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Studies on the Coagulation of Chicken Blood

XIII. Dietary Factors Limiting the π-Factor Level: Identification and Function of Phytoene

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Further studies of the fat soluble factors correcting diet-induced π-factor deficiency in chickens are reported. Phytoene was identified as the active factor in corn oil and cod liver oil. The minimum requirement for complete correction of severe deficiencies was 2×10^{-4} μg per g of body weight when given orally for 3 days in succession. After intravenous injections of phytoene a lag period of about 10 min was observed. A rapid increase in π-factor concentration ensued, reaching the maximum level about 35 min after the injection.

Unidentified, active factors present in lard, chicken liver fat, and whale liver oil, appear to be metabolically related to phytoene. Phytoene may be the precursor of the lard factor. Both seem to be precursors of two unidentified factors in chicken liver fat. One of the latter and the whale liver oil factor are probably identical and possibly the ultimate metabolite responsible for the observed effect, presumably a stimulation of π-factor biosynthesis.

In a preceding paper[1] maximum level of the π-factor component of the labile factor activity in chicken plasma was shown to depend on a simultaneous dietary supply of (1) choline, (2) an unidentified, water soluble factor present in pancreas powder, and (3) either one of a group of mutually replaceable, unidentified fat soluble factors present in corn oil, cod liver oil, lard, and whale liver oil.

The identity of the corn oil (and cod liver oil) factor, its function and possible relationship to the unidentified factors in lard and whale liver oil, are the subjects of the present paper.

MATERIAL AND METHODS

For details and references to details of the materials and methods used in this study, the preceding paper should be consulted.[1]

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Spectrophotometry. A Beckman Model DU and (later) Model DK-2 spectrophotometer were used. All fractions were dissolved in U.V. transparent hexane. Apart from phytoene prepared from tomatoes, none of the most active, chromatographic subfractions contained sufficient material for a reasonably accurate, gravimetric determination of the solutes and evaluation of the purity of the product. A tentative association of biological activity with a characteristic U.V. absorption spectrum proved successful only with the most active subfractions of corn oil and cod liver oil.

Further details on special methods and materials used are given in the text.

EXPERIMENTS AND RESULTS

Elution pattern of the active factors in corn oil, cod liver oil, lard, and whale liver oil

Previous work [1] had shown that the active factors in the above fats were recovered in the epiphasic part of the sterol-poor fraction of the unsaponifiables. The corn oil (and cod liver oil) factor could be separated from the active factors in lard and whale liver oil by elution chromatography on activated alumina. In the present study the optimal conditions for elution of the active factors were studied in greater detail by changing the polarity of the eluent more gradually.

By standardization of the column material and the chromatographic procedure the active factor in each fat was recovered in the same main fractions in separate runs, usually in the same, or neighbouring subfractions at similar loading of the column. Activated alumina [2] was used (1 g per 2—3 g of original fat). Column dimensions varied, with a diameter/height ratio of about 1/10. The epiphasic part of the sterol-poor fraction of the unsaponifiables of each fat (fractions —11 [1]) was applied in hexane or light petrol. Each eluent (5—7 ml per g of adsorbent) was collected in 4—5 subfractions which were tested for activity,[2] separately or combined. The characteristic elution patterns of the active factors are seen from Tables 1 and 2.

Table 1. Elution pattern of the active factor in corn oil and in cod liver oil.

Eluent	Test sample	Corn oil		Cod liver oil	
		Dose[a]	Response[b]	Dose[a]	Response[b]
2 % (v/v) benzene in light petrol (LP)	All sub-fractions	5	≤17	1	≤17
5 % benzene in LP	Head-fraction	5	≤17	1	≤17
	Mid-fractions I/II	5	100	1	100
	Tail-fraction	5	100	1	100
8 % »	Head-fraction	5	100	1	100
	Mid-fractions I/II	5	100	1	100
	Tail-fraction	5	≤17	1	≤17
13 % » and subsequent eluents (*cf.* Table 2)	All sub-fractions	10	≤17	2	≤17

[a] Equivalent dose (in g) of original fat.

[b] π-factor concentration after supplement, in percent of maximum.[1] Basic diet: S—11.2, S—11.2 A, S—21.2 A, or S—36.2 A.[1] Pre-test levels below 17 %.

Table 2. Elution patterns of the active factors in lard and whale liver oil.

Eluent	Test sample	Lard		Whale liver oil	
		Dose[a]	Response[b]	Dose[a]	Response[b]
Prior eluents (*cf.* Table 1) and 30 % (v/v) benzene in light petrol (LP	All sub-fractions	10	≦17	3	≦17
0.6 % acetone in LP	Head-fraction	10	≦17	3	≦17
	Mid-fractions I/II	10	100	3	≦17
	Tail-fraction	10	≦17	3	≦17
1.5 % »	Head-fraction	10	≦17	3	≦17
	Mid-fractions I/II	10	≦17	3	100
	Tail-fraction	10	≦17	3	≦17
5 % » , and 20 % methanol in ethyl ether	All sub-fractions	15	≦17	5	≦17

[a] and [b]: see footnotes to Table 1.

The corn oil factor and the cod liver oil factor (Table 1) appeared in the same subfractions (5 % benzene following 2 %; when more abundant also in the first 8 % benzene eluates), suggesting identity. The non-identity of this factor and the active factors in lard and in whale liver oil was confirmed (Table 2). In addition a clear-cut separation of the latter factors was obtained. The lard factor was confined to one or two of the central sub-fractions of the 0.6 % acetone eluate following 30 % or 40 % benzene, whereas the whale liver oil factor was recovered only in one or two of the central subfractions of the 1 ½ % acetone eluate following 0.6 % acetone.

Thus, *three* distinct molecular species have a closely similar biological effect.

Identification of the active factor in corn oil and cod liver oil

Further purification of the active factor in corn oil and cod liver oil was attempted by large-scale chromatography of pooled samples of the epiphasic unsaponifiables[1] (fractions −11), fractional molecular distillation, and rechromatography. The relation between biological activity and characteristic light absorption bands was studied. An association of the π-factor stimulating activity with an U.V. absorption peak at about 285−286 mμ with shoulders at about 275−276 and 297−298 mμ, became increasingly apparent as the purity of the fractions improved. The most purified fractions had equal activities at equal extinctions at 285 mμ, whereas additional peaks, or secondary shoulders at about 233 and 325 mμ were unrelated to the biological potency of the fractions. Typical absorption spectra of some of these fractions are shown in Fig. 1. (The ordinate scale is arbitrary because of the extremely small amount of solutes in these fractions and gives only the *relative* extinction of *equipotent* fractions). The absorption spectrum of a corresponding, chromatographic fraction of tomato extract, active (see below), but lacking the absorption bands at about 233 and 325 mμ, is inserted for comparison.

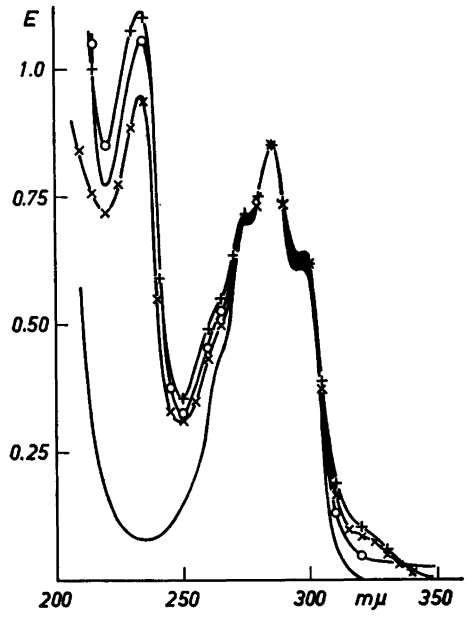

Fig. 1. U.V. absorption spectra of equipotent phytoene fractions. Fractions from cod liver oil (O), corn oil (×), and (+); and from tomatoes (97 % pure).

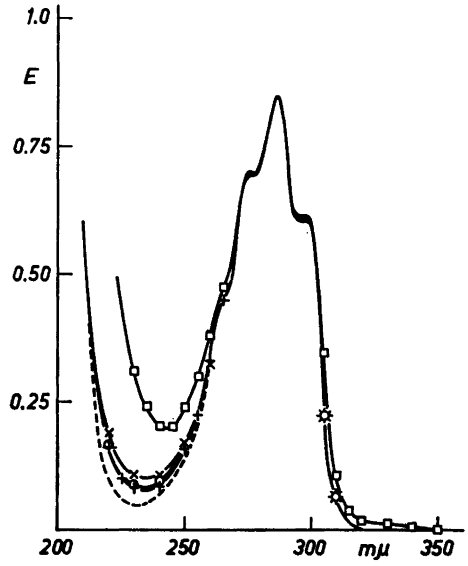

Fig. 2. U.V. absorption spectra of equipotent fractions of phytoene from tomatoes. Phytoene fractions: 97 % pure (+), To—34 and 96 % pure (O), To—42 and 94 % pure (×), To—43 and 80 % pure (□). Pure phytoene [3] (— — —).

At this time phytoene originally prepared from tomatoes and carrot oil [3] had just been characterized as a colorless carotenoid with a central chromophore of three conjugated double bonds [4] and a characteristic absorption spectrum with a main peak at 286 mμ and shoulders at 276 and 298 mμ. Tomato extract prepared according to Rabourn *et al.*[3] proved active (Table 3), and the extract was fractionated by column chromatography on alumina as for the active fractions described above. Active fractions of tomato extract were eluted from *unactivated* alumina with 5 % benzene in light petrol. The first sub-fraction gave the characteristic absorption spectrum of phytoene. In subsequent subfractions the absorption spectrum of phytofluene [5] became more dominant. More polar constituents of the extract (carotenes and lycopene) were eluted with 10 % and 25 % benzene and with ethyl ether, and proved inactive (Table 3). A better separation of phytoene and phytofluene was obtained in large scale work (extract of 10 kg of tomatoes) with activated alumina.[2] Biological activity was associated only with the phytoene spectrum and was proportional to the extinction at 286 mμ. Phytofluene fractions with no spectral evidence of phytoene contamination showed no demonstrable activity when tested at more than 100 times the minimum dose of phytoene for maximum response. The effect of some of these fractions is recorded in

Table 3. Effect of tomato extract and derived chromatographic fractions.

Column	Eluent	Test sample	Daily dose[a]	Response[b]
		Whole extract	10	100
Unactivated alumina	Light petrol (LP)	Combined fractions	20	≤17
	5 % benzene in LP	Head-fraction	20	100
			1	100
			0.1	57
		Mid-fraction I	20	100 (33)
		Mid-fraction II	20	37 (20)
		Tail-fraction	20	≤17 (23)
	10 %, 25 % benzene in LP, and ethyl ether	All sub-fractions	20	≤17
Activated alumina	2 % benzene in LP	Combined fractions	20	≤17
			Daily dose[c]	
	5 % benzene in LP	Head + mid-fractions	0.18 phytoene	83
		Tail-fraction (To−34)	0.13 »	37
			0.18 »	83
			0.20 »	100
			0.22 »	100
			2.0 »	100
	10 % benzene in LP	Mid-fraction I (To−42)	0.20 »	100
		Mid-fraction II (To−43)	0.16 »	67
			0.18 »	87
	15 % benzene in LP	Tail-fraction	3.8 phytofluene	23 (27)
	25 % »	Mid-fraction II	25 »	≤17
			0.126 phytoene	33
		Rechromatographed phytoene (97 % pure)	0.134 »	40
			0.160 »	67

[a] Equivalent dose (in g) of tomatoes.
[b] See footnote *b* in Table 1. Pre-test levels above 17 % given in brackets.
[c] In μg per kg of body weight.

Table 3, and some of the corresponding absorption spectra of the phytoene fractions are given in Fig. 2. As in Fig. 1 the ordinate scale is arbitrary and gives the relative extinction of equipotent fractions. The spectrum of pure phytoene [3] is inserted for comparison. The purest fractions obtained regularly in this work had $E\,{}^{1\,cm}_{1\,\%} = 820-825$ at 286 mμ. The content of phytoene and phytofluene in the fractions was calculated from the extinction values reported in the literature, *viz.* $E\,{}^{1\,cm}_{1\,\%} = 850$ at 286 mμ for phytoene,[3] and $E\,{}^{1\,cm}_{1\,\%} = 1350$ at 348 mμ for phytofluene.[6] The best preparations of phytoene obtained from tomatoes were thus about 97 % pure.

The relation between dose and effect on the plasma level of π-factor was established by feeding graded amounts of the most purified phytoene fractions

to chickens of different body weight (between 650 and 1400 g). In a medium range the response increased linearly with the dose. When the phytoene content of highly purified fractions from corn oil and cod liver oil was estimated from extinction values at 286 mμ, the experimental points fitted in with the results obtained with phytoene from tomatoes and gave the dose-response curve shown in Fig. 3. With this evidence it seemed possible to conclude that the effect of corn oil and cod liver oil on the plasma level of π-factor in chickens is due to the content of phytoene.

Some additional experiments were carried out with a preparation of synthetic phytoene kindly supplied by Prof. B. C. L. Weedon, Queen Mary College, London. The preparation was stated to "contain a mixture of all possible stereoisomers about the central triene unit with the all-*trans*-isomer predominating".[7] In repeated tests with this preparation the response increased with the relative increase in dose as with phytoene prepared from natural sources. However, calculations of the *absolute* doses, based on extinction values at 286 mμ and $E^{1\%}_{1cm} = 850$, showed that the synthetic preparation had only 15—16 % of the activity of the compound isolated from tomatoes. This figure may be a "reasonable estimate"[7] of the content of the natural isomer which most certainly is the central *cis*-isomer.[8] It has not yet been possible to obtain samples of the pure, synthetic isomers for biological testing. The results obtained so far seem to indicate that the effect on the plasma level of π-factor may be limited to the isomer with the central *cis*-configuration.

π-Factor response to intravenous injection of phytoene

In all previous experiments the purification of active principles in established, essential dietary components was guided by assay of the partic-

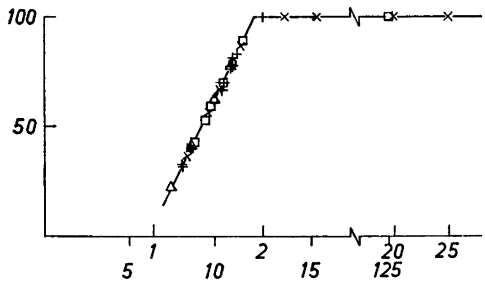

Fig. 3. Dose-response curve for phytoene. Ordinate: π-factor concentration in % of maximum. Abscissa: dose in μg per 10^4 g of body weight; upper scale: phytoene from cod liver oil (O), corn oil (×), tomatoes (+); lower scale: synthetic phytoene (□); in relative units: phytoene fraction from corn-fed chicken liver (△).

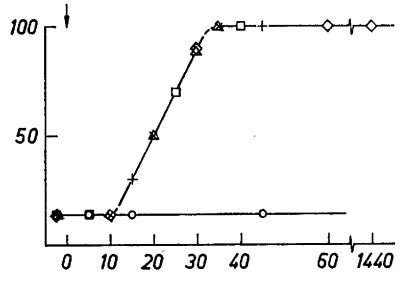

Fig. 4. Response to intravenous injection of phytoene. Ordinate: as in Fig. 3. Abscissa: time in min. Arrow indicates time of injection. Experimental chickens (+, ×, □, ◇, △). Control (O).

ular coagulation factor before and after *oral* ingestion of the fractions obtained.[1,2,9] The preparation of phytoene of 96—97 % purity encouraged studies on the effect of *intravenous* administration of this compound on the π-factor level. Pilot experiments showed that the route of administration was not critical. The action of phytoene is thus not mediated *via* the intestinal flora. Studies on the time course of this action were then carried out.

For these experiments phytoene (in hexane solution) was mixed with 0.05 g Tween 80 and 0.4 ml ethanol. (Pure hexane was used in control experiments). The solvents were blown off under nitrogen and a clear solution obtained on addition of 0.5 ml saline. This volume, supplying 2—3 times the minimum oral dose for maximum effect, was injected into the jugular vein

Table 4. In vivo versus in vitro effects of phytoene.

Dose of phytoene[a]	Blood sample[b]	Labile factor factor activity		π-factor concn.[c]
		Total	π-factor	
0	Pre-injection	100	4	≦17
	5, 15, and 45 min	100	4	≦17
6	Pre-injection	87	2	≃13
	15 min	89.5	4.5	30
	45 min	100	15	100
5	Pre-injection	87	2	≃13
	10 min	87	2	≃13
	20 min	92.5	7.5	50
	35 min	100	15	100
5	Pre-injection	87	2	≃13
	5 min	87	2	≃13
	25 min	95.5	10.5	70
	40 min	100	15	100
6	Pre-injection	87	2	≃13
	10 min	99	2	≃13
	30 min	86	13.5	90
	60 min	100	15	100
	24 h	100	15	100
5	Pre-injection	100	2	≃13
	20 min	86	8	50
	30 min	89.5	14	88
	35 min	89.5	16	100
In vitro addition, doses in μg per 10^3 ml of blood: 0, 0.7, 3.0, 8.9.		100	4	≦17

[a] In μg per 10^4 g of body weight. [b] Time after intravenous injection. [c] In % of maximum.

after the first blood sample was taken. Subsequently, 2—4 samples of blood were drawn from the contralateral vein or the carotid arteries at different time intervals. The π-factor levels in samples drawn within one hour were then determined simultaneously. In another set of experiments phytoene in 0.02 ml Tween-saline was added *in vitro* to π-factor deficient, oxalated blood before separation of the plasma, to give concentration of phytoene up to (and beyond) that obtained by intravenous administration. Even distribution of the injected dose in a blood volume of 7—8 % (v/w) was assumed. The results obtained in one series of experiments with seven different chickens are summarized in Table 4 and Fig. 4.

In vitro addition of phytoene to blood was without effect on the labile factor activity and the level of π-factor. Intravenous injection of the control solution likewise had no effect. After injection of phytoene no changes in π-factor concentration occurred during the first 10—11 min. A rapid increase ensued, and the maximum level was reached 33—35 min after injection. No further change in π-factor concentration occurred during the following 24 h with the phytoene doses tried here. In three of the present experiments the increase in total labile factor activity of the plasma was all due to the increase in π-factor concentration. In two experiments simultaneous (but unrelated?) changes were observed in the activity of the remaining components of the labile factor complex.

Possible relation of phytoene to the active factors in lard and whale liver oil

(1) *Dose-response relationships.* The effect of purified fractions containing the active factor in lard or whale liver oil increased linearly with increasing doses as shown for phytoene from tomatoes, corn oil, and cod liver oil. The absolute amounts of solutes administered could not be determined accurately because of the small quantities present in the purified fractions. Attempts to relate U.V. absorption peaks or bands with biological activity were unsuccessful, but by suitable choice of abscissa scales different subfractions containing the same factor, gave results which fitted the same response curve. The *relative* content of an active factor in different fractions may thus be determined.

For a comparison of the effect of phytoene (synthetic, or derived from tomatoes, corn oil, or cod liver oil) with that of lard factor and of whale liver oil factor, the abscissa scales were chosen to make the curves coincide at the minimum dose for maximum response. Fig. 5 shows that the slopes of the set of dose-response curves thus obtained, are different. The observed differences in the elution pattern of the factors are evidently associated with differences in the response to *relative changes* in dose reflecting dissimilarities in mode of action.

(2) *Phytoene and lard factor as precursors of common, active metabolites.* It seemed reasonable to assume that phytoene, lard factor, and whale liver oil factor were metabolically interrelated, and that one of these factors might be a common conversion product of the two others. To test this hypothesis chickens fed on diet S-21.2A were transferred to diets supplying adequate

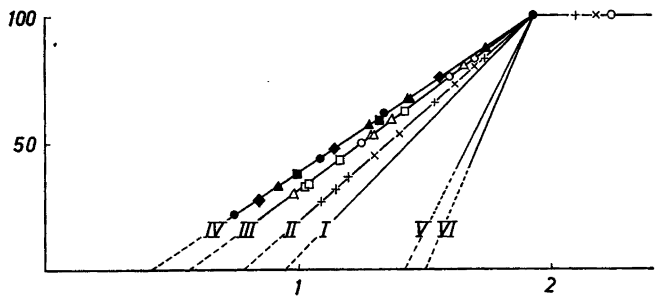

Fig. 5. Dose-response curves. Ordinate: as in Fig. 3. Abscissa: dose of active factors, relative scales. I: phytoene (*cf.* Fig. 3). II: lard factor; abdominal fat, lard, and active fractions from normal pigs (+), active fractions from corn- and carrot-fed pigs (×). III: conversion product I; active fractions of liver fat from chickens fed lard (○), corn (△), cod liver oil (□). IV: whale liver oil factor (conversion product II); active fractions of whale liver oil (◆), liver fat from chickens fed lard (●), corn (▲), cod liver oil (■). V: alloöcimene (2,6-dimethyloctatriene(2:4:6)). VI: cosmene (2,6-dimethyloctatetraene (1:3:5:7)).

amounts of corn oil, cod liver oil, or lard for maintenance of maximum π-factor level. The chickens were killed after 1—2 weeks on these diets. Pooled livers were stored in dry ice, cut in slices for saponification (under nitrogen) with methanol and 60 % KOH, and the unsaponifiables were isolated and fractionated as described.[2] The activity of the unsaponifiables was recovered only in the epiphasic part of the sterol-poor fractions. No activity was demonstrable in the unsaponifiables of chickens maintained on diet S-21.2A when tested

Table 5. Active factors in epiphasic unsaponifiables of liver fat (Li—11) from chickens fed lard (La).

Alumina column eluent	Test sample(s)	Daily dose[a]	Chicken weight	Response[b]
Light petrol (LP), and 5 % and 8 % benzene in LP	All sub-fractions	25	—	≤17
	Head- + mid-fractions I/II	25	—	≤17
13 % benzene in LP	Mid-fraction III	11	790	50
		15	790	83
	Tail-fraction	25	—	≤17
20 % and 40 % benzene in LP	All sub-fractions	25	—	≤17
0.6 % acetone in LP	All sub-fractions	25	—	≤17
1.5 % acetone in LP	Head-fraction	25	—	≤17
	Mid-fraction I	7	990	44
		9	1010	62
		20	1040	100
	Tail- + mid-fractions II/III	25	—	≤17
6 % acetone in LP, and 20 % methanol in ether	All sub-fractions	25	—	≤17

[a] Equivalent dose (in g) of original liver. [b] See footnote *b* in Table 1.

Table 6. Active factors in epiphasic unsaponifiables of liver fat (Li – 11) from chickens fed cod liver oil (CLO), and yellow corn (Ma).

Alumina column eluents	Test sample(s)	CLO – Li – 11			Ma – Li – 11		
		Daily dose[a]	Chicken weight	Response[b]	Daily dose[a]	Chicken weight	Response[b]
2 % benzene in light petrol (LP)	Combined sub-fractions	25	–	≤17	25	–	≤17
5 % benzene in LP	Head-fraction	25	–	≤17	25	–	≤17
	Mid-fraction I	25	–	≤17	25	990	28
					5	910	22
	Mid-fraction II	25	895	33	8	1080	62
					8	990	78
	Mid-fraction III	25	–	≤17	25	–	67
	Tail-fraction	25	–	≤17	25	–	≤17
8 % benzene in LP	All sub-fractions	25	–	≤17	25	–	≤17
13 % benzene in LP	Head + mid-fraction I	25	–	≤17	25	–	≤17
					19	1120	44
	Mid-fraction II	22	1070	35	20	1060	53
		28	1090	53	29	1200	80
		22	950	33	25	1010	31
	Mid-fraction III	28	1060	44	40	1170	59
	Tail-fraction	25	–	≤17	25	–	≤17
20 %, 40 % benzene; 0.6 % acetone; in LP	All sub-fractions	25	–	≤17	25	–	≤17
1.5 % acetone in LP	Head-fraction	25	990	≤17	25	860	≤17
					5	750	57
		22	1050	38	5	660	67
	Mid-fraction I	28	1010	59	8	880	87
					25	1100	100
	Tail- + mid-fractions II/III	25	1080	≤17	25	1090	≤17
6 % acetone in LP, and 20 % methanol in ethyl ether	All sub-fractions	25	–	≤17	25	–	≤17

[a] Equivalent dose (in g) of original liver. [b] See footnote b in Table 1.

at more than double doses. The active fractions (−11) of the unsaponifiables of the three types of chicken liver (corn-, cod liver oil-, and lard-) were fractionated by chromatography as above (activated alumina, 50 g per 150−200 g of original liver). All fractions were tested for π-factor stimulating activity.

(a) *Lard-liver* (La-Li). The characteristic elution pattern for the active factors in lard-liver are seen from Table 5. An active factor was recovered in the late mid-fraction of the 8−13 % benzene eluate, another in the early mid-fraction of the 0.6−1.5 % acetone eluate. Neither corresponded to the active factor in lard which is recovered in the central (or late) mid-fraction of the 0.6 % acetone eluate following 30 % or 40 % benzene. The two active fractions thus appeared to contain conversion products (I and II) of lard factor, the elution pattern and dose-response relationship of one of which (II) were identical with those of the whale liver oil factor (*cf.* Table 2 and Fig. 5).

(b) *Cod liver oil-liver* (CLO-Li) *and corn-liver* (Ma-Li). As shown by the elution patterns (Table 6) three separate, active main fractions were obtained. The active 2−5 % benzene eluate fraction of CLO-Li gave spectral evidence of phytoene in trace amounts. The U.V. absorption spectrum of the most active of the corresponding Ma-Li fractions was that characteristic of an impure phytoene, and the dose-response relationship of this fraction was as for the purest phytoene tested (*cf.* Fig. 3).

The active 8−13 % benzene eluate fractions were indistinguishable from conversion product I of lard factor, both in elution pattern and in dose-response relationship (*cf.* Fig. 5).

The third active fractions of these liver fats were indistinguishable from whale liver oil factor (conversion product II) in the same respects (*cf.* Fig. 5).

(c) *Comments.* The above experiments showed that the livers of chickens fed on diets containing lard, cod liver oil, or corn oil contain at least two different factors which like phytoene and the lard factor correct the reduced plasma level of π-factor in chickens with inadequate supplies of these fats. The latter chickens were markedly deficient in these factors. As phytoene and the lard factor are the only active constituents of corn (and cod liver oil), and lard, respectively, it must be assumed that the unidentified chicken liver factors are common conversion products of phytoene and the lard factor.

Neither of these products, nor the lard factor have been prepared sufficiently pure and in sufficient quantities to allow an estimation of minimum requirements for maximum effect. Thus, it cannot be stated which of the products may be regarded as the later metabolite and the physiologically more active factor. In Fig. 5 the slopes of the dose-response curves for the four categories of active factors, and the theoretically inactive fraction of each, increase in the order: conversion product II (whale liver oil factor) < conversion product I < lard factor < phytoene. The inactive fraction may represent losses during absorption and/or conversion of the compounds to a physiologically active factor. The dose-response curves of two active monoterpenes (based on data presented in a preliminary paper [10] and inserted for comparison) were even steeper than those above. When this is seen in relation to the observed minimum dose for maximum effect (cosmene: 4.4×10^{-1}, alloöcimene: 1.4×10^{-1}, and phytoene: 2×10^{-4}. μg/g) it appears probable that phytoene and lard factor

are first transformed into conversion product I, and subsequently into conversion product II (the whale liver oil factor).

(3) *Possible relation of lard factor to phytoene.* The absence of unchanged lard factor in the livers of lard-fed chickens indicated that its conversion to conversion product I might be too rapid to allow a demonstration of lard factor as a possible intermediate in the transformation of phytoene to conversion product I.

Determinations of the content of lard factor in different samples of lard showed only minor variations with a tendency to slightly lower values in the winter months. A few attempts were made to increase this content by increasing the supply of dietary phytoene. This was done in cooperation with the Experimental Station of Andelsslagteriet, Hillerød, and Slagteriernes Forskningsinstitut, Roskilde, both Denmark. Supplementary feeding with dried alfalfa had no effect on the content of lard factor in the abdominal fat of pigs fed on the conventional barley-milk rations. Substitution of yellow corn for barley, however, raised the content of the active factor to about 240 %, and pigs with free access to carrots had about 480 % of the activity obtained by feeding the conventional rations. To ensure that this increased activity was due to increase in lard factor and not to unchanged phytoene, samples of abdominal fat from the corn- and carrot-fed pigs were minced, ground with anhydrous sodium sulfate, and the fat extracted with chloroform and saponified. The

Table 7. Activity of pig's abdominal fat, and chromatographic fractions of the sterol-poor unsaponifiables (Fl—10).

Pig's ration	Test sample(s)	Daily dose[a]	Chicken weight	Response[b]
Conventional[c]	Abdominal fat	1.5	790	32
		2	780	66
Conventional + alfalfa[d]	»	1.5	760	33
Corn substituted for barley	»	1	880	73
		1.5	820	100
Conventional + carrots (*ad. lib.*)	»	0.5	850	80
Alumina column eluents[e]				
2 %, 8 %, 15 %, 40 % benzene in LP	All sub-fractions	5	—	≤17
0.6 % acetone in light petrol (LP)	Head + mid-fraction I	5	—	≤17
	Mid-fraction II	5	—	100
	Mid-fraction III	5	—	100
	Tail-fraction	5	—	≤17
1.5 %, and 6 % acetone in LP, and 20 % methanol in ethyl ether	All sub-fractions	5	—	≤17

[a] Dose, or equivalent dose of original fat, in g.
[b] See footnote *b* in Table 1.
[c] Barley-milk rations of Slagteriernes Forskningsinstitut, Roskilde, Denmark.
[d] 0.5—1.5 kg of alfalfa fodder per day.
[e] Chromatography of Fl—10 from pigs fed corn, and carrots.

sterol-poor fraction of the unsaponifiables was applied to a column of activated alumina, and all fractions obtained by stepwise elution tested for activity. The results are summarized in Table 7. No activity was detected in the fractions expected to contain phytoene (2—8 % benzene), or the conversion products I (8—15 % benzene), or II (0.6 %—1.5 % acetone). The activity was confined to the central and late mid-fractions of the 0.6 % acetone eluate following 40 % benzene, in which the lard factor normally appears.

DISCUSSION

The present paper reports on the first, successful identification of a member of a family of specific, previously unidentified dietary factors which may limit the plasma level of particular coagulation factors in chickens. [1,2,9] Phytoene appears to be the only active component of corn oil, cod liver oil and tomato lipids correcting the deficiency in the π-factor component of the labile factor activity induced by feeding of special, experimental diets. This may represent the first demonstration of a specific function of this carotenoid in an animal system. The possibility that the activity of the phytoene fractions might reside in a minor impurity (<3 %) cannot be completely disregarded, but seems remote considering its close association with features characteristic of phytoene. Moreover, the established minimum oral dose of the best preparations for maximum response is 2—3 orders of magnitude *below* the oral vitamin K requirement for maximum prothrombic activity in chickens (*viz.* 0.2 μg or about 4×10^{-4} μmole *versus* 27 μg of menadione or about 1.6×10^{-1} μmole, per kg of body weight [11]). Phytoene might thus be formally classified as a coagulation vitamin or pro-vitamin, but it is not known if a complete lack of π-factor represents a sufficiently grave impairment of the coagulation mechanism and hemostasis in chickens to allow a use of either of these concepts in their classical sense.

The specificity of phytoene for the observed effect is remarkable. More unsaturated carotenes were not responsible for the activity of crude lipid fractions, and phytofluene, containing one additional double bond and thus a conjugated pentaene structure,[8] was inactive when tested a hundred-fold dose. A central triene configuration thus seems essential for biological activity. Moreover, preliminary experiments with synthetic preparations of phytoene indicate that only one (or a few) of the possible central stereoisomers (probably the central *cis*-compound) may have biological activity. A reconciliation of this specificity with the apparently similar action of phytoene-free, chromatographic fractions of the active animal fats was attempted by assuming a metabolic interrelationship between the responsible factors and phytoene. The reported experiments on the active fractions of animal fats are compatible with the view that both phytoene and the active factor in lard may serve as precursors of common, active metabolites. Furthermore, the reported experimental feeding of pigs indicate that dietary phytoene may be the precursor of lard factor. The failure of detecting lard factor in the liver fat of corn oil-, and lard-fed chickens indicate that its conversion (to conversion product I) may be too rapid to allow accumulation of substantial amounts of intermediate and residual lard factor, respectively.

Present evidence thus indicates the metabolic sequence: phytoene → lard factor → conversion product I → conversion product II (whale liver oil factor), but the inadequacy of the present information is only too apparent. Additional evidence might be obtained in further feeding experiments when sufficient quantities of the unidentified factors become available. The suggestion that the whale liver oil factor is the ultimate, active conversion product and most directly responsible for the increase in π-factor level, is also tentative and based only on evidence given by the dose-response curves. It implies that the minimum dose for maximum effect of phytoene, lard factor, and the conversion products I, and II, should decrease in this order. The present fractions are still too impure, and the content of solutes was too small to give reliable information on these points.

The conditions for elution of the unidentified factors do, however, give fragments of information regarding possible molecular events of the interconversions. Conversion of phytoene to lard factor is associated with an increase in polarity, the further formation of conversion product I with a slightly smaller decrease, and the subsequent generation of conversion product II with a new increase in polarity. This is indicated by the elution patterns and the position of the active factors relative to that of known carotenoids. Lard factor was retained on the columns under conditions for complete elution of phytofluene, eluted ahead of conversion product II, which in turn was eluted ahead of a compound with a sharp, main absorption peak at about 498 mμ and secondary maxima at about 378 and 423 mμ, presumably ζ-carotene.[8] Conversion product I was located well above phytoene, and between two bands with brilliant yellowish fluorescence, both showing a sharp, main absorption maximum at 367—368 mμ and secondary maxima at 349—350 and 386—387 mμ. The fluorescent compounds were present in the unsaponifiables of cod and whale liver oils, as well as in chicken liver unsaponifiables irrespective of the dietary source of fat. Neither of them have been identified. Both were eluted ahead of phytofluene. Inspection of the U.V. absorption spectra of active fractions of the animal fats have not revealed characteristic peaks to be tentatively associated with the unidentified, active factors. A gross contamination with inactive, non-transparent compounds might well conceal such peaks. The characteristic absorption peak of phytoene has not been seen in the U.V. spectra of the most active fractions of lard factor. The central triene unit may thus be involved in and lost during conversion of phytoene to lard factor. A cyclization of phytoene in analogy with that of squalene [12] might be a possible tentative model which might help to explain the specific properties of this carotenoid. Further speculation on the metabolism of phytoene must await further investigations.

The rapid, but delayed response to intravenous injection of phytoene, and the lack of effect of *in vitro* addition are compatible with an *indirect* action of this dietary factor. π-Factor is presumably of protein nature,[1] and phytoene is thus not simply a co-factor for a preexisting π-factor apoprotein. The very similar time-dependence of the response (prothrombic activity) of vitamin K deficient chickens to intravenous injection of vitamin K_1,[13] indicated a close correspondence in the basic mode of action of these two substances. Vitamin K is apparently not a structural part of the prothrombin

molecule,[14] but has been assigned the role of a co-factor of an apoenzyme catalyzing prothrombin biosynthesis.[15] By direct application of the newer notions of structural, operator, and regulatory genes[16] to protein synthesis in animals generally, one might now consider vitamin K to act as an extrinsic effector substance neutralizing the repressor of the operon which codes the specific messenger-RNA for prothrombin polypeptide biosynthesis, or as a co-factor at some intermediate stage in a subsequent conversion of nascent prothrombin polypeptide chains into the final coagulation factor. Papers favouring the latter view have been published recently.[17,18]*

These conceptions might also apply to the mechanism of action of vitamin K on the plasma levels of the δ-, φ_1-, φ_2-, and $\varkappa$-factors in chicken plasma and also to that of the unidentified dietary factors required for maximum level of φ_2-factor,[9] $\varkappa$-factor,[2] and π-factor.[1] However, the additional possibilities that one or more of the dietary factors may be structural parts of the coagulation factors, or co-factors for the respective, preexisting coagulation factor apoproteins cannot be completely disregarded. The latter of these possibilities has been disproved only with respect to the effect of phytoene on the π-factor component of the labile factor activity, but the apparent precursor character of phytoene causes a further complication of the matter. From the present experiments it cannot be concluded whether the observed effect represents a *de novo* biosynthesis of a complete coagulation factor or merely a conversion of an extrinsic precursor into an active co-factor for a preexisting coagulation factor apoprotein. However, the protein nature of π-factor[1] and the specific increase in this component of the labile factor activity following resupplementation with phytoene, suggest that the effect is a stimulation of specific protein biosynthesis by a phytoene metabolite.

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* *Note added in proof.* The former view was expressed in a paper[19] published after this manuscript was submitted.

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