CVI. OBSERVATIONS ON CERTAIN FACTORS NECESSARY FOR THE NORMAL NUTRITION OF THE RAT.

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INTRODUCTION.

IN course of attempts made to isolate vitamin B₁ [Guha and Drummond, 1929], it was observed early in 1928 that if in these experiments the animals were kept in a state of more or less stunted growth by alternate administration and withdrawal of vitamin B₁ concentrates over a period of 10–12 weeks, the subsequent prolonged administration of vitamin B₁ concentrates of known potency (as determined by pigeon curative tests and growth tests on younger rats) did not permit continued good growth in these animals. Growth was not effectively restored by increasing the doses of vitamin B₁ and B₂ concentrates (the latter being supplied as alkaline-autoclaved marmite). But if they were replaced by relatively small quantities of whole brewer's yeast, dried at 35°, or of marmite, the animals responded with much more continued growth [Guha and Drummond, 1929, p. 896, footnote]. Even so, growth ceased after some time at a point well below the normal. At this stage, milk was tried, partly empirically and partly with the classical experiments of Hopkins [1912] in mind, with distinctly beneficial results. These preliminary observations suggested the possibility that in yeast and in milk we might be dealing with factors other than the known vitamins. As, however, other explanations might well be given for the observed results, these preliminary experiments were followed up and an investigation carried out during the last 3 years with reference to the following points.

(1) Can the observed effect be ascribed to a better protein in the supplement or to increased amounts of the known vitamins A, B₁, B₂, C, D or E?

(2) Is the increased growth associated with increased food intake? If so, would it be possible to have increased growth with restricted food intake?

(3) To extract, if possible, the new factor (or factors) from natural sources and define its chemical character.

In this investigation over one hundred rats were used, some of which were kept under observation for experimental periods exceeding 35 weeks. Extensive food-intake records were kept. No attempt was made to find if the

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absence of the new factor (or factors) would affect breeding and lactation. It should be emphasised that entirely regular results were not obtained, but the effect of the factors concerned was well outside the limits of individual biological variations and was sufficiently regular to warrant the conclusions reached. It may also be pointed out that part of this work was carried out at University College, London, and part at the Biochemical Laboratory, Cambridge, with animals reared on different diets. The maximum attainable weights of the stock rats also differ. Still, the results obtained at the two laboratories agree fairly, and the effects of the absence or administration of the new factors are similar. Unfortunately, not many experiments could be carried out on the extraction of the factors concerned and no suitable method has yet been devised for this purpose. A preliminary report of this work has appeared elsewhere [Guha, 1930].

Since 1926 a number of papers have appeared bearing more or less directly on the same subject [Osborne and Mendel, 1926; Palmer and Kennedy, 1927; Evans and Burr, 1927, 1; Hunt, 1928; Reader, 1928, 1929, 1930; Coward, Key and Morgan, 1929; Mason, 1929; Chick and Copping, 1930]. This work is discussed later in relation to the present investigation.

EXPERIMENTAL.

The rats were kept in separate cages with screened bottoms, and supplements were given, wherever necessary, in separate dishes and, in general, the procedure described by Guha and Drummond [1929] was followed. The rats used at University College, London, were raised on a diet of the following composition:

					%
Maize meal	...	...	...	...	63
Whole wheat	...	...	...	...	20
"Casein" (B.D.H. "light white")	...			...	9
Dried yeast powder	...	...	...	...	4
Calcium carbonate	...	...	...	...	3
Common salt	...	...	...	...	1

Some sheep-liver was supplied once a week. These rats were normally capable of attaining a maximum weight of 300 g. for males and about 210 g. for females, which usually took about 30–34 weeks. The rats at the Cambridge laboratory were bred on a dietary of brown bread, fresh milk and whole wheat, and usually reached a maximum weight of 350–360 g. for males and about 250 g. for females. The animals used at University College and those at Cambridge are designated as "A Class" and "B Class" rats respectively. They were all of the black and white strain. In the following experiments 2 drops of cod-liver oil were given daily to each rat in a dish, unless otherwise mentioned.

A. *Experiments showing the effect of vitamin B₁ and B₂ preparations, yeast, marmite and milk, on rats, whose growth had been artificially stunted over a considerable period.*

Fig. 1 illustrates cases of A Class rats, in which, owing to the alternate administration and withdrawal of vitamin B₁ preparations, the rats were kept at a subnormal weight over considerable periods. These rats were throughout on a basal diet composed of 75 % rice starch, 21 % "light white casein"

Fig. 1.

Curve (1). ↑ 0.5 cc. of a vitamin B₁ concentrate from wheat germ *plus* 1 cc. of 50 % alkalised marmite; ↓ the dose of each doubled; ⚡ the dose of each trebled; × 0.5 g. dried brewer's yeast daily.

Curve (2). ↑ 0.5 g. fresh marmite daily; ↓ 15 cc. of milk in addition.

(B.D.H.) and 4 % salt mixture (McCollum). In addition, they received daily doses of 2 drops of cod-liver oil of proved potency and 1.0 cc. of a 50 % solution of alkaline-autoclaved marmite [Guha and Drummond, 1929]. After the expiration of 10–12 weeks from the beginning of experimental feeding, the administration of a vitamin B₁ concentrate, in the form of a fuller's earth extract obtained from wheat germ [Guha and Drummond, 1929] or of a charcoal concentrate obtained from brewer's yeast by the acid-alcohol method of Kinnersley and Peters [1928], could not maintain continued growth. Doubling or trebling the dose of the vitamin B₁ concentrate and of the alkalised marmite produced only a temporary response. The increased effect of brewer's yeast (dried in the laboratory at 35° by means of an electric fan), and of fresh untreated marmite over vitamin B₁ and B₂ preparations, and of fresh pasteurised milk over marmite was quite noticeable. That the vitamin B₁ concentrates used in these experiments were highly potent in small doses (0.5 cc.) was shown by growth tests on younger rats. The activity of the fuller's earth extract was also checked by curative tests on pigeons, the day-dose being found to be of the order of 0.1–0.15 cc. That autoclaved marmite affords a very potent preparation of vitamin B₂ has also been demonstrated

[Hassan and Drummond, 1927] and has been confirmed by the writer on numerous occasions [Guha, 1931].

B. Experiments demonstrating the increased effect of dried brewer's yeast over that produced by relatively large doses of vitamin B₁ and B₂ preparations on normal growing rats.

While the effects of yeast and milk, described in Section A, were suggestive and were actually first observed as a side-issue in our work on the fractionation of vitamin B₁, it was obvious that the fact that these observations were made under conditions in which growth was artificially inhibited over a fairly long period would complicate the conclusions. Experiments were, therefore, carried out with young growing A Class rats, which were given vitamin B₁ and B₂ preparations of known potency from the start of the experimental feeding period. In addition to vitamins A and D, some of these animals were also provided with a vitamin E concentrate from wheat germ oil. Here also growth usually fell off after about 9 or 10 weeks and while increased doses of vitamins B₁ and B₂ temporarily stimulated growth, it could be effectively restored by a relatively small dose of dried brewer's yeast (Fig. 2). This did

Fig. 2.

- Curve (1). 0.5 cc. of fuller's earth preparation from wheat germ *plus* 1 cc. of 50 % alkalised marmite from the beginning of experiment; ↑ dose of each supplement doubled; ↓ dose of each quadrupled; ⚡ 0.5 g. dried brewer's yeast daily.
- Curve (2). 0.5 cc. of fuller's earth concentrate from wheat germ *plus* 1 cc. of 50 % alkalised marmite from the beginning of experiment; ↑ dose of each supplement doubled; ↓ 1 g. yeast residue in addition; ⚡ 0.2 g. yeast ash in addition; × the above supplements replaced by 0.5 g. dried brewer's yeast.

not occur on feeding the ash of yeast or the residue of yeast obtained after extraction with boiling water. The supposition, that in these experiments yeast exerted its effect by means of its protein content, is rendered improbable by the fact that the same basal diet as described in Section A was used, which contained commercial "light white casein," a comparatively uninjured protein. The observations of Osborne and Mendel [1925], that rats required larger

amounts of "vitamin B" at later stages of the growing period, might receive a new interpretation in the light of these experiments.

C. The effect of milk on the growth of rats receiving vitamin B₁ and B₂ preparations.

It was found that when rats were exhibiting subnormal growth after being on vitamin B₁ and B₂ preparations for 9 or 10 weeks, 8–15 cc. of fresh unskimmed milk would produce a resumption in growth (Fig. 3). This appears

Fig. 3.

Curve (1). 0.5 cc. of fuller's earth preparation from wheat germ *plus* 2 cc. of 50 % alkalised marmite from the beginning of experiment; ↑ 8 cc. of unskimmed milk.

Curve (2). Same supplements as represented in Curve (1); ↑ 10 cc. of unskimmed milk.

to be far out of proportion to the amounts of vitamins B₁ and B₂ that milk contains, especially since, as has been shown in the previous sections, the effect of increased doses of vitamin B₁ and B₂ concentrates is not so marked. It would appear, therefore, that milk contains the additional factor present in yeast.

D. Effect of milk on the growth of rats fed on a diet containing yeast.

The observation that milk could supplement vitamins B₁ and B₂ led the writer to investigate what effect, if any, milk would have if the diet were already supplemented by yeast. Although in these early experiments, "light white casein" formed the protein of the basal diet, a distinct effect was observed if relatively large quantities of milk (15–25 cc.) were fed in addition. The experiments of Coward, Key and Morgan [1929] have since shown that "light white casein" contains some factor not present in "Glaxo vitamin-free casein." These findings had an obvious relation to those of the writer, and, therefore, in all subsequent experiments, "Glaxo vitamin-free casein" was substituted for the "light white" variety. It may, however, be stated here that "Glaxo casein," "light white casein" and purified caseinogen, as prepared by dis-

solving commercial caseinogen in dilute sodium hydroxide and precipitating by hydrochloric acid, show some difference in physical properties. While this question could not be investigated in detail for lack of time, it would appear that amongst these "light white casein" is most nearly present in the natural condition. It is probable that, for nutritional experiments involving the use of purified caseinogen, the original method of purification by Hammarsten, as adopted by Svedberg, Carpenter and Carpenter [1930] for the preparation of pure caseinogen for molecular weight determinations, would be the best. The use of ammonia instead of sodium hydroxide would probably lessen the risk of de-amination and racemisation.

In the following experiments B Class rats were used. The preliminary results were obtained with a basal diet composed of "Glaxo casein," commercial sucrose, yeast and salt mixture (Osborne and Mendel), without any added fat. In subsequent experiments, however, the following diet was used:

	%
Commercial sucrose	60
"Glaxo vitamin-free casein"	21
Palm kernel oil	8
Dried yeast	7
Salt mixture (Osborne and Mendel)	4

Daily doses of 2 drops of cod-liver oil were given separately to each rat in a dish, according to the usual procedure. However, in some of these experiments cod-liver oil (2.5 %) was incorporated in the diet with equally good results. In such a case, however, the diet should be made up every week. The palm kernel oil, which in some experiments was replaced by an equal amount of olive oil, was added in order to provide some fat in the diet [Burr and Burr, 1929]. On such a dietary the weight of the B Class male rats became steady or nearly so in the region of 200–220 g. after an experimental feeding period of 10–12 weeks. The corresponding weight of the female rats was 150–160 g. When the rats attained this stage, fresh "certified" milk in varying doses (10–25 cc.) had a pronounced effect, which could not be produced by (1) increasing the daily dose of cod-liver oil to 4 drops, (2) increased allowances of vitamin B₁ and B₂ preparations and yeast, (3) orange juice (5 cc. daily), (4) olive oil (6 drops daily), (5) lactalbumin (1 g. daily) in the form of "albumactin," (6) the addition of the following amino-acids to the diet in the stated proportions:

	%
Cystine	1
<i>D</i> -Lysine dihydrochloride	0.5
Tryptophan	1
Histidine monohydrochloride	1
Arginine hydrate	1
Tyrosine	1

Both wheat embryo oil (6 drops daily) obtained by extraction with cold ether, and wheat germ, heated at 130–170° for 3 hours—a treatment, which,

Fig. 4.

Curve (1). Basal diet as described in Section D; ↑ 15 cc. fresh certified milk daily.

Curve (2). Basal diet as described in Section D; ↑ 15 cc. fresh certified milk daily.

Fig. 5. Basal diet as described in Section D.

Curve (1). Body weight steady after 11 weeks' feeding on basal diet; ↑ 4 drops of cod-liver oil added daily; ↓ 0.5 g. dried yeast added daily; ⬇ 5 cc. of orange juice daily; × 15 cc. fresh certified milk daily.

Curve (2). Body weight steady after 12 weeks' feeding on basal diet; ↓ 1 g. lactalbumin fed daily; ⊙ the mixture of essential amino-acids incorporated in the diet; ↑ 6 drops of olive oil daily; ⬆ 15 % heated wheat germ incorporated in the diet; × 15 cc. fresh certified milk daily.

according to Evans and Burr [1927, 2], does not seriously affect vitamin E of the embryo—when introduced into the diet in a proportion of 15 % produced only a slight stimulation of growth. These experiments are illustrated

in Figs. 4 and 5. The effect of milk on the growth of males is more marked than on that of females. This difference in response between males and females has been noticed throughout this investigation and is probably to be correlated with the normally greater rate of growth of the males.

These experiments indicate that the factor present in milk cannot be identified with vitamin A, C, D, E or any of the factors present in yeast, and also that the deficiency in growth in the absence of this substance cannot be attributed to a deficiency of the so-called essential amino-acids investigated. The possibility cannot, however, be excluded that some other amino-acid or a peptide like that recently described by Dakin, West and Howe [1930], which the body may be unable to synthesise, may be the factor concerned.

E. *The sources of the new factor.*

The method by which this factor was assayed in different materials consisted in feeding these to the rats after their growth had stopped as the result of a feeding period of 10 weeks on the basal dietary described in the foregoing section. A weekly increase of 10 g. body weight for 3-4 weeks was considered as the standard of good growth. It has to be mentioned that even milk does not produce sustained growth for a very long time and the milk requirements also vary considerably from animal to animal. The results obtained with some materials are given in the following table.

Nature of material	Dose	Result
Egg-yolk	1-1.5 g.	Good growth
Egg-white	2 g.	Fairly good growth
Pig-liver	2 g.	Slight growth
Sheep-liver	2 g.	Slight growth
Spinach (dried at 35°) ...	1-1.5 g.	Good growth
Grass (dried at 35°) ...	1-1.5 g.	Good growth
Alfalfa	1-1.5 g.	Fairly good growth
Certified Grade "A" milk	10-25 cc.	Good growth

The fact that milk contains considerable quantities of this factor suggested the possibility of its extraction from "Glaxo" milk powder. But neither the ethereal extract of this powder (15 drops of the melted fat) nor the powder itself in daily doses of 1 g. was capable of producing growth. It is possible that the substance was injured in the drying process. The cream of milk was not much more effective than milk itself.

Both spinach and grass (dried at 35° by means of an electric fan) were extracted with ether for 6 hours in a Soxhlet in an atmosphere of nitrogen. But the extracts were almost entirely inactive, while the residues produced only slight growth.

Finally, it has to be mentioned that both "light white casein" and milk show very varying degrees of activity. Considering the possibility of seasonal variations a scrutiny was made of the records, but the evidence obtained was not conclusive. However, it appears possible that if the experiments were carried out under more standardised conditions with a fixed source of milk

supply, such a correlation might be revealed. Spinach and grass appear to be somewhat more active than milk, considering dry weights, and it seems plausible to suppose that the ultimate source of the milk factor is the food of the cow. A sample of carotene, kindly provided by Dr Moore, in a daily dose of 1 mg. gave negative results. Likewise, chlorophyll, when incorporated in the diet in the proportion of 0.15 %, proved entirely inactive.

F. The stability of the factor present in milk to heat.

The following table summarises the results, obtained very roughly.

Treatment	Time	Result
Boiling	40 mins.	Approx. half destroyed
Boiling	2½ hrs.	At least 75 % destroyed
Autoclaving at 12-13 lbs. pressure	15 mins.	Less than half destroyed
Autoclaving at 18 lbs. pressure	2½ hrs.	Wholly inactivated

G. The question of food consumption.

Very extensive food-intake records were kept, in some cases throughout the experimental period. These records show that, in many cases, when growth was resumed after administration of milk, the food consumption did not rise

Fig. 6.

Curves (1) and (2). Basal diet as described in Section D; ↑ 15 cc. fresh certified milk daily.

Curve (3). Shows the food intake of the animal represented in Curve (1) before and after administration of milk.

Curve (4). Food-intake curve corresponding to Curve (2).

appreciably. Two such typical cases are shown in Fig. 6. Though in some other cases, the food intake rose, the fact, that it is possible to get resumption of

growth without greatly increased food consumption, suggests that this factor exerts its influence more directly on the assimilation of the food ingested than on appetite. If this is so, its effect should be reflected in a positive nitrogen balance.

DISCUSSION.

It is not necessary to examine in detail all the papers referred to before. It is significant that all those investigators, working, sometimes, under considerably different conditions, observed the inadequacy of the known factors for the normal nutrition of the rat. In some of this work, however, it is not clear whether the limiting factor might not have been "vitamin B," while in some other cases specially "purified" proteins were used and the possibility could not be excluded that the effect of the supplement given was merely a good protein effect. Preparations of the third factor in yeast, called vitamin B₄, have recently been obtained by Reader [1930]. The experiments described in this paper have a great resemblance to those of Coward, Key and Morgan [1929]. They were led from a study of the relative qualities of "light white casein" and "Glaxo casein" in the basal diets of rats for vitamin A and B tests to the discovery that the former variety of caseinogen contained some factor, in which the latter variety was deficient. They subsequently traced this substance in milk and in many other materials. The writer's observations, however, started with milk. It appears probable that we are dealing here with the same substance. It should be stated, however, that Coward has found ox-liver to be rich in the factor, while, according to my observations, pig-liver and sheep-liver appear to be deficient in it. Coward and her collaborators have also observed that it can be extracted from wheat embryo by 90 % alcohol and by ether. My preliminary attempts to extract it from spinach and grass have so far failed. Attention should also be called to the work of Mason [1929], who independently showed that lettuce provided some additional factor for growth and, by a critical analysis, proved that this factor was not identical with vitamin E, which is present in large quantities in lettuce [Evans and Bishop, 1923].

The experiments described in this paper appear to eliminate the effect of all the known vitamins. That this factor cannot be identical with vitamin E is also shown by the fact that wheat germ heated under conditions in which vitamin E is not destroyed [Evans and Burr, 1927, 2] loses its activity. That untreated wheat germ is rich in this factor has been shown by Coward. The protein effect would appear to be excluded by the negative results obtained with lactalbumin and with the essential amino-acids tested. The idea that it might be some inorganic material seems to be similarly excluded by the observation that the factor in milk can be inactivated by autoclaving. That the deficiency described here cannot be of the type encountered by Burr and Burr [1929] is shown by the practically negative effect of olive oil. However, no evidence about its chemical nature has yet been obtained.

It appears that the factor present in yeast may also be present in milk, but the factor present in milk is not present in appreciable quantities in yeast. The two factors, therefore, seem to be different. It is possible that the observation of earlier workers that "complete" synthetic rations produce good growth was due to an insufficiently long observational period. It is also felt that the growth curves would to a very considerable extent depend on the dietary on which the stock had been reared and also on the strain of rats used.

SUMMARY.

1. Accumulated evidence is presented to show that yeast contains a factor other than vitamin B₁ or B₂, and that milk contains a factor other than those present in yeast. Both the milk factor and the yeast factor are apparently required for the normal growth of rats. But even then entirely normal growth is not sustained.

2. The factor present in milk is different from other known vitamins, A, D, C or E.

3. The deficiency of the milk factor cannot be corrected by adding lactalbumin or the essential amino-acids to the diet.

4. Food-intake records show that increased growth on the administration of milk may occur without appreciably increased food intake.

5. The stability of the milk factor to heat has been investigated. The fact that it can be inactivated by autoclaving argues against the inorganic nature of this substance.

6. The factor present in milk is also present in egg-yolk, egg-white, spinach, grass and alfalfa, but much less so in pig- and sheep-liver.

7. Carotene or chlorophyll cannot replace the milk factor in the diet.

8. No satisfactory method has yet been devised for extracting the substance.

9. A method of assay of this factor is indicated.

10. Male rats show a much more marked response to this factor than females.

11. Milk and "light white casein" show appreciable variations in potency.

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REFERENCES.

- Burr and Burr (1929). *J. Biol. Chem.* **82**, 345.
Chick and Copping (1930). *Biochem. J.* **24**, 1764.
Coward, Key and Morgan (1929). *Biochem. J.* **23**, 695.
Dakin, West and Howe (1930-31). *Proc. Soc. Exp. Biol. Med.* **28**, 2.
Evans and Bishop (1923). *J. Metabol. Res.* **3**, 233.
—— and Burr (1927, 1). *Proc. Soc. Exp. Biol. Med.* **24**, 740.
—— ——— (1927, 2). The antisterility vitamine fat-soluble E. Memoirs of the University of California, vol. **8**.
Guha (1930). *Chem. Ind.* **49**, 850.
—— (1931). *Biochem. J.* **25**, 931.
—— and Drummond (1929). *Biochem. J.* **23**, 880.
Hassan and Drummond (1927). *Biochem. J.* **21**, 653.
Hopkins (1912). *J. Physiol.* **44**, 425.
Hunt (1928). *J. Biol. Chem.* **79**, 723.
Kinnorsley and Peters (1928). *Biochem. J.* **22**, 419.
Mason (1929). *J. Nutr.* **1**, 311.
Osborne and Mendel (1925). *J. Biol. Chem.* **63**, 233.
—— ——— (1926). *J. Biol. Chem.* **69**, 661.
Palmer and Kennedy (1927). *J. Biol. Chem.* **75**, 619.
Reader (1928). *Chem. Ind.* **47**, 1247.
—— (1929). *Biochem. J.* **23**, 689.
—— (1930). *Biochem. J.* **24**, 77.
Svedberg, Carpenter and Carpenter (1930). *J. Amer. Chem. Soc.* **52**, 241.