A GROWTH DEFICIENCY DISEASE, CURABLE BY WHEAT GERM OIL*

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(Received for publication, October 12, 1934)

The advances made in the purification of the vitamins have provided an improved method of approach toward the study of the number, as well as nature, of the essential food factors. Because of the uncertain composition of the vitamin B complex, it is as yet very difficult to test for additional water-soluble components of the ultimate purified diet. The fat-soluble group, however, appears less complex, and presents a promising field for the investigation of the possible existence of additional vitamin-like factors.

A satisfactory basal ration may be supplied by fat-free casein, sucrose, and salts; to these constituents may be added a fat-free yeast to provide the water-soluble components necessary for the rat, an animal which is able to synthesize vitamin C. Through the use of carotene, irradiated ergosterol, and a linolate (1), a diet may be provided with fat-soluble vitamins A and D, and the essential unsaturated fatty acid (vitamin F), in practically pure form. The nature of the metabolic factor in lard reported by Wesson (2) is obscure, but its omission does not appear to affect growth. The remaining consideration is vitamin E, to which a subtle late growth stimulus has been attributed by Evans (3). Since this effect is not reported to become well evident until after the 8th month of life, and is marked only after a year, the absence of the vitamin should cause sterility but should not influence growth until the maturity "plateau" is reached.

* Presented to the faculty of the School of Hygiene and Public Health of the Johns Hopkins University in partial fulfilment of the requirements for the degree of Doctor of Science in Hygiene, May, 1933.
Thus, by supplementing a fat-free diet with practically pure sources of vitamins A and D, and the essential unsaturated fatty acid (vitamin F), it is possible to test accurately for any unrecognized fat-soluble vitamins that might affect early or middle growth. With this purpose in view, the following investigation was undertaken.

**Preparation of Diet and Feeding Technique**

The purified diet used was as follows:

**Basal Ration**

- **Casein (hot alcohol-, and ether-extracted)** 20.0
- **Yeast (ether-extracted)** 10.0
- **Salt Mixture 185 (McCollum)** 4.0
- **Sucrose (commercial)** 66.0

**Supplements**

- **Carotene**—An ethyl laurate solution of crystalline carotene, fed at 2 drops per day = 0.08 mg. of carotene per rat per day. This solution was prepared fresh every week.
- **Irradiated Ergosterol**—An ethyl oleate solution of the irradiated resin, incorporated in the diet at 30 drops per kilo, was used. This was equivalent to 6 drops per kilo of viosterol 250 D.
- **Ethyl Linolate**—A distilled oil, fed at 3 drops per day = 65 mg. per rat per day.

The casein was prepared by washing the commercial product in dilute acetic acid, distilled water, and 95 per cent alcohol. It was then extracted successively in Soxhlet extractors with hot 80 to 90 per cent alcohol, hot 95 per cent alcohol, and with anhydrous ether, each extraction being for 24 hours. The yeast used was the dried product of the Northwestern Yeast Company, but was extracted with anhydrous ether for 24 hours in a Soxhlet extractor. Commercial cane sugar, which contains inappreciable fat, supplied the carbohydrate. The ethyl linolate was prepared from technical linoleic acid by esterification and careful vacuum distillation. Since the saturated and singly unsaturated fatty acids appear to have no specific rôle in nutrition (1), purified

1 Supplied through the courtesy of Dr. Charles E. Bills, Mead Johnson and Company, Evansville, Indiana.

2 The author is indebted to Dr. Warren M. Cox, Jr., of Mead Johnson and Company, for some of the oil used.
preparations of ethyl laurate and ethyl oleate were secured as solvents for the carotene and irradiated ergosterol, respectively.

By means of preliminary assays, it was shown that the carotene, irradiated ergosterol, and ether-extracted yeast were above the optimal amounts. In the absence of data on the optimal preventive dosage for the essential unsaturated fatty acid, the ethyl linolate was fed at 65 mg. per rat per day, the adequacy of which had been demonstrated in a previous experiment.

The animals were kept on wire screens in individual cages. In order to insure a ready consumption of the supplements, the carotene and ethyl linolate were administered with a small portion of ration in the evening. By morning this had been consumed, and ample diet for the day was put in the feed cups.

**EXPERIMENTAL**

*Growth Deficiency*—A group of rats was started upon the experimental diet when weaned, that is, when the animals were about 25 days old and weighed 40 to 50 gm. From the very start of the experiment the growth of these animals was compared with the composite growth curves of rats on the laboratory stock diet (Fig. 1).

The results were surprising. In the experimental females, growth was slightly below normal for the first 5 weeks on the diet, slow at the 10th week, and very slow or checked by the 14th week. By the 18th week, all had definitely reached a plateau or suffered a slight decline. At this time the weights ranged from 140 to 170 gm., while the stock animals of the same age weighed about 190 to 210 gm. and were on a slow ascent (Fig. 1).

Similarly, the males grew at a little less than the normal rate for the first 5 weeks, were slow at the 12th, very slow or stationary at the 16th, and had all reached a plateau or experienced a slight decline in weight between the 18th and 22nd weeks (Fig. 1). It appeared, therefore, that the sequence of growth retardation was the same as that of the females, except that the greater growth potential of the males required about 2 weeks more in order for them to reach an analogous stage of plateau or slight decline. At the point of plateau and slight decline, i.e. the 18th to 22nd week, the weights of the experimental males ranged from 198 to 243 gm. On the other hand, the normal males weighed approximately 300 to 350 gm. by the 18th week and were still gaining.
Although male rats were continued upon the diet for 36 weeks, in the additional period they remained stationary or declined as much as 15 gm. from the weights attained at the 22nd week. The stock males, on the other hand, had grown to their maturity weights of 350 to 450 gm. by the 36th week after weaning (Fig. 1).

In the case of the experimental females, some animals were maintained on the diet for a year or slightly longer. Instead of ultimately increasing in weight, the rats slowly declined to weights of 120 to 135 gm., amounting to losses of 30 to 45 gm. from the maximum weights that they had reached at the 14th to 16th week. The stock females, on the contrary, attained their normal weights of 200 to 250 gm. at about the 32nd week after weaning, and maintained themselves at those weights (Fig. 1).

After about 30 weeks on the diet the females became progressively poorer in appearance, so that after the 40th week they were in a miserable condition. In comparison with stock animals they appeared slightly inferior in length, but more strikingly subnormal through lack of body fat. Indeed, when grasped in the hand, the animals felt like little more than fur, bones, and viscera.

There was also a striking decrease in vigor. The animals did not run about their cages; they scarcely moved except when disturbed or in need of food. When the animals did move, their motions were stiff and slow—accompanied in some cases by a
decided arching of the back. If they were lying on the feed pans when the latter were unhooked for refilling, they would not jump off quickly like vigorous rats. Instead they would put the fore legs down and slowly drag the hind legs after them. Frequently they would fall while trying to step down to the wire screen.

The estrous cycles of the animals often appeared irregular, exhibiting a peculiar interruption between the preestrous and estrous stages. In the case of an animal continued on the diet until it had reached a very poor state, repeated mating tests were negative. The vaginal smears revealed that ovulation apparently had ceased, with the animal probably remaining in diestrus. This effect, no doubt, was not specific, but rather a manifestation of the poor nutritive state.

**Failure of Any Known Early Growth Vitamin to Cure Deficiency**—As early as the 12th to 14th weeks it was quite evident that the diet was not complete for early and middle growth. Although the rats at no time showed signs of any recognized deficiency, additions of various known vitamins were made. All supplements were given between the 14th and 23rd weeks on the diet. The animals used were those in which the growth curves had reached a plateau when the experimental diet alone was fed, or were still level after the addition of a negative supplement.

The complicity of the vitamin B complex was ruled out by the negative results of substituting 10 per cent unextracted North-western yeast for the ether-extracted product, and the lack of benefit derived from feeding an additional 1 gm. per day of North-western and Anheuser-Busch yeasts. Vitamins A and D were likewise eliminated by the absence of improvement after increasing the carotene dosage from 2 drops per day to 3 drops per day, or after adding cod liver oil (Mead Johnson and Company) at 3 drops per day, or viosterol at 1 drop per 3 days. Finally, a deficiency of the essential unsaturated fatty acid (vitamin F) was disproved by the negative effect of trebling the dosage of ethyl linolate, of substituting lard at 0.5 gm. per day for the linolate, or of supplementing with the same amount of lard. The failure of lard to improve the condition also demonstrated that Wesson's metabolic factor (2) was not concerned.

Inasmuch as a casein factor reported by Coward, Key, and Morgan (5) and by Guha (6) was suspected, the untreated com-
commercial casein was substituted for the extracted product of the experimental diet. This change likewise conferred no benefit.

**Demonstration of Curative Factor**—Since none of the vitamins included in the plan of the diet was concerned in the subnormal growth, various natural foods were fed to make certain that the animals were not permanently stunted. Striking resumption of growth followed the administration of wheat germ, egg yolk, and to a slightly less degree, the addition of lettuce. Although a significant effect might not be detected after 1 week, the response was quite definite at the end of the 2nd week. In the males the gain was from 10 to 25 gm. in the first 2 weeks, followed by smaller gains for many succeeding weeks. By the 10th week on the supplement growth had become slow at about 275 to 300 gm., but a gradual gain was continuing.

In the female the response was 5 to 15 gm. by the end of the 2nd week, with growth continuing at 3 to 6 gm. a week until the 10th or 12th week. By this time the animals had reached 190 to 205 gm., weights within the lower range of normal figures, and remained on the customary maturity plateau (Fig. 2). In appearance they were essentially normal. A comparison with the control experimental animals, of the same age but restricted to the experimental diet alone, indicated that the improvement was in size, body fat, vigor, and general well being.

The striking character of the response, as well as its regularity, left no doubt as to the existence of an unrecognized effect of some

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3 Furnished through the courtesy of the Russell-Miller Milling Company, Minneapolis.
principle upon early and middle growth, and upon the maintenance of well being.

Distribution of Factor—In order to learn more of the factor, its distribution was studied. The data are summarized in the following tabulation. A good response was such as that described in the previous section; namely, a gain of 5 to 15 gm. in the females, and of 10 to 25 gm. in the males at the end of 2 weeks, with growth continuing at a slower rate for at least 8 weeks afterward. Fair and poor were degrees of approach to the condition of absence of growth or slight decline, which was considered a negative response.

<table>
<thead>
<tr>
<th>Food per day</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butter fat (0.5 gm.)</td>
<td>Negative-fair*</td>
</tr>
<tr>
<td>Casein, commercial (as 20% of diet)</td>
<td>Negative</td>
</tr>
<tr>
<td>Chlorophyll (5 drops of Merck's alcoholic solution)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Cod liver oil (3 drops)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Egg yolk (2 gm.)</td>
<td>Good</td>
</tr>
<tr>
<td>Lard (0.5 gm.)</td>
<td>Negative</td>
</tr>
<tr>
<td>Lettuce, fresh (10 gm.)</td>
<td>Fair</td>
</tr>
<tr>
<td>Liver, fresh (2 gm.)</td>
<td>Negative-poor*</td>
</tr>
<tr>
<td>Milk, pasteurized (25 cc.)</td>
<td>Negative-fair*</td>
</tr>
<tr>
<td>Wheat germ (1 gm.)</td>
<td>Good</td>
</tr>
<tr>
<td>Xanthophyll (0.08 mg.)</td>
<td>Negative</td>
</tr>
<tr>
<td>Yeast (1 gm.)</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

* The response in these was irregular, being negative during the first 4 to 5 weeks and showing intervals of improvement thereafter. Since these are animal products, it is likely that the variation was due to differences and changes in the diets of the source animals.

Nature and Properties of Factor—Wheat germ was selected as a starting material for investigation into the nature and properties of the factor. When fed at a level equivalent to 7 gm. per day of wheat germ, the ash proved ineffective. Therefore, it was probable that the active substance was of organic nature.

The solubility of the principle was determined by making hot extracts of wheat germ with water, 70 per cent alcohol, 95 per cent alcohol, acetone, and ether. Although a slight response was secured from water and 70 per cent alcohol, much better growth was promoted by the other concentrates, particularly that from the ether extract. The wheat germ oil from ether extraction...
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gave a good response when fed at 0.5 and 1 cc. per day. Since boiling alcohol extracts, held at a temperature of 79° for 2 hours, were active, the factor did not appear to be very heat-labile.

By reason of the inactivity of the ash and the potency of concentrates, the vitamin-like nature of the beneficial principle was demonstrated. Furthermore, in both distribution and properties the growth factor appeared similar to vitamin E.

Relation of Growth Factor to Vitamin E—The behavior of the principle upon saponification was determined by feeding the unsaponifiable fraction and the fatty acids secured from wheat germ oil. The fractions were separated from both hot saponification (1½ hours at 79°) and cold saponification (24 hours at 25°), in a manner similar to the general procedure described by Burr and Burr (1). Since 0.5 gm. per day of wheat germ oil had proved effective, the fractions were fed equivalent to their amounts in 0.5 gm. of the oil. In both cases the fatty acids were inactive, while the unsaponifiable concentrates permitted a slow but definite growth. The response, however, was not so good as that secured by the equivalent amount of wheat germ oil. The results indicated that the growth factor, like vitamin E, withstood saponification and went into the unsaponifiable fraction, but with some loss in activity.

A few preliminary efforts were made to investigate further the relation of the growth factor to vitamin E by a study of the growth potencies of different samples of wheat germ oil (prepared by ether extraction). When fed prophylactically as a supplement to the experimental diet, the unsaponifiable fraction of one oil caused a slightly improved growth. Consequently, the results furnished no distinction between vitamin E and the growth factor.

However, an unsaponifiable concentrate was also prepared from a second batch of wheat germ oil. This sample of oil appeared considerably different from the first by reason of its darker color and higher solidification point, being completely solid at 8°. Two male rats were fed from weaning upon the experimental diet but with the addition of the concentrate equivalent to 2 gm. per week of wheat germ oil, an amount in excess of the fertility requirement of 250 mg. per week (1).

The growth curves revealed that these animals were always sub-normal in size and were still at least 100 gm. underweight at the
end of the 28th week. Instead of remaining at a plateau, however, as in the animals on the experimental diet alone, the growth curves of the rats continued on a very slow ascent. The vitamin E potency of the extract had been demonstrated by the fact that the males were fertile as late as the 28th week on the diet, which was the date of the last mating. By means of mating tests, it was proved that controls not receiving the concentrate were sterile when tested, which was in some cases the 18th week, and in others the 24th week on the diet. Since the potency of the extract was demonstrated, this constituted a case in which early growth had not been improved, despite the definite presence of some vitamin E. Thus some evidence was presented for a possible distinction between the growth and fertility factors in wheat germ oil, as well as a variation in the potencies of concentrates from different samples of oil. Further experiments on this point have been planned.

**Vitamin E and Xanthophyll**

Incidental to the work on wheat germ oil, two male rats were fed from weaning upon the experimental diet, but with the addition of an ethyl laurate solution of xanthophyll, equivalent to 0.08 mg. per day of the pigment. After 18 weeks the weight curves had reached a plateau at 210 to 220 gm., thus demonstrating that this quantity of xanthophyll did not provide the early and middle growth factor of wheat germ oil, even when fed from the time of weaning. In view of the suggestion by von Euler and Klussmann (7) that a relationship might exist between vitamin E and xanthophyll, mating tests were attempted. The negative results of these tests suggested that the animals were sterile, a fact confirmed by the extremely small size and degenerated appearance of the testes at autopsy. Olecott and Mattill (8) have recently reported the absence of a simple relationship between vitamin E and xanthophyll in the female rat; this experiment confirmed the lack of such a connection in the male rat.

**Possibility of Additional Fat-Soluble Factors**

Since the highest weights reached by the cured animals were only the lower range of normal figures attained by the animals on the stock diet, it seemed probable that one or more additional fat-soluble growth factors might exist. In a single case of preliminary
investigation on this point, fresh liver at 2 gm. per day was given as a further supplement to a cured female rat with a growth curve that had reached a plateau at 205 gm., after the addition of wheat germ. No change, however, was noted during a month's feeding. Due to the age of the animal, no effect could be expected from the water-soluble early growth factor in liver, such as has been reported by Mapson (9). The negative result of this supplement suggested that liver contained no additional factor limiting late growth.

**DISCUSSION**

Since the growth stimulation produced by the curative foods is quite different from the late growth effect attributed to vitamin E, the response cannot be explained upon the basis of the properties ascribed to the known vitamins. By reason of its distribution, general properties, and partition into the unsaponifiable fraction of wheat germ oil, the active substance appears quite similar to vitamin E. If the marked growth effect of the curative foods is really due to vitamin E, the experiment demonstrates a new method for the assay of this vitamin, that is, by growth response rather than by prevention or cure of sterility.

However, it is noteworthy that the females may even cease to ovulate, thereby preventing the very testing for vitamin E deficiency. In the case of two male rats that received a concentrate of the unsaponifiable fraction of wheat germ oil, the animals were proved potent, although distinctly subnormal in weight.

Mention of additional fat-soluble factors has occasionally been made. Mason (10) believed that the beneficial effect of lettuce was not due to its vitamin E content alone. Coward and coworkers (5) and Guha (6) produced a deficiency disease through the use of a special extracted and heat-treated casein, but they did not prove that the curative principle was not vitamin E. In the present experiment, however, growth failed on a diet containing an extracted but unheated casein, and no potency was found in the untreated commercial casein—a product which was unextracted and had never been exposed to temperatures above 55°.4

In view of the demonstrated growth effect of wheat germ oil, it seems pertinent to inquire as to the completeness of various assay
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diets, particularly that for vitamin A testing. Coward and her associates (11) have reported on the discrepancies produced by using different types of casein. During the course of a preliminary vitamin A assay for the present investigation, it was found that the addition of ample carotene over a long period of time did not permit the rats eventually to reach normal weights. In fact, improved growth appeared to result upon the further addition of wheat germ oil, but not upon increasing the carotene or supplementing with ethyl linolate. From these indications it appears that the growth factor of wheat germ oil cannot safely be omitted from nutritional experiments of long duration.

The use of fat-free diets supplemented by carotene, irradiated ergosterol, and linolate, constitutes a useful technique for the study of fat-soluble factors in nutrition, as well as an infallible diet for the production of vitamin E sterility. Further studies with this type of diet are contemplated as a means of investigating the possible existence of other fat-soluble factors.

**SUMMARY**

1. The preparation of a highly purified diet has been described. This was accomplished by obtaining the protein, carbohydrate, salts, and yeast of the basal ration in a fat-free state, and then supplementing with sources of the fat-soluble vitamins A and D, and the essential unsaturated fatty acid (vitamin F), in a practically pure form.

2. Such a diet failed to promote normal early and middle growth in the rat; prolonged feeding (40 to 50 weeks) to female rats produced a deficiency disease marked by a significant loss of weight, loss of vigor, emaciation, muscular disturbance, and a general lack of well being. In this poor nutritive state the females sometimes showed a disturbance in the estrous cycle, with consequent cessation of ovulation.

3. The condition was readily curable by certain foods, notably wheat germ, wheat germ oil, and egg yolk, the addition of any one of which soon restored growth and well being.

4. The preparation of active concentrates by extraction with ether and other fat solvents indicated the vitamin-like nature of the beneficial factor.

5. In distribution and general properties the growth principle resembled vitamin E.
6. The production of the deficiency and its cure by wheat germ oil and by other substances permits one of two conclusions: (a) that vitamin E is necessary not only for reproduction and late growth stimulation, but also for normal early and middle growth, and for the maintenance of well being; or (b) that wheat germ oil contains an unrecognized fat-soluble growth factor. Some evidence favors the latter view.

The writer takes pleasure in acknowledging the advice and kindly criticism of Professor E. V. McCollum, at whose suggestion this investigation was undertaken.

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