TABLE V
Summary of Data for AOM Oxygen Absorption and Bomb Methods

Type of fat	AOM		Oxygen absorption at 100C. hr		Bomb at 100C and 50 lb pressure		Bomb at 100C and 100 lb pressure	
Meat fat	1	1	1.3	1.3	1.5	1.5	.....	.....
Meat fat	1	1	1.4	1.3	1.7	1.4	.....	.....
Meat fat	38	45	16.3	17.7	13.3	16.1	14.2	15.8
Meat fat	26	30	11.5	11.6	14.1	14.3	13.7	14.7
Meat fat	40	40	4.8	4.8	12.2	11.2	11.7	13.1
Meat fat	50	40	5.1	5.1	12.2	11.9	13.2	11.7
Meat fat	47	53	25.4	25.5	23.8	22.9	20.8	23.1
Meat fat	87	87	25.8	26.4	28.4	32.1	23.8	25.8
Meat-vegetable fat	48	40	13.6	11.8	20.7	17.8	18.3	19.2
Meat fat	....[a]	....[a]	16.3	17.6	15.7	15.7	13.2	13.1
Meat-vegetable fat	145	150	15.9	14.1	38.0	39.2	35.5	32.2
Vegetable	55	50	23.4	23.0	19.7	21.7	17.3	19.7
Vegetable	57	55	16.9	17.9	13.7	15.1	14.9	14.1
Vegetable	108	106	22.5	22.6	29.2	24.7	23.8	25.7
Vegetable	135	140	41.0	44.5	40.4	39.4	35.7	39.1
Meat-vegetable fat	96	100	31.6	31.5	44.9	39.6	34.9	34.4
Meat-vegetable fat	10	14	15.6	15.1	14.2	14.3	11.2	12.6
Meat-vegetable fat	100	95	27.1	27.6	31.3	27.9	26.5	26.8
Meat-vegetable fat	106	100	29.9	33.8	32.2	32.4	27.3	29.6

Because of great activity the first two samples were not tested at 100 lb pressure but at 25 lb pressure. End point came at 1.6 and 1.7 hr.
[a] Foamed out of tube.

of 10C results in about one-half reaction time with a satisfactory precision.

The foregoing investigations were undertaken prior to the proposal of the modified ASTM Bomb method (4). Since that time, a series of commercially available fats and shortenings have been compared by the AOM, Eckey Oxygen Absorption at 100C and Modified ASTM Oxygen Bomb procedures. The data are contained in Table V.

The following conclusions are possible from interpretation of these data. There was a high degree of correlation between the oxygen absorption methods (Eckey and Bomb) which might be expected, since the principles of measurement are similar. A marked increase in oxygen pressure resulted in only a slight decrease in the time required for making bomb determinations. No single, all inclusive, correlation was obtained between the AOM and either of the oxygen absorption methods. This might be anticipated since the end points of the methods are based on different phenomena.

While it is believed from our knowledge of the stability of the shortening used in this study that oxygen absorption may serve as a better index of actual shelf stability than AOM, this cannot be established without carefully conducted storage studies. These should be carried out in conjunction with evaluation of chemical methods and organoleptic panel findings on a variety of fats and oils. A research project in this area is now in progress at our laboratory and is expected to provide answers to many of the questions raised here.

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Effect of Diet and Encephalomalacia on the Fatty Acid Composition of the Brain of Young and Old Chickens[1]

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Abstract

Encephalomalacia was induced in chickens more than 64 days old by feeding a high linoleic acid diet with an antioxidant (ethoxyquin) for 64 days and then deleting the antioxidant.

The cerebella of young chickens fed linoleic acid contained greater proportions of linoleic acid, arachidonic acid, and fatty acids with retention times corresponding to C-22 polyunsaturated fatty acids than chickens fed low linoleic acid diets. The cerebella of chickens with encephalomalacia were higher in linoleic acid and an unknown acid than the cerebella from control chickens fed antioxidant. Other fatty acids were not significantly affected by the disease.

The cerebella of hens fed a high linoleic acid diet for 12 weeks, starting from 500 days old, contained a higher proportion of linoleic acid and C-20 triene than hens fed a low linoleic acid diet. In contrast to chicks, the % of arachidonic acid or fatty acids with retention times greater than arachidonic were not affected by diet.

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Introduction

ENCEPHALOMALACIA is a disease characterized by cerebellar degeneration. It occurs in young chickens fed diets low in biologically active antioxidants and high in linoleic acid (1). The brains of chickens fed linoloeic acid contain higher proportions of linoleic acid and arachidonic acid than brains of chickens fed low-linoleic acid diets (2,3,4). It has been suggested (1) that the initial cause of encephalomalacia is peroxidation of fatty acids of the linoleic acid family [i.e., fatty acids have the structure $CH_3(CH_2)_4$-$(CH{=}CH{-}CH_2)_{2\text{-}6}(CH_2)_xCOOH$] in the brain as a result of increased concentrations of such fatty acids in the brain concomitant with depletion of antioxidant from this tissue.

Destruction of a fatty acid by peroxidation should result in a decreased amount of that fatty acid. In the present studies the fatty acid composition of the brains of normal chickens, and those with encephalo-

malacia, was determined in an attempt to detect peroxidation by a decreased proportion of one or more unsaturated fatty acids.

Under laboratory conditions the greatest incidence of encephalomalacia occurs when the chickens are 10 to 20 days old (2,7); in the field the highest incidence is 10 days later (8). To our knowledge the disease has not previously been observed in birds more than 50 days old. When 180-day old pullets were fed a purified low-antioxidant, high-linoleic acid diet for 16 weeks no encephalomalacia was observed although egg production, fertility, and hatchability were drastically reduced (9).

The reason for the resistance of older birds to encephalomalacia could be a result of the metabolic inertia of the brain lipid of older animals (10,11,12), i.e. the fatty acid composition of an adult brain may not be affected by dietary linoleic acid. One of the objects of the present study was to determine whether adult brain tissue can be influenced by diet. Based on the "*in vivo* peroxidation" hypothesis, chickens fed high linoleic acid diets, but protected against encephalomalacia during their growth period by an antioxidant, should develop encephalomalacia when older if the protective antioxidant is removed from the diet. Such a study was conducted using the antioxidant Santoquin [2] as it is rapidly excreted (13), and it is effective in the prevention of encephalomalacia (1).

Experimental Procedure

Experiment 1. Day-old Nichols pullets were fed Diet S-35-E (Table 1). When the birds were 21 days old Santoquin was deleted from the diet of 70 birds. It was deleted from another group of 28 birds when they were 64 days old. All birds were examined daily for symptoms of encephalomalacia: ataxia, head retraction, and "bicycling" motion of legs. See Figure 1.

Birds from Experiment 1 which had gross evidence of encephalomalacia were decapitated; the cerebella were removed and immediately frozen by pressing against aluminum foil held against solid CO_2. They were store at −15C until analyzed for fatty acids. Cerebella were removed from control birds fed Santoquin at the same time.

TABLE I
Experimental Diets

Ingredients	Chick Diet S-35-E	Hen Diet S-25
Isolated soybean protein	35.00	25.00
Safflower oil or hydrogenated coconut oil [a]	.....	15.00
Coconut oil	6.00	.....
Ethyl linoleate (75%) [b]	6.00	.....
Salts A [c]	6.00	.....
Na $SeO_3 \cdot 5H_2O$	0.0001	0.0001
Hen salt mixture [d]	.....	5.00
Limestone	.....	2.80
Methionine hydroxy analogue [e]	0.80	0.40
Cellulose	.....	8.00
Vitamin mixture [c]	0.50	0.60
Choline chloride	0.20	0.25
Vitamin A (50,000 I.U./g)	0.05	0.05
Vitamin D_3 (7,500 I.C.U./g)	0.03	0.05
Santoquin	0.05	0.075
Vitamin E (20,000 I.U./lb)	.....	0.50
Glucose (Cerelose)	q.s. 100	q.s. 100

[a] Hydrol—Durkee Famous Foods, Chicago, Ill.
[b] Nutritional Biochemical Co., Cleveland, O. To prevent rancidity while in the diet 0.1% of the antioxidant Tenox 6 was added to the ethyl linoleate.
[c] Machlin, L. J., and R. S. Gordon, Poultry Sci., *37*, 1460 (1958).
[d] This supplied as % of the diet; $Ca_3(PO_4)_2$, 2.8; K_2HPO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.25; $Fe(C_6H_5O_7) \cdot 6H_2O$, 0.14; $ZnCO_3$, 0.015; KI, 0.004; $CuSO_4 \cdot 5H_2O$, 0.002; H_3BO_3, 0.009; $CoSO_4 \cdot 7H_2O$, 0.0001; $MnSO_4$, 0.065; NaCl, 0.70.
[e] Registered trade mark of the Monsanto Chemical Co., for calcium DL-2-hydroxy-4-methylthiobutyrate.

[2] Santoquin, registered trademark of the Monsanto Chemical Co. for ethoxyquin, 1,2-dihydro-6-ethoxy 2,2,4-trimethylquinoline.

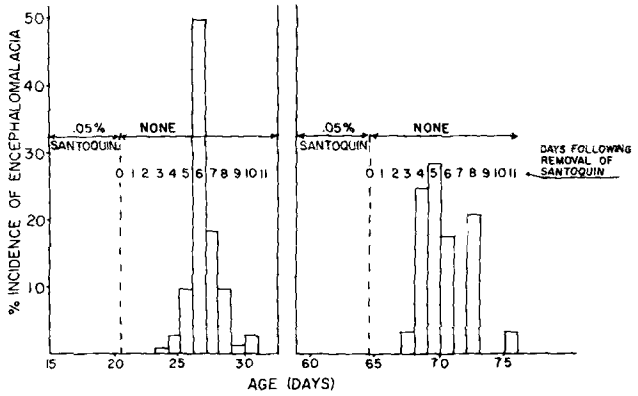

FIG. 1. Demonstration of encephalomalacia in chickens older than 67 days. All were fed a high linoleic acid diet (S-35-E) containing .05% Santoquin. Santoquin was removed from the diet of one group 21 days old and from another 64 days old.

Experiment 2. White Leghorn pullets in their second laying year were fed 12 weeks on a purified diet containing either safflower oil or hydrogenated coconut oil as the sole fat source (Table I). Four birds in each group were then killed and cerebella removed for fatty acid analysis. The fats were protected from oxidation by adding 0.075% Santoquin to the diet.

Fatty Acid Analysis

An approximately 10% tissue homogenate in distilled water was prepared at 0C. Mild saponification was done by adding 4 ml of 50% KOH to 4 ml of the 10% homogenate and heating at 50C under nitrogen for 24 hr. The mixture was acidified with concd HCl to pH 2 and extracted with purified pentane. The pentane was evaporated in vacuo at 25C and the fatty acids esterified by refluxing at 50C for 3 hr in 4.0 ml absolute methanol containing 0.5% dry HCl (w/w) and 0.5 ml of 2,2-dimethoxypropane (14). After adding 4.5 ml of distilled water the esters were extracted with purified pentane and micro distilled (15) on a cold finger at 60C and 0.15 mm Hg. for 1 hr. The purified esters were dissolved in pentane and stored at −15C under nitrogen until analyzed. Storage for as long as 3 months had no effect on the acid composition (2).

The gas chromatographic column used is essentially as described by Lipsky (16). A 2 m x ¼ inch stainless steel tube was packed with 20% LAC-3-R-728 on 60-80 mesh Chromosorb W and used at a column temperature of 180C and an injection block temperature of 290C. Gas flow was 130 ml helium per min. An Aerograph Model A 90-0 was used with a 1 mv Brown recorder, a disc integrator, and a hot wire detector. Results are expressed as % by wt of total fatty esters calculated from areas under the curves in the chromatograms. The retention times of known methyl esters were compared to the methyl esters from tissue samples to identify the major peaks. No pure methyl ester of a fatty acid with a retention time longer than behenic acid was used. The ratio of the retention time of all peaks relative to methyl stearate was calculated. Fatty acids are referred to by notation in which identification is given by carbon chain lengths, followed by the number of double bonds per molecule, if any. Quotation marks indicate that positive identification of a peak was not achieved.

Results and Discussion

Encephalomalacia in birds older than 67 days. When Santoquin was removed from the diet of birds

being fed a high linoleic acid diet (S-35) for 21 days or 64 days, 100% incidence of encephalomalacia was observed within 11 days following removal of the Santoquin (Fig. 1). In almost all cases, death with hemorrhage in the cerebella occurred within 48 hr after the initial symptoms. In no case did chickens receiving Santoquin show any evidence of the disease. This is the first time encephalomalacia has been observed in birds older than 50 days, and demonstrates a convenient method for producing the disease for biochemical studies.

Former failure to produce encephalomalacia in older birds fed low antioxidant, but high linoleic acid diets (9) was probably because of a low content of fatty acids of the linoleic acid family in the cerebella produced by low dietary intake of linoleic acid during early life when brain lipids were synthesized at a rapid rate, and/or sufficient antioxidant protection from retention of dietary tocopherols deposited in the brain during early life.

TABLE II

Fatty Acid Composition of Cerebella of Young Chickens; Effect of Dietary Linoleic Acid and Encephalomalacia

Fatty Acid	Ratio of Retention Time	Dietary Linoleic Acid		
		None	4.8%	
		Normal[a]	Normal[b]	Encephalomalacia[c]
		Fatty Acids in Cerebella (%)[d]		
	(18:0=1.00)			
14:0	0.35	1.1±0.16	1.5±0.25	1.3±0.08
16:0	0.59	28.6±2.20	28.1±0.68	27.3±0.84
16:1	0.66	2.9±0.28	2.6±0.37	1.0±0.39
18:0	1.00	17.4±0.82[f]	18.6±0.30[f,g]	19.6±0.18[g]
18:1	1.15	25.3±0.92[f]	21.7±0.80[g]	22.6±0.71[g]
18:2	1.40	0.3±0.09[f]	2.0±0.14[g]	3.5±0.20[h]
18:3	1.88	1.1±0.11[f]	0.7±0.06[g]	0.9±0.05[g]
"20:1"[e]	2.23	1.0±0.18[f]	0.2±0.07[g]	0.3±0.06[g]
"20:2"	2.51	7.2±0.96[f]	0.0 [g]	0.0 [g]
"20:3"	2.70	0.7±0.25[f]	0.4±0.13[g]	0.8±0.06[f]
20:4	3.10	5.1±0.24[f]	9.3±0.05[g]	9.3±0.20[g]
"22:3"	4.30	1.5±0.22[f]	0.0 [g]	0.0 [g]
"22:4"	5.25	1.1±0.32[f]	4.1±0.26[g]	3.6±0.33[g]
"22:5"	6.00	1.6±0.43[f]	4.4±0.29[g]	3.9±0.22[g]
"22:6"	7.99	5.5±0.67[f]	4.0±0.38[g]	3.7±0.23[g]

[a] 6 replicates.
[b] 5 replicates.
[c] 8 replicates.
[d] Average % by wt ± standard error (standard deviation).
[e] Quotation marks are used when other fatty acids may have the same etention time and therefore the fatty acid indicated is merely suggestive.
[f,g,h] Numbers with different letters are significantly different ($p<.05$).

Fatty acid composition of chick cerebella. Table II ummarizes the effect of dietary linoleic and encephlomalacia on the % composition of cerebella fatty cids. Confirming earlier reports (2,6,7), the cereella of chicks fed linoleic acid contained higher proortions of linoleic acid (18:2) and arachidonic acid 20:4). In the present studies, fatty acids with retenion times greater than 20:4 were readily measured. These peaks have not been identified, however, by xtrapolation of the log retention times of known atty acids and comparison with other published reorts (17); they would correspond to C-22 acids aving 3, 4, 5, or 6 double bonds and will be referred o as such tentatively. Such fatty acids are known to ccur in brain lipids (18,19,20). The biochemical gnificance of these acids will be difficult to assess ntil the peaks have been identified. However, our bservation that dietary linoleic resulted in increased 22:5" would agree with the previous reports of eiser *et al.* (21,22), who has shown that the chicken an convert 18:2 to pentaenoic acid. It was subequently found (23) that 22:5 was the pentaenoic id involved. In addition, Horwitt (24) reports at the cerebella lipids of chicks fed corn oil are igher in 22:5 than chicks fed diets low in 18:2.

The cerebella of chickens with encephalomalacia contained a higher proportion of 18:2 and "20:3" than control chickens fed the same level of dietary linoleic acid with an antioxidant. There is no apparent explanation for this observation. There were no significant differences in the percent 20:4, "22:4," "22:5," or "22:6." A difference of 5% in 20:4 could have been detected. However, for "22.4," "22.5," and "22.6," the variation was so large that a difference of over 18% would have been necessary for statistical significance. It is quite likely that peroxidation of much less than 18% of an unsaturated fatty acid would be sufficient to disrupt the lipid structure which contains it. Peroxidation causes changes in the structure of polyunsaturated fatty acids from the *cis* to the *trans* form and from unconjugated to conjugated double bond systems. Furthermore, β lipoproteins are denatured by lipohydroperoxides (25) and, therefore, generation of a small amount of hydroperoxide could disrupt a relatively large lipoprotein-containing structure in a cell. The present studies, therefore, may be too insensitive to detect the peroxidation process. In order to prove the "*in vivo* peroxidation theory," procedures which measure the longer chain unsaturated fatty acids with greater precision are necessary or, preferably, more accurate methods should be developed for detection of the products of peroxidation in tissues.

Fatty acid composition of hen cerebella. Studies with old hens (Table III) demonstrated that the fatty acid composition of the cerebella can be influenced by diet. The cerebella of hens fed a diet containing 15% safflower oil contained more 18:2 and "20:3" and less 10:0 and 14:0 than cerebella from hens fed 15% hydrogenated coconut oil as the sole source of fat. This indicates that there is no blood-brain barrier to linoleic (or shorter chain acids) as has been suggested by studies with the rat (26). In contrast to the observation with the young chicks, none of the fatty acids with retention times greater than 20:3 were influenced by diet. Apparently these longer chain acids are contained in lipid structures which have extremely slow turnover rates, in contrast to the shorter chain acids which may be concentrated in structures with appreciable turnover rates, and therefore are susceptible to dietary influences. The proportion of 20:4 and "22:4" in the cerebella of the hens was approximately the same as that observed in young chickens, and the proportion of "22:6" was even higher in older chickens. Therefore, if peroxidation of these fatty acids in young chickens is the

TABLE III

Fatty Acid Composition of Cerebella of Hens[a]

Fatty Acid	Ratio of Retention Time	Dietary Supplement[b]	
		15% Hydrogenated Coconut Oil	15% Safflower Oil
	(18:0=1.00)		
10:0	0.21	0.2±0.06	0.0±0.03**[c]
12:0	0.24	0.5±0.08	0.3±0.10
14:0	0.35	1.0±0.11	0.7±0.07*
16:0	0.59	24.1±0.28	23.7±1.12
16:1	0.66	2.9±0.23	4.3±0.60
18:0	1.00	17.8±0.38	18.6±0.41
18:1	1.15	24.8±0.90	22.9±0.84
18:2	1.40	1.0±0.20	3.0±0.37**
18:3	1.90	1.2±0.09	1.1±0.05
"20:3"	2.72	0.3±0.13	0.8±0.07**
20:4	3.11	8.0±0.27	8.2±0.72
"22:4"	5.31	5.0±0.47	4.0±0.36
"22:5"	6.00	0.3±0.22	0.2±0.18
"22:6"	7.99	9.1±1.28	9.0±0.99

[a] Hens had been fed commercial diets for approximately 500 days. They were fed the experimental diets for 12 weeks and then decapitated.
[b] Added to diet S-25.
[c] One asterisk indicated probability of less than 0.05; two less than 0.01 that difference is significant.

cause of encephalomalacia, one would expect the disease to occur in the hen as well as in the young chicken. However, since the proportion of "22:5" in the cerebella of hens is much less than that of the cerebella of chicks fed linoleic acid, it is possible that the peroxidation of this fatty acid or similar fatty acids is responsible for the development of encephalomalacia. Since dietary linoleic acid increased "20:3" in the cerebella we assume that this "20:3" belongs to the linoleic family in regard to the position of doubled bonds. Such a 20:3 acid has been found in liver tissue (27) and probably exists in other tissues as well.

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• *Letter to the Editor*

The Use of Iodine Vapor as a General Detecting Agent in the Thin Layer Chromatography of Lipids[1]

A SULPHURIC ACID SPRAY, followed by heating, is commonly recommended as a detection system of general utility in thin-layer chromatography. Although highly sensitive and capable of giving additional information through the development of colors peculiar to certain compounds or lipid classes, the corrosive nature of the spray makes the plates difficult to handle and not readily photographed when a permanent record is desired.

In this laboratory, iodine vapor has found favor as a general detecting agent for the following reasons: it is sensitive; corrosive residues do not remain on the plates; the plates can be photographed readily in color or in black and white, and additional information can be gained from the behavior of iodine in the spots.

Limits of sensitivity were established with the following compounds: inositol phosphate, glycerol phosphate, tripalmitin, triolein, egg lecithin, dipalmitoyl lecithin, β-sitosterol, and a corn oil sterol fraction. The compounds were spotted on plates using a concentration range of 50 to 1 μg. The plates were set in covered chromatography jars containing iodine crystals in a glass dish. An exposure time of 60 min was found to be sufficient when the temperature in the laboratory was between 70 and 80F; color formation starts within 5 sec of exposure. The plates were then removed and the location of the colored spots fixed by scraping around their periphery with a sharp pencil. All the above compounds could be detected at the 1 μg level. When 1 μg spots of each were placed on the origin and the plate developed in hexane:ether:acetic acid 90:10:1, only the sugar phosphates could not be located readily at the 1 μg level.

The observation that the brown iodine color left certain spots more readily than others suggested the possibility of classifying compounds on this basis. The data obtained with 5, 10, and 20 μg spots exposed to iodine vapor for 40 min are summarized in Table I.

TABLE I

Compound	Color after removal from vapor				
	1 min	1 hr	2 hr	4 hr	6 hr
Glycerol	M-Y[a]	V-P-Y	V-P-Y	V-P-Y	V-P-Y
Glycerol phosphate	M-Y[a]	P-Y	V-P-Y	V-P-Y	V-P-Y
Inositol	M-B[a]	V-P-Y	C	C	C
Inositol phosphate	P-B[a]	V-P-Y	C	C	C
Tripalmitin	Y	P-Y	P-Y	V-P-Y	V-P-Y
Triolein	V-D-B	D-B	D-B	D-B	D-B
Trilinolein	V-D-B	V-D-B	V-D-B	V-D-B	V-D-B
66% Tripalmitin 33% triolein	D-B	B-Y	M-B-Y	S-Y	S-Y
33% Tripalmitin 66% Triolein	D-B	B	B	B	B
66% Tripalmitin 33% Trilinolein	D-B	D-B	D-B	D-B	D-B
Dipalmitoyl kephalin	M-Y	M-Y	S-Y	S-Y	S-Y
Dipalmitoyl lecithin	D-Y	M-Y	M-Y	M-Y	M-Y
Egg lecithin	V-D-B	D-B	D-B	D-B	D-B
Lyso egg lecithin	M-Y	M-Y	B	B	B
β-sitosterol	S-Y	S-Y	S-B	S-B	S-B
Corn oil sterol fraction	V-D-B	V-D-B	V-D-B	V-D-B	V-D-B

B = brown; Y = yellow; D = dark; S = strong; M = medium; P = pale; V = very pale; C = colorless.
[a] Spot had pale white center surrounded by brown ring.

Sugars and sugar phosphates tended to lose their color quickly, and saturated fats were observed to lose color more rapidly than unsaturated fats. This characteristic served to differentiate between the triglyceride mixtures. The well-known ability of lecithin to bind iodine was demonstrated by the retention of color by both the saturated and unsaturated lecithins. At no time, however, was the dipalmitoyl lecithin as deeply colored as the egg lecithin. As expected, lyso egg lecithin behaved in an intermediate manner. Dipalmitoyl kephalin resembled tripalmitin rather than dipalmitoyl lecithin. The plant sterols tested developed a permanent dark brown color. The β-sitosterol spots, however, did not become brown until 2 hr after removal from the iodine vapor.

Bromine vapor was tried as a possible substitute for iodine vapor and proved unsatisfactory. A 4% aqueous solution of Thiodene, as a spray, was also tested as a supplement to iodine vapor. It decreased rather than increased the sensitivity and is not recommended as an adjunct to iodine vapor.

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