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Studies on the Coagulation of Chicken Blood

II. Unidentified Nutritional Factors, Essential for Maximum Level of $\varkappa$-factor

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By nutritional experiments it has been shown that the level of $\varkappa$-factor in chicken plasma depends on the composition of the diet, and that the $\varkappa$-factor level is determined by the simultaneous presence of a set of nutritional factors, six of which are of unidentified nature. Besides vitamin K, nutritional factors in barley, wheat bran, pancreas powder, corn oil, dried skimmed milk, and alfalfa are required for maximal $\varkappa$-factor activity. A diet is described which is adequate for maintaining the plasma $\varkappa$-factor concentration at a maximal level during the experimental periods. When the diet is fed to chickens with low levels of $\varkappa$-factor, their $\varkappa$-factor concentration is raised to the maximal level.

The extraction and preliminary concentration of the active principles in the essential ingredients of this diet is described. One of these factors is a lipo-soluble compound. It is non-saponifiable and may be a hydrocarbon containing a few polar groups. The other five factors are water-soluble compounds, probably containing nitrogen.

It is suggested that the factors influence the $\varkappa$-factor level of plasma by affecting the formation of $\varkappa$-factor. They may be catalysts for this process or essential parts of the $\varkappa$-factor molecule.

It was described in a previous paper [1] how mixtures of vitamin K-deficient and coumachlor plasmas could be used to distinguish between three different "types" of coagulation factors ($\varkappa$-, δ- and φ-factors). The concentrations of δ- and φ-factors appeared to be limiting for the coagulation rate of vitamin K-deficient plasmas, whereas the coagulation rate of coumachlor plasmas was limited by the concentrations of $\varkappa$- and φ-factors. A method for comparison of $\varkappa$-, δ- and φ-factor levels in different plasmas was outlined. Application of this method to plasmas from chickens on slightly different diets revealed some variations in the levels of these factors, indicating that their concentrations in plasma might depend on nutritional factors other than vitamin K.

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The present paper deals with: 1) the design of a diet which can raise the plasma ϰ-factor concentration to a maximum in chickens, where the ϰ-factor concentration is low, and is adequate for maintenance of the ϰ-factor concentration at the maximal level, and 2) extraction and preliminary concentration of the active principles in the essential ingredients of this diet.

MATERIALS AND METHODS

The materials and method for measurement of ϰ-factor activity were described in a previous paper [1].

Feeding of chickens. Chickens were raised on a commercial diet [2] for 2—3 weeks and thereafter on the same commercial diet or on various experimental diets (Table 1) for no less than 2—3 weeks. Variations in the plasma levels of ϰ-factor were thereafter followed in *individual* chickens by determination of ϰ-factor activity at intervals of 5—10 days. The effect of different diets and of changes in the composition of diets on the ϰ-factor activity could thus be followed for several weeks.

Testing of concentrates. By omission of an essential ingredient from a diet, adequate for maintenance of maximal ϰ-factor level, a reduced level of ϰ-factor was induced in 2—4 weeks. Chickens on such "deficient" diets were then used to trace the active principles of the various essential food ingredients through the extraction and fractionation procedures.

Extraction residues were tested for activity by feeding a diet where extracted material was substituted for the corresponding unextracted material in the complete diet. The effect on the ϰ-factor level was examined after 6—9 days.

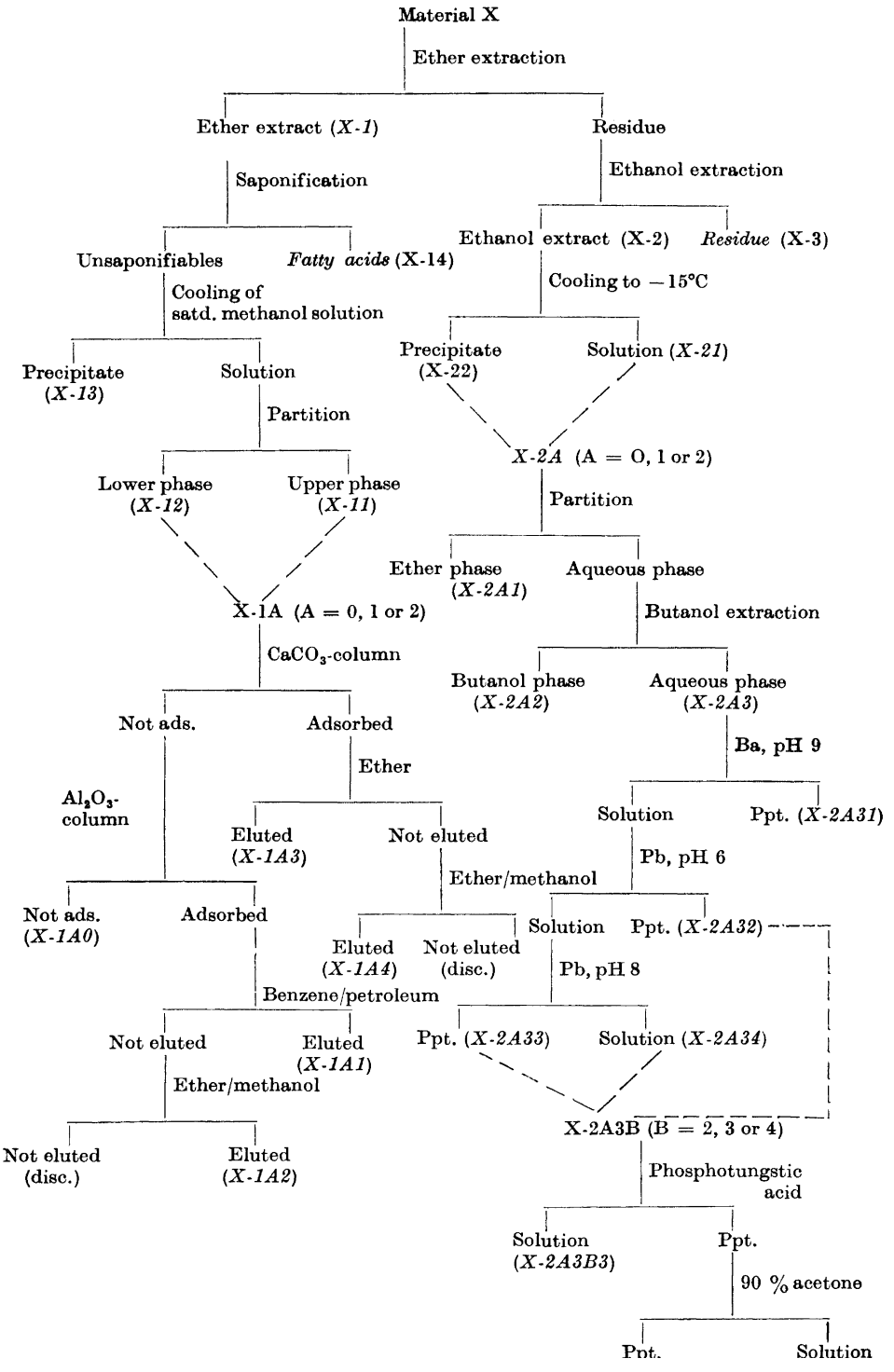
Table 1. Composition of exploratory diets.

Ingredients	Composition of diets in grams					
	S-02.2A	S-04.2A	S-05.2A	S-06.2A	S-21.2A	S-25.2A
Casein, Vitamin Test [a]	70	—	—	—	—	—
Casein, Stege [b]	—	70	—	—	150	170
Gelatine	30	30	30	—	80	80
Pancreas powder [c]	—	—	70	100	80	—
Dried skimmed milk	—	—	—	100	—	—
Salt mixture[c]	50	50	50	50	50	50
Vitamin-sucrose mixture [d]	105	105	105	105	105	105
Ground corn	200	200	200	200	—	400
Ground barley	200	200	200	200	—	—
Ground oats	100	100	100	—	—	—
Wheat bran	80	80	80	80	—	—
Dried alfalfa	60	60	60	60	60	60
Refined peanut oil	50	50	50	50	50	50
Sucrose	55	55	55	55	425	85

All chickens received vitamins A and D as described by Dam and Søndergaard [2].

a) From Genatosan Ltd, Loughborough, England.
b) From A/S Dansk Mejeri Industri & Export Kompagni, Stege, Denmark.
c) As described by Dam and Søndergaard [2].
d) As described by Sørbye and Kruse [1].

Diagram 1. General scheme for extraction and fractionation.



Extracts and fractions were prepared as aqueous solutions, emulsions, oils, or mixed with sugar and made into pellets. They were fed as supplements to the deficient diet for 4 (3—5) days in succession. The *x*-factor level was then examined on the 5th (4th—6th) day.

General extraction and fractionation procedures

Extraction of essential food ingredients. All food ingredients, which proved essential for maintenance of maximal *x*-factor activity, were subjected to extraction in a Soxhlet extractor. The materials were first extracted with ethyl ether for 20 h. If the active factor could not be extracted with ether, the ether-extracted material was next extracted with hot 96 % ethanol for 24 h. All active extracts were subjected to further fractionation.

Fractionation of ether extracts (Fractions 1).

Saponification was performed with 5 ml of methanol and 1 ml of 60 % potassium hydroxide per g of extract by refluxing under nitrogen for 1/2—3/4 h.

Unsaponifiable matter was isolated after addition of water to the soap solution by extraction trice with ether. The combined ether extracts were washed free from alkali and dried over sodium sulfate.

Fatty acids (fraction 14) were isolated from the ether extracted soap solution after acidification by extraction with three lots of ether. The extracts were washed free of acid and dried over sodium sulfate.

The bulk of sterols were separated from the rest of the unsaponifiable matter by dissolving the unsaponifiables in a minimum of hot methanol. The main part of the sterols separated out after storage overnight at —15°C, was filtered off and washed with cold methanol (fraction 13).

Partition. The sterol-poor, unsaponifiable matter was dissolved in a small amount of light petroleum (b.p. 40—70°C). To the petroleum solution were added 9/10 vols. of methanol and 1/10 vol. of water. The mixture was shaken and allowed to separate. The lower phase was drawn off, and the same procedure was repeated 9 more times with the upper phase. The final upper phase was washed free from methanol, dried over sodium sulfate and concentrated to a suitable volume (fraction 11). The lower phases were combined and extracted with ether after addition of water. The ether extract was washed, dried over sodium sulfate and finally dissolved in ether, or in petroleum containing a trace of ethanol (fraction 12).

Chromatography. When the activity of the unsaponifiable matter had been located to the upper- or lower-phase material, or to both, further fractionation was attempted by chromatography. The active fractions (fraction 1A) were dissolved in light petroleum and first applied to a column of calcium carbonate * (1 g of adsorbent for 2—3 g of original oil or ether extract). The column was then washed with light petroleum (6-7 ml per g of adsorbent). The material, adsorbed on the column, was eluted with ether (fraction 1A3) and/or with mixtures of ether and methanol (fraction 1A4).

The material, washed out of the calcium carbonate column by light petroleum, was concentrated and chromatographed on alumina ** (1 g of adsorbent per 1—2 g of original oil or ether extract). The column was washed with light petroleum (6-7 ml per g of adsorbent), giving fraction 1A0, and eluted with a benzene-petroleum mixture (fraction 1A1), ether and/or ether-methanol (fractions 1A2).

Fractionation of ethanol extracts (fractions 2).

Ethanol extracts, containing the active principle of an essential dietary component, were stored overnight at —15°C and thus separated into a cold-ethanol soluble (fraction 21) and a cold-ethanol insoluble part (fraction 22). In many cases the active principle was

* $CaCO_3$ (Merck, for chromatography) was heated at 125°C for 16-20 h before use.

** Al_2O_3 (Merck, for chromatography) was washed free from alkali and U.V. adsorbing material by percolation with hot water and with hot methanol. The washed product was dried at 160°C for 48 h. Before use it was activated at 190°C for 20 h.

concentrated in one of these fractions only. Usually both fractions were distributed separately in a water:ethanol:ether solvent system as follows: to the solution (fraction 21) or suspension (fraction 22) of the fractions in ethanol were added 3 vols. of ether and 3 vols. of distilled water. The mixtures were shaken and allowed to separate.

Ether layers (fractions 2A1) were evaporated to dryness under reduced pressure, and emulsified in 15 % aqueous ethanol with small amounts of Tween 80. (Ethanol was added to all aqueous fractions to prevent deterioration on storage).

Aqueous phases were concentrated on a steam bath and extracted twice by shaking with 1/2—1/3 vol. of *n*-butanol. The combined *butanol extracts* (fractions 2A2) were concentrated by evaporation under reduced pressure, washed with ether and finally dissolved in 15 % aqueous ethanol containing trace amounts of Tween 80.

The *butanol extracted solutions* (fractions 2A3) were freed from butanol by extraction with ether, concentrated and finally dissolved in 15 % aqueous ethanol.

In these studies all or the major part of the activity of an ethanol extract was found in the butanol-extracted aqueous solutions, and further fractionation of the aqueous solutions was attempted by *fractionation with metal salts.*

Aqueous fractions carrying an active principle (fraction 2A3) were further purified by precipitation with barium hydroxide: A solution of barium hydroxide was added until pH was 9.5—10. The resulting precipitate was separated by centrifugation after storage at 5°C overnight. The precipitate was regenerated by addition of dilute sulfuric acid to pH 2.5 or slightly below, heating on a steam bath for 1 h and filtration. The filtrate was adjusted to pH 6 with sodium hydroxide. To the solution was finally added 15 % ethanol (fraction 2A31).

Precipitation with lead acetates. The supernatant after precipitation with barium hydroxide was brought to pH 6 with dilute sulfuric acid. A solution of neutral lead acetate (10 %) was added until precipitation was complete. The precipitate was separated by centrifugation after storage at 5°C overnight. The precipitate was suspended in water and regenerated by addition of dilute sulfuric acid to bring pH below 2.5, heating on a steam bath for one hour and filtration. The filtrate was then treated with hydrogen sulfide gas to remove the last traces of lead. Excess hydrogen sulfide was removed by heating on a steam bath, and pH was brought to 6 with sodium hydroxide. The solution was filtered and 15 % ethanol added (fraction 2A32).

The supernatant after precipitation with neutral lead acetate at pH 6 was brought to pH 8 with barium hydroxide. A solution of basic lead acetate (10 %) was added until precipitation was complete. pH 8 was maintained by continuous addition of barium hydroxide solution. The resulting precipitate was separated by centrifugation after storage at 5°C overnight. The precipitate was regenerated as described for the neutral lead acetate precipitate and dissolved in 15 % aqueous ethanol (fraction 2A33).

The supernatant after precipitation with basic lead acetate was also regenerated in the same way as the two fractions precipitated with neutral and basic lead acetates and finally dissolved in 15 % aqueous ethanol (fraction 2A34).

When the active factor was concentrated in one or more of the fractions not precipitated by barium (fractions 2A3B), further fractionation of the active fraction(s) was attempted by

Precipitation with phosphotungstic acid. The active fraction(s) (2A3B) was dissolved in 5 % sulfuric acid, and a 10 % solution of phosphotungstic acid was added until precipitation was complete. After storage at 5°C overnight the solution was centrifuged. The precipitate was treated twice with acetone-water (9:1) and separated into a soluble fraction (2A3B1) and a fraction, insoluble in 90 % acetone (fraction 2A3B2). Both these fractions and the filtrate after precipitation with phosphotungstic acid (fraction 2A3B3) were regenerated by addition of barium hydroxide solution to pH 9—9.5, heating on a steam bath for one hour and filtration. Excess barium in the filtrate was precipitated by addition of dilute sulfuric acid and the excess of sulfuric acid was finally neutralized with sodium hydroxide. After filtration and concentration, 15 % ethanol was added to all solution.

EXPERIMENTS AND RESULTS

Through a series of experiments it became apparent that *x*-factor concentration in plasma from chickens was related to the chicken diet. The first

Table 2. $\varkappa$-factor activity of chickens on exploratory diets.

Chicken No.	Diet	$\varkappa$-factor level in % of maximal
5924	Commercial	80
6209	»	45
8024	»	75
8044	»	40
1990	S-21.2A	95
8335	»	55
6325	S-02.2A	25
6874	»	65
8839	»	60
7168	S-25.2A	40
7169	»	70
7170	»	70
2406 * First test	S-21.2A	100
2406 * Second test	S-21.2A	78
2406 * Third test	S-21.2A	73
2406 * Fourth test	S-21.2A	68

* The blood samples were taken at intervals of seven days.

experiments seemed to indicate that the $\varkappa$-factor levels of chickens on different diets were markedly different, but it was soon realized that the $\varkappa$-factor level of chickens, fed the same diet, also showed similar, individual differences (Table 2). Chickens with a high level of $\varkappa$-factor in the first plasma sample usually showed decreasing levels of $\varkappa$-factor in subsequent plasma samples taken at intervals of seven days. The supply of vitamins was above usual requirements with these diets, and it was therefore suspected that the observed variations in $\varkappa$-factor level might reflect variations in the available amounts of *other nutritional factors* which were not provided in adequate amounts with these diets.

A change of diet often resulted in a change of $\varkappa$-factor activity of the plasma (Table 3). In some cases, a dietary change was followed by an initial increase in $\varkappa$-factor concentration. On prolonged administration of the new diet the $\varkappa$-factor level, however, again declined. This course of events was also observed after supplementation of a vitamin K-deficient diet with Synkavit. The $\varkappa$-factor concentration first rose to the maximal level. On prolonged feeding of this supplemented diet, the $\varkappa$-factor level dropped below maximal. An adequate diet was finally worked out [diet No. S-06.2A). This diet was able to raise the $\varkappa$-factor concentration in all chickens to a level which appeared to be the *maximal level of* $\varkappa$*-factor* in chicken plasma, and to maintain the $\varkappa$-factor concentration at this maximal level. Diet No. S-06.2A apparently provided adequate supply of all nutrients, essential for maximal $\varkappa$-factor activity. Plasma from chickens raised on this diet, therefore, served as a reference, representing maximal or 100 % activity of $\varkappa$-factor.

After establishment of the adequate diet (S-06.2A), the importance of the various ingredients for maximal $\varkappa$-factor activity could be determined.

Table 3. Influence of dietary changes on ϰ-factor activity.

Chicken No.	Sequence of diets	Length of dietary periods	ϰ-factor level at end of period, in %
6323	No. S-02.2A	6 weeks	80
	No. S-04.2A	1 week	55
8717	Commercial	6 weeks	50
	No. S-05.2A	1 week	90
		2 weeks	70
		3 weeks	65
1981	Vitamin K-deficient [a])	2 weeks	17 [c])
	Do. + Synkavit [b])	1 week	100
	(10 mg per kg of diet)	2 weeks	80
		3 weeks	70
8715	Commercial	6 weeks	35
	No. S-02.2A	1 week	85
	No. S-06.2A	1 week	100
		2 weeks	100
9044	No. S-21.2A	4 weeks	not measured
	No. K-13.3	1 week	100
		2 weeks	100
		3 weeks	100
		4 weeks	100

a) as described by Dam and Søndergaard [2].
b) Bicalcium-2-methyl-1,4-naphthohydroquinone biphosphate (Roche).
c) Thromboplastin coagulation time of plasma was 84 min/100, compared to 24—25 for normal plasma.

A series of diets (Table 4) were prepared and tested, each lacking one of the ingredients of diet No. S-06.2A. After 2—4 weeks on these diets, chickens normally had ϰ-factor levels below 65—75 % of maximal. Diet No. K-13.3 was an exception. Chickens on this diet were able to maintain their ϰ-factor activity at 100 %. The active factor in corn could thus be supplied by corn oil alone, and ether extracted corn was found unessential for maximal ϰ-factor activity in these studies. The low ϰ-factor activity produced by diet K-5.2 proved that corn oil could not be replaced by refined peanut oil. The low ϰ-factor activity produced by diets K-6 and K-11 showed that the essential material supplied by pancreas powder was not supplied by similar amounts of dietary casein.

It was thus evident that barley, wheat bran, corn, pancreas powder, dried skimmed milk and alfalfa were supplying nutrients essential for maximal ϰ-factor activity.

The diets K-1, K-2, K-5.2, K-6 or K-11, K-12, and K-14 were adopted as basal diets since chickens, given these diets, would have plasma ϰ-factor levels

Table 4. Composition of experimental diets.

Ingredients	Composition of diets in grams							
	K-1	K-2	K-5.2	K-6	K-11	K-12	K-13	K-14
Casein, Vitamin Test [a]	—	—	—	150	—	—	—	—
Casein, Stege [b]	—	—	—	—	150	—	—	—
Pancreas powder [c]	150	150	150	—	—	150	150	150
Dried skimmed milk	100	100	100	100	100	—	100	100
Salt mixture [c]	50	50	50	50	50	50	50	50
Vitamin-sucrose mixture [d]	105	105	105	105	105	105	105	105
Ground corn	250	250	—	250	250	250	—	250
Ether-extracted ground corn	—	—	200	—	—	—	—	—
Ground barley	—	200	200	200	200	200	200	200
Wheat bran	100	—	85	85	85	85	85	85
Dried alfalfa	60	60	60	60	60	60	60	—
Refined peanut oil	—	—	50	—	—	—	—	—
Corn oil	—	—	—	—	—	—	50	—
Sucrose	185	85	—	—	—	100	200	60
Vitamins A and D[2]								

a—d *cf.* footnote to Table 1.

specifically sensitive to the active principles in barley, wheat bran, corn oil, pancreas powder, dried skimmed milk and alfalfa, respectively.

Extraction of the active principles from the various sources could thus be attempted and studies of their behavior during fractionation procedures started.

1. *Concentration of the active principles in barley.* In Table 5 are summarized the results from testing of the various fractions obtained from barley. It will be seen that the active factor was not extracted by ether (By-1), and that the residue after ethanol extraction was inactive (By-3). After partition of the ethanol extracts (By-21 and By-22), the ether phases (By-211 and By-221) were without activity. Butanol extracts (By-212 and By-222) of the lower phases were not significantly active. All activity was found in By-223, whereas By-213 was inactive. This indicated that the active factor was completely precipitated from the original ethanol extract by cooling to —15°C.

Further fractionation of By-223 revealed that the active factor could not be precipitated by barium at pH 9 (By-2231) and not with neutral lead acetate at pH 6 (By-2232), but was precipitated by basic lead acetate at pH 8 (By-2233) with no demonstrable activity being left in the filtrate (By-2234).

After treatment of By-2233 with phosphotungstic acid the filtrate fraction (By-22333) was inactive, indicating that the active factor had been completely precipitated. The slight activity of By-22331, but pronounced activity of By-22332, showed that the active factor formed a compound with phosphotungstic acid which was almost insoluble in 90 % acetone.

2. *Concentration of the active factor in wheat bran.* In Table 6 are summarized the results from testing of the various fractions obtained from wheat bran. It will be seen that the active factor was not extracted by ether (Hk-1), and

Table 5. Effect of barley fractions fed as supplements to diet No. K-1.

Supplement	Daily amounts, in equivalents of unfractionated barley	x-factor level in %	
		Before feeding of supplement	After feeding of supplement
By-1	30 g	70	50
By-3	20 % of diet	70	70
By-211 + By-221	20 g	65	55
By-212 + By-222	20 g	55	60
By-213	20 g	60	50
By-223	20 g	50	100
By-2231	15 g	65	60
By-2232	15 g	60	60
By-2233	15 g	65	100
By-2234	15 g	70	65
By-22331	15 g	60	65
By-22332	15 g	65	100
By-22333	15 g	65	65

that the residue after ethanol extraction was inactive (Hk-3). After partition of the ethanol extracts (Hk-21 and Hk-22) the ether layers (Hk-211 and Hk-221) did not contain the active factor. Butanol extracts (Hk-212 and Hk-222) of the lower phases were inactive. The butanol extracted solutions carried all activity. The slight activity of Hk-213, but pronounced activity of Hk-223, showed that the main part of the active factor had been precipitated by cooling the original ethanol extract to −15°C.

Further fractionation of Hk-223 revealed that the active factor could not be precipitated by barium at pH 9 (Hk-2231). It was not precipitated by neutral

Table 6. Effect of wheat bran fractions fed as supplements to diet No. K-2.

Supplement	Daily amounts, in equivalents of unfractionated wheat bran	x-factor level in %	
		Before feeding of supplement	After feeding of supplement
Hk-1	20 g	55	50
Hk-3	8 % of diet	70	55
Hk-211 + Hk-221	10 g	65	65
Hk-212 + Hk-222	10 g	65	60
Hk-213	10 g	60	65
Hk-223	10 g	50	100
Hk-2231	8 g	—	70
Hk-2232	8 g	65	70
Hk-2233	8 g	—	65
Hk-2234	8 g	70	100
Hk-22341	8 g	70	55
Hk-22342	8 g	75	100
Hk-22343	8 g	65	65

Table 7. Effect of pancreas powder fractions fed as supplements to diet No. K-6.

Supplement	Daily amounts, in equivalents of unfractionated pancreas powder	x-factor level in %	
		Before feeding of supplement	After feeding of supplement
Pa-3	15 % of diet	65	60
Pa-211 + Pa-221	10 g	75	60
Pa-212 + Pa-222	10 g	60	55
Pa-213	10 g	55	100
Pa-223	10 g	60	100
Pa-2031	10 g	70	65
Pa-2032	10 g	65	60
Pa-2033	10 g	75	100
Pa-2034	10 g	60	70
Pa-20331	10 g	75	60
Pa-20332	10 g	75	100
Pa-20333	10 g	65	70
Pa-20333	10 g	90	75

lead acetate at pH 6 (Hk-2232), nor with basic lead acetate at pH 8 (Hk-2233), but remained in the final filtrate (Hk-2234).

After treatment of Hk-2234 with phosphotungstic acid, the filtrate fraction (Hk-22343) was inactive, indicating that the active factor had been completely precipitated. No activity was found in Hk-22341, but pronounced activity in Hk-22342, showing that the active factor formed a compound with phosphotungstic acid which was insoluble in 90 % acetone.

3. *Concentration of the active factor in pancreas powder.* In Table 7 are summarized the results from testing of the various fractions obtained from ether extracted pancreas powder.

The active factor was not present in the residue after ethanol extraction (Pa-3). After partition of the ethanol extracts (Pa-21 and Pa-22), the ether layers (Pa-211 and Pa-221) were without activity. Butanol extracts (Pa-212 and Pa-222) of the lower phases were inactive. The butanol extracted solutions

Table 8. Effect of corn oil fractions fed as supplements to diet No. K-5.2.

Supplement	Daily amounts, in equivalents of unfractionated corn oil	x-factor level in %	
		Before feeding of supplement	After feeding of supplement
Ma-11	5 g	70	100
Ma-12	5 g	60	100
Ma-13	8 g	75	70
Ma-14	5 g	—	75
Ma-100 + Ma-101	5 g	75	65
Ma-102	5 g	60	100
Ma-103	5 g	70	60

Table 9. Effect of dried skimmed milk fractions fed as supplements to diet No. K-12.

Supplement	Daily amounts, in equivalents of unfractionated dried skimmed milk	κ-factor level in %	
		Before feeding of supplement	After feeding of supplement
Tm-1	10 g	50	60
Tm-3	10 % of diet	60	50
Tm-211 + Tm-221	10 g	65	65
Tm-212 + Tm-222	6 g	65	80
Tm-213	6 g	80	100
Tm-213	6 g	55	100
Tm-223	10 g	60	100
Tm-2031	6 g	—	60
Tm-2032	7 g	65	100
Tm-2033	7 g	75	70
Tm-2034	7 g	75	70
Tm-20321	7 g	70	85
Tm-20322	7 g	70	100
Tm-20323	7 g	80	65

carried all activity. Pa-213 and Pa-223 were both active and thus showed that the active factor had been only partly precipitated from the original ethanol extract by cooling to −15°C. The two fractions were therefore combined and designated Pa-203.

Further fractionation of Pa-203 revealed that the active factor could not be precipitated by barium at pH 9 (Pa-2031), and not with neutral lead acetate at pH 6 (Pa-2032). The precipitate obtained by addition of basic lead acetate at pH 8 (Pa-2033) contained the active factor, and very little activity, if any, was left in the filtrate (Pa-2034).

After treatment of Pa-2033 with phosphotungstic acid the filtrate was inactive (Pa-20333). The active factor had been completely precipitated. Activity was found in Pa-20332, but not in Pa-20331, showing that the active factor formed a compound with phosphotungstic acid, which was insoluble in 90 % acetone.

4. *Concentration of the active factor in corn oil.* In Table 8 are summarized the results from testing of the various fractions obtained from corn oil.

After saponification of corn oil and fractionation of the unsaponifiable matter, the active material was concentrated in the fractions Ma-11 and Ma-12. This meant that the active factor in corn oil was unsaponifiable and did not belong to the sterols. The sterol fraction (Ma-13) and the fatty acids (Ma-14) were inactive.

The two active fractions (Ma-11 and Ma-12) were combined (Ma-10) and chromatographed. After washing this solution through a calcium carbonate column the adsorbed material was eluted with ether (Ma-103) and found to be inactive. The active factor was adsorbed on an alumina column. The fraction passing through this column by washing with light petroleum (Ma-100) and the material eluted with 30 % benzene in light petroleum (Ma-101) were inac-

Table 10. Effect of alfalfa fractions fed as supplements to diet No. K-14.

Supplements	Daily amounts, in equivalents of unfractionated alfalfa	x-factor level in %	
		Before feeding of supplement	After feeding of supplement
Lu-1	5 g	60	60
Lu-3	6 % of diet	70	60
Lu-211 + Lu-221	3 g	75	65
Lu-212 + Lu-222	4 g	—	70
Lu-213	4 g	70	65
Lu-223	5 g	60	100
Lu-2231	4 g	65	60
Lu-2231	4 g	80	65
Lu-2232	4 g	65	65
Lu-2233	4 g	65	65
Lu-2234	4 g	85	100
Lu-22341	4 g	—	70
Lu-22342	4 g	65	100
Lu-22343	4 g	70	65

tive. The active factor could be eluted with 10 % methanol in ethyl ether (Ma-102).

5. *Concentration of the active factor in dried skimmed milk.* In Table 9 are summarized the results from testing of the various fractions obtained from dried skimmed milk. The active factor was not extracted with ether (Tm-1), and the residue after ethanol extraction was inactive (Tm-3). After partition of the ethanol extracts the ether layers (Tm-211 and Tm-221) were inactive. Butanol extracts (Tm-212 and Tm-222) of the lower phases contained some activity, but the main part remained in the auqeous phases (Tm-213 and Tm-223). The latter fractions were both active, and the active factor had thus only been partly precipitated by cooling the original ethanol extract to −15°C. The two fractions were combined and designated Tm-203.

Further fractionation of fraction Tm-203 revealed that the active factor could not be precipitated by barium at pH 9, but was precipitated by addition of neutral lead acetate at pH 6 (Tm-2032). No further activity was precipitated by basic lead acetate at pH 8 (Tm-2033), and no activity remained in the final filtrate (Tm-2034).

After treatment of Tm-2032 with phosphotungstic acid, the filtrate was inactive (Tm-20323). The active factor had thus been precipitated completely. Some activity was found in Tm-20321, but the main part was in Tm-20322, showing that the active factor formed a compound with phosphotungstic acid which was slightly soluble in 90 % acetone.

6. *Concentration of the active factor in alfalfa.* In Table 10 are summarized the results from testing of the various fractions obtained from dried alfalfa. The active factor could not be extracted with ether (Lu-1), and no activity was left in the residue after ethanol extraction (Lu-3).

After partition of the ethanol extracts (Lu-21 and Lu-22) the ether layers (Lu-211 and Lu-221) were found to be inactive. Butanol extracts (Lu-212 and Lu-222) of the lower phases were inactive. Of the butanol extracted solutions, only Lu-223 was active, whereas Lu-213 had no demonstrable activity. This showed that the active factor had been precipitated completely by cooling the original ethanol extract to −15°C.

Further fractionation of Lu-223 revealed that the active factor could not be precipitated by barium at pH 9 (Lu-2231). The precipitate obtained by addition of neutral lead acetate at pH 6 (Lu-2232) was inactive. So was the precipitate obtained by addition of basic lead acetate at pH 8 (Lu-2233). The active factor remained in the final filtrate (Lu-2234).

After treatment of Lu-2234 with phosphotungstic acid, the filtrate was inactive (Lu-22343). The active factor had been precipitated completely. Lu-22341 was inactive, whereas Lu-22342 carried all activity, showing that the active factor in alfalfa had formed a compound with phosphotungstic acid which was insoluble in 90 % acetone.

DISCUSSION

The experiments demonstrate that the level of $\varkappa$-factor in chicken plasma depends on dietary factors, and that $\varkappa$-factor concentration cannot by dietary means be raised above a certain level, which is regarded as the maximal level of $\varkappa$-factor in chicken plasma. $\varkappa$-factor concentrations below the maximal level have, so far, always turned out to be due to dietary deficiencies.

The design of an adequate diet — able to maintain $\varkappa$-factor at the maximal level during the experimental periods — and of deficient diets — producing lower levels of $\varkappa$-factor — permitted a study of the active principles in the various nutrients of the complete diet, which were found to be essential for maximal $\varkappa$-factor concentration in plasma. So far, work on extraction and preliminary concentration of the active principles has shown that fractions, carrying the activity, may be separated from the bulk of inactive material by conventional methods. The activity of each essential nutrient thus seems to be due to its content of a special *nutritional factor* which is not found in comparable quantities in the other ingredients of the adequate diet. Thus, the effect of corn in the diet is due to a fat-soluble substance, present in corn oil. It is non-saponifiable and does not seem to be sterol. From its behavior on partition and chromatography one might expect it to be a hydrocarbon with a relatively small number of polar groups.

The effects of the other five essential ingredients are due to alcohol-extractable, water-soluble substances. None of the factors can be precipitated from aqueous solutions by barium hydroxide. Some, however, form insoluble compounds with lead, whereas others are not precipitated from aqueous solutions on addition of lead salts. All the water-soluble factors form precipitates with phosphotungstic acid which show, at most, a limited solubility in 90 % acetone. One might therefore expect these factors to be nitrogen-containg compounds with a relatively complex structure.

It seems logical to conclude that the $\varkappa$-factor level in chicken plasma is controlled by a set of nutritional factors, each of which may act as a limiting factor for $\varkappa$-factor formation.

Depending on their previous dietary situation chickens may have greater or smaller stores of these factors. Only when these stores have been exhausted, the $\varkappa$-factor will drop and indicate a dietary insufficiency. One should therefore expect that diets may have "hidden deficiencies". If, *e.g.*, a certain diet limits $\varkappa$-factor level to 20—30 % of maximal through the amount of one essential dietary factor, the chicken will be able to build up stores of other essential dietary factors, even if these factors are provided in amounts below that required for maximal $\varkappa$-factor level. On admission of adequate amounts of the limiting factor, it might be expected that $\varkappa$-factor would show a rise, even up to 100 %. With this increased output of $\varkappa$-factor, the stores of the other essential factors, not adequately provided by the diet, would sooner or later be exhausted, whereby one of these factors would become the limiting factor for the $\varkappa$-factor level. Consequently, the $\varkappa$-factor concentration would gradually drop to a level determined by the dietary supply of this factor. This might be the explanation for the transient increases observed after changing diets. The experiment, in which a vitamin K-deficient diet was supplemented with Synkavit, was particularly illustrating. After Synkavit supplementation a rise to 100 % was observed. Then the $\varkappa$-factor level again declined, indicating that dietary factors, other than vitamin K, had become limiting factors for the $\varkappa$-factor level.

In studies of this nature one must obviously always be prepared to find $\varkappa$-factor deficiencies due to dietary insufficiencies not observed previously. The demand for dietary factors in addition to those described may in the present studies have been covered by reserves obtained from the egg, or provided by the earliest diet, or even supplied through the activities of the intestinal flora. The present work only indicates that at least six unidentified nutritional factors, in addition to vitamin K, are involved directly or indirectly in the formation of $\varkappa$-factor. The reports by Howard and co-workers [3,4] may in this connection be seen as another example of relations between dietary factors and blood coagulation factors. These workers demonstrated that plasma from scorbutic guinea-pigs was not activated by glass, showed decreased blood thromboplastin formation and had prolonged Quick time, but normal coagulation time with Russel's viper venom-cephalin. These symptoms were prevented by vitamin C. The reports indicate a role of vitamin C in the formation of the glass activable factor(s), which act in the intrinsic coagulation system, and of a factor (or factors) acting in the extrinsic system.

The present work gives no information as to the mode of action of vitamin K and the six unidentified factors in determining the $\varkappa$-factor level of plasma. However, each of these nutritional factors probably plays a specific part in the mechanism for formation of $\varkappa$-factor, since none of the six unidentified factors can replace vitamin K or any of the other five, and vitamin K cannot replace any of the six unidentified factors. In the same sense as vitamin K is regarded as a vitamin for formation of $\varkappa$-factor, prothrombin, proconvertin and other coagulation factors, it might be justified to regard the six unidentified nutritional factors as vitamins or co-vitamins for formation of $\varkappa$-factor.

Whether or not they are of importance for formation of other proteins or coagulation factors is not known at present. Their classification as true vitamins will of course, depend on determination of the quantities in which they are required, or rather by deciding whether they serve as catalysts for essential steps in the formation of $\varkappa$-factor or as structural units in the $\varkappa$-factor molecule. In the latter case the designation vitamin might not be proper. These problems must await the preparation of the pure compounds. Much work remains to be done before this eventually becomes possible.

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