Effects of *trans* Fatty Acid Isomers upon Essential Fatty Acid Deficiency in Rats.* (22699)

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Recently it has been found that diets containing hydrogenated fats cause degeneration of spermatogenic tissue in weanling rats(1). Hydrogenated fats contain considerable proportions of *trans* isomers of the unsaturated fatty acids(2). Therefore it became of interest to learn if known *trans* isomers of the unsaturated acids have any effect upon spermatogenic tissue and if they are deposited in the animals. The most obvious unnatural fatty acid isomers to test for this effect are the *cis, trans* and *trans, trans* isomers of linoleic acid. These acids have been demonstrated to possess no essential fatty acid activity(3,4) and they are not oxidized by lipoxidase. Moreover, the all *trans* isomer of linolenic acid has been found to act as an effective competitive inhibitor of lipoxidase oxidation of linoleate(5), and it might be expected that the *trans* isomers act as competitive inhibitors of enzyme reactions involving essential fatty acids. Elaidic acid might also be suspected of antagonism to the essential fatty acids by the same reasoning. Therefore it was decided to test these substances for their overall effects on essential fatty acid (EFA) deficiency and upon the development of spermatogenic tissue. The quantities of these acids available did not allow a detailed or full investigation of their effects, but some effects have been observed in a few animals.

Methods. Male 15-day-old rats were fed a depletion diet containing 18% casein, 67% sucrose, 4% Wesson salts, 4% celluflour, 1% hydrogenated coconut oil, 1% cholesterol, 2% sulfasuxidine, and 3% urea plus required vitamins. The inclusion of cholesterol has been demonstrated to accelerate EFA deficiency(6). At the end of 3.5 months, all rats had developed severe EFA deficiency symptoms. The rats were then divided into pairs and were given a diet containing 18% casein, 73% sucrose, 4% salts, 4% celluflour, and 1% hydrogenated coconut oil plus the required vitamins. The fatty acid esters were then administered as oral supplements at the rate of 0.1 ml per day for 3 weeks. The rats were weighed and scored biweekly during the period of supplementation. At the end of this period, they were killed, and the left testis was removed from each for histological examination. The carcasses were extracted with boiling ethanol and petroleum ether. The lipid was saponified, the unsaponifiable matter was removed, and the free fatty acids recovered. The fatty acids from the odd-numbered rats were separated into "saturated" and "unsaturated" acids by crystallization from 10 volumes of petroleum ether per weight of acids at —40°. Fatty acids from even numbered rats were esterified with methanol for use in determining the content of *trans* unsaturation. The values are expressed as elaidic acid. Ethyl elaidate (*trans*-9-octadecenoic acid ethyl ester) was prepared from elaidic acid (m.p. 44°). Ultraviolet absorption was $k_{268} = 0.025$ and $k_{233} = 0.18$. Ethyl linolelaidate (*trans*-9, *trans*-12-octadecadienoic acid ethyl ester) was prepared by the method of Kass and Burr(7). Infrared spectrum showed no *cis* double bonds to be present. Ultraviolet absorption indicated the presence of about 7% conjugated diene acids. $k_{268} = 0.18$, $k_{233} = 6.7$. Methyl *cis*-9, *trans*-12-linoleate was prepared by Dr. O. S. Privett. The preparation had strong absorption bands at 10.28 μ (*trans*) and 3.3 μ (*cis*). $k_{233} = 0.52$ and $k_{268} = 0.19$. Ethyl linoleate (nat. *cis*-9, *cis*-12-octadecadienoic acid ester) was prepared by low temperature crystallization of safflower oil fatty acids. Infrared spectrum showed evidence of a trace of *trans*

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TABLE I. Effects of Fatty Acid Supplements upon E.F.A.-Deficient Rats.

Rat No.	Supplement	Wt gain, g	Score change*	Testis/body wt, %	Spermatogenic tissue†	Carcass fatty acids, g	*Trans* acids, %‡	Total *trans* acids, g‡	Gain/g *trans* acid‡
1	Ethyl elaidate	48	2.0		1	12.8			
2		40	− .5		2	12.2	1.4	.17	258
3	" linolelaidate	27	2.0	.13	1	20.8			
4		10	2.0	.22	1	13.5	2.7	.36	50
5	Linolelaidate +	71	0	.28	3	18+			
6	linseed oil	74	−1.5	.30	3	29.6	3.9	1.15	63
7	*cis,trans* linoleate	21	2.0	.34	3	12.6			
8		9	3.0	.20	2	9.8	3.0	.29	51
9	*Idem* + linseed oil	51	− .5	.26	4	32.3			
10		63	−2.0	.18	2	25.5	2.3	.59	97
11	Linolenate	42	− .5	.27	2	28.1			
12		92	1.5	.29	2	28.7	.9	.26	259
13	Linoleate	56	−2.5	.24	2	27.1			
14		50	−4.5	.31	4	33.5	1.2	.40	132
15	Linseed oil	62	−2	.35	4	22.7			
16		61	1.5	.28	4	29.3	0	0	0
17	None (fat-free)	31	1.0	.29	2	11.5			
18		36	.5	.22	1	19.0	0	0	0
19	Deficient initial					4.0			
20	controls					6.1	1.4	.1	

* Scores of 0-3 for scaliness of feet, 0-4 for scaliness of tail, and 0-2 for roughness of hair coat were added.

† Degree of recovery was graded from 0 to 5 as follows: Degree 0: Tubules appear atrophic and contain only syncytium of Sertoli cells with few scattered spermatogonia. 1: Spermatogonia and spermatocytes present, but epithelium of seminiferous tubules contain only one or 2 layers of germinal cells. Polynucleated large round cells in advanced stages of dissolution may occur. Spermatids and sperm present. 2: Spermatogonia, spermatocytes and some spermatids present. Many spermatids are in stages of degeneration, and polynucleated cells may be formed by fusion of degenerating spermatids. Mature sperm are absent, whereas scattered maturing sperm may be seen. 3: Spermatogonia, spermatocytes, spermatids and a few sperm present. 4: Epithelium of seminiferous tubules appears normal, but number of sperm in lumen of tubules is less than normal. 5: Complete recovery.

‡ Calculated as elaidic acid.

unsaturation. $k_{233} = 0.65$ and $k_{268} = 0.04$. Methyl linolenate (*cis*-9, *cis*-12, *cis*-15 octadecatrienoic acid methyl ester) was prepared by Dr. L. C. Norcia. Its iodine value was 251.5, $k_{233} = 1.6$ and $k_{268} = 0.09$. The infrared spectrum detected the presence of a small amount of *trans* unsaturation. Linseed oil had low ultraviolet absorption ($k_{233} = 0.4$ and $k_{268} = 0.08$) and its infrared spectrum showed no evidence of *trans* unsaturation.

Results. Growth. The fat-free diet allowed a weight gain during the period of supplementation only about half that caused by linseed oil or linolenate supplementation, and about two-thirds that caused by linoleate. Compared with rats fed fat-free diet, those fed linolelaidate or *cis, trans* linoleate grew very slowly. This growth inhibition was overcome in both cases by supplements of linseed oil. Elaidate allowed growth better than that allowed by the fat-free diet. Thus the single *trans* double bond in a monoenoic acid does not inhibit growth, whereas in the nonconjugated diene, linoleate, one or more *trans* double bonds induces inhibition. Previous studies of the action of these compounds with much older rats did not demonstrate actual inhibition of growth, but these acids were found unable to stimulate growth in the older deficient rats(3,4). In the present study, the effect is more striking.

Skin condition. The skin symptoms were worsened by supplementation with linolelaidate or *cis, trans* linoleate. Previous experiments with older rats had demonstrated the inability of these unnatural isomers to cure skin symptoms. The worsening of the skin symptoms was prevented by linseed oil when it was given with the unnatural isomers.

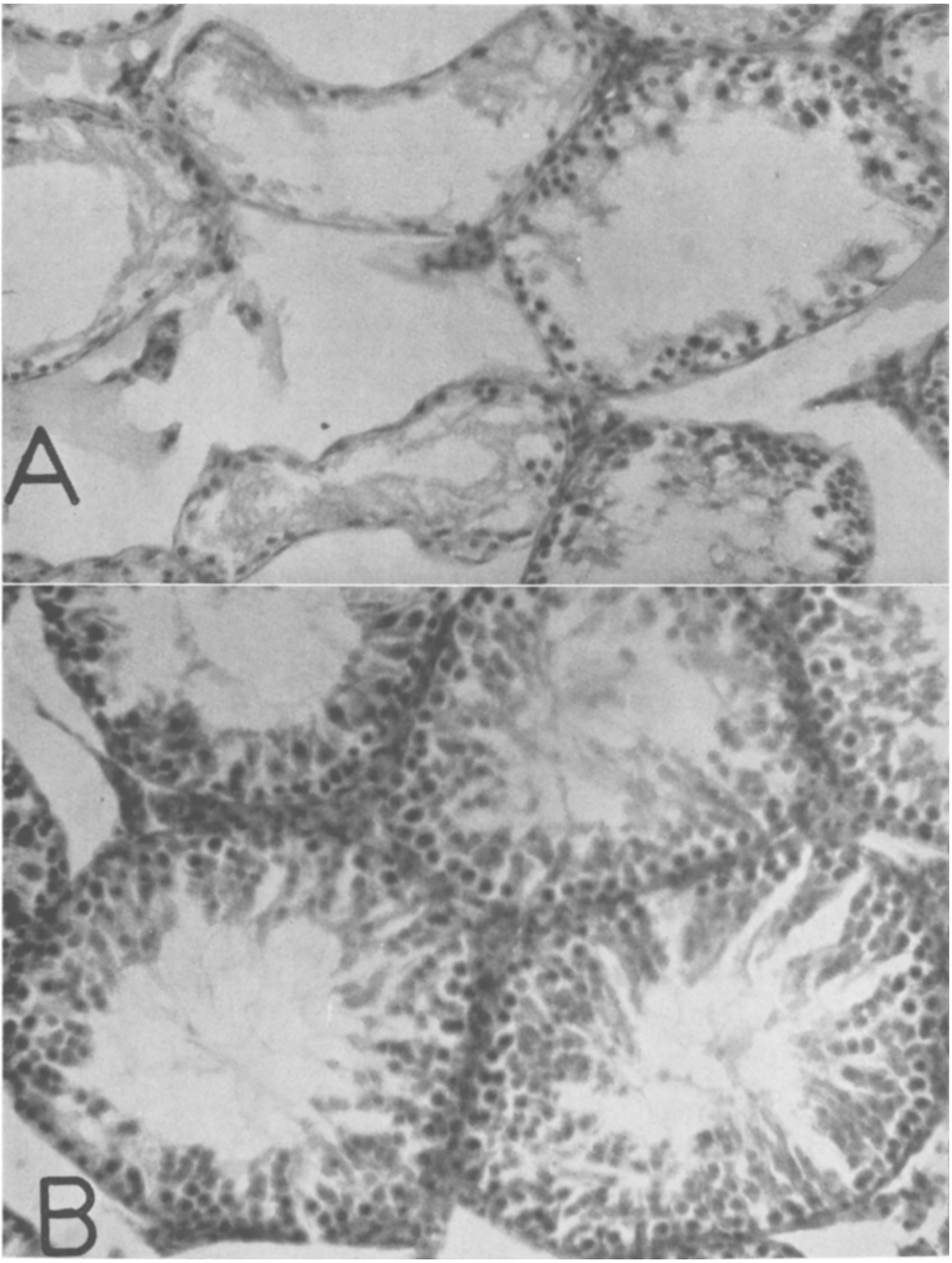

FIG. 1. A. Degeneration of spermatogenic tissue as result of EFA deficiency accelerated by cholesterol. Section of testis from rat No. 18. B. Recovery of spermatogenic tissue as consequence of supplementation with linseed oil. Section of testis from rat No. 16. Hematoxylin-Eosin stain. Magnification 67×.

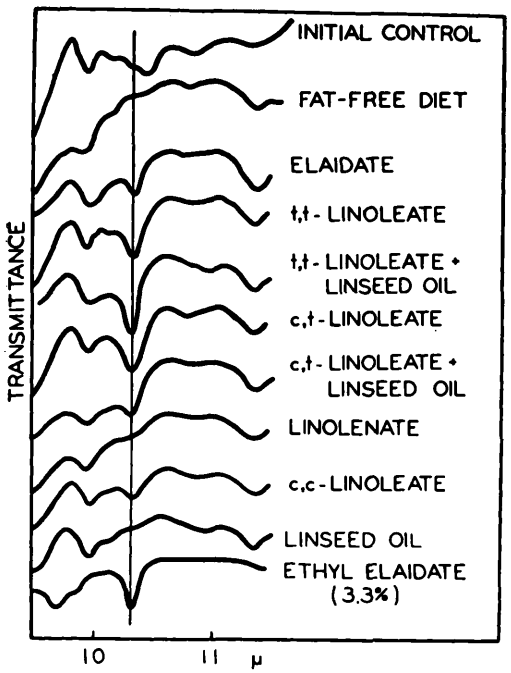

FIG. 2. Infrared spectra of fatty acid ethyl esters from rats fed various supplements. Spectra measured on 50% solutions of ester in carbon disulfide and compared with 3.3% ethyl elaidate + 46.7% ethyl oleate in carbon disulfide (lower curve).

Improvement of skin condition was significant only in the rats fed linoleate.

Testis weight. Testis weights of rats fed the *trans* isomers of linoleate were low. The ratio of testis weight to body weight was somewhat low in rats fed linolelaidate, in one rat fed *cis, trans* linoleate, in one fed *cis, trans* linoleate plus linseed oil and in one fed the fat-free diet. In all other cases only minor variations were found.

Histology of testis. That cholesterol increases the depletion of essential fatty acids is also indicated by the severe degeneration of spermatogenic tissue in the depleted rats. In a previous experiment weanling rats fed a fat-free diet 18 weeks had almost normal testicular tissue upon histological examination. but in the epididymi considerable numbers of degenerate spermatogenic cells were present (1). In the present study spermatozoa were not found in the epididymi of any of the rats. The epididymi were empty or contained only a few degenerate spermatogenic cells. Degenerated testes and the effect of feeding linseed oil are shown in Fig. 1. Supplementation of the fat-free diet with either elaidate or linolelaidate did not result in recovery of the spermatogenic tissue (Table I). Best recoveries were found in rats supplemented with linseed oil, and in one rat fed linoleate. Linolenate had no effect upon the spermatogenic tissue. The effect of *cis, trans* linoleate upon recovery of spermatogenic tissue is questionable.

Deposition of trans acids. The infrared spectra of methyl esters of the fatty acids of one rat from each group showed differences (Fig. 2). At the end of the depletion period there was *trans* unsaturation in the fatty acids of the rat. The sample taken from the fat-free rat at the end of the period of supplementation showed no evidence of *trans* double bonds, nor did the sample from the rat fed linseed oil. Fatty acids from the rats fed linoleate or linolenate had 1.2 and 0.9% of *trans* acids respectively, but the fatty acids from rats fed the unnatural *trans* isomers contained two or three times this amount. When the weight gain is related to the amount of *trans* unsaturation (expressed as elaidic acid), it is clear that the *trans* monoenoic acid does not have the growth inhibiting effect of *cis, trans* or *trans, trans* dienoic acids. It also appears that all the *trans* acids inhibited fatty acid synthesis.

Summary. Feeding weanling male rats a diet free of essential fatty acid (EFA), containing 1% cholesterol, 2% sulfasuxidine, and 1% hydrogenated coconut oil for 3.5 months induced an EFA deficiency accompanied by severe testicular degeneration. The degeneration was partially corrected by supplementation with linoleate or linseed oil for 3 weeks. However, sperm were not found in the epididymis in any case. *Trans* isomers of linoleic acid were unable to induce recovery of the testis. The *trans* isomers inhibited growth, worsened the skin condition, and were found to be deposited in the animal fat.

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Histopathological Changes by Acetoacetate in Normal and Scorbutic Guinea Pigs.* (22700)

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Acetoacetate, which on repeated injection has been reported from this Laboratory to induce experimental diabetes by causing inactivation of insulin(1), hyperglycemia accompanied with reduced glucose tolerance(2), depletion of blood GSH(3), depletion of thiamin, riboflavin and niacin(4,5), has recently been found to cause hypovitaminosis C(6), which is also known to bear a close relation with diabetes(7,8). It has also been shown that disturbance in carbohydrate metabolism brought about in scurvy(9) is further aggravated by continued injection of acetoacetate in gradually increasing doses(10). Now because acetoacetate causes depletion of blood ascorbic acid when injected in high doses and histopathology in scurvy has been shown by Mukherjee and Banerjee(11) to reveal widespread degenerative changes in liver, spleen, kidney, testes and suprarenal cortex of chronic and acute scorbutic guinea pigs and since β-hydroxy butyric acid, another member of the ketone bodies has been shown by Nath *et al.*(12) to bring about histological changes in the pancreas, it seemed desirable to study the histopathological changes of different tissues of normal as well as scorbutic guinea pigs injected with acetoacetate in gradually increasing doses.

Methods. Fifty male guinea pigs weighing from 300 g to 400 g were divided into 4 groups, each group containing animals of approximately corresponding weights. The last group of animals served as the control, each animal being fed 5 mg ascorbic acid daily supplementing the recommended scorbutic diet (Group I—Control). Group II animals received diet like that of Group I and plus daily injections of acetoacetate (Sodium salt solution of concentration 200 mg/cc at pH 7.2) intraperitoneally, in increasing amounts. The initial dose was 175 mg/kg, increased by 40 mg/kg every third day so that final dose after 21 days was 415 mg/kg (Group II—Acetoacetate injected). Scurvy was produced in animals of Group III by feeding scorbutic diet as recommended by Banerjee(13). Animals of Group IV were fed the scorbutic diet and injected daily with acetoacetate in dosage similar to that in Group II (Group IV—Scorbutic and superimposed with acetoacetate injection). Animals were housed in individual cages. Paired feeding technic was followed to obviate any results due to inanition. The animals of Group II served as the control for this purpose, as it was seen from a preliminary experiment that the diet intake of this group was found to be the lowest as the experiment advanced. The animals of this group were fed the scorbutic diet *ad libitum* and the amount consumed by each was noted every day. The same amount of diet was fed, to every animal of corresponding weight in all the other groups, on the subsequent day. Just after decapitation different tissues of the animals of each group were removed and fixed in Zenker-formol solution. After dehydration

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