swelling of mitochondria from the deficient group. On the other hand, EDTA inhibited swelling of liver mitochondria from both groups of rats. These data would indicate that ATP influences mitochondrial swelling in a manner related to its role as a phosphate donor.

Summary. Comparison of rates of *in vitro* swelling of liver mitochondria from control rats and from rats fed diets deficient in essential fatty acids were studied under a variety of conditions. Higher swelling rates of EFA deficient mitochondria were observed using 0.30 M sucrose medium. Addition of ATP or serum albumin inhibited swelling of normal but not that of EFA deficient mitochondria. Respiratory inhibitors prevented swelling of both groups of mitochondria. Cholate accentuated the swelling pattern of mitochondria from both groups of rats. The fatty acid composition of lipid from mitochondrial, microsomal and supernatant fractions from both groups of livers were also presented.

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Linoleic Acid as Causative Agent of Encephalomalacia in Chickens Fed Oxidized Fats.* (25626)

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Chickens fed certain partially oxidized fats manifest an edemic condition known as "toxic fat" disease(1,2,3). Our original purpose was to determine whether the feeding of heated cottonseed oil will result in this disease. It was found that ingestion of heated cottonseed oil does not cause "toxic fat" disease but does result in a high incidence of encephalomalacia even with dietary levels of Vit. E usually sufficient to prevent this disease. High levels of Vit. E or a supplement of the antioxidant Santoquin† (herein after referred to by generic term ethoxyquin) completely prevented encephalomalacia. Evidence is presented which demonstrates that increased requirement for Vit. E was caused by presence of linoleic acid in the diet. This is in agreement with other reports(4,5,6). Fats high in linolenic acid but

* Preliminary report presented in *Fed. Proc.,* 1959, v18, 535.

† Registered trademark of Monsanto Chemical Co., for 1,2-dihydro-6-ethoxy, 2,2,4-trimethyl quinoline.

TABLE I. Experimental Diets.

Ingredient	ES-45	ES-35
	%	
Isolated soybean protein*	45.	35.0
Fats	20.	4 to 16
Salts A†	6.	6.0
Microcel E‡	2.	
Glycine	1.4	1.0
Choline chloride mixture (25%)	.8	.8
Methionine hydroxy analog (MHA)§	.7	.7
Vit. mixture (triturated on glucose)‖	.5	.5
Vit. A conc. (10,000 I.U./g)¶	.1	.10
" D_3 " (7,500 ")	.03	.03
Aureomycin conc. (10 mg/lb)	.1	.10
Procaine, penicillin (50%)	.004	.004
NF 180**	.05	.05
Glucose (cerelose)	to 100	to 100

* Assay Protein C-1, Archer-Daniels-Midland Co., Cincinnati, O.

† Contains in g/60 g the following compounds: $CaCO_3$, 15; K_2HPO_4, 9; Na_2HPO_4, 7.3; $Ca_3(PO_4)_2$, 14; NaCl, 8.8; $MgSO_4 \cdot 7H_2O$, 5; ferric citrate, 0.5; $MnSO_4 \cdot 4H_2O$, 0.42; KI, 0.04; $ZnCO_3$, 0.02; $CuSO_4 \cdot 5H_2O$, 0.02.

‡ Johns-Manville, N.Y.

§ Registered trademark of Monsanto Chemical Co. for calcium *di*-2-hydroxy, 4-methylthiobutyrate.

‖ This supplies in mg/kg of finished diet: vit. B_{12}, 0.03; biotin, 0.3; menadione, 1; pyridoxine HCl, 8.; folic acid, 4.; riboflavin, 16.; calcium pantothenate, 20.; thiamine HCl, 24.; nicotinic acid, 100. This mixture and salts "A" can be obtained from Nutritional Biochemicals Corp., Cleveland, O.

¶ A stabilized preparation prepared by Dawes Lab., Chicago, Ill.

** Registered trademark of Hess and Clark for furazolidine feed preparation.

low in linoleic acid had no effect on Vit. E requirement.

Methods. Cockerels from a Vantress-Arbor Acre cross were reared in electrically heated, wire-floored cages maintained in air-conditioned rooms provided with continuous illumination. Diets were fed *ad lib.* All birds were examined daily and those showing definite signs of encephalomalacia (ataxia, "cycling" motion of legs, head retraction, etc.) were removed. The brains of some affected birds were examined histologically. In almost all cases cerebellar lesions typical of those previously reported for encephalomalacia(7) were observed. In the first 2 experiments the chicks were fed a corn-soybean diet containing 7.7 I.U. Vit. E‡/kg for 7 to 10 days. They were then fed the experimental rations containing 20% heated cottonseed oil. The basal diet (S-45, Table I) contained no source of Vit. E. In all other studies a purified diet containing 4% "stripped" lard as the only source of Vit. E (less than .066 I.U. E/kg diet) was fed for 7 to 10 days. The birds were then fed experimental diets for 11 to 22 days. All fats were stabilized after heating with 0.1% Tenox VI.§ Determination of peroxide level, as the TBA (thiobarbituric acid) value(8,9), of dietary fat at beginning and end of experiments demonstrated that no oxidative rancidity had occurred during this period. In addition to dietary source of Vit. A (10,000 I.U./kg) an emulsion of Vit. A acetate was added to drinking water as suggested by Bieri *et al.*(10). The livers of chickens receiving experimental diets, including those showing symptoms of encephalomalacia, contained 63 to 194 I.U. Vit. A/g when analyzed at end of experimental period. It is therefore believed that the studies were not complicated by Vit. A deficiency.

Results. In Exp. 1 (Table II) all birds died or showed definite signs of encephalomalacia when fed heated aerated, refined cottonseed oil. In agreement with Naber(2) no birds showed symptoms of "toxic fat" disease. Since encephalomalacia can be prevented by Vit. E or certain antioxidants(8,11), the next studies were designed to determine levels of Vit. E and/or antioxidant required to prevent

TABLE II. Effect of Feeding Heated, Aerated Cottonseed Oil on Incidence of Encephalomalacia.*

Fat source *Exp. 1*	Incidence of encephalomalacia or death†
Cottonseed oil (Wesson oil)	0/4
Idem heated‡ 95°C, 13 days	14/14
" " 170-180°C, 4 days	14/14

* All birds were fed corn-soybean meal diet containing approximately 7.7 I.U. vit. E/kg for 10 days. They were then fed experimental diets for 22 days. Diet S-45 with addition of 8.8 I.U. vit. E/kg was used as the basal.

† Numerator is No. of chickens which either died or showed definite symptoms of encephalomalacia after start of experiment. Denominator is No. of chickens started on each treatment.

‡ 2 to 4 kg were heated in stainless steel beakers with air bubbling through pot at 500-700 ml/min.

‡ As d α tocopherol acetate.

§ This product contains 10% BHT and 10% BHA. Available from Eastman Chemical Products, Inc., Kingsport, Tenn.

the disease when 20% heated cottonseed oil was fed. The requirement for ethoxyquin was between 0.0125% and 0.02% of the diet, (Table III). The antioxidant BHT at a level of 0.05% had no effect on the disease. The requirement for Vit. E was more than 44 but less than 88 I.U./kg. This is much higher than any previously reported requirement.

TABLE III. Effect of Vit. E and Ethoxyquin on Encephalomalacia Induced by Feeding of Heated Cottonseed Oil.

Fat source	Vit. E (I.U./kg)	Ethoxyquin (%)	Incidence of encephalomalacia
*Exp. 2**			
Cottonseed oil	22		0/11
Heated cottonseed oil†			11/11
Idem	22		7/11
"	220		0/11
"		.05	"
"	22	.05	"
" + .05% BHT‡			10/11
Exp. 3a§			
Cottonseed oil			0/10
Heated cottonseed oil†			9/10
Idem	44		2/10
"	88		0/10
"	176		"
"		.0125	1/10
"		.0250	0/10

* Chicks fed a corn-soybean diet containing 7.7 I.U. vit. E/kg for 7 days, then fed experimental rations for 15 days.

† Heated and aerated at 170-180°C for 4 days.

‡ 2,6-ditertiary-butyl-p-cresol.

§ All birds fed vit. E deficient diet for 8 days, then fed experimental diets for 15 days. 0.1 ppm of selenium (as $Na_2Se_2O_3 \cdot H_2O$) was added to basal diet. After heating, 0.1% of Tenox 6 (an antioxidant containing 10% BHT and 10% BHA) was added to the fat.

Studies were then conducted to determine whether feeding of other heated fats would also result in a high incidence of encephalomalacia. In all cases analysis of heated fats for Vit. E(12) demonstrated that essentially all the Vit. E had been destroyed. The results summarized in Table IV show that the encephalomalacia-inducing ability of heated cottonseed oil was not unique and that in general the incidence of encephalomalacia was correlated with linoleic acid content of fats. Linseed oil which is much more unsaturated than cottonseed oil resulted in a lower incidence of encephalomalacia.

TABLE IV. Correlation of Linoleic Acid Content of Various Oxidized Fats with Encephalomalacia.

Dietary fat	Iodine No.	Linoleic acid (%)	Encephalomalacia (index)*
Safflower oil	140	72.4	152 (2)
Cottonseed "	114	54.8	100 (3)
Corn "	127	55.7	57 (2)
Peanut "	101	29.0	14 (1)
Linseed "	180	18.0	16 (3)
Olive "	84	7.0	0 (1)
Tallow	40	2.2	0 (1)
Cod liver oil	165	2.3	0 (1)

* Index = $\frac{\%\text{ encephalomalacia in test fat} \times 100}{\%\text{ encephalomalacia in cottonseed oil group}}$.

No. in parenthesis indicates No. of experiments conducted.

Fatty acids of cottonseed oil were then fractionated after conversion of the triglycerides to the methyl esters by interesterification with methanol using sodium methylate as catalyst. The methyl esters were fractionally distilled using a 2 foot Vigreaux column (2-3 theoretical plates) operating at pressure of approximately 0.1 mm mercury. The fractions were analyzed by gas chromatography and fed to 1 week old chickens. The results are given in Table V. They show that encephalomalacia results from feeding of distilled methyl esters and that incidence of encephalomalacia was correlated with linoleic acid content of the fraction fed. As fractional distillation procedure did not separate oleic and linoleic acid to any appreciable extent, in the next study these fatty acids were fed separately. These results are given in Table VI

TABLE V. Fractionation of Cottonseed Oil.*

Fraction #	Linoleic	Oleic	Palmitic	Stearic	Incidence of encephalomalacia†
	——— % ———				
1	32.4	5.2	53.9	.7	0/3
2	36.4	11.3	49.0	1.3	1/3
3	64.1	20.5	12.3	2.4	1/3
4	72.5	23.4	.3	3.3	3/3
Residue	67.3	25.4		5.3	2/3

* Distilled at 0.1 mm mercury pressure with 2 ft Vigreaux column. All fractions fed at 8% of diet (ES-35) for 7 days, after 7 days on vit. E-free diet (ES-35 containing 4% stripped lard).

† Numerator is No. of birds showing definite symptoms of encephalomalacia. Denominator is No. of birds/treatment.

TABLE VI. Incidence of Encephalomalacia in Chickens Fed Purified Fatty Acids.

Dietary fat	Incidence of encephalomalacia*
None (Diet ES-35)	0/10
5% linoleic acid†	6/6
10% oleic " ‡	0/8
20% " "	0/4

* Numerator is No. of birds showing definite symptoms of encephalomalacia. Denominator is No. of birds/treatment.

† Obtained from Hormel Fn., Austin, Minn. Lot 15-M. Iodine number is 172.5 (theoretical 172.4). A single peak observed with gas chromatography.

‡ Obtained from Fisher Chemical Co. Catalog No. A-222. Contains less than 5% polyunsaturated fatty acids.

and clearly demonstrate that feeding of linoleic but not oleic acid resulted in encephalomalacia.

Feeding of heated cod liver oil also did not result in encephalomalacia. This fat contains arachidonic and clupanodonic acid. These highly unsaturated compounds could have been destroyed during the heating process. To avoid such destruction a sample of cod liver oil was saponified(13) in the presence of 0.1% ethoxyquin. The soap solution was extracted for 24 hours with ether(13). This removed all Vit. E. The soaps were then converted to fatty acids by addition of acid and fed. At the end of 20 days, 10 out of 16 birds fed 12% cod liver oil fatty acids had encephalomalacia.

Discussion. These studies demonstrate that addition of linoleic acid to diets low in Vit. E and other biologically active antioxidants results in encephalomalacia. Fats high in linolenic acid were less effective than fats high in linoleic acid. Our observations as those of Century and Horwitt(14), confirm the work of Dam *et al.*(4), who reported that feeding of linoleic acid, but not linolenic acid, results in encephalomalacia. However, our work is not complicated by a selenium deficiency or by possible oxidation of fatty acids in the diet and gut.

Although not definitely proven, arachidonic acid also appears to exaggerate Vit. E deficiencies. Dam(15) showed that feeding of fatty acid fractions known to be high in arachidonic acid promoted encephalomalacia. In the present study, cod liver oil subjected to procedures which removed Vit. E but which did not destroy arachidonate caused encephalomalacia. In addition Moore and his co-workers(16) have shown that cod liver oil acts as a Vit. E antagonist in the rat. Century and Horwitt(16), however, found that cod liver oil did not promote encephalomalacia. Since no precautions were taken in their experiments to prevent oxidation of fatty acids after addition to the diet, destruction of some or all of the arachidonic acid before absorption could have accounted for their results.

The effect of linoleic and presumably arachidonic acid in promoting Vit. E deficiency symptoms could be the result of: (1) destruction of Vit. E in the diet and in the gut during oxidation of these fatty acids, (2) increased destruction of Vit. E in tissues due to presence of high levels of these fatty acids and their subsequent autoxidation, (3) disruption of some cell structure and/or metabolic process as a result of autoxidation of linoleic and/or arachidonic acid in the tissue.

Blaxter *et al.*(17) reported that dystrophy of calves fed cod liver oil is prevented by methylene blue without a concomitant increase in tocopherol content of the tissues. Jensen *et al.*(18) found that eggs from turkeys fed fish oil failed to hatch unless DPPD (N,N′ diphenyl-p-phenylenediamine) was added to the diet. Addition of DPPD did not cause increased tocopherol deposition in the egg yolk. Draper and Csallany(19) demonstrated quite clearly that the effect of DPPD in curing muscular dystrophy in the rabbit was not due to conservation of Vit. E in the tissues or in the diet. Most of our diets were essentially devoid of Vit. E. Moreover, destruction of Vit. E in the diet (and presumably in the gut) was prevented by addition of an antioxidant. These observations indicate that destruction of Vit. E in the diet, gut or tissue is not a likely explanation for our observations. We think a more reasonable hypothesis is that addition of linoleic and/or arachidonic acid to the diet increases the content of these fatty acids in the tissue. Their subsequent autoxidation following depletion of antioxidants from tissues results in disruption of a critical cell structure which is the

beginning of a sequence of events which results in encephalomalacia.

The observation that linolenic acid does not promote encephalomalacia would be explained if linolenic acid was not a part of the critical lipid structures involved, or if deposition of this particular fatty acid in the tissues was not increased significantly by its addition to the diet.

Summary. Feeding of heated and aerated unsaturated fats to chickens fed Vit. E low diets resulted in high incidence of encephalomalacia. Incidence of encephalomalacia was correlated with linoleic acid, but not the linolenic acid content of fats fed. Feeding of highly purified linoleic acid resulted in 100% incidence of encephalomalacia. When 20% heated cottonseed oil was the fat source between 44 and 88 I.U./kg of Vit. E or between .0125% and 0.025% of the antioxidant, ethoxyquin was required for prevention of the disease.

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Chelation and Iron Metabolism I. Relative Iron Binding of Chelating Agents and Siderophilin in Serum.* (25627)

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Iron absorption, transport, storage and function are mediated in the organism by the nature of the iron combination. It may be anticipated that knowledge of the factors governing iron complex formation and interaction can lead to new approaches to problems of iron therapy(1,2,3,4). The present study concerns the relative iron-binding strengths of the plasma iron-binding protein siderophilin and various synthetic iron-binding chelating agents in rabbit serum.

Materials and methods. The synthetic chelate compounds, beta hydroxyethyliminodiacetic acid,‡ nitrilotriacetic acid,‡ dibetahydroxyethylglycine,‡ 1,2-propylenediaminetetraacetic acid,§ betahydroxyethylethylenediaminetriacetic acid§ (HOEDTA), ethylenediaminetetraacetic acid‖ (EDTA), and trans-1,

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