thetic estrogens(8). The inhibitory action of the ovaries on tumor growth may well be of a more complex nature. The tumor system employed in this study will serve as an instructive model in elucidating the regulatory mechanism of various hormones in cancer.

Summary. The growth of solid Ehrlich tumor, as compared with body weight, was studied in relation to gonadal and adrenal function in Ha/ICR Swiss mice. Both tumor weight and body weight were much greater in males than in females, and castration reversed the differences. Adrenalectomy was inhibitory for tumor growth in females, but not in males. Body weight was decreased by adrenalectomy in both sexes. Hydrocortisone was generally inhibitory for both tumor weight and body weight, but increased tumor weight in females with sham surgery. Estrogen treatment in castrated females significantly reduced tumor growth, but not body weight. Testosterone administration increased body weight in castrated male mice, but had little effect on tumor weight. These findings are discussed in the light of the hormonal responsiveness of the tumor.

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Exudative Diathesis and Muscular Dystrophy Induced in the Chick by Esters of Polyunsaturated Fatty Acids. (29476)

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The incidence and severity of vitamin E deficiency syndromes in chicks have been shown to be directly related to increasing levels of polyunsaturates when fed with diets low in vit. E(1,2). But, to our knowledge these syndromes have never been induced in chicks fed varying levels of polyunsaturates in diets containing supposedly adequate levels of vit. E and other related dietary protectants.

In a previous study(3) at our laboratory, however, it was observed that chicks fed the most unsaturated fractions of ethyl esters of fatty acids prepared from menhaden *(Brevoortia tyrannus)* fish oil in a diet containing supposedly adequate levels of vit. E and related dietary protectants developed exudative diathesis and muscular dystrophy as early as 9 days. The diets contained 44 ppm dl-α-tocopherol acetate, 167 ppm ethoxyquin, 0.3 ppm Se, and 0.9% sulfur amino acids. But, since this observation was incidental to another study, there was a question as to whether it might have been an artifact. Also, in the event that it was a correct observation, there was a question as to whether these disorders in some manner were due only to the ethyl ester form of the fractions or were related to the oxidation of the oil in the feed.

Experimental. Ethyl esters and triglycerides. Unfractionated ethyl esters were prepared from commercial, light, cold-pressed menhaden oil by a rapid alcoholysis method.

Four equal-volume fractions of ethyl esters were obtained by the "cyclic" molecular distillation technique of Cawley, Barnitz, and

Jackson, as reported by Embree(4). A forerun distillate and a pot residue, obtained during the distillation, each of which amounted to about 4%, were discarded. The apparent mean degree of unsaturation of each fraction, as indicated by the iodine value, increased from 78 in Fraction 1 to 284 in Fraction 4.

Reconstituted triglycerides were prepared from the most unsaturated fraction (4th cut) of molecularly distilled fatty acids.‡

After being collected, all of the oil samples were stored in amber-colored bottles under nitrogen and were refrigerated throughout the experiment.

Basal ration. A basal premix consisted of 46 parts of soybean meal (50% protein), 33.78 parts of glucose,§ 5 parts of cellulose,‖ 5 parts of mineral mixture,¶ 5 parts of vitamin mixture** (which contributed 44 I.U. of vit. E per kilogram of diet), 0.2 parts of methionine hydroxy analogue, .0167 parts of ethoxyquin, and .0022 parts of penicillin by weight for a total of 95 parts. The remaining 5 parts of oil fraction were added daily during the feeding period to bring all diets to 100 parts by weight.

The soybean meal at a 46% level in all diets was calculated to contribute approximately 0.3 ppm Se based on Scott's analyses (5), 0.4% methionine, and 0.3% cystine to the basal premix, which was also fortified by addition of 0.2% methionine hydroxy analogue to make the total equivalent content of sulfur amino acids about 0.9%.

Chicks. Day-old white Arbor Acre cockerels were randomly allotted into 3 groups—6 chicks to a group, placed into brooder-starter batteries, and fed for 15 days each of the diets. Each pen of 6 chicks was group-weighed at the conclusion of the experiment. At that time, the birds were examined externally for swollen jowls and discoloration under the skin. Necropsies to detect symptoms of exudative diathesis and muscular dystrophy were performed only on those chicks that died during the experiment.

Chemical analyses of fish oil samples. A single series of peroxide values of Fraction 4 oil after mixing with the feed and subsequent extraction was determined during a 24-hour period at 2-hour intervals. The mean iodine values of the oil fraction were also determined in the mixed feed at zero time and after the 24th hour. Official methods of the American Oil Chemists' Society were used for these analyses.

The mean iodine values and the fatty acid composition by GLC analyses were determined of the 4 ethyl ester fractions and of the reconstituted triglycerides from Fraction 4. The GLC analyses for the chemical composition of the oils were made with 9′ × ¼″ packed column containing 17.0 g of 7% diethylene glycol succinate polyester on 100-110 mesh Anakrom ABS (acid and base washed, siliconized diatomaceous earth) in a Research Specialties Company 600 Series instrument equipped with a beta-ionization detector. The temperature of the column was 185°C, the argon carrier gas was at 20 psi, and the detector was operated at 800 volts.

Results and discussion. Incidence of exudative diathesis and muscular dystrophy. The results (Table I) verify previous observations that the highly unsaturated ethyl esters of menhaden oil fatty acids (Fraction 4) are very potent at the 5% level in inducing exudative diathesis with an accompanying high incidence of mortality. By the 9th day, 50% of the chicks had developed the first symptoms. By the 15th day, all chicks fed the ethyl esters of Fraction 4 (or their triglycerides) were affected.

‡ Prepared by L. W. Lehman at the BCF Technological Laboratory, Seattle, Wash.

§ Cerelose, Corn Products Co., Argo, Ill.

‖ Solka Floc, Brown Co., Berlin, N. H.

¶ The mineral mixture contributed to each kilogram of diet 16 g $CaCO_3$, 17.5 g $CaHPO_4$ (Dynafos), 5 g NaCl (iodized), 5 g KCl, 5 g $MgSO_4 \cdot H_2O$, 0.8 g $FeC_6H_5O_7 \cdot 3H_2O$, 0.3 g $MnSO_4 \cdot H_2O$, 0.2 g $ZnSO_4 \cdot 7H_2O$, 0.1 g $Al_2(SO_4)_3 \cdot 18H_2O$, 0.04 g $Na_2SiO_3 \cdot 9H_2O$, 0.02 g $CuSO_4 \cdot H_2O$, 0.01 g $Co(CH_3COO)_2 \cdot 4H_2O$, and 0.1 g $(NH_4)_2MoO_4 \cdot 4H_2O$.

** The vitamin mixture fortified each kilogram of diet with 7 mg thiamine HCl, 7 mg riboflavin, 8 mg pyridoxine HCl, 60 mg niacin, 30 mg calcium pantothenate, 2 mg folic acid, 2 mg menadione, 29 μg vit B_{12}, 220 μg biotin, 9000 I.U. vit A, 450 I.C.U. vit D_3, 44 I.U. dl-alpha tocopherol acetate, and 1.65 g choline Cl.

TABLE I. Effect of Ethyl Ester Fractions of Menhaden Oil and Reconstituted Triglycerides.

Treatments*	Avg wt of chicks, g	No. live chicks exhibiting exudative diathesis		No. dead chicks	No. dead chicks exhibiting muscular dystrophy
		Swollen jowl	Discoloration under skin		
Basal	228	0	0	0	0
Corn oil	253	0	0	0	0
Tallow	211	0	0	0	0
Ethyl esters:					
Unfractionated	238	0	1	0	0
Fraction—1	211	0	0	0	0
—2	212	0	0	0	0
—3	198	0	2	0	0
—4	153	5	17	7	3
Reconstituted triglycerides from Fr 4A†	113	8	18	14	1

* Eighteen chicks per treatment on test for 15 days. Additions to basal diet were at 5% level, replacing 5% of glucose.

† Because the original supply of Fraction 4 was exhausted, a new batch had to be made.

White striations of the muscles were noted in 3 of the 7 dead chicks that had been fed Fraction 4. Since the chicks that died were the only ones necropsied, no further considerations were possible in this experiment.

The incidence of exudative diathesis apparently becomes greater as the fractions increase in unsaturation. Only 1 chick showed symptoms of exudative diathesis when fed the diet containing the unfractionated ethyl esters, no chicks were affected in those groups fed Fractions 1 or 2, and 2 chicks were affected of those fed Fraction 3.

Causative agents. All chicks of the groups fed the reconstituted triglycerides from the most highly unsaturated fatty acid fraction (Fraction 4) developed exudative diathesis and one of the dead chicks showed symptoms of muscular dystrophy. The incidence of mortality was slightly greater with the chicks fed the triglycerides than with those fed Fraction 4. These results rule out the possibility that the observed disorders were due only to the ethyl ester form of the fatty acid.

The average peroxide values of Fraction 4 ethyl esters extracted from feeds varied from 4.2 to 9.6 with no indication of a rising trend in 24 hours (Table II). Ethoxyquin at the 167 ppm level in the diet appeared to be adequate to inhibit any substantial formation of peroxide. In addition, there was no real difference between the iodine values of the oil fraction in the mixed feed at zero time and at the 24th hour. No rancid odors were noted in the feed during the 24-hour feeding period. These results rule out oxidation products of the oils in the feed as possible causative factors of the abnormalities noted in this study.

TABLE II. Peroxide Value* of Fraction 4 Ethyl Esters After Esters Were Mixed with the Feed and Subsequently Extracted for Analyses.

Time after mixing (hr)	Peroxide value†
0	4.7
2	4.2
4	4.4
6	7.1
8	5.2
10	6.0
12	9.7
14	7.0
16	5.4
18	6.2
20	6.3
22	5.9
24	5.3

* American Oil Chemists' Society Official Method Cd 8-53, 1962.

† Avg of 2 or more determinations expressed as milliequivalents of peroxide per kg of sample.

The mean iodine number and the peroxide value as well as the fatty acid composition of the 4 fractions of ethyl ester and of the reconstituted triglycerides from Fraction 4 are presented in Table III. As expected, the

TABLE III. GLC Analysis for Fatty Acids of Fractions of Ethyl Esters.

Fatty acid	Relative amount of the fatty acid in: Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fr 4A triglycerides
	Area %				
C:2 bonds					
12:0	.23	—	—	—	—
13:0	.12	—	—	—	—
14:0	19.5	6.7	trace	trace	.1
Unk	—	trace	—	—	—
15:0	2.1	.8	trace	—	—
Unk	—	trace	trace	—	—
16:0	24.5	23.1	16.0	1.7	1.2
16:1	22.5	20.4	10.0	.7	.6
16:2	4.2	3.5	1.9	trace	.1
16:3	4.7	3.6	—	—	—
17:0	2.2	1.0	1.3	trace	.3
18:0	2.1	3.4	5.9	3.9	3.4
18:1	6.7	15.0	21.3	12.1	12.7
18:2	4.4	3.5	2.2	1.0	1.7
Unk	—	1.9	2.9	—	—
Unk	—	—	1.4	.5	—
18:3	—	.7	1.4	1.1	1.9
Unk	—	.8	2.1	—	—
18:4	2.4	3.6	5.2	2.4	—
Unk	—	—	—	1.5	—
Unk	—	—	—	.6	—
20:0	1.3	.5	1.1	.6	—
20:1	1.5	1.3	2.3	3.9	6.7
20:3 (?)	—	—	—	.6	—
20:4	—	.8	1.4	2.5	2.3
20:5	1.7	7.1	17.0	33.8	24.3
21:Unk	—	—	—	—	1.2
22:1	—	.7	1.1	3.1	4.9
22:4 (?)	—	—	.7	2.0	2.5
22:5	—	—	.8	5.5	4.3
22:6	—	1.0	3.2	21.0	20.0
22:Unk	—	—	—	—	4.4
24:1 (?)	—	—	trace	.8	1.4
Iodine No.	78	109	170	284	294
Peroxide values	2.5	2.0	2.1	5.0	4.0

longer chain length, higher unsaturated esters tended to be concentrated in the 3rd and 4th fractions. Certain compounds of unknown structure were also present in these fractions.

Stoffel and Ahrens(6) showed that menhaden body oil contained C_{20} and C_{22} pentaenoic and C_{22} hexaenoic acids of the linolenic acid-type rather than the linoleic acid-type. It is therefore possible that the syndromes described in these experiments may be influenced by the linolenic-type polyunsaturation in fatty acids with very long carbon chains.

In view of the current interest in feeding polyunsaturates to both humans and animals, it is important to point out that to equal the levels of the Fraction 4 fed to produce experimentally the syndromes discussed here would require feeding a diet containing at least 25% of total menhaden oil. This exceeds by far the levels of whole fish oil that would normally be fed. The chances, therefore, of the general consumption of excess amounts of the factors involved in these physical disorders are quite improbable.

Summary and conclusions. Exudative diathesis, muscular dystrophy, and depressed growth with accompanying high incidence of mortality were observed in chicks fed certain fractions of molecularly-distilled polyunsaturated fatty acids obtained from menhaden oil. These fractions were fed daily as either ethyl esters or reconstituted triglycerides in supposedly adequate nutritional diets containing 0.3 ppm Se, 44 ppm dl-α-tocoph-

erol acetate, 0.9% sulfur amino acids, and 167 ppm ethoxyquin. The chicks developed exudative diathesis as early as the 9th day of experiment when fed the ethyl esters or reconstituted triglycerides obtained from the most unsaturated fractions of fish oil. The observed disorders are not due to the ethyl ester form of the fatty acids or to the oxidation of the oil in the feed.

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Effect of Red Cell Age on Its Susceptibility to Lysis by Rabbit Hemolytic Factor. (29477)

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A hemolytic factor capable of lysing human and other mammalian red cells *in vitro* has been previously isolated from rabbit erythrocytes(1-3). This factor requires exogenous ATP, Mg^{++}, and Na^{+} or K^{+} for its lytic activity, but is not dependent on glycolysis, choline esterase, or active ion transport. In addition, the action of this factor on intact erythrocytes is completely inhibited by preincubation with purified human stroma in the presence of the above cofactors. It appears, therefore, that its substrate is some component of the red cell membrane and that lysis of the cell is the result of membrane degradation.

Since it has been demonstrated that some relationship exists between the age of the red cell and its susceptibility to osmotic(4), immunologic(5), and primaquine-induced hemolysis(6) it was of interest to investigate the effect of red cell age on its lysis by rabbit hemolytic factor.

Materials and methods. *^{59}Fe citrate:* Abbott Laboratories; 100 microcuries per ml.

Glycine-2-^{14}C: Prepared by the Isotopes Specialties Co.; specific activity of 4.2 mC per mM.

Buffered saline: 99 parts isotonic saline + 1 part isotonic Tris (hydroxymethyl) aminomethane-Histidine · HCl buffer, pH 7.0.

Incubation medium. Sucrose (0.25 M) containing adenosine triphosphate (0.001 M), $MgCl_2$ (0.002 M), glutathione (0.0016 M), Ethylenediamine tetraacetic acid (0.0002 M), and potassium phosphate buffer, pH 7.0 (0.01 M).

Hemolytic factor from rabbit erythrocytes. Partially purified rabbit hemolytic factor was prepared by ammonium sulfate fractionation of hemolyzed rabbit red cells as previously described and the hemolytic titer of each preparation was determined before use as described under "Hemolysin Test"(2), substituting washed rat for human red cells.

Glutamic oxaloacetic transaminase activity (GOT). Assayed by the method of Steinberg and Astrow(7).

Specific radioactivity of hemoglobin (Hgb). Hgb concentration was determined by measuring the optical density of stroma-free hemolysates at 540 mμ in a Beckman model DU Spectrophotometer. The Hgb was precipitated (carrier Hgb added where necessary) 3 times from CO_2-saturated water with acetone at 0°. ^{59}Fe was measured in a Tracer Lab well-type scintillation counter and ^{14}C in an automatic Nuclear D-47 gas-flow counter. Radioactivity was expressed as CPM per mg of Hgb.

Experimental and results. Radioactively tagged red blood cells were obtained from