CXV. GROWTH AND REPRODUCTION ON A LOW FAT DIET

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THE low fat diet employed by the Burrs [Burr & Burr, 1929; 1930; Burr *et al.* 1932] in their work on the essential fatty acid has served as a model in the search for new fat-soluble dietary essentials. The diets used in such investigations have, without sufficient evidence, been generally referred to as "fat-free", "rigorously fat-free" etc. Failures to disclose the existence of new factors of a lipid nature are qualified, therefore, by the absence of quantitative data on the fat contents of these diets. In addition, there exist in the literature suggestions, both implied in the results reported and specifically stated, that fat-soluble factors other than vitamins A, D and E, and the essential fatty acid, are required by the rat [Burr & Burr, 1929; Mason, 1929; Coward *et al.* 1929; Guha, 1931; Mapson, 1932; Blumberg, 1935; Marchesi, 1935; Olcott & Mattill, 1937; Martin, 1939].

When analysis disclosed that several components of so-called "fat-free" diets contained appreciable amounts of lipid, the preparation of a diet lower in lipids than any previously fed was successfully attempted. The analytical findings, the preparation of the diet and the results of the subsequent feeding experiments are reported in this paper.

Analysis of dietary components and preparation of a low fat diet

"Fat-free" diets have generally been composed of a salt mixture, sucrose, casein (ether- or alcohol-extracted or both), ether-extracted yeast and pure or concentrated sources of vitamins A (or carotene), D and E, and the essential fatty acid. Since the degree of purity of the fat-soluble essentials used in these diets is generally apparent, and since methods for purifying them are available, attention was first turned to the lipid contents of extracted yeast and casein.

Analysis of dietary components. The Liebermann saponification method for fat determination as described by Leathes & Raper [1925] was found most suitable for the analysis of yeast. By acidifying and extracting the entire saponified mixture, a measure of all light petroleum-soluble substances was obtained.

Table I. *Lipid content of dried yeast before and after ether extraction*

Brand of yeast	Lipid content %		
	Unextracted	Ether-extracted 24 hr.	Ether-extracted 48 hr.
Northwestern Yeast Foam Tablet Powder	4.2	3.7	3.7
Anheuser-Busch Strain C	5.7	5.5	5.5
Fleischmann No. 1550	5.8	4.6	4.5

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Three brands of dried yeast, unextracted and extracted for periods of 24 and 48 hr. in the Soxhlet apparatus with c.p. anhydrous ethyl ether, were examined. The average figures (Table I) show differences in the yeasts both with respect to total lipids and the % lipids removed by ether extraction; but in each case it is clear that most of the fatty substances could not be removed by this method. Whether this was due to their inaccessibility to the solvent, or to their existence in insoluble chemical combinations was not determined.

In order to eliminate the possibility of the conversion of sugars or amino-acids into light petroleum-soluble substances during the strong alkali hydrolysis, control experiments were made with sucrose and casein but with negative results. Furthermore, ether-extracted yeast cells stained for fat with Sudan III gave positive results, as evidenced by the appearance of many bright red globules. Thus the assumption that ether-extracted yeast furnishes a "fat-free" source of the vitamin B complex was shown to be erroneous.

Two methods, the Liebermann saponification [Leathes & Raper, 1925] and the Shaw [1920] adaptation of the Roesse-Gottlieb procedure, were investigated as to their accuracy in determining the lipid content of casein. Since the Liebermann method gave much lower values owing to the formation of a fat-including coagulum, the method of Shaw was accepted as superior in this case. The results of analyses made on samples of casein subjected to extraction with hot or cold alcohol and ether, or to successive extractions with these solvents, are given in Table II. Ether extraction of crude casein is much less

Table II. *Lipid content of extracted casein*

Casein	Treatment	Lipid content %
Crude	None	1.45
Crude	None*	1.50
E.E.	Ether-extracted*	1.48
CA	Cold alcohol-extracted†	0.24
CA 1	Cold alcohol + 24 hr. warm ether extraction‡	0.17
CA 2	Cold alcohol + 48 hr. warm ether extraction	0.13
CA 3	Cold alcohol + 72 hr. warm ether extraction	0.12
CA 4	Cold alcohol + 24 hr. hot 95 % alcohol extraction	0.025
CA 5	Cold alcohol + 24 hr. warm ether and 24 hr. hot 95 % alcohol extraction	0.02

* Figures taken from Shaw [1920].

† Acetic acid-washed and continuously extracted in a Lloyd extractor with cold 70 % and cold 95 % alcohol for 24 hr. each.

‡ This and all subsequent extractions carried out in Soxhlets.

efficient than cold 70 and 95 % alcohol extraction, which removes approximately 83 % of the lipids. Furthermore, it is seen that the removal of lipid from this cold alcohol-extracted casein is more readily accomplished by continuous extraction with hot 95 % alcohol than with warm anhydrous ethyl ether. The successive application of these two last-named solvents does not seem to reduce the lipids to a significantly lower level than extraction with hot 95 % alcohol

alone. Indeed, in both of these cases the results of the analysis are in the range of the blank determinations, but since there generally seemed to be one or two droplets of lipid in the weighing vessels no corrections were made. It would appear from Table II that many "fat-free" diets have contained milk fat in addition to yeast fat.

Since no lipid was found in commercial sucrose analysed by the Liebermann method, the sucrose used in "fat-free" diets has probably not been a source of contamination.

Of the known fat-soluble essentials used in these diets, provitamin A and vitamin D have recently been included in pure forms; vitamin E has, with a few exceptions, been added as the crude unsaponifiable matter of wheat germ oil, and the unsaturated fatty acid has been supplied (in long term experiments) by a distillate containing a mixture of esters.

Preparation of a low fat diet. (Low fat diet is used throughout the remainder of this paper to refer to a diet whose lipid content is small.) Following unsuccessful attempts to remove lipids completely from yeast, a satisfactory source of the vitamin B complex was extracted in the following manner. 100 g. Northwestern Yeast Powder were added to 1 l. 50 % alcohol and the mixture was rapidly stirred for 2 hr. The mixture was allowed to stand for 1 hr. and the extract was then filtered through No. 52 Whatman filter paper. Most of the alcohol was removed by concentrating the filtrate on the water pump at 40–50° to a volume of about 200 ml. The concentrate was shaken for 3 min. in a separating funnel with an equal volume of c.p. anhydrous ethyl ether. This extraction was repeated. Liebermann analysis showed the fat content of the resulting defatted yeast extract in terms of whole yeast to be 0.01 %.

The extract was assayed for growth, reproduction and lactation on vitamin B complex-deficient rats. The extract from 100 g. of yeast added to 900 g. of diet produced excellent growth in depleted male rats, an average gain of 300 g. over a period of 17 weeks. Female animals that had gained 120 g. in 6.5 weeks and were then mated produced and weaned good litters. The results obtained with the extract at this level were comparable in every respect with those obtained on 10 % whole yeast.

The preparation of a low fat casein is indicated in Table II (Casein CA5). While ether extraction in addition to hot alcohol extraction had no apparent effect on the lipid content, this step was included as an added precaution. The hot 95 % alcohol extraction was carried out in all-glass Soxhlets.

Methyl linoleate was prepared from cottonseed oil by the method of Rollett [1909]. This procedure was facilitated by substituting a 1:1 light petroleum (B.P. 30–60°): carbon tetrachloride mixture for ligroin in recrystallizing the tetrabromostearic acid. The purity of the preparation was attested by the melting-point of the tetrabromide, 114–115°, the figure obtained by Rollett, and the iodine number of the ester, 172.3, the theoretical value being 172.2.

A method for preparing a highly potent concentrate of vitamin E for long-term feeding experiments where the use of the pure vitamin is not yet feasible has been reported [Mackenzie *et al.* 1938]. Although this concentrate had proved potent in single 3 mg. doses in rats reared on a typical lard-containing vitamin E-deficient diet, there was no assurance that the same level would suffice on a low fat diet. It was assayed at a 5 mg. level on female rats maintained on the low fat diet (described below) without vitamin E and found to be effective.

As the remaining components necessary for the completion of the diet, sucrose, cystine (as a supplement to the casein), salts, calciferol and carotene, presented no problems, the following diet was assembled.

Sucrose ¹	...	...	...	...	...	...	63.85
Casein (extracted with cold 70 % and 95 % alcohol, warm ether and hot 95 % alcohol)	...	...	...	...	...	...	20.00
Cystine	...	...	...	...	...	...	0.05
Salt mixture, McCollum 51* + 1.6 % MnSO ₄ , 2H ₂ O	...	...	...	...	...	...	6.10
Yeast extract† equivalent to 10 g. yeast	...	...	...	...	...	...	
Carotene, 20 mg. per 900 g. diet	...	...	...	...	...	...	
Calciferol,‡ 0.2 mg. per 900 g. diet	...	...	...	...	...	...	

* CaCO₃ 1.5, KCl 1.0, NaCl 0.5, NaHCO₃ 0.7, MgO 0.2, KH₂PO₄ 1.7, Fe citrate 0.5.

† Extract prepared from Northwestern Yeast Foam Tablet Powder.

‡ We are indebted to Dr C. E. Bills of Mead Johnson & Company, Evansville, Indiana, for the calciferol.

Supplements

Methyl linoleate, 25 mg. per rat 6 times weekly.

Vitamin E concentrate, 5 mg. per rat twice weekly.

Mixing the diet and care of animals. The yeast extract was poured on the casein and evaporated at room temperature before an electric fan. The carotene and calciferol were dissolved in c.p. anhydrous ethyl ether and immediately evaporated on separate portions of the casein-yeast extract mixture. The various components of the diet were then thoroughly mixed. The diet was prepared fresh every 3–5 days and stored in the ice box between feedings.

All-metal individual cages with raised mesh bottoms were used to house the animals. Food was supplied *ad libitum*, the supply being replenished daily, except once each week when the animals were allowed to consume their ration entirely, thus ensuring the complete intake of all components. Supplements were administered in the morning on a small amount of the basal diet and were always consumed by evening.

The maximum lipid content of the basal diet is 0.0078 %, of which 0.0022 is due to carotene and calciferol and 0.0056 to impurities in the casein and yeast extract. Of the two supplements, the methyl linoleate is pure, while the vitamin E concentrate contains at the most 70 % of extraneous matter.

The daily consumption of lipid, based on the average food intake of 10 g. per rat per day, is approximately 0.027 g., of which 0.025 g. is methyl linoleate. Thus the lipid content of the complete diet (basal plus supplements) may be given as 0.27 %. Similarly, the maximum non-vitamin lipid content of the complete diet is 0.0156 %, and of the diet without vitamin E, 0.0056 %.

Feeding experiments on the low fat diet

Animals obtained from the laboratory stock colony¹ were placed on the experimental diet when they were about 21 days old and weighed 35 g. The composite growth curves of one group of 10 male and one group of 10 female rats maintained on the diet for a period of 44 weeks are given in Fig. 1. The curves for the low fat and the stock females show an insignificant superiority in weight on the part of the low fat animals. The curve for males on the low fat diet diverges from the stock male curve at 12 weeks and is about 30 g. below at 44 weeks. The significance of this difference is questionable in view of the great individual variations within both groups.

The average time of cessation of growth (defined as a failure to gain more than 5 g. during the ensuing 6 weeks) for the female rats was 22 weeks. Some females maintained a constant weight until the experiment was terminated, while others after remaining at a constant weight for 7–12 weeks gained as much

¹ The McCollum stock diet is composed of wheat (soft) 20.0, maize (yellow) 20.0, rolled oats 20.0, flaxseed oil meal 10.0, casein (crude) 3.5, whole milk powder 25.0, CaCO₃ 0.5, NaCl 1.0, Fe citrate 0.0011, CuSO₄, 5H₂O 0.0004.

as 15 g. Eight male rats ceased to grow after an average of 32 weeks, while 4 were still growing at 40 weeks. Of the 8 males, 3 subsequently gained 15-35 g. and 5 held a constant weight for their remaining 8 weeks on the experiment. The erratic behaviour of the animals with respect to the time and duration of constant weight maintenance could not be correlated with different batches of diet.

Fig. 1. Average growth curves of male and female rats receiving the low fat diet ———, the low fat vitamin E-deficient diet , and the stock diet - - - - -.

The adequacy of the diet for growth was further tested on 4 groups of rats by doubling the intake of all vitamins and cystine, by increasing the vitamin E concentrate four-fold, by giving supplements of fresh calves' brain, and by replacing the low fat diet by the stock diet. With the exception of the experiments in which the vitamin and the cystine intakes were doubled, these changes were made with rats that had maintained constant weights for 6 weeks or longer. The growths of the control and test animals had been similar prior to the changes in diet. The results of these changes, summarized in Table III, were in all cases negative.

Table III. *Growth response as a result of supplementing or replacing the low fat diet for 4- and 8-week periods*

Change in diet	No. of rats	Sex	Av. time of change weeks	Av. time of cessation of growth weeks	Av. wt. at cessation of growth g.	Av. wt. at change g.	Av. wt. increase over wt. at cessation of growth	
							4 weeks	8 weeks
Doubling all vitamins and cystine	3	♂	19	—	—	261	12	—
Controls on low fat diet	3	♂	20	—	—	260	12	—
Doubling all vitamins and cystine	2	♀	21	—	—	162	4	—
Controls on low fat diet	2	♀	20	—	—	182	6	—
Vitamin E concentrate, 40 mg. per week	3	♀	25	18	213	212	2	—
Controls on low fat diet	3	♀	31	25	191	191	4	—
Fresh brain, 1 g. daily	5	♀	28	21	199	196	2	6
Controls on low fat diet	5	♀	28	22	205	204	3	5
Stock diet	2	♂	36	30	279	278	10	14
Controls on low fat diet	4	♂	38	30	304	302	10	10
Stock diet	6	♀	37	30	196	193	1	1
Controls on low fat diet	6	♀	36	27	204	201	1	1

Since brain is an exceptionally rich source of lipids, 4 male and 4 female rats were placed at weaning on the low fat diet with the addition of 1 g. of fresh brain per rat daily and maintained on this regimen for 30 weeks. Their growth did not significantly differ from that of animals on the low fat diet.

The fertility of females on the low fat diet was demonstrated by the delivery of 14 litters by 5 rats mated to stock males. Following the second and third parturitions at 15 and 21 weeks, the behaviour of the mothers and the young was observed. Several days before the termination of pregnancy the mesh bottoms were removed from the cages and the females were placed on a bed of filter paper clippings previously extracted with hot 95 % alcohol. In all cases the females built nests and showed solicitude for their young, as was manifested by retrieving and hovering. Though the young were normal in appearance, many died during the first 3 days. Doubling the vitamin and cystine intakes on the 13th day of the 3rd pregnancy with 3 of the rats did not substantially improve the results. When young were living at 21 days they were, with the exception of those from one litter, poor in weight and appearance. The details of this experiment are summarized in Table IV.

Table IV. *Reproductive behaviour of females grown and maintained on the low fat diet*

Rat no.	First gestation*		Second gestation				Third gestation			
	No. of living young	Av. wt. at birth g.	No. of living young	Av. wt. at birth g.	Fate of young	Av. wt. at 21 days g.	No. of living young	Av. wt. at birth g.	Fate of young	Av. wt. at 21 days g.
C 5	9	4.1	7†	4.7	Dead 1 day	—	5‡	5.8	5 weaned	31.0
C 8	8	5.2	9	5.2	Dead 8 days	—	7‡	5.6	2 weaned	20.5
C 18	7	4.1	8	4.5	Dead 2 days	—	10‡	4.1	Dead 3 days	—
C 24	6	4.7	10	4.6	Dead 1 day	—	11	4.8	1 weaned	19.0
C 30	6	5.5	9	4.9	5 weaned	23.5	—	—	—	—

* Lactation not observed following 1st gestation.

† In all 2nd and 3rd gestations, litters were reduced to 6 young on day following delivery.

‡ All vitamins and cystine doubled on 13th day of pregnancy.

Results obtained by exchanging the young of the low fat diet mothers with those of stock diet females suggested that milk production on the low fat diet was probably inadequate.

Four male and 4 female second generation animals weaned by females C5, C8 and C30, which had grown up on the low fat diet (Table IV), were kept on this diet for a period of 16 weeks, at which time the average weight for males was 228 g. and for females 165 g. The appearance of these animals was normal.

Following their last gestation, the female rats used for reproduction were continued on the low fat diet for 10–20 weeks. Despite the nutritional strain of pregnancy, and in some cases lactation, they showed no signs of abnormality.

Motile sperms were found in the epididymides of male rats after they had been 44 weeks on the low fat diet.

Feeding experiment on the low fat vitamin E-deficient diet

As the possibility existed that the vitamin E concentrate used in the complete low fat diet might, in addition to vitamin E, contain another factor essential for the rat, 10 male and 8 female rats were reared on the low fat diet without vitamin E. The growth of these animals, though somewhat inferior to that of the controls, gave no indication that any factor other than vitamin E was lacking. The average growth curves are given in Fig. 1. The average time when weight became stationary for the 8 females was 16 weeks, and for 8 of the males 23 weeks. Two of the males had not ceased growing at 37 weeks. As was the case with the animals on the complete diet, there was an inexplicable tendency in both males and females to resume growth after maintaining a constant weight for 6–10 weeks. This occurred in about half of the animals and the gains varied between 10 and 20 g. in 9–12 weeks.

After the deficiency of vitamin E in the diet had been checked by resorption gestations on several females, 4 female rats whose weights were stationary were given 20–40 mg. of the vitamin E concentrate weekly. An average gain of 18 g. in 12 weeks resulted, as contrasted with an average gain of 1 g. for their 4 controls. This gain corresponds rather closely to the difference between the growth curves of female rats with and without vitamin E.

The oestrous cycle was followed on 6 rats from the 8th to the 23rd week of the experiment. The average length of the cycle for 5 of the rats was 4.7 days. For the 6th animal the average was 5.8 days. The prolongation of the cycles in this case occurred during postoestrus.

At the 40th to the 42nd week of the experiment some of the vitamin E-deficient animals showed the first symptoms of the paralysis mentioned by Evans [1932] and later described by Blumberg [1935], Ringsted [1935] and Burr *et al.* [1937]. There was an unmistakable spreading of the hind legs accompanied by a lowering of the posterior abdominal region. This abnormality was observed in 5 of 8 males and 1 of 4 females. Thirteen male and 5 female rats on the low fat diet with added vitamin E concentrate showed no signs of the paralysis at 42–44 weeks.

DISCUSSION

Inspection of the "fat-free" diets used by others in nutritional investigations, and consideration of the analytical findings reported in this paper, show that in addition to the known fat-soluble dietary essentials such diets have included light petroleum-soluble substances of unknown significance. The diet used in this experiment contained at the most, exclusive of the fat-soluble essentials, 0.0156 % of lipid. Of the fat-soluble essentials, vitamin E alone was added in an impure form, and here the contamination did not exceed 7 mg. of lipid matter per rat per week. The disadvantages resulting from the use of a concentrate instead of the pure vitamin were partially mitigated by running a parallel experiment on the low fat diet without vitamin E, thus reducing the non-vitamin lipid to 0.0056 %. The symptoms of avitaminosis-E would hardly be expected to obscure an additional nutritional deficiency.

The growth of animals maintained on the complete low fat diet for as long as 11 months was good and compared favourably with growth on the stock diet. Modifying the low fat diet, even to the extent of replacing it by the stock diet, did not significantly affect the weights of the animals. The differences between the average growth curves of animals on the complete and vitamin E-deficient diets suggested an uncomplicated vitamin E deficiency. Furthermore, the

response of vitamin E-deficient females to the vitamin E concentrate corresponded to the difference between the growth curves and was of the order of magnitude of the growth response obtained by Evans *et al.* [1938] with pure vitamin E. These results, obtained on the complete and vitamin E-deficient low fat diets, furnish no evidence for the existence of an additional fat-soluble growth factor.

Female rats on the experimental diet were fertile. Since it is not likely that the vitamin E concentrate carried a second factor necessary for reproduction, additional evidence is furnished against the existence of a hitherto unrecognized fat-soluble essential. The subsequent normal behaviour of the females used for reproduction and the fair growth of second generation animals point in the same direction. However, negative results in an experiment of this kind can never be conclusive. While the probability of the existence of an additional fat-soluble growth (and to a lesser degree reproduction) factor has been considerably reduced, it must be borne in mind that experiments in which different basal diets are employed may yield opposite results.

It seems probable that the inferiority of young born to females on the low fat diet was primarily due to inadequate milk production. If this was the case, a subminimal supply of the vitamin B complex can be excluded as the cause, since good lactation was obtained in animals used for assay of the yeast extract. One or more of the fat-soluble vitamins may have been involved, though doubling them did not rectify the condition. Similar results on low fat diets have been reported by Evans *et al.* [1934], and Olcott & Mattill [1937]. The former workers found the addition of 25 % lard or butter fat beneficial. While this suggests that a fat-soluble factor, or fat as such, is necessary for normal lactation, in none of these cases has the complicity of cystine or methionine, shown by Daggs & Tomboulia [1935] and Daggs & Lidfeldt [1938] to be potent lactagogues, been excluded.

The failure of Olcott & Mattill [1937] to observe the paralysis reported by Evans [1932], Blumberg [1935], Ringsted [1935], and Burr *et al.* [1937] was probably due to the comparatively short duration of their experiments. On the low fat vitamin E-deficient diet used in this experiment, the first symptoms of the paralysis were observed in some of the animals at 40–42 weeks. Heretofore the evidence that the paralysis is a result of vitamin E-deficiency has rested on the preventive action of wheat germ oil. The absence of symptoms from control animals receiving a highly potent vitamin E concentrate is an advance in the identification of the active substance.

SUMMARY

1. Evidence has been presented indicating the presence of fat in several components of "fat-free" diets.

2. The preparation of a diet lower in lipids than any previously reported has been described. The total lipid content of this diet is approximately 0.27 %. The maximum non-vitamin lipid content of the diet is 0.0156 %, and of the diet without vitamin E concentrate, 0.0056 %. The other fat-soluble essentials were added in pure form.

3. This low fat diet was found satisfactory for good growth and reproduction.

4. The results of feeding experiments on the complete and vitamin E-deficient diets furnish no evidence for the existence of a hitherto unknown fat-soluble factor necessary for growth or reproduction in the rat.

5. The early symptoms of paralysis observed in adult rats on a vitamin E-deficient diet were prevented by a highly potent concentrate of vitamin E.

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