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Studies on the Coagulation of Chicken Blood

XII. Assay and Dietary Dependence of the π-Factor Component of the Labile Factor Activity

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A method is presented for quantitative determination of the concentration of the π-factor component of the labile factor activity in oxalated chicken plasmas. The principle of the method is a "titration" of the π-factor activity with a selective adsorbent.

The π-factor level is shown to depend on the composition of the experimental diets, but to be unaffected by coumarin drugs. Diet-induced deficiencies in π-factor, correctable by supplementary feeding, could be attributed to inadequate supply of either (1) choline, or (2) an unidentified water soluble factor present in pancreas powder, or (3) unidentified fat soluble factors present in the unsaponifiables of corn oil, cod liver oil, lard, or whale liver oil; or to a combination of these three categories of dietary defects. A stable, maximum level of π-factor is obtained when choline, and the pancreas factor, and either one of the fat soluble factors are supplied in adequate amounts. Preliminary work on the extraction and purification of the unidentified factors is reported.

In preceding studies on the labile factor activity of oxalated chicken plasmas, *i.e.* the activity of plasma factors limiting the coagulation rate of stored, oxalated, normal chicken plasmas, it was shown that this activity was of complex nature.[1–5] The heterogeneity of the labile factor activity was revealed in adsorption studies using a series of crystalline compounds. Sixteen different categories of labile factor activity adsorbents were gradually recognized as selective adsorbents for sixteen different components of the labile factor activity measured in coagulation tests with chicken brain thromboplastin as assay accelerator. The combined effect of these sixteen factors was finally shown to account for all of the labile factor activity of chicken plasmas.[6]

In these studies, the total labile factor activity of chicken plasmas showed great variability, and no obvious relation between dietary composition and

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labile factor activity could be made out. The great number of possibly independent variables determining the labile factor activity indicated that this relationship might be complex, and that the ultimate causes of the observed variations might be more readily revealed by studies on the individual component factors. The relative activity of single factors, expressed as percent of total, showed similar variations in separate experiments, and plasmas tested simultaneously varied markedly in activity of particular factors expressed as percent of total activity of a common reference plasma.

The heterogeneity of the labile factor activity and the complex activity interrelationships [6] of the components presented some problems for quantitative assay of individual members of the labile factor family. Thus, a determination of the *concentration* of a particular factor could not be based on a direct comparison of the *activity* adsorbed by a selective adsorbent in plasmas tested simultaneously since this activity depended not only on the concentration of the factor itself, but in most cases also on the concentrations of one or two of the remaining factors (factors with synergistic effects).[3-6] Another inconvenience was that separate experiments were not directly comparable. The dilution curves relating coagulation time to labile factor activity could differ both in slope and position with different samples of substrate plasma,[2] the relative sensitivity of the substrate plasmas to different factors might also vary, and slight differences in the potency of the accelerator solution (thromboplastin) might be difficult to avoid. The latter problems could be eliminated if an unchanging source of reference plasmas were accessible. At present this only represents an additional problem.

Previously, the assay of the φ_1-, φ_2-, $\varkappa$-, and δ-factors had met with analogous problems (only far less accentuated because of the merely dual nature of the φ-factor activity measured in the Stypven-cephalin assay system,[7] and the homogeneity of the $\varkappa$-, and δ-factor activities [8]). Assay by "adsorbent titration" was shown to eliminate these problems [8,9] allowing determination of the concentration of these factors in absolute terms, *viz.* the equivalent concentration of standard preparations of special adsorbents.

In the present study the "adsorbent titration" technique has been tried for quantitative assay of one of the components of the labile factor activity, *viz.* the π-factor. This term was introduced to name the labile factor activity component which was selectively removed from Sr-carbonate preadsorbed, oxalated plasmas by relatively small amounts of Pb-carbonate, Pb-oxalate, and only a few other adsorbents.[3] π-Factor was characterized further by a complete lack of activity in test systems where Stypven or Stypven-cephalin replaced brain thromboplastin as assay accelerator.[3] Moreover, it had a unique, synergistic relationship to two of the other, discernible components of the labile factor activity resistant to Sr-carbonate adsorption, *viz.* the γ-, and μ-factors, selectively adsorbed from Sr-carbonate preadsorbed plasmas by Ba-phosphate and Bi-oxalate, respectively.[3,6] Additional evidence for the discrete nature of the π-factor was obtained in the present work. It will be shown that the concentration of π-factor in chicken plasma is limited by the supply of a possibly unique combination of dietary factors, the identity of most of which is a problem for further investigations.

Table 1. Experimental diets, composition in g per kg.

Ingredients	S-02.2A	S-10.6A	S-20.6A	S-11.2	S-11.2A	S-11.5A	S-11.6	S-21.2A	S-30.A	S-35.2A	S-36.2A	S-40.2	S-40.4A
Casein, Vitamin Test*	70	200	—	150	150	150	150	—	—	—	—	—	—
Casein, Stege*	—	—	200	—	—	—	—	150	—	—	—	80	80
Gelatine	30	80	80	80	80	80	80	80	—	—	—	—	—
Pancreas powder*	—	—	—	80	80	80	80	80	200	250	250	220	220
Ground corn	200	—	—	—	—	—	—	—	—	400	—	—	—
Ether-extracted ground corn	—	—	—	—	—	—	—	—	—	—	400	—	—
Ground barley	200	—	—	—	—	—	—	—	—	—	—	—	—
» oats	100	—	—	—	—	—	—	—	—	—	—	—	—
» wheat bran	80	—	—	—	—	—	—	—	—	—	—	—	—
Dried alfalfa	60	60	60	—	60	60	—	60	60	60	60	—	60
Refined peanut oil	50	—	—	50	50	40	—	50	—	50	50	50	30
» lard	—	40	40	—	—	—	40	—	—	—	—	—	30
Cod liver oil	—	20	20	—	—	20	20	—	—	—	—	—	—
Salt mixture*	50	50	50	50	50	50	50	50	50	50	50	50	50
Vitamin mixture*	5	5	5	5	5	5	5	5	5	5	5	5	5
Cystine	—	5	5	—	—	—	—	—	—	—	—	—	—
Sucrose	155	540	540	585	525	515	575	525	685	185	185	595	525
Vitamins A and D*													

Composition of the commercial diet "No. 66": ground barley 240; ground wheat, 200; wheat bran, 250; alfalfa meal, 60; dried brewers yeast, 10; meat and bone meal, 100; fish meal, 50; dried skim milk, 70 ; Deltafor (vitamin D source from Philips A/S, Copenhagen), 10; sodium chloride, 5; calcium carbonate, 5; total 1000.

* For details see Ref.[9], Table 1.

MATERIALS AND METHODS

The materials and methods used in this study were for the main part as described in detail previously.[2,3,9-11]

The test system for determination of the labile factor activity [2] was modified in the later part of this work to minimize test plasma manipulations. The 0.20 ml volume of the one-in-twenty dilution of test plasma was replaced by 0.01 ml of *undiluted* test plasma which was diluted with buffer for preparation of the reference curve.[2]

Diets. The composition of the semi-purified diets used in this work is given in Table 1. The composition of the special commercial diet (No. 66) was as described by Dam *et al.*[12]

Extraction and preliminary purification of unidentified dietary factors was carried out according to the general fractionation scheme described previously.[11]

Reproducibility of coagulation time determinations. As in previous work replicate determinations of coagulation times up to 40–50 sec differed by no more that 0.1 sec. In most cases this corresponds to a methodical error of about 1 % or less in labile factor activity. This degree of accuracy is essential and was acquired during the first few years of the author's occupation with coagulation problems.

EXPERIMENTS AND RESULTS

Assay of π-factor

As already mentioned considerable variations in π-factor activity had been observed in plasmas tested simultaneously. A reliable method for quantitative determination of the *concentration* of π-factor in chicken plasmas, suitable for routine work, was an obvious prerequisite for an inquiry into the causes of such variations and the possible dietary dependence of π-factor.

(1) *Assay by "adsorbent titration"*. The labile factor activity adsorbed from individual, Sr-carbonate preadsorbed, oxalated chicken plasmas was always proportional to the concentration of the selective adsorbent used, until a maximum, partial adsorption of the labile factor activity had been obtained (*i.e.* complete adsorption of a component factor).[3-5] The minimum concentration of adsorbent required for this effect was unaffected by prior removal of other components of the activity, and it was pointed out[3] that this value (the "adsorbent titre") might prove proportional to, and thus represent a measure of the plasma concentration of the adsorbable factor.

Quantitative determination of the π-factor concentration by "titration" of the π-factor activity with a selective π-factor adsorbent thus seemed a possibility. The crucial question was whether the adsorption capacity per unit concentration of the π-factor adsorbent was constant, a characteristic of the adsorbent, and thus, suitable as an assay "unit". Information on this point was obtained in comparative studies on the adsorption of π-factor activity in different samples of chicken plasma, all preadsorbed by Sr-carbonate as described previously.[3] For such studies *identical* assay reagents were required (*cf.* p. 2143). Thus, only adsorption experiments carried out simultaneously could be compared. Technical problems prevented studies of more than four samples of plasma in each experiment. The activity of aliquots of each plasma adsorbed by graded concentrations of Pb-oxalate,was expressed in percent of the activity of the most active, untreated plasma.

In experiments with plasmas from chickens fed on diets later characterized as adequate for maximum level of π-factor, the adsorbent "titres" of individual plasmas were identical, *viz.* 15 mg/ml, but the adsorption curves were usually not parallel (Fig. 1). The *activity* adsorbed per unit concentration is thus not a characteristic of the adsorbent.

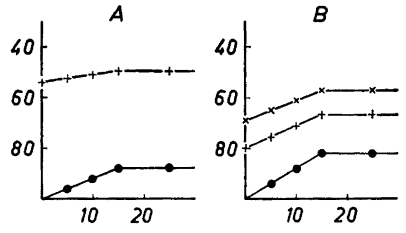

Fig. 1. Adsorption of labile factor activity by Pb-oxalate. Ordinates: labile factor activity of adsorbed plasma aliquots (the most active, untreated plasma = 100) Abscissae: adsorbent (Pb-oxalate) in mg/ml Symbols: (●—●), (+—+), and (×—×) denote individual plasmas. A and B are representative experiments.

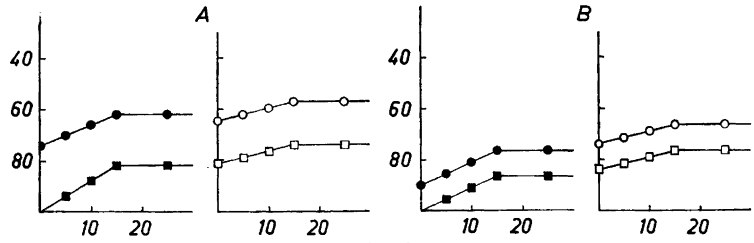

Fig. 2.

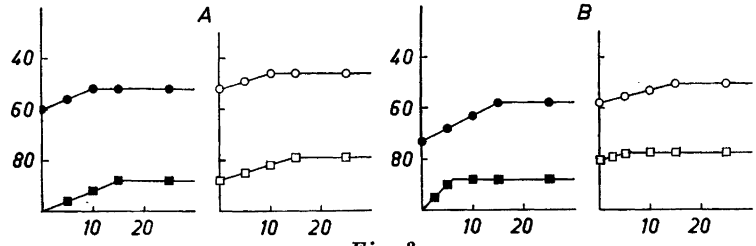

Fig. 3.

Figs. 2 and 3. Adsorption of π-factor activity before and after preadsorption of synergistic factors. Ordinates and abscissae as in Fig. 1. Symbols: squares and circles denote individual plasmas, filled symbols *before*, open symbols *after* pretreatment with Ba-phosphate (80 mg/ml) + Bi-oxalate (12 mg/ml). A and B are representative experiments.

The observed differences in the slope of the curves were, however, only caused by differences in the plasma concentrations of the γ- and μ-factors, both of which influence the specific activity of π-factor.[3,6] This was concluded from studies of the effect on the adsorption curves of a complete removal of the synergistic factors by prior treatment of the plasma samples with adequate amounts of mixture of Ba-phosphate [3] and Bi-oxalate.[4] Whether converging (or diverging), or parallel *before*, the curves for adsorption of π-factor activity in different plasmas were invariably parallel *after* preadsorption of the γ- and μ-factors (Fig. 2).

Finally, experiments with plasma pairs of different adsorbent "titres" proved that in the absence of differences in the content of synergistic factors all observed differences in total π-factor activity were associated with proportional differences in the adsorbent "titre" (Fig. 3). Thus, the previous reservation that the latter differences might be attributed to differences in the content of *other* plasma components, inert in this assay, but adsorbed in competition with π-factor,[3] proved untenable.

The Pb-oxalate "titre" is accordingly a reliable measure of the plasma concentration of π-factor. It is independent of changes in the concentration of the remaining components of the labile factor activity, and independent of small differences in the sensitivity of individual substrate plasma samples and in the potency of the thromboplastin reagent used in separate experiments. A value of 15 mg/ml of the Pb-oxalate preparation used (or 30 mg/ml of Pb-carbonate [3]), appears to represent the maximum plasma concentration (100 %) of π-factor in normal chickens.

Table 2. "Adsorbent titration" of π-factor.

Test plasma*	Concn. of PbC_2O_4 (mg/ml)	Coagulation time in min/100 Concn. of test plasma 100 %	50 %	20 %	buffer	Labile factor activity %	π-Factor concn.** PbC_2O_4-titre mg/ml	% of max.
I	0	36.0	42.2	52.3	70.0	78	15	100
	2.5	36.3				75.5		
	5	36.6				73		
	10	37.2				68		
	15, 20	37.8				63		
II	0	34.8	40.9	50.6	70.0	90	≤2.5	≤17
	2.5, 5, 10, 20	35.1				87		
III	0	34.0	39.8	49.4	—	100	6.5	43
	2.5	34.5				94		
	5	35.0				88		
	10, 20	35.3				84.5		

* Oxalated plasma preadsorbed by Sr-carbonate (20—25 mg/ml).[3]

** The minimum increment in adsorbent concentration (2.5 mg/ml) prevented a graduation of π-factor concentrations below 17 %.

A typical, simultaneous assay of the π-factor concentration in three experimental plasmas is recorded in detail in Table 2.

(2) *Studies on a possible, alternative procedure.* In "adsorbent titration" of π-factor activity all observations are concerned with the effect of a selective *removal* of the factor. A prior separation of π-factor from all other components of the labile factor activity (*e.g.* by selective adsorption and subsequent elution) would allow a more direct determination of the π-factor concentration in different plasma samples. This procedure would undoubtedly be more time-consuming than the "adsorbent titration" assay and require a reliable source of references plasmas for comparison of separate experiments. Only a limited series of experiments were carried out for comparison with the "titration" method during preliminary studies on the elution of adsorbed π-factor activity.

Pilot experiments indicated that labile factor activity adsorbed by Pb-oxalate could be eluted by use of phosphate or citrate buffers. The activity of the eluates was measured after removal of the eluent solutes by dialysis in the cold (40 h at 0—2°C), either against oxalated saline and isotonic buffer, or against distilled water for subsequent lyophilization and dissolution in buffer. The apparent recoveries were discouragingly low, rarely exceeding 5—6 %. An inactivation of the eluted factor is compatible with the labile nature of the activity. A pronounced lability in aqueous solutions has been reported for purified human factor V, even at 0°C,[13] and also for purified bovine factor V.[14] However, irreversible adsorption, or adsorption-induced conformational changes in π-factor may also be partly responsible for the low recoveries.

Table 3. Elution of adsorbed π-factor activity.

	Elution sequences	Coagulation time*			Heat labile effect of eluate*	Preparation technique. (Plasmas from chickens on diet S-35.2A)
		Eluate	Heated** eluate	Buffer control		
I	a) 3+1 ml 0.1 M phosphate pH 6.6	64	64	64	0	8 ml $SrCO_3$ plasmas and 130 mg PbC_2O_4. Adsorbent prewash: 2 × 6 ml oxalated saline. Dialysis of eluates (40 h) against oxalated saline and buffer
	b) 3+1 ml 0.3 M phosphate pH 6.6	61.9	64.2	64.2	2.3	
	c) 3+1 ml 0.5 M phosphate pH 6.6	64.4	64.4	64.4	0	
	d) 3+1 ml 0.8 M phosphate pH 6.6	64.8	64.8	64.8	0	
II	a) 2 × 2½ ml 0.02 M Na-citrate	59	60	60	1	5 ml $SrCO_3$ plasma and 90 mg PbC_2O_4. Adsorbent prewash: 2 × 5 ml oxalated saline. Dialysis of eluates (40 h) against distilled water, lyophilization and dissolution in 2½ ml buffer
	b) 2 × 2½ ml 0.10 M Na-citrate	60	60.2	60.2	0.2	
	a) 2 × 2½ ml 0.05 M Na-citrate	60.8	62	62	1.2	
	b) 2 × 2½ ml 0.20 M Na-citrate	62.3	62.3	62.3	0	
III	a) 2 × 2½ ml 0.02 M citrate pH 6.6	72.7	72.9	72	0.2	
	b) 2 × 2½ ml 0.05 M citrate pH 6.6	72	73	72	1	
	c) 2 × 2½ ml 0.10 M citrate pH 6.6	74.6	74.6	72.2	0	
	a) 2 × 2½ ml 0.1 M phosphate pH 6.6	72.4	72.4	72.4	0	
	b) 2 × 2½ ml 0.2 M phosphate pH 6.6	71.2	72.4	72.4	1.2	
	c) 2 × 2½ ml 0.4 M phosphate pH 6.6	72.5	72.5	72.5	0	
IV	a) 4 ml eluate dialysed against oxalated saline and buffer	51.2	54	54	2.8	12 ml $SrCO_3$ plasma and 200 mg $PbC_2O/$. Adsorbent prewash: 2 × 8 ml oxalated saline and 2 × 4 ml 0.1 M phosphate pH 6.6. Elution with 2 × 4 ml 0.3 M phosphate pH 6.6
	b) 4 ml eluate dialysed against aq. dist., lyophilized, and dissolved in 4 ml buffer	52.8	54	54	1.2	

* in min/100. (Test system: stored plasma, test solution, thromboplastin, $CaCl_2$(35 mM), each 0.20 ml).
** 90 sec. 95°C.

Despite the obvious shortcomings in technique some useful informations were obtained in these experiments. The results presented in Table 3 show that: (1) whatever remained of the eluted activity at the time of testing, this activity was non-dialyzable and completely destroyed by heating to 95°C for 90 sec (I—IV); (2) elution with citrate and phosphate buffers was equivalent (III) and optimal at 0.3 M phosphate of pH 6.6 (I), and at 0.05 M Na-citrate (II) or a somewhat lower concentration of citrate buffer of pH 6.6 (the activity of citrate eluates was occasionally masked by the presence of a heat-stable inhibitor, III); (3) a considerable part of the activity is lost by lyophilization (IV). Thus, it may be assumed that π-factor is of protein nature.

Simultaneous assay of π-factor by adsorption-elution and adsorbent "titration" is recorded in Table 4, showing that: (1) in the range of proportional adsorption the recovered activity was proportional to the amount of adsorbent used (I and II); (2) in the range of maximum partial adsorption of labile factor activity the recovered activity was independent of the amount of adsorbent (I and II); (3) the recovered activity was identical with plasma pairs of identi-

Table 4. Determination of π-factor concentration. "Adsorption-elution" *versus* "adsorbent titration".

	Chicken diet	Source of eluates: Plasma volume	Source of eluates: Adsorbent concn. (mg/ml)	Preparation of eluates	Effect of eluate *cf.* Table 3	Results of "adsorbent titration": PbC_2O_4-titre	Results of "adsorbent titration": % of max
I	S-35.2A	5 ml	5	Prewash: 2 × 5 ml oxalated saline. Elution: 2½ ml 0.03 M Na-citrate. Eluates dialyzed (aq. dist.), lyophilized, and dissolved in 2½ ml buffer.	0.4	15 mg/ml	100
		5 ml	10		0.8		
		5 ml	15		1.3		
		5 ml	20		1.3		
II	S-35.2A	5 ml	5	Prewash: 2 × 6 ml oxalated saline and 2 × 3 ml 0.1 M phosphate pH 6.6 Elution: 3 ml 0,3 M phosphate pH 6.6	0.3	15 mg/ml	100
		5 ml	10		0.6		
		5 ml	15		1		
		5 ml	20		1		
	S-21.2A	5 ml	18	Eluates dialysed etc. as in I. Final volume 2½ ml	0.1	≤2.5 mg/ml	≤17
	S-02.2A	5 ml	18		0.5	7.5 mg/ml	50
III	S-35.2A	5 ml	17	As in I	2	15 mg/ml	100
	S-40.4A	5 ml	17		2	15 mg/ml	100
	S-21.2A	5 ml	17		0.2	≤2.5 mg/ml	≤17
	S-21.2A	5 ml	17		0.2	≤2.5 mg/ml	≤17
IV	S-02.2A	8 ml	16	Prewash as in II. Elution: 0.3 M phosphate pH 6,6 (0.5 of plasma vol.). Dialysis: oxalated saline and buffer. Final eluate volume: 0.6 of original plasma.	0.2	≤2.5 mg/ml	≤17
	S-35.2A	8 ml	16		1.2	15 mg/ml	100
V	S-35.2A	7 ml	16		2.3	15 mg/ml	100
	S-02.2A	7 ml	16		0.4	≤2.5 mg/ml	≤17

cal adsorbent "titre" (III); (4) with plasmas of different "titres" the recovered activities were roughly proportional to the "titres" (II-V).

The results obtained by adsorption-elution are thus in apparent conformity with those of adsorbent "titration", but this may be of doubtful significance because of the low recoveries obtained by adsorption-elution. The latter technique needs considerable improvement.

The relative simplicity and rapidity of the adsorbent "titration" assay encouraged the use of this method for routine analysis. As a further advantage the results obtained in separate experiments could then be compared by reference to "titres" of a standard preparation of an adsorbent instead of activities relative to that of a reference plasma, determined simultaneously.

Dietary dependence of the π-factor.

The "adsorbent titration" technique was used to study the causes of observed variations in π-factor concentration. Plasma from chickens fed on certain diets showed decreasing or uniformly low levels of π-factor in successive blood samples whereas that of chickens on other diets was maintained at a high and stable level throughout the experimental periods. As shown in Table 5

Table 5. π-Factor levels (% of max.) induced by different diets.

Plasma sample No.*	Diets													
	S-02.2A	S-10.6A	S-20.6A	S-11.2	S-11.6	S-11.2A	S-11.5A	S-21.2A	S-35.2A	S-36.2A	S-40.2	S-40.4A	no.66	S-30.A
1	33	≦17	≦17	23	100	≦17	100	≦17	100	≦17	≦17	100	≦33	≦33
2	23	≦17	≦17	≦17	100	≦17	100	≦17	100	≦17	≦17	100	23	≦33
3	≦17	–	–	–	100	–	100	≦17	100	–	–	100	–	–

* Successive blood samples taken at intervals of 5–9 days. First sample after 3–5 weeks on the experimental diets.[11]

diets such as S-11.6, S-11.5A, S-35.2A, and S-40.4A, met all demands for maintenance of π-factor at a concentration which apparently represents the maximum, normal level of π-factor in chicken plasma. On the other hand, diets lacking one or more of the ingredients of these diets (S-02.2A, S-10.6A, S-11.2, S-11.2A, S-20.6A, S-21.2A, S-30.A, S-36.2A and S-40.2), and a special commercial diet (No. 66) were inadequate in this respect.

The essential ingredients of the adequate diets were sorted out. The presence of pancreas powder (lacking in diets S-02.2A, –10.6A and –20.6A) was essential. In addition either of the fat sources: corn oil (difference S-35.2A/S-36.2A), lard (difference S-40.4/S-40.2), or cod liver oil (difference S-11.5A/S-11.2A), was required for maximum level of π-factor. Dried alfalfa (lacking in S-11.6) and casein (lacking in S-35.2A and –40.4A) were not essential parts of the adequate diets.

Table 6. Effects of supplementary feeding.

Basic diet	Daily supplement (fed for 3–5 days)	π-Factor concn. in % of max.	
		Pre-test	After suppl.
S-30.A	1 g peanut oil	≤33	<33
S-30.A	1 g cocoa-nut oil	37	<33
S-30.A	1 g corn oil	≤33	100
S-21.2A	1 g »	≤17	100
S-21.2A	2 g lard	≤17	100
S-21.2A	1 g cod liver oil	≤17	100
S-11.2A	Unsaponifiables from 1.5 g whale liver oil	≤17	100
S-11.2A	2 g wheat germ oil	≤17	100
S-20.6A	4 g pancreas powder	≤17	100
no. 66	4 g pancreas powder	≤33	23
no. 66	1 g cod liver oil	≤33	43
no. 66	2 g dried yeast*	≤33	83
no. 66	2 g dried yeast* + 1 g cod liver oil	≤33	100

* "Waldhof".

Chickens fed on the inadequate diets responded to supplementary feeding with the missing essential ingredient. The π-factor level was raised to 100% within 3 days. Representative experiments are recorded in Table 6.

The π-factor level of chickens fed on the special commercial diet (No. 66) could not be raised to maximum by supplementary feeding with pancreas powder or cod liver oil (or corn oil). Feeding of dried yeast as the only supplement raised the π-factor level to 80–90 % of maximum, and when combined with supplements of corn oil or cod liver oil, maximum level of π-factor was obtained consistently.

The above evidence that the π-factor level in chicken plasma depends on the supply of essential dietary ingredients, raised the question whether this effect was due to changes in the over-all composition of the diets, or to the action of specific dietary *factors*. In the analogous studies concerning the coagulation factors reduced below optimal levels in vitamin K deficiency and/or after coumarin drug administration in chickens, it had been found that specific, unidentified dietary factors in addition to vitamin K were involved in the control of the plasma levels of κ-factor,[11] and of φ_2-factor, but not of φ_1-factor.[9] Evidence that the plasma level of π-factor might be controlled similarly, possibly by a unique combination of dietary factors, is presented below.

Dietary factors, essential for maximum level of π-factor

(1) *Coumarin drugs and π-factor level.* The effect of a coumarin drug (coumachlor [10]) was studied in chickens fed on diets adequate for maintenance of π-factor at the maximum level. After administration of the drug for 3

Table 7. Coumachlor and π-factor concentration.

Chicken diet	Coumachlor dose	Coagulation time*		π-Factor concn. (%)	
		Pre-test	After drug	Pre-test	After drug
S-40.4A	0.01 mg/g × 3	26	115	100	100
	0.01 mg/g × 3	23	150	100	100
	Control	26	25	100	100

* in min/100. Test system: plasma, buffer, thromboplastin, and $CaCl_2$ (30 mM), 0.2 ml each.

days in succession there was no measurable effect on the plasma concentration of π-factor. The thromboplastin accelerated coagulation time of the plasmas increased to 4—7 times the value for normal chickens. Representative experiments are recorded in Table 7. The complete lack of effect of a potent antagonist of vitamin K indicates that vitamin K is not involved in the control of the plasma level of π-factor.

(2) *The active factor in yeast.* The effect of supplementation with yeast was only observed in chickens fed on the special, commercial diet (No. 66). In chickens fed on the semi-purified, inadequate diets (S-11.2A, or S-10.6A) yeast supplementation had no or at most a very slight effect. Presumably, the factor supplied by yeast was present in adequate amounts in the semi-purified diets. Mixtures of synthetic vitamins were accordingly tested for the yeast effect, as such or in combination with cod liver oil, and in amounts corresponding to a daily intake of 40—50 g of the adequate diets. The results are given in Table 8.

It is apparent that the effect of yeast supplementation could be mimicked by a combination of all the B-vitamins (vit. mixt. I); that inositol and *p*-aminobenzoic acid (lacking in vit. mixt. II), and a mixture of thiamine, riboflavin, nicotinic acid, pantothenate, pyridoxine, biotin, and folic acid (vit. mixt. III) were inactive; and that the whole effect of the complete vitamin B mixture

Table 8. Effects of synthetic B-vitamins.

Supplement to diet No. 66	Daily dose, fed for 3—5 days	π-Factor concn. after suppl.[d]
Vitamin mixture I[a]	Corresponding	87
Vit.mixt. I + cod liver oil	to	100
Vitamin mixture II[b]	40—50 g	83
Vitamin mixture III[c]	of diet	≤33
Choline (chloride)	no.	87
Choline + cod liver oil	S-11.5A	100

[a] Containing in mg: choline chloride, 80; inositol, 35; *p*-aminobenzoic acid, 10; thiamine hydrochloride, 0.24; riboflavin, 0.32; nicotinic acid, 4; calcium pantothenate, 0.96; pyridoxine, 0.28; biotin, 0.008; folic acid, 0.16.

[b] as I, but inositol and *p*-aminobenzoic acid omitted.

[c] as II, but choline chloride omitted.

[d] in % of maximum. Pre-test levels below 33 %.

Table 9. Effect of pancreas powder fractions.

Test sample	Daily dose[a]	Response[b]
Pa-3	4—6	≤17
Pa-21	2	80
Pa-22	2	50 (27)
Pa-201	3	≤17
Pa-202	3	≤17
Pa-203	1.5	70
Pa-2031	3	50 (50)
Pa-2032	3	97
Pa-2033	3	100
Pa-2034	3	≤17 (27)
Pa-20301	4	≤17
Pa-20302	4	100
Pa-20303	4	≤17

[a] equivalent dose (in g) of original pancreas powder
[b] π-factor concentration after supplementation with test sample, in percent of maximum. Basic diet: S-02.2A, S-10.6A, or S-20.6A. Pre-test levels above 17 % given in brackets.

could be attributed to choline alone. The low levels of π-factor in chickens fed on diet No. 66 is thus explainable as mainly due to inadequate supply of dietary choline.

No attempts have as yet been made to determine the minimum requirement of choline for maintenance of maximum levels of π-factor in chicken

Table 10. Purification of the fat-soluble factors. I.

Procedure	Fraction	Corn oil		Cod liver oil		Lard		Whale liver oil	
		Dose[a]	Response[b]	Dose[a]	Response[b]	Dose[a]	Response[b]	Dose[a]	Response[b]
Methanol extraction	Extract	2	100	½	100				
	Residue	2	≤17	½	≤17				
Saponification and fractionation of the unsaponifiables	Fatty acids (-14)	2	≤17	½	≤17	3	≤17		
	Sterols (-13)	2	≤17	½	≤17	3	≤17	6	≤17
	Epi-phasic (-11)	2	100	½	100	2	100	2	100
	Hypo-phasic(-12)	2	≤17	½	≤17	3	≤17	5	≤17
Molecular distillation of unsaponifiables[c]	1st distillate							1.5	≤17
	Residue of 1st distn.							1.5	100
	Residue of 3rd distn.							1.5	100

[a] equivalent dose (in g) of original fat.
[b] π-factor concentration after supplementation with fraction, in percent of maximum. Basic diet: S-11.2, S-11.2A, S-21.2A or S-36.2A. Pre-test levels above 17 % given in brackets.
[c] Kindly supplied by A/S Collett & Co., Oslo.

plasma, nor to study the effect of other constituents of yeast or other donors of labile methyl groups. The further work was confined to the analogous effects obtained by (1) supplementation of the diets S-02.2A, S-10.6A, or S-20.6A with pancreas powder, and (2) supplementation of the diets S-11.2, S-11.2A, S-21.2A, S-30.A, S-36.2A, or S-40.2 with corn oil, liver oils, or lard.

(3) *Studies on the active principles in pancreas powder, corn oil, liver oils, and lard.* (a) *The active factor in pancreas powder* (Pa). For information on the nature of this factor the general extraction, fractionation and testing procedures described in detail previously,[11] were followed. As seen from Table 9 the active principle was extracted from ether extracted pancreas powder with hot, 96 % ethanol in a Soxhlet extractor, leaving an inactive residue (Pa-3). Both the cold-soluble (Pa-21) and the cold-insoluble (Pa-22) fractions were active. Partition of the recombined extracts (Pa-20) between ether and water gave an inactive upper phase (Pa-201), and additional inactive material (Pa-202) was removed by butanol extraction of the lower phase, concentrated by evaporation. The active, butanol extracted solution (Pa-203) was fractionated by successive precipitations. The active factor was not precipitated by Ba-ions at pH 9 (Pa-2031). Addition of neutral Pb-acetate to the filtrate at pH 6 caused partial precipitation of the active factor (Pa-2032). The remaining part of the activity was precipitated by addition of basic Pb-acetate at pH 8 (Pa-2033), the final filtrate (Pa-2034) being inactive. Addition of phosphotungstic acid to the combined active fractions (Pa-2030) in acid solution gave an inactive filtrate (Pa-20303). The active factor was recovered from the insoluble (Pa-20302), but not from the soluble (Pa-20301) fractions prepared by extraction of the precipitate with 90 % acetone.

(b) *The active factors in corn oil, cod liver oil, lard, and whale liver oil.* A preliminary purification of these mutually replaceable factors was attempted by liquid-liquid extraction and saponification, and a fractionation of the unsaponifiables[11] by separation of the bulk of sterols, partition of the sterol-poor fraction between light petrol and 90 % methanol, and by column chromatography of the active fractions.

Table 11. Purification of the fat-soluble factors. II.

Procedure	Eluate fractions	Corn oil		Cod liver oil		Lard		Whale liver oil	
		Dose[a]	Response[b]	Dose[a]	Response[b]	Dose[a]	Response[b]	Dose[a]	Response[b]
Alumina column chromatography of the epi-phasic fractions(-11)	Light petroleum (LP)	2	27 (33)	0.8	≤17	5	≤17	3	≤17
	Benzene/LP = 1/4 (v/v)	2	100	0.8	100	5	≤17	3	≤17
	Acetone/LP = 1/9 (v/v)	2	≤17	1	≤17	5	100	3	100
	Ethyl ether (E)	2	≤17	1	≤17	5	≤17	3	≤17
	Methanol/E = 1/9 (v/v)	2	≤ 17	1	≤17	5	≤17	3	≤17

[a] and [b]: see footnotes to Table 10.

As shown in Table 10 the active principle in corn oil (and in cod liver oil) could be separated from the bulk of inactive material on removal of about 8 % of the fat by continuous extraction with methanol. After saponification the active principles of all fats were recovered only in the epiphasic fraction of the sterol-poor part of the unsaponifiables. After enrichment of vitamin A by molecular distillation of the unsaponifiables from whale liver oil, the active factor was present only in the residue.

Chromatography on columns of activated alumina [11] of the active epiphasic fractions of the fats revealed that the active factors were non-identical. Table 11 shows that whereas the active principle in corn oil and in cod liver oil was completely eluted with 20 % benzene in light petrol, the corresponding fractions of lard and of whale liver oil were inactive in the doses tested. The active factors in the latter fats were completely eluted with 10 % acetone in light petrol. These factors are thus slightly more polar than the active factor in corn oil and in cod liver oil, but still predominantly of non-polar (possibly hydrocarbon) nature.

Further studies are reported in a following paper.

DISCUSSION

Present work provides evidence that "adsorbent titration" is a reliable technique for quantitative assay of the π-factor component of the labile factor activity in chicken plasmas. Separation of protein components of the coagulation mechanism by use of adsorbents has a tradition of more than fifty years. The use of crystalline adsorbents as tools for *differential* and *quantitative* analysis of possibly complex coagulation activities is, however, not traditional and may be met with a certain reservation. The relation of the components of the chicken coagulation mechanism discernible by this technique, to presently accepted components of the human coagulation mechanism is not known, and the possible consequences as to the complexity of the coagulation mechanism in vertebrates in general, cannot be readily evaluated.

The present method was suggested by simple adsorption experiments in which a *qualitative* differentiation of the labile factor activity was obtained. It was inferred that highly specific adsorption "sites" in certain categories of crystalline adsorbents allowed a selective or mutually independent removal of discrete components of this plasma activity.[2–5] By the present observations that the *specific* adsorption capacity of a selective π-factor adsorbent is constant under the experimental conditions of current interest and may be used as assay "unit", assay of the π-factor component by "adsorbent titration" seems finally established as a *quantitative* method.

The methods for assay of the $\varkappa$-, δ-, φ_1-, and φ_2-factors of the chicken coagulation mechanism, developed previously,[8,9] are essentially similar. Thus, differential analysis of (complex) coagulation activities by "adsorbent titration" seems to be a rather versatile technique and a valuable supplement to present methods for quantitative assay of discrete components of the coagulation mechanism. It may be of special importance for such work in species where the advantages of existing sources (or means for preparation) of substrate plasmas for *specific* assay of single coagulation factors cannot be exploited because of species-specificity phenomena.

The complex nature of the labile factor activity in chicken plasma leaves relatively little "space" for a quantitative evaluation of each component factor. This seems to be partly compensated for by the acquired precision of the method. Certain sources of methodical errors generally associated with coagulation studies in other species, are absent. "Contact activation" is thus a negligible variable in work with chicken plasma due to the gross or total deficiency in the factors XII and XI (as well as IX).[15,16] Moreover, the choice of assay "units" makes results obtained in separate experiments independent of possible variations in the assay conditions. Increased attention may accordingly be paid to minor differences in labile factor activity beyond the maximum experimental error of the method (1 %). Assuming that the π-factor component accounts for at least 12 % of the total activity, replicate determinations of the π-factor concentration may not vary more than ± 4 % of the mean. Changes of 10 % (of the maximum level) or more in successive plasma samples, therefore, probably represent changes, the final causes of which may eventually be elucidated. This is exemplified by the reported studies on the relation between dietary composition and plasma level of π-factor, offering a partial explanation of the variations in total labile factor activity observed in chickens.

As in the analogous studies of the $\varkappa$-and φ_2-factors,[9,11] a maximum plasma level of the π-factor is ensured only by adequate supplies of particular dietary components, the effects of which have been traced through a series of fractionation steps. Thus, the plasma level of the π-factor seems to be controlled by a set of specific dietary factors which may prove to be unique. At least it is different from those concerned with the $\varkappa$-, φ_1,- and φ_2-factors and comprises choline, an unidentified, water soluble factor (present in pancreas powder), and either one of a group of unidentified, fat soluble factors, unsaponifiable and of predominantly non-polar character. The group of fat soluble factors is of particular interest in this connection. The identity of one of these factors and its possible relation to the others is the subject of a following paper.

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