tibility of hamsters was about twice that of mice.

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## Effect of Intravenous Injection of Oxidized Methyl Esters of Unsaturated Fatty Acids on Chick Encephalomalacia.* (26093)

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Diets high in unsaturated fats and deficient in Vit. E are known to cause chick encephalomalacia(1,2,3) which can be prevented by addition to the diet of tocopherols or specific synthetic antioxidants(4,5,6) but not by selenium(7,8). As autoxidation of unsaturated fats can readily occur in an environment devoid of antioxidants, encephalomalacia may represent the consequence of consuming peroxidized fat or may represent the result of peroxidation *in vivo*. Furthermore, it has recently been observed that inclusion of various oxidized fatty acids(9) or fat(10) in the diet accelerates the incidence of the disease. The present study was designed to clarify the effect of oxidized fatty acids on development of encephalomalacia.

*Methods.* To compare the effect of various oxidized fatty acids on incidence of cerebellar disorders, 4 groups of 4 to 8 one-week-old male chicks (New Hampshire Columbian cross) were fed *ad libitum* for 2 weeks a purified diet(9) which contained 10% corn oil‡ and 4 groups the same diet supplemented with 8 mg % DL-$\alpha$-tocopherol. The diets were prepared daily. The oxidized fatty acids consisted of the hydroperoxide of methyl linoleate(11), the reduced hydroperoxide of methyl linoleate(12), and methyl 12-oxo-*cis*-9-octadecenoate prepared from methyl ricinoleate(13). These preparations were emulsified in chick serum (10 mg/ml) at approximately 1000 rpm for 1 minute at 0°C with a Teflon pestle homogenizer and 1 ml injected intravenously. After injection, the birds were allowed access only to water. When symptoms of cerebellar disorders were observed, the birds were sacrificed for histological examination(9).

In another study, week-old chicks were divided into 12 groups of 6 to 12 birds each and fed *ad libitum* Vit. E deficient diets. These diets contained 35.30 or 11.77% soy (ADM) protein, with or without inositol, ascorbic acid and varied amounts of stripped corn oil§ substituted into the diet at the expense of glucose. The diets were prepared daily. Birds which had not shown symptoms of encephalomalacia after 1 week were injected intravenously with 10 mg of methyl linoleate hydroperoxide emulsified in 1 ml of serum. The effect of dietary pretreatment on the severity of cerebellar disorders induced by intravenous injection of the lipohydroperoxide could thus be compared.

Four groups of 16 chicks which had been kept on the high protein, Vit. E deficient diet (treatment 2) were also injected as follows: one, 1 ml of low density lipoproteins ($S_f$ 20-400 class) obtained from chick serum(14); two, 1 ml of the low density lipoproteins which had been denatured by heating for 1 minute at 100°C; three, 1 ml emulsified methyl linoleate hydroperoxide (10 mg/ml serum); and four, 10 mg linoleic acid hydro-

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‡ Mazola, furnished through courtesy of Corn Products Refining Co., Argo, Ill.

§ Corn oil stripped of vit. E by high vacuum distillation, furnished through courtesy of Distillation Products, Inc., Rochester, N. Y.

TABLE I. Effect of Intravenous Injection of Oxidized Methyl Esters of Unsaturated $C_{18}$ Fatty Acids on Cerebellar Disorders.

| Group | Lipid inj. | Cerebellar disorders: Total chicks: No supplement | Cerebellar disorders: Total chicks: 8 mg % tocopherol |
|---|---|---|---|
| 1 | Methyl linoleate hydroperoxide | 5/8 | 0/8 |
| 2 | Reduced methyl linoleate hydroperoxide | 0/4 | 0/4 |
| 3 | Methyl 12-oxo-*cis*-9-octadecenoate | 0/4 | 0/4 |
| 4 | Methyl linoleate | 0/4 | 0/4 |

peroxide(15) in 1 ml serum.

*Results.* Intravenous injection of as little as 10 mg of the hydroperoxide of methyl linoleate into chicks kept on a diet which contained 10% corn oil induced cerebellar disorders such as ataxia, tremors, and retropulsive movement 1-5 hours after injection (Table I). No cerebellar disorders were noted after injection in birds kept on the diet which had been supplemented with 8 mg % $\alpha$-tocopherol. Injection of reduced hydroperoxide, methyl 12-oxo-*cis*-9-octadecenoate, or fresh methyl linoleate caused no cerebellar disorders. Thus, of the oxidized fatty acids tested, only lipoperoxide initiated symptoms of cerebellar disorders. Pathological examination of tissue of the cerebellum indicated that injection of methyl linoleate hydroperoxide caused considerable cerebellar edema or congestion of the capillaries. No detectable necrosis or hemorrhage, which has been considered to accompany the so-called nutritional encephalomalacia, was noted.

The presence of the mixed tocopherols in corn oil(16) might have partially prevented destruction of the cerebellum, since 8 mg % of additional $\alpha$-tocopherol completely prevented the cerebellar disorders caused by injection of methyl linoleate hydroperoxide. That the mixed tocopherols naturally present in corn oil influenced the development of cerebellar disorders was shown in chicks kept on diets which contained corn oil stripped of Vit. E. Response to injection of lipohydroperoxide into chicks kept on such Vit. E deficient diets was found to be greatly influenced by dietary composition.

Diets which caused a high incidence of nutritional encephalomalacia also caused severe destruction of the cerebellar tissue after injection of methyl linoleate hydroperoxide. A high dietary protein level greatly accelerated the rate of incidence of nutritional encephalomalacia (Table II). Furthermore, rate of incidence depended on the level of

TABLE II. Effects of Composition of Diets on Chick Nutritional Encephalomalacia and on Cerebellar Disorders Caused by Injection of Methyl Linoleate Hydroperoxide.*

| Treatment No. | Soy (ADM) protein, % | Stripped corn oil, % | Ascorbic acid, mg % | Inositol, mg % | Feed intake 7 days, g | Wt gain 7 days, g | Effect of intrav. inj. of methyl linoleate hydroperoxide in survivors: No. chicks with encephalomalacia | Total No. chicks | No. chicks with cerebellar disorders | Chicks showing hemorrhage and necrosis: No. | Chicks showing hemorrhage and necrosis: %‡ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 35.30 | 2 | 0 | 0 | 146 | 96 | 0/12 | 6 | 4 | 1 | 25 |
| 2 | 35.30 | 10 | 0 | 0 | 142 | 96 | 5/12 | 6 | 6 (2)† | 6 (2) | 100 |
| 3 | 35.30 | 20 | 0 | 0 | 140 | 59 | 10/12 | 2 | 2 | 2 | 100 |
| 4 | 11.77 | 2 | 0 | 0 | 144 | 52 | 0/12 | 6 | 1 | 0 | 0 |
| 5 | 11.77 | 10 | 0 | 0 | 129 | 45 | 0/12 | 6 | 5 | 1 | 20 |
| 6 | 11.77 | 20 | 0 | 0 | 129 | 46 | 2/12 | 6 | 4 | 2 | 50 |
| 7 | 35.30 | 10 | 75 | 0 | 136 | 96 | 2/6 | 4 | 4 (2) | 0 | 0 |
| 8 | 35.30 | 10 | 0 | 30 | 141 | 82 | 4/6 | 2 | 2 (2) | 2 (2) | 100 |
| 9 | 35.30 | 10 | 75 | 30 | 129 | 89 | 0/6 | 6 | 6 (2) | 1 | 17 |
| 10 | 11.77 | 10 | 75 | 0 | 129 | 51 | 0/6 | 6 | 5 | 0 | 0 |
| 11 | 11.77 | 10 | 0 | 30 | 129 | 47 | 0/6 | 6 | 6 (1) | 0 | 0 |
| 12 | 11.77 | 10 | 75 | 30 | 133 | 49 | 0/6 | 6 | 5 | 0 | 0 |

* 0.30 or 0.10% of glycine was added to high and low protein diet(9), respectively. Supplements to basal diet were made at expense of glucose on weight basis. † ( ) indicates shock-like symptoms developed in chicks. ‡ Out of No. which showed cerebellar disorders.

stripped corn oil; a high fat, high protein diet resulted in the highest incidence of encephalomalacia. Although the presence of either ascorbic acid or inositol alone was not effective in preventing a high incidence, the presence of both ascorbic acid and inositol depressed the incidence of encephalomalacia.

When the chicks which had not developed symptoms of encephalomalacia were injected with emulsions of methyl linoleate hydroperoxide, all of the chicks which had been kept on diets high in protein and fat (treatments 2, 3, 8) showed cerebellar ataxia and various degrees of degeneration, necrosis and hemorrhage of the cerebellar tissue after injection. Engorged capillaries were scattered through the necrotic tissue and sometimes accompanied an increase of the glia cells. No striking sign of organization in the malacic area was noted. These histological findings could be considered to represent an acute and a relatively early stage of the cerebellar lesion. Injection of methyl linoleate hydroperoxide did not cause lesions in the cerebrum, optic lobes, basal ganglia, medulla, or spinal cord. Most of the chicks, in treatments other than 2, 3, 6, or 8 which showed cerebellar disorders after injection of methyl linoleate hydroperoxide, revealed only dilation of the capillaries or cerebellar edema with disruption and separation of the cellular and fibrillar elements.

We noted that unless chicks showed positive symptoms of cerebellar disorders, no noticeable cerebellar lesions were present. Furthermore, symptoms of encephalomalacia were not precipitated by removal of feed from the chicks. Cerebellar disorders and the subsequent cerebellar lesions appeared to be induced only as a result of injection of methyl linoleate hydroperoxide. Although the emulsion of methyl linoleate hydroperoxide was injected slowly, some chicks died immediately in shock. Autopsy of these chicks indicated that only chicks which had been kept on a diet high in protein and fat (treatments 2, 8) showed noticeable cerebellar necrosis or hemorrhage. Chicks which had been supplemented with Vit. E did not go into shock even after rapid injection of methyl linoleate hydroperoxide. It therefore appeared that physiological conditions brought on by dietary pretreatment predisposed chicks toward shock.

As it has previously been reported that methyl linoleate hydroperoxide selectively or preferentially denatures serum $\beta$-lipoproteins *in vitro* (11), it was possible that denatured $\beta$-lipoproteins in the emulsion of methyl linoleate hydroperoxide might have caused cerebellar disorders after injection. However, injection of a 1 ml solution of heat denatured lipoprotein did not cause cerebellar disorders or shock. Thus, the initiation of cerebellar disorders appeared to be solely due to injection of the hydroperoxide of methyl linoleate. The hydroperoxide of linoleic acid was as effective as the hydroperoxide of methyl linoleate.

*Discussion.* Cerebellar disorders can be developed in chicks by 2 different but related means: (1) by injection of lipohydroperoxide and (2), by feeding of a diet high in unsaturated fat and deficient in Vit. E. The extent of cerebellar damage seemed to depend on dietary level of Vit. E, protein, fat, inositol, ascorbic acid and possibly other dietary factors.

The emulsion of methyl linoleate hydroperoxide may have decomposed after injection or other oxidative changes in the hydroperoxide may have occurred with time. However, the fact that injected reduced methyl linoleate hydroperoxide or a long chain carbonyl compound, methyl 12-oxo-*cis*-9-octadecenoate did not induce encephalomalacia seems to suggest that lipohydroperoxide, not the oxidized products derived from the fatty acids *in vivo*, is the initiating agent in encephalomalacia.

Nutritional encephalomalacia may develop as a result of both functional and organic disturbances in cerebellar tissue by lipohydroperoxide. When a sufficient but still immeasurable level of lipohydroperoxides has accumulated in the blood or some tissue site, they may act preferentially on the circulatory system in the cerebellum. Although 10 mg of hydroperoxide was sufficient to precipitate cerebellar disorders when injected, less lipohydroperoxide may be sufficient to induce

nutritional encephalomalacia. It is possible that a sufficient amount of lipohydroperoxide may accumulate from dietary sources in the blood of Vit. E deficient chicks to induce encephalomalacia, although most of the lipohydroperoxide may be destroyed during intestinal absorption. Nutritional encephalomalacia may also be accentuated by dietary prerequisites, such as Vit. E deficiency, a high fat, high dietary protein level, and absence of dietary ascorbic acid and inositol.

We have previously shown that methyl linoleate hydroperoxide inhibits lipid absorption in rats(11). However, when diluted with fresh methyl linoleate, some lipohydroperoxide seemed to be absorbed. Furthermore, the amount of lipohydroperoxide absorbed may not necessarily be proportional to the peroxide content of ingested fat. It is also possible that during Vit. E deficiency unsaturated fatty acids in serum and tissue lipoproteins may be peroxidized *in vivo* to give an additional amount of lipohydroperoxides to induce encephalomalacia.

It has recently been shown that the low density lipoproteins contain a major part of total serum tocopherol(17). Furthermore, we have recently shown that low density lipoproteins are very easily peroxidized *in vitro* and that hematin compounds greatly accelerate formation of lipohydroperoxide in isolated low density lipoproteins which resulted in rapid denaturation of the lipoproteins(18). Therefore, diets deficient in Vit. E, or containing high amounts of unsaturated fatty acids may not only be more susceptible to peroxidation, but may also favor *in vivo* peroxidation of serum low density lipoproteins. The aggravating effect of oxidized fatty acids or heated oils on encephalomalacia may be due to 1) their activity as prooxidants, 2) their peroxidation and destruction of Vit. E in the diet, and 3) their ability to cause lipoproteins to be more susceptible toward peroxidation *in vivo*.

*Summary.* Cerebellar disorders developed in chicks 1-5 hours after intravenous injection of 10 mg of the hydroperoxide of methyl linoleate or of linoleic acid emulsified in 1 ml of serum. No cerebellar disorders were noted, after injection, in birds kept on an identical diet but supplemented with 8 mg % $\alpha$-tocopherol. Injection of reduced hydroperoxide, methyl 12-oxo-*cis*-9-octadecenoate or fresh methyl linoleate caused no cerebellar disorders indicating only lipohydroperoxide initiated symptoms. The severity of cerebellar disorders induced by injection of lipohydroperoxide was greatly influenced by dietary pretreatment. The cerebellum of chicks which had been kept on high protein, high fat, Vit. E deficient diets was damaged more extensively by injection of lipohydroperoxide than the cerebellum of chicks kept on adequate diets. These data suggest that nutritional encephalomalacia may be initiated by accumulation of sufficient, though still immeasurable, levels of lipohydroperoxide *in vivo*.

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## A Solar Daily Variation in Oxygen Consumption of the Embryonated Egg.* (26094)

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For a number of years this laboratory has pursued an extensive comparative study of the persistent metabolic rhythms in algae, angiosperms, invertebrates, and vertebrates. These metabolic rhythms possess basic, component, solar and lunar daily variations which predominate or underlie the phase-labile, adaptive rhythms of special processes when the latter are present. The occurrence of such variations is called extrinsic rhythmicality because the periods, and at least to some extent, the amplitudes and forms, are derived in direct response to some subtle geophysical rhythm(1). The disclosure of these variations in all organisms studied suggests that this is a universal phenomenon. If there were present such fluctuations in the metabolic state of the embryonated egg, they might constitute a variable whose importance has not been recognized to date. Therefore, the following study was undertaken to learn (i) if systematic fluctuations did indeed occur and (ii) something of their character were this the case.

*Materials and methods.* $O_2$-consumption of individual eggs was measured with a Brown automatic recording respirometer(2, 3) in constant illumination, temperature, humidity, pressure, $CO_2$- and $O_2$-tension, etc. It was necessary to modify the respirometer chamber somewhat. A glass tumbler having a volume of 110 cc was fitted with a No. 11 rubber stopper. To the upper end of this stopper was sealed a plastic (Saran) sack of about 60 cc capacity which was the oxygen reservoir. The rubber stopper was penetrated by a 26-gauge hypodermic needle which served as a capillary connection between the collapsible oxygen reservoir and the chamber beneath. In operation 10 cc of water, a vial of KOH solution, and a small glass frame were placed in the bottom of the tumbler. The egg was rested air sack uppermost on the 4 tips of the glass support. The flask was stoppered, leaded until it just submerged, and suspended beneath the recorder in the water bath of a barostat at 38 $\pm$ .05° C. The unit was hermetically sealed and aspirated to a constant pressure of 28.7 in. Hg.

Eggs were obtained periodically from a local commercial source between Nov. 1959, and April, 1960. Before its oxygen consumption was recorded, each egg was allowed to warm to room temperature and was then placed in a forced-draft incubator at 38°C at about 4 P.M. C.S.T. The day on which incubation was initiated was designated Day 1. During the morning or afternoon of Day 2 or 3 the egg was transferred to the respirometer in which incubation was continued until termination of the experiment on Day 7. The egg was then opened and inspected. If an embryo did not appear to have developed normally its record was discarded. Ordinarily the oxygen supply was depleted on Day 7. Some data for Day 8 were obtained by opening the barostat for a 10-minute interval on Day 7 and replenishing the supply of oxygen and of $CO_2$-absorbent. This study is restricted to a 4-day period in development. The onset of the period is determined by the sensitivity of the recorder and the end, by the capacity of the $O_2$-reservoir.

*Results.* About 1800 hours of respiration

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