

## ON THE FATTY ACIDS ESSENTIAL IN NUTRITION. III\*

By GEORGE O. BURR, MILDRED M. BURR, AND ELMER S. MILLER

(From the Department of Botany, University of Minnesota, Minneapolis)

(Received for publication, April 16, 1932)

After it was demonstrated (1) that a deficiency disease was caused by the lack of fatty acids in the diet, a study of the well known natural fatty acids was undertaken. In the second paper of this series (2) it was shown that none of the saturated fatty acids occurring in hydrogenated coconut oil was effective in curing the disease and promoting renewed growth of the animal. Pure methyl linolate is highly effective in curing sick animals and all oils which contain appreciable amounts of this acid are likewise good.

Since butter (30 per cent oleic acid) gave very poor results at the high level of 300 mg. daily (3 per cent of the diet) it was postulated that oleic acid is entirely negative and the small effects due to butter were due to traces of linoleic acid. But a sample of commercial methyl oleate gave good results and the value of oleic acid was left uncertain. Other common fatty acids which are now being studied are linolenic, arachidonic, and eleostearic. It is the object of this paper to report the results of some studies of these acids.

### *Diet and Technique*

The constant temperature room is now maintained at 26.0° ± 1° the year round. The same cages and diets described in the first paper (1) are used for all work unless specifically stated to be changed. The maintenance diet, Diet 550-B, contains 12 per cent pure casein, 84.1 per cent sucrose, and 3.9 per cent salt mixture

\* This work was supported by grants from the Medical Research Fund of the University of Minnesota, the National Research Council, and the Institute of American Meat Packers. Reported before the American Society of Biological Chemists at Montreal, 1931.

(McCollum Salt Mixture 185) (3). This is supplemented daily with 0.65 gm. of ether-extracted Northwestern dry yeast, and the non-saponifiable matter from 70 mg. of highest grade cod liver oil (Patch) and from 35 mg. of wheat germ oil. All known vitamins seem to be supplied in excess. The drinking water is distilled and contains 0.27 mg. of KI per liter.

Rats are weaned when 21 days old and put on the low fat diet. They must weigh over 36 gm. on weaning day. The weight curves reach a plateau when the rats are about 150 gm. in weight and when it has been established that they have reached their maximum weight and are actually declining slightly they are used as curcs. Positive results are marked by a clearing of the skin, improvement of hair coat, and renewed growth both in length and weight. Increase in weight is used as the quantitative measure of the effectiveness of an oil or fatty acid.

#### EXPERIMENTAL

*Linoleic Acid*—In the preceding paper (2) 5 drops daily of methyl linolate were used. When larger or smaller doses are used (10 or 3 drops) marked differences of rate of response can be seen (Chart I). By similar studies on oils it has been demonstrated that maximum effects are reached at the 10 drop level. The pure methyl linolate was prepared from corn oil by the method of Rollett (4).

*Oleic Acid*—The preparation of oleic acid free from appreciable quantities of contaminating acids presents some difficulties. The method of Lapworth *et al.* (5) was used for olive oil. This procedure requires the separation of lead salts, preparation and purification of barium oleate, and finally esterification and distillation. This preparation is presumably free from linoleic acid and contains about 2 per cent palmitic acid, which does not interfere with our tests.

Since butter gave almost negative results as a curative fat, oleic acid was also prepared from it. Melted and filtered butter was saponified and esterified in the usual way and the esters subjected to fractional distillation. All of the lower fatty acid esters were removed at 140° and 3 mm. pressure. The residue was saponified and the oleic acid was purified by the same technique as used for olive oil. Yield, 75 cc. of methyl oleate from 800 gm. of butter.

The methyl oleate was fed to six rats, three receiving the olive oil acid and three the butter acid. The curves given in Chart II show that no growth resulted from the feeding of these esters over a period of 50 days. The hair coat, skin, and tail showed no improvement. There is no evidence that oleic acid has any curative effect although it may arrest the downward trend of the animals. It seems, therefore, that the slight positive effects noted

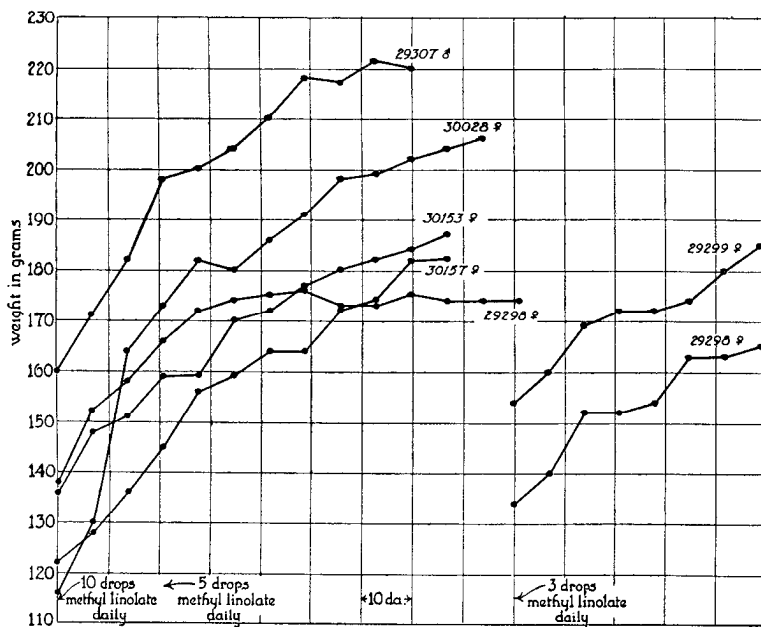


CHART I. Weight curves showing the growth response of rats to different quantities of methyl linolate. Along with the renewed growth there is rapid clearing of the skin.

for butter (2) are due to acids more unsaturated than oleic, probably linoleic.

*Linolenic Acid*—Although linolenic acid is usually absent from stored fats such as lard and tallow, it may be deposited in the fat depots if the food supply furnishes much of the acid (6). Levene and Rolf (7) have shown that in liver lecithin linolenic acid exceeds linoleic acid. However, Turner (8) found no linolenic acid in sheep liver.

Pure methyl linolenate was prepared from linseed oil by the method of Rollett (9). The hexabromide was recrystallized until it melted at 180–181° (uncorrected). This assured the elimination of all but traces of other fatty acids and their bromides. The hexabromide was then debrominated, esterified, and distilled at less than 1 mm. The water-clear ester was stored *in vacuo* until used. Special precautions were taken to prevent oxidation after each sealed tube was opened. The methyl linolenate was fed at a low level only. Results with three rats are given in Chart

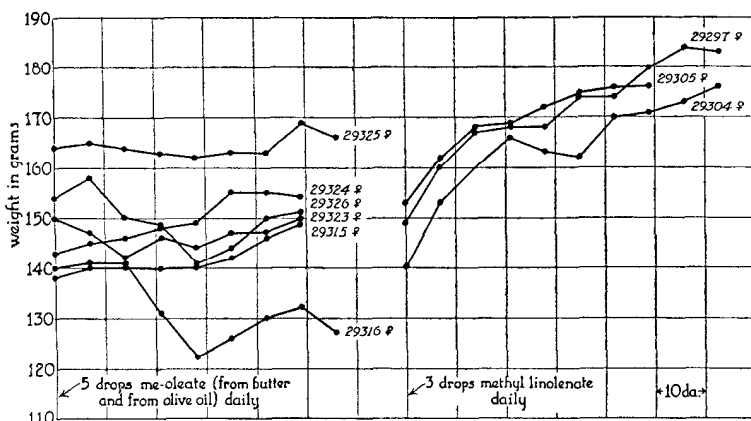


CHART II. Weight curves showing the growth response of rats to methyl oleate and methyl linolenate. Rats receiving methyl oleate show no renewed growth or clearing of the skin. Methyl linolenate quickly cleared the skin and the growth rate equaled that due to a like quantity of methyl linolate.

II. The gain in weight is almost identical with that for 3 drops of methyl linolate and the skin clears with great rapidity. It seems that linolenic acid can replace linoleic acid completely in the curing of rats suffering from a deficiency of fat.

*α-Eleostearic Acid*—Tung (China wood) oil is not ordinarily considered edible, but it has no harmful effects on rats when fed in small quantities. It is composed largely of the glyceride of *α*-eleostearic acid. A small amount of other unsaturated acids is present but linoleic and linolenic acids have not been reported present. *α*-Eleostearic acid melts at 48° and may be readily

changed into the  $\beta$  acid which melts at  $71^\circ$ . Since they readily absorb only 2 molecules of bromine the eleostearic acids were formerly considered isomeric with linoleic acid. The recent work of Böeseken and coworkers (10) shows that there are three double bonds in the eleostearic acids and that they are isomeric with linolenic acid.  $\alpha$ -Eleostearic acid and its glycerides absorb oxygen very rapidly from the air and it is of interest to know whether this

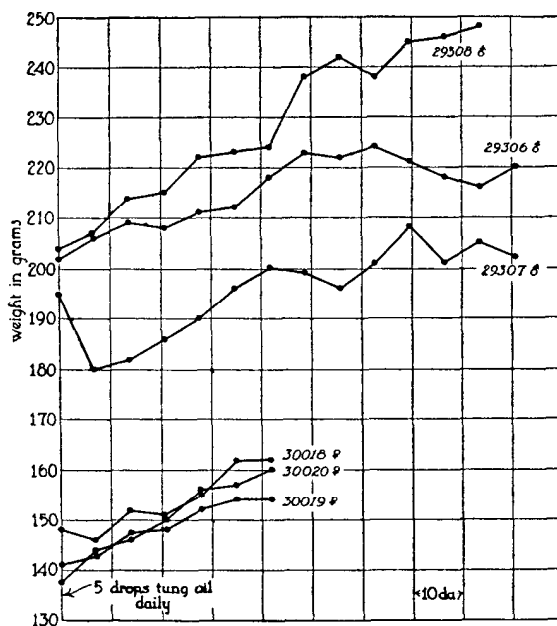


CHART III. Weight curves showing the slow response of rats to tung oil. The skin cleared very slowly.

acid which does not occur in animals can replace the more common linolenic acid. Tung oil was first fed to six rats. Slow but positive cures were effected. The skin gradually improved. The very gradual growth (Chart III) indicated that a trace of impurity rather than  $\alpha$ -eleostearic acid was causing the response.

Pure  $\alpha$ -eleostearic acid, m.p.  $44-45^\circ$ , was prepared from Florida tung oil.<sup>1</sup> This was given to rats in 5 drops doses for 2 weeks, but

<sup>1</sup> This oil was kindly furnished by Dr. J. S. Long, Lehigh University. It was the 1928 crop and had been kept under nitrogen.

they did not eat it well and the experiment was of little value. No tendency toward improvement was seen (Chart IV).

Methyl- $\alpha$ -eleostearate was then prepared by two methods. According to the first the acid was dissolved in an equal volume of absolute methyl alcohol and enough methyl alcoholic hydrogen chloride added to make a 4 per cent solution of HCl. This was left under  $\text{CO}_2$  at room temperature overnight. The ester was purified by washing with dilute  $\text{Na}_2\text{CO}_3$ , distilled water, and  $\text{CaCl}_2$  brine. The ester was finally taken up in ether, dried over anhydrous  $\text{Na}_2\text{SO}_4$ , and finally recovered *in vacuo*.

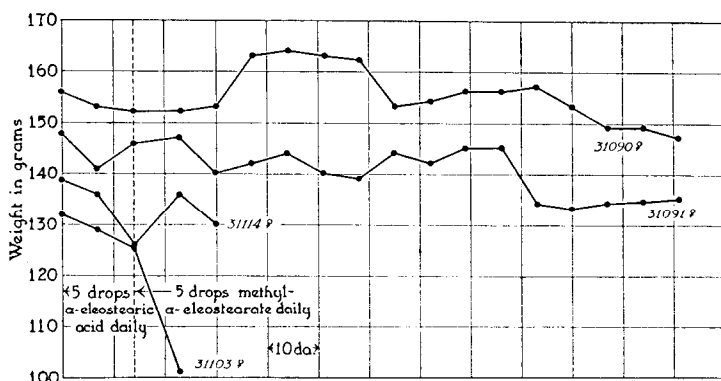


CHART IV. Weight curves showing the response of rats to  $\alpha$ -eleostearic acid and to methyl- $\alpha$ -eleostearate. This acid fails to improve the skin or increase the weight.

This preparation is of high quality without distillation. Distillation must be avoided since it causes a rearrangement into the isomeric form, methyl- $\beta$ -eleostearate. The  $\alpha$ -eleostearate was sealed *in vacuo* and fed by dropping from a syringe so that the air was always excluded.

The other preparation was made by the very mild reagent, diazomethane.<sup>2</sup> The reaction goes smoothly and completely to give a high quality product requiring little or no purification.

Since there is some confusion in the literature concerning the

<sup>2</sup> We are indebted to Professor Lee I. Smith of the Department of Organic Chemistry for the first preparation made and for the detailed technique used by us.

rearrangement of  $\alpha$ -eleostearic acid into  $\beta$ -eleostearic acid we checked our esters by saponifying small samples and recovering the free acid. After a single crystallization from alcohol the melting point was always between 43–45°. But when the methyl ester was distilled at 5 mm. pressure and the distillate was saponified, the  $\beta$  acid was recovered. After a single crystallization from alcohol it melted at 67–68°.

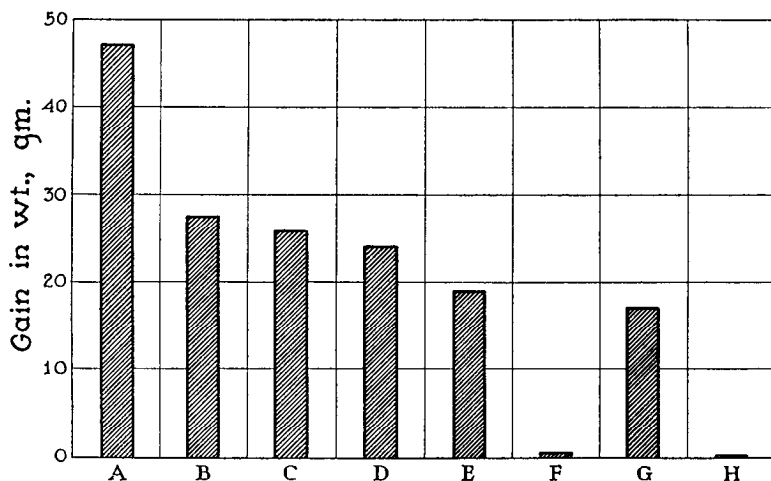


CHART V. A summary of the results given in the previous charts. The columns give the average maximum gains in weight of groups receiving the supplements over a period of 40 days. Column A, 7½ drops of methyl linolate daily; Column B, 3 drops of methyl linolate daily; Column C, 3 drops of methyl linolenate daily; Column D, 3 drops of methyl linolate plus methyl linolenate (1:1 mixture) daily; Column E, 3 drops of methyl linolate plus methyl linolenate (1:1 mixture) plus 10 per cent methyl arachidonate daily; Column F, 5 drops of methyl oleate (from butter and from olive oil) daily; Column G, 5 drops of tung oil (90 per cent eleostearic acid) daily; Column H, 5 drops of methyl- $\alpha$ -eleostearate daily.

All of the preparations gave the same nutritional results. Some curves are shown in Chart IV. There is no evidence of improvement, either in weight or in skin condition.

#### DISCUSSION

A general summation of the comparisons of oleic, linoleic, linolenic, and  $\alpha$ -eleostearic acids is given in Chart V. In all cases

where considerable growth took place the skin cleared and the rats were generally improved. A better muscle tone is always noticeable after a rat has been cured.

By this work oleic acid has been definitely grouped with the saturated acids as ineffective in the curing of rats subnormal because of the lack of fat. This substantiates the arguments put forth in the second paper of this series (2) that it is possible for animals to synthesize from carbohydrates large amounts of fat and still suffer from a fat deficiency. The review of the literature will not be repeated here but it seems clear that warm blooded animals synthesize only the saturated acids and oleic acid and that they are dependent upon the food supply for linoleic and linolenic acids. One of these two acids must be ingested by the rat if it is to survive and our findings indicate that they are interchangeable in the tissues. Further work is being done on the relative values of the two.

The comparison of whole tung oil with methyl- $\alpha$ -eleostearate is interesting. Since the  $\alpha$ -eleostearic acid does not have any curative effect it is evident that there is an acid in tung oil in small amounts which causes the renewed growth. Similar effects were seen when 15 drops of butter were fed daily to rats (2). Since pure oleic acid and the saturated fatty acids are ineffective, small amounts of undetermined acids are assumed to be present. These acids are probably linoleic or linolenic.

A mixture of linoleic and linolenic esters is of no more value than either of the esters alone (Chart V, Column D). This is interesting since tissues normally have a mixture of the two. When methyl arachidonate was added as 10 per cent of the mixture the animals uniformly showed less response (Chart V, Column E). The reason for this is not at all clear. Lard contains appreciable amounts of arachidonic acid and it is one of the best curative fats. Liver and liver fat are rich sources of arachidonic acid. Both have been used by us as preventives for the fat deficiency and have proved highly effective. Since there is no reason to attribute toxic effects to small amounts of arachidonic acid it seems probable that some of the purified arachidonic acid which we have fed has been altered in the process of preparation.

## CONCLUSIONS

1. Both linolenic acid and linoleic acid are effective in curing rats suffering from a fat deficiency. They seem to be about equal in value and can replace each other in the tissues.

2. Oleic acid is ineffective in the curing of sick rats and is classed with the saturated acids.

3.  $\alpha$ -Eleostearic acid, an isomer of linolenic acid, is ineffective in curing sick rats. This might be attributed to its high melting point.

4. Tung oil, like butter, has enough undetermined unsaturated acids to effect slow cures.

5. Mixtures of linoleic and linolenic esters are no more effective than a single ester, while the addition of a preparation of methyl arachidonate has a slight unexplained depressing effect.

## BIBLIOGRAPHY

1. Burr, G. O., and Burr, M. M., *J. Biol. Chem.*, **82**, 345 (1929).
2. Burr, G. O., and Burr, M. M., *J. Biol. Chem.*, **86**, 587 (1930).
3. McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 63 (1918).
4. Rollett, A., *Z. physiol. Chem.*, **62**, 410 (1909).
5. Lapworth, A., Pearson, L. K., and Mottram, E. N., *Biochem. J.*, **19**, 7 (1925).
6. Ellis, N. R., and Isbell, H. S., *J. Biol. Chem.*, **69**, 239 (1926).
7. Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, **67**, 659 (1926).
8. Turner, K., *Biochem. J.*, **24**, 1327 (1930).
9. Rollett, A., *Z. physiol. Chem.*, **62**, 422 (1909).
10. Böeseken, J., *J. Soc. Chem. Ind.*, **48**, 71T (1929).