject.

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