C. THE INFLUENCE OF DIFFERENT SAMPLES OF "CASEIN" ON VITAMIN TESTS.

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IN a recent paper Coward, Key and Morgan [1929] showed that rats which had ceased to grow on a diet containing all the recognised essentials for growth (dextrinised rice starch, "vitamin-free casein" (Glaxo), agar-agar, salt mixture, dried yeast, and cod-liver oil) resumed growth when "light-white casein" (British Drug Houses) was substituted for the Glaxo "vitamin-free casein." Similar resumption of growth was obtained when the casein had been extracted with cold alcohol and ether (4 times). This suggested that the extracted "light-white casein" contained some factor necessary for growth which was not contained in the "vitamin-free casein." The fact that some rats grow vigorously when "vitamin-free casein" is the only source of protein in their diet (an experience of this and of many other laboratories) suggests that these rats had acquired reserves of some factor from previous feeding and had been able to draw upon such reserves when the factor was absent from the diet. It would rather discredit the suggestion that the "vitamin-free casein" was either damaged or an inadequate protein.

Whatever the explanation, it seemed desirable to make a direct comparison of the two forms of casein as the source of protein in order to see how much different rats varied in their growth on these two caseins and how much the difference in the casein might affect an assay of vitamin A or vitamin B.

I. VITAMIN A TESTS.

The presence of 2-3 % of fat (and possibly, therefore, a certain amount of vitamin A) in "light-white casein" (B.D.H.) necessitated its extraction before using it for a vitamin A test. For these experiments, therefore, the "light-white casein" was extracted once with cold alcohol and four times with cold ether, each time standing for about 24 hours with occasional shaking. The extracted casein is named casein B in these experiments; the Glaxo "vitamin-free casein" is named casein G.

Five different litters containing 4, 2, 6, 4 and 4 rats respectively were prepared in the ordinary way for a vitamin A test, except that half of the rats

of each litter were given casein B and half were given casein G. Vitamin D was supplied by giving each rat irradiated ergosterol, about 0.00005 mg. per day, in olive oil. Growth ceased in all the rats within a reasonable time and doses of the same cod-liver oil were administered to each one as it became steady in weight.

The results are seen in Fig. 1. The growth-curves of rats from any one litter are immediately above or below each other. The following observations may be made and conclusions drawn.

(a) The rats receiving casein B have become steady in weight in a reasonable time and have responded to doses of cod-liver oil. Casein B was therefore practically free from vitamin A.

Fig. 1. Rats of five litters, half of each given extracted "light-white casein" and the other half given "vitamin-free casein" in a vitamin A-free diet. There is evidence, in the behaviour of the rats given the latter casein, of a deficiency other than of vitamin A.

(b) During the preparatory period the rats of the first two litters (*i.e.* rats H 256, 255, 248) receiving casein G have grown very similarly to their litter mates (H 257, 258, 249) receiving casein B. Therefore, these two litters probably had sufficient reserves of the new factor (if such it be) or, alternatively, casein G must have been as adequate a protein as casein B.

(c) The rats of the last three litters receiving casein G (*i.e.* rats H 346, 347, 348; G 210, 211; H 341, 342) have not grown as well as their litter mates receiving casein B (H 349, 350, 351; G 212, 214; H 343, 344). Therefore these three litters had lower reserves of the new factor (if such it be) or alternatively, casein G must be a less adequate protein than casein B. The

difference in behaviour of different litters given casein G supports the view that they had different reserves of an unknown factor rather than the view that casein G is biologically inadequate for growth.

(d) The response to doses of cod-liver oil given by rats receiving casein B is in all litters much greater than that given by rats receiving casein G. The primary response of rats H 256, 255, 248 to 0.02 g. cod-liver oil appears to be the making up of a deficiency of vitamin A, but the later decline in weight appears to be the manifestation of another deficiency.

(e) The response given by rats H 346, 347, 348; G 210, 211; H 341, 342 to doses of 0.01 g. cod-liver oil is variable and, in general, slight. On comparison with the results obtained by the same dose on the rats receiving casein B, it would appear that the response of the former rats was limited by a deficiency of some factor other than vitamin A, a deficiency which had manifested itself in the preparatory period of some of the rats before the giving of cod-liver oil was begun.

Discussion.

The differences in growth obtained by the use of the two kinds of casein here described may be attributed to (a) the presence of some definite factor, hitherto unrecognised, in one sample and its absence from the other, or (b) some biological property (greater digestibility?) of one sample, not shared by the other.

The question can be definitely settled in favour of the first suggestion only by obtaining an extract of the "light-white casein" of a potency similar to that of the casein itself. We have not, so far, been able to make an extract of such potency [Coward, Key and Morgan, 1929]. But, in view of the fact that the growth on the purer (or poorer?) casein seems to vary according to litters, rather than according to individuals, it would appear that such variations were due to variations in reserves of some factor obtained from previous feeding rather than to a deficiency of the protein as such.

Whatever the explanation proves to be, this experiment clearly demonstrates that any dose of cod-liver oil can bring about very different responses in the growth of rats according to the kind of casein which is their source of protein. In the experiment described in this paper, the sample of cod-liver oil would have been considered a rich source of vitamin A if the test had been carried out on the diet containing casein B. On the other hand, it would have been considered a poor source of vitamin A if it had been carried out on a diet containing casein G. Thus it is obvious that the definition in the United States Pharmacopœia of a unit of vitamin A activity as the "minimum daily dose of cod-liver oil required to cause a gain in weight of from 10–20 g. within a period of 35 days under the conditions of growth and diet specified in this assay" is insufficient, since the only condition required of the casein is that it should be "rendered free from vitamin A."

When a test for a vitamin is dependent on the growth-promoting power

of that substance, every substance essential for growth, other than that under examination, should be supplied in sufficient amounts in the basal diet of the test animals. Even when a growth-promoting substance can be stored in the tissues of the animals (as vitamin D can be, and as this unknown factor appears to be) it is not safe to assume that the reserves are large enough to carry the animal through the test.

We consider, however, that given a colony of reasonable uniformity (*i.e.* in regard to, reserves of growth-promoting factors) any uniform sample of casein may still be used, provided that the test be strictly comparative. We find that a large dose of cod-liver oil gives better growth than a small one, even when using casein G, although the maximal growth may not be obtained. This is not an effect of amount of fat. All oils tested in this laboratory are so diluted with olive oil that one drop of solution contains the required dose of cod-liver oil.

To make a result obtained in one laboratory comparable with one obtained in any other, it would be necessary to agree on a material containing vitamin A to be used by all workers as a standard of reference. The response given by the substance under test could then be compared with the response given by the standard preparation of vitamin A in the same laboratory. Adopting the amount of vitamin A activity in a given amount of the standard preparation as the unit, the result obtained could then be expressed in units, and results from different laboratories could be compared. This must apply whatever may be the source of protein, or indeed of any of the constituents of the diet.

II. VITAMIN B TESTS.

The greater growth-promoting potency of "light-white casein" compared with "vitamin-free casein" has lately become evident in this laboratory in vitamin B tests also. The basal diet for such tests has, until recently, consisted of:

"Vitamin-free casein" (Glaxo)	...	15 %
Dextrinised rice starch	...	79
Agar-agar	...	2
Salt mixture (Steenbock's)	...	4

5-6 drops of a sample of good cod-liver oil are given to each rat twice a week. The rats are kept on screens to prevent coprophagy. Three or four are kept together in a cage during the preparatory period; they are kept singly during the test period.

It was in a routine test of dried yeast that the influence of the two forms of casein in vitamin B tests was first observed. After 11 days' preparatory period on the diet described, five rats were given the dried yeast as 0, 2, 5, 8 and 10 % respectively of the diet. After 10 days none of the rats had grown. This was so unusual that it was suspected that the litter of rats was suffering from the same deficiency that had been noted in many vitamin A tests.

Accordingly, "light-white casein" unextracted, and shown to be free from both vitamins B₁ and B₂ by Coward, Key and Morgan [1929] was substituted for the "vitamin-free casein" in the diet of the three rats receiving 0, 5 and 10 % dried yeast respectively. The result is seen in Fig. 2 (a). The control, not receiving dried yeast, continued to lose weight and died in 3 days. Those given 5 and 10 % dried yeast, respectively, immediately began to grow rapidly, and

Fig. 2. Evidence of a deficiency in "vitamin-free casein" from a vitamin B test: (a) "vitamin-free casein" in vitamin B-free diet; no growth for 10 days after the addition of dried yeast. "Light-white casein" substituted in the diet of two rats resulted in much greater growth than obtained with the original casein; (b) "vitamin-free casein" in vitamin B-free diet throughout. Rats of different unknown history of previous feeding. Very irregular response to graded doses of dried yeast; (c) "light-white casein" in vitamin B-free diet throughout. Allowing for an interruption of the growth of rat L 53 owing to a respiratory infection, much more regular response (graded to the dose of dried yeast) was obtained than in the use of "vitamin-free casein."

only slackened their rate of growth when the doe on 5 % dried yeast had reached 140 g., and the buck on 10 % dried yeast had reached 210 g. The rat receiving 2 % dried yeast and "vitamin-free casein" throughout the experiment, continued to lose weight for a further 7 days and then resumed very slow growth. The rat (a buck) receiving 8 % dried yeast and "vitamin-free

casein" throughout the experiment, began to grow, but at a much slower rate than the doe receiving 5 % dried yeast and "light-white casein." Moreover, it only attained a weight of 104 g. by the end of the experiment.

In a second experiment, rats from a different source, but prepared on the same basal diet containing "vitamin-free casein," were given similar doses of vitamin B, *i.e.* they were given 0, 2, 5, 8 and 10 % dried yeast respectively, and maintained on this casein throughout the experiment. The response was immediate, though not proportional to the dose. It must be concluded that these rats had definite, though different, reserves of the recently recognised factor, or, alternatively, that they had different powers of dealing with the protein (Fig. 2 (b)).

A third experiment was begun at the same time. A group of rats (three from one litter and two from another) was given the vitamin B-free diet containing "light-white casein" from the beginning of the experiment. After 12 days they were given 0, 1, 2, 4 and 8 % dried yeast respectively. Except that the rat receiving 4 % dried yeast developed a temporary respiratory infection during part of the test period, the rats gave a much more regular response to the dose given than did the rats receiving "vitamin-free casein" (Fig. 2 (c)).

A similar experiment has been performed with marmite, giving a similar result. Three rats were given the vitamin B-free diet containing "vitamin-free casein," and were steady in weight in 12 days. They were then given daily for 20 days 0.1, 0.2, 0.4 g. marmite respectively. None of them grew at all, either maintaining or very slightly losing weight. The two receiving 0.1 and 0.4 g. marmite, respectively, were then given "light-white casein" in place of the "vitamin-free casein." The former, a buck, grew very slowly, 9 g. in 45 days, the latter, a doe, grew 74 g. in 45 days. The third, a buck, continued to receive 0.2 g. marmite and "vitamin-free casein," and grew 16 g. in 45 days.

An experiment in which all the rats received the diet containing "light-white casein" throughout, gave regular results, those on 0.1 g. marmite maintaining weight, those on 0.2 and 0.4 g. respectively giving growth graded to the dose.

A test of a particular extract of yeast, started about this time, began to give very irregular results, the responses given to 0.4 g. varying from - 1 to + 17 g. in 15 days. The response given to 0.6 g. varied from - 5 to + 25 g. in 15 days. The substitution of "light-white casein" for the "vitamin-free casein" of the basal diet made a great difference. The rats which had not grown at all began to do so, mostly at once, and the final growth curves were remarkably similar (Fig. 3).

Discussion.

Greater uniformity of results has been obtained in vitamin B testing by the use of "light-white casein" than by the use of "vitamin-free casein." A serious failure to respond to high doses of dried yeast included in the vitamin B-free diet made it appear that the particular rats used for the test

were suffering from some deficiency hitherto unsuspected in the vitamin B-free diet. Further tests with the same dried yeast on (a) rats from a different source, and therefore with a different history of pre-experimental feeding, and (b) rats given "light-white casein" throughout the whole test, proved that the dried yeast had its usual vitamin B potency. The different results obtained in the three tests suggest that either the "light-white casein" supplied some factor not supplied by the "vitamin-free casein," and the different litters of rats had different reserves of the unknown factor, or the different litters of rats had different powers of utilising the "vitamin-free casein."

Fig. 3. The influence of the sample of casein in a vitamin B test on the regularity of response to doses of a dried yeast extract.

Whatever may prove to be the explanation of the results, it is evidently desirable to use "light-white casein" for the basal diet of rats in vitamin B testing. As this form of casein contains no vitamin B₁ or B₂, it is not necessary to extract it before use.

SUMMARY.

1. The growth response of rats, given cod-liver oil after they have ceased to grow in a preparatory period, may vary greatly according to the sample of casein used in the basal diet.

2. The difference due to the different caseins in vitamin A testing is so great, that an estimate based simply on the growth response of a number of animals in relatively undefined conditions (as in the United States Pharmacopœia cod-liver oil test) must give widely different results in the hands of different workers. Vitamin A tests (of food substances at least), to be of value, must be comparative, and it is suggested that all workers should use a common standard of reference.

3. Evidence is given which supports the view that "light-white casein" (B.D.H.), even after extraction with alcohol and ether, contains some growth-promoting factor which is not necessarily present in other samples of casein.

4. For vitamin B testing "light-white casein" (B.D.H.) as the source of protein gives more uniform results than "vitamin-free casein" (Glaxo). As it contains neither vitamin B₁ nor B₂, it does not need to be extracted before use in vitamin B testing.

REFERENCE

Coward, Key and Morgan (1929). *Biochem. J.* **23**, 695.