106. FAT-DEFICIENCY DISEASE OF RATS. THE RELATIVE CURATIVE POTENCIES OF METHYL LINOLEATE AND METHYL ARACHIDONATE WITH A NOTE ON THE ACTION OF THE METHYL ESTERS OF FATTY ACIDS FROM COD LIVER OIL

By ELEANOR MARGARET HUME,[1] LESLIE CHARLES ALFRED NUNN,[2] IDA SMEDLEY-MACLEAN and HANNAH HENDERSON SMITH

From the Lister Institute, London, S.W. 1

(*Received 9 March 1940*)

In a previous communication [Hume *et al.* 1938] experiments were described in which the relative potencies of methyl linoleate and linolenate in curing the fat-deficiency disease of rats were determined. The finding of Burr *et al.* [1932] was confirmed that the presence of either of these acids in a fat-free diet is able to promote the weight increase of rats but, in contrast with the results of Burr & Burr, it was found that linolenic acid had only about one-sixth of the potency of linoleic acid.

The highly unsaturated acids which have so far been shown to be characteristic of animal fat are the C_{20} acid (arachidonic) with 4 double bonds, and the C_{22} acid with 5 double bonds; it was shown by Nunn & Smedley-MacLean [1938] that arachidonic acid was absent from the livers of rats which had been fed for about 7 months on the fat-deficient diet, but was present in those which, after such a depletion period, had received for 5 weeks a daily dose of 14 mg. methyl linoleate.

Investigation of the activity of arachidonic acid was therefore of interest. Two apparently conflicting statements as to its biological activity appear in the literature. Burr *et al.* [1932] replaced 10% of a mixture of methyl linoleate and linolenate by methyl arachidonate, and found that the mixture containing the arachidonic acid was less effective than the original mixture. Turpeinen [1938], on the other hand, working in Evans's laboratory, claimed that methyl arachidonate was 3 times as potent as the linoleate in promoting weight increase, but he gave no detailed account of its effect on the skin condition. An investigation of the comparative effects of methyl linoleate and methyl arachidonate has therefore been carried out and is now described. The results appear to establish conclusively the superiority of methyl arachidonate in promoting weight increase but not in curing the skin lesions.

Preparation of methyl arachidonate.

The raw material used at first was pig's liver in which the highly unsaturated acid is mainly arachidonic [Hartley, 1909; Brown, 1928]. The freshly minced liver was added in 1 kg. portions to 2·5 l. of 4*N* HCl and the mixture boiled for 3 hr. in an atmosphere of N_2; the solid residue was then filtered off and

[1] Member of the Scientific Staff, Medical Research Council.

[2] Grocers' Research Scholar of the Lister Institute.

heated with 10% alcoholic KOH for 1 hr. in an atmosphere of N_2, and part of the alcohol was then distilled off; the residual solution was acidified and extracted with benzene. The benzene solution of the fatty acids was dried for a short time with anhydrous Na_2SO_4, and Br was added in slight excess. The solid bromide which separated was extracted with ether and ethyl acetate; methyl arachidonate was obtained from it by debromination and esterification [Ault & Brown, 1934].

The accumulation in this way of a supply of methyl arachidonate sufficient for the feeding experiments would have been a laborious process, but it was subsequently possible to make more rapid progress through the kind help of Messrs Allen & Hanburys who furnished a supply of the crude fat extracted from ox suprarenal glands, which has been shown to be a rich source of arachidonic acid [Ault & Brown, 1934]. The crude fat was saponified with alcoholic KOH, and from this the acid was prepared as described above for pig's liver. The I.V. for the preparations of methyl arachidonate used for feeding were 312, 315, 320 and 290 (theory for $C_{21}H_{34}O_2$=319).

Biological technique

The biological technique was as described in the previous communication [Hume *et al.* 1938], with the following modifications.

Vitamins A and D were given in the form used in the second part of the previously reported investigation, vitamin D being given as irradiated ergosterol.

Vitamin E in the present experiments was given as *dl*-α-tocopherol, kindly supplied by Roche Products, Ltd. A 4% solution in paraffin oil, with the addition as stabiliser of 0·01% of ψ-cumoquinol (the gift of Prof. A. R. Todd), was given once a week, not later than the 9th week of experiment, in an amount corresponding to 0·8 mg. tocopherol, to all rats. The only available evidence that the dose was a sufficient one was the normal post-mortem appearance of the uterus, which was completely devoid of the brown coloration described by Martin & Moore [1939] as characteristic of even the virgin rat's uterus in vitamin E deficiency.

The rats received the fat-free diet from weaning, and dosage with the test material was begun not earlier than the 145th day and was continued for 5 weeks.

Both weight increase and cure of skin symptoms were used as criteria, and were assessed in the same way as before.

The rate of weight increase in rats receiving the fat-free diet, even when adequately supplemented with a more than minimum dose of essential unsaturated fatty acid, is not rapid, but it is not impossible that, after the long period of depletion, the normal rate of weight increase cannot be resumed [Clarke & Smith, 1938]. The possibility that insufficiency of choline may be acting as a limiting factor is being examined.

Animal experiments

For the comparison of methyl arachidonate with methyl linoleate 45 rats were used, of which 26 received supplements of arachidonate, 11 of linoleate and 8 served as untreated controls.

The experiment was carried out in two stages: in the first, daily doses of 42 and 14 mg. of the two materials were compared. The number of animals receiving 42 mg. of arachidonate had to be reduced to 2 through insufficiency of material, and the number receiving 14 mg. daily of arachidonate, therefore,

includes 5 which had received 42 mg. daily for the first 4 days of dosing. The results for weight increase and for cure of symptoms are set out in Tables 1A and 2A. The average values for weight increase with the larger dose are derived

Table 1. *Total weight increase (g.) in 35 days of young rats which had received a diet devoid of fat for about 140 days, and were then given various doses of methyl linoleate or methyl arachidonate or no dose*

			Methyl linoleate		Methyl arachidonate			
A Litter no.	Sex	Negative controls	Daily 42 mg.	Daily 14 mg.	Daily 42 mg.	Daily 14 mg.	Every 2nd day 14 mg.	Every 4th day 14 mg.
4591	♂	−15	31	.	.	37*	.	.
	♂	.	.	.	.	19	.	.
4599	♂	3	.	.	.	30*	.	.
4596	♂	11	.	.	.	.	.	.
	♀	6	32	18	.	25*	.	.
	♀	.	.	.	.	28	.	.
	♀	.	.	.	.	18	.	.
4600	♂	7	.	13	.	24*	.	.
	♂	.	.	.	.	16	.	.
	♀	9	.	11	.	17*	.	.
	♀	.	.	.	.	1	.	.
4604	♂	4	.	.	.	.	.	.
	♀	6	44	18	12	20	.	.
	♀	.	.	.	30	18	.	.
Average		6	35	15	21	21	.	.
B								
4619	♂	.	.	10	.	36	18	5
	♀	.	.	18	.	24	17	−6
4626	♂	.	.	.	.	33	7	4
	♀	.	.	20†	.	19	15	6
	♀	.	.	10	.	.	.	.
Average		.	.	14	.	28	14	4

* Had received 42 mg. daily for 4 days.
† Rat recovering from pneumonia.

from too few animals to give an indication. The average values for the smaller dose indicate some superiority of the arachidonate in promoting weight increase, but for cure of skin symptoms, the results for methyl linoleate appear if anything slightly superior.

In order to obtain if possible a more accurate quantitative result, a second experiment was made in which 2 litters of 8 rats each were divided equally into 4 groups of 4, of which 1 group received 14 mg. of linoleate daily, and the other 3 groups received 14 mg. (1 drop) of arachidonate every day, every other day and every 4th day, respectively (see Tables 1B and 2B). The average values for weight increase in this series, combined with the values obtained in the first series (Table 1A), indicate that the arachidonate had about twice the value of the linoleate. The average increase of 14 g. over the experimental period was the same for those receiving 14 mg. of linoleate daily, as for those receiving 14 mg. of arachidonate every other day; the values for the other doses fall sufficiently well into line with this result. It is to be noted that the response to 14 mg. daily of methyl arachidonate approached the maximal values obtained by the authors with the fat-free diet when supplemented with unsaturated esters.

Table 2. *Healing of skin lesions of the ankles of rats which had received the fat-free diet for about 140 days, and were then dosed for 35 days with varying doses of methyl linoleate or methyl arachidonate or no dose. Same animals as in Table 1*

			Methyl linoleate		Methyl arachidonate			
A Litter no.	Sex	Negative controls	Daily 42 mg.	Daily 14 mg.	Daily 42 mg.	Daily 14 mg.	Every 2nd day 14 mg.	Every 4th day 14 mg.
4591	♂	θ	++	.	.	++*	.	.
	♂	.	.	.	.	+	.	.
4599	♂	θ	.	.	.	++*	.	.
4596	♂	θ	.	.	.	.	.	.
	♀	θ	+	+	.	+*	.	.
	♀	.	.	.	.	+	.	.
	♀	.	.	.	.	θ	.	.
4600	♂	θ	.	++	.	+*	.	.
	♂	.	.	.	.	+	.	.
	♀	θ	.	+	.	+*	.	.
	♀	.	.	.	.	+	.	.
4604	♂	θ	.	.	.	.	.	.
	♀	θ	++	+	++	θ	.	.
	♀	.	.	.	+	θ	.	.
B								
4619	♂	.	.	+	.	+	θ	θ
	♀	.	.	+	.	+	+	θ
4626	♂	.	.	.	.	+	θ	θ
	♀	.	.	+	.	+	θ	θ
	♀	.	.	+	.	.	.	.

Three degrees of healing are recognized: complete ++, partial +, none θ.
* Had received 42 mg. daily for 4 days.

For cure of skin symptoms in this second experiment also there was no superiority of the arachidonate; in 35 days, with a daily dose of 14 mg., healing was incomplete with both materials; with a dose of 14 mg. arachidonate every other day, there was no healing at all in 3 cases out of 4.

Prolonged action of methyl linoleate and methyl arachidonate

It was recorded in the previous communication [Hume *et al.* 1938, Table II] that the beneficial effect on weight increase and skin lesions of a daily dose of 80 mg. methyl linoleate could be observed long after the dose had been stopped. It seemed of interest to ascertain whether this was also the case with methyl arachidonate. The 16 rats used in the second experiment were therefore maintained on the unsupplemented diet for 12 days after dosage had ceased. The total weight increase for a group of 4 rats in the 12 days was greater for those which had received 14 mg. of linoleate or arachidonate every day than for those which had received this dose of arachidonate every 2nd or 4th day. Slight continued improvement of skin symptoms could also be detected in the rats which had received 14 mg. daily of linoleate or arachidonate, but with the others no improvement could be seen.

Action of cod liver oil esters in supplementing the fat-free diet

In the previous communication [Hume *et al.* 1938] it was reported that the methyl ester of the highly unsaturated acid, docosahexaenoic acid, isolated from cod liver oil by Farmer & van den Heuvel [1938], had little or no action in curing the skin lesions of the fat-deficiency disease, but that it did promote weight increase in rats whose weights had become stationary on the fat-free

diet. It seemed, therefore, to be of interest to ascertain what prophylactic action the whole fatty acid fraction of cod liver oil might have when given at the start of the experimental period on the fat-free diet.

Preparation of the methyl esters of cod liver oil acids. Cod liver oil was saponified and the methyl esters of the total fatty acids were prepared in the usual way. No division into saturated and unsaturated acids was made.

Biological experiment. Six rats, all females belonging to the same litter, received the fat-free diet as described above, but three of them received also in addition a daily supplement of methyl esters of cod liver oil acids. The amount was gradually increased from 1 drop (0·019 g.) at the beginning of the experiment to 4 drops by the 37th day, at which amount it remained until the 150th day when it was increased to 8 drops. On the 230th day whole cod liver oil was given instead of the methyl esters until the 260th day when the animals were killed. On the 230th day the weights of the 3 animals receiving the supplement of esters were 169, 174 and 174 g., and of the negative control animals 156, 161 and 166 g. The animals with supplements began to develop this slight advantage within the first few weeks of the experiment, and maintained it throughout.

All of the animals, without exception, showed dryness of the ankles, and all of them, except one receiving cod liver oil esters, showed ringing of the tail. The potency of the cod liver oil esters against the fat deficiency disease was thus very slight, and seemed to be less than that of the methyl ester of docosahexaenoic acid, but it should be remembered that the maximum dose of cod liver oil esters was about 150 mg. daily, while that of the pure docosahexaenoate was 60–100 mg. The docosahexaenoate constitutes not more than 5% of the total methyl esters of cod liver oil acids, so that the dose of docosahexaenoate given with the cod liver oil esters was very small.

Summary

1. Methyl arachidonate was prepared from pig's liver and from ox suprarenal glands.

2. The material was tested curatively on rats receiving the fat-free diet of Burr *et al.* in simultaneous comparisons with methyl linoleate.

3. In confirmation of the work of Turpeinen, methyl arachidonate was found to be more active than methyl linoleate in promoting weight increase; its activity in curing skin lesions was, however, no greater than that of methyl linoleate.

4. Methyl arachidonate, like methyl linoleate, unless given in very small doses, continued to exercise a beneficial effect for some time after dosage had been suspended.

5. Methyl esters of cod liver oil fatty acids were given prophylactically to young rats on the fat-free diet. The activity in promoting weight increase and in giving protection against skin lesions was very slight.

REFERENCES

Ault & Brown (1934). *J. biol. Chem.* **107**, 607, 615.
Brown (1928). *J. biol. Chem.* **80**, 455.
Burr, Burr & Miller (1932). *J. biol. Chem.* **97**, 1.
Clarke & Smith (1938). *J. Nutrit.* **15**, 245.
Farmer & van den Heuvel (1938). *J. Soc. chem. Ind., Lond.*, **57**, 24.
Hartley (1909). *J. Physiol.* **38**, 353.
Hume, Nunn, Smedley-MacLean & Smith (1938). *Biochem. J.* **32**, 2162.
Martin & Moore (1939). *J. Hyg., Camb.*, **39**, 643.
Nunn & Smedley-MacLean (1938). *Biochem. J.* **32**, 2178.
Turpeinen (1938). *J. Nutrit.* **15**, 351.