CXIII. THE NEED FOR A STANDARD OF REFERENCE IN VITAMIN A TESTING.

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INCREASING numbers of workers are realising that greater accuracy is obtained in growth tests for vitamins by considering, not the growth curves of single animals, but the mean growth curves of groups of animals [Chick and Jackson, 1932; Coward *et al.*, 1930; 1931; Sherman and Batchelder, 1931]. In this laboratory, the vitamin A content of any sample of cod-liver oil has been determined by finding the mean increase in weight in 5 weeks of a group of rats each of which was given the same daily dose of the oil after it had ceased to grow on a diet deficient in vitamin A. During the last two years, a simultaneous test of a particular sample of cod-liver oil serving as a standard of reference has been made with each sample of oil tested. Results obtained from the successive tests of this oil have indicated that the average response of groups of rats to the same amount of a given sample of cod-liver oil may fluctuate greatly from month to month, and some evidence of this has already been published [Coward, Dyer and Morton, 1932]. The examination of a series of cod-liver oils showed that the intensity of absorption at $328\text{ m}\mu$ was the physical measurement which agreed best with the biological estimate of vitamin A, but for certain oils even this agreement was bad. The agreement was better when the relative vitamin A potency of different oils was estimated by simultaneous biological examination of the standard oil, rather than by examinations made at different times. Thus it was evident that in order to make biological estimates in terms of a common standard, such as the carotene recently proposed as an international standard, simultaneous tests of the unknown substance and the standard must always be made.

Further tests on the oil used as a standard of reference emphasise still more the need of a standard of reference in every estimation. In all, 13 tests have been made on the same oil, using the same technique, the same daily dose of the oil (2 mg.) and rats as nearly alike as our colony could supply. There has been no change in the laboratory workers. The variations in the apparent vitamin A value of this sample of cod-liver oil are given in Table I. To obtain each of the figures, about 10 rats (5 bucks and 5 does) were given a vitamin A-free diet until they became steady in weight. Each rat was then given a daily dose of 2 mg. of our standard oil for 5 weeks. From the curves of response drawn up in the laboratory, which relate mean increase in weight to dose of vitamin A given, the abscissae corresponding to the mean increases in weight of the groups of rats were determined. The average of the abscissae for the bucks and does in successive tests gives the relative potency of the oil at different times.

Table I. *Variations in the apparent vitamin A value of one sample of cod-liver oil in successive tests on different groups of about 10 rats.*

Date of test	Average of abscissae (weighted means) of curves of response (for bucks and does) relating mean increase in weight in 5 weeks of a group of rats to dose of vitamin A
June-July, 1931	0.93
August, 1931	1.05
October, 1931	1.18
November, 1931	0.73
Jan.-Feb., 1932	1.52
March, 1932	1.29
April-May, 1932	1.11
June, 1932	2.64
July-Aug., 1932	3.48
August, 1932	2.30
Aug.-Sept., 1932	1.33
September, 1932	2.03
Sept.-Oct., 1932	1.58

The first seven values were reported in the paper by Coward, Dyer and Morton [1932]. It was shown that the different values could be attributed to errors of random sampling, *i.e.* considering the standard deviation of the response of the animals, these variations could occur without change in the general conditions of the experiments. But in June, 1932, some change took place which induced in the rats a greater response to 2 mg. of the oil than had ever before been given. In the test made in July-August, 1932, the apparent value of the oil was five times its apparent value in November, 1931. It is unbelievable that the actual vitamin A value of the oil could increase. It must, therefore, be admitted that equal doses of vitamin A can bring about very different responses in groups of rats at different times. It follows that equal responses in groups of rats at different times do not prove that the doses given are of equal vitamin A potency. The need for making a simultaneous test on a standard of reference whenever a substance is tested for vitamin A is obvious.

(Experiments are in progress in many laboratories concerning the use of a particular sample of carotene as a standard of reference for vitamin A. This sample is issued by the National Institute for Medical Research, Hampstead, on behalf of the Permanent Standards Committee of the League of Nations.)

Possible causes of the variation in response to the same dose of vitamin A.

The variation in response to 2 mg. oil A was gradual, though it was evidently not seasonal (Table I). The differences in response may have been due to (a) differences in the vitamin A-free diet of the animals, (b) differences in the animals themselves, (c) the influence of some undetermined factor, possibly in the diet of the breeding colony.

(a) *Variation in vitamin A-free diet.* The only change in the constituents of the vitamin A-free diet that we can detect during the time that the 13 tests of oil A were being made, was in the caseinogen, but this does not account for the sudden rise in the apparent value of oil A in June, 1932. Special batches of "light white casein" are allocated to our use by the British Drug Houses and delivered as required, 1 cwt. at a time. One batch was exhausted and another set aside for us in June, 1932, but the June test on oil A was finished before the first hundredweight of the second batch was delivered to us in July. Thus, so far as we can tell, there were no variations in the caseinogen to account for the

variations in the apparent vitamin A value of the oil. The amount of vitamin D given to the rats each week (8 units per rat) has not been changed, and from tests of the vitamin B content of several samples of dried yeast, though not of all, we think that the dried yeast has not varied appreciably during the tests on oil A. Thus it seems impossible to account for the variations in response to the same dose of oil A on the ground of changes in the basal diet of the rats.

(b) *Variations in the test animals.*

1. *The degree of correlation between the rate of growth in the preparatory period (i.e. the time during which the rat is fed on a vitamin A-free diet only, until it ceases to grow) and the growth during the 5 weeks when the rat was given a daily dose of cod-liver oil has been determined.* It had occasionally occurred to us that when a rat grows slowly in the preparatory period, its response to a given dose of vitamin A is slower than that of a rat which grows rapidly in the preparatory period. A measure of the rate of growth in the preparatory period was made by determining the increase in weight of the rat in the first 5 weeks of feeding on the vitamin A-free diet only. Most of the rats grew for 6 or 7 weeks before becoming steady in weight, so that the rate of growth during the first 5 weeks seemed to be a fair estimate of the "growth potential" of the rat. The degrees of correlation between the rate of growth in the preparatory period and the growth during the 5 weeks' test period for the bucks and does respectively were found to be:

- 0.033 for the bucks (63);
- 0.135 for the does (82).

These figures should be at least + 0.208 and + 0.183 respectively to indicate a positive correlation ($P = 0.1$, i.e. the probability that a correlation should arise by random sampling from an uncorrelated population). The figure – 0.135 for the does indicates approximately a 1 in 2 chance of a definite relationship, but as the sign is negative, it indicates that the greater the rate of growth in the preparatory period, the slower the rate of growth in the test period and *vice versa*. Thus, in this colony of rats, there is no indication that a greater rate of growth in the preparatory period of feeding on the vitamin A-free diet only is accompanied by a greater response to a dose of vitamin A when this is given to the rat.

2. *The degree of correlation between the weight of the rat at the beginning of the test period (i.e. at the time the rat ceased to grow and was first given a dose of cod-liver oil) and the increase in weight in 5 weeks during which it was given a daily dose of 2 mg. of oil A has been determined.* As we discard male rats which do not cease to grow before they attain a weight of 120 g. and female rats at 110 g. our investigation does not cover a very wide range of initial weights. It was found that:

- $r = -0.194$ as determined from 63 bucks;
- $r = -0.095$ as determined from 82 does.

For a probability of $P = 0.1$ that there is no correlation, r would have to be – 0.208 ($n = 63 - 2$ bucks) to indicate that the higher the initial weight the less (on account of the *minus* sign) would be the response. Similarly, r for the does would have to be – 0.183. Thus the probability that differences in weight at the beginning of the test period would affect the results under the conditions and limitations of these tests appears to be small.

When, however, the correlation between the initial weight of the rat and the abscissa of the curve of response corresponding to the increase in weight of the rat is determined, it is found that:

$$r = -0.2472 \text{ for the bucks;}$$

$$r = -0.2456 \text{ for the does.}$$

Whence $P = 0.025$ for the bucks, and $P = 0.05$ for the does.

This method of determining a correlation indicates that the initial weight of the rat influences the response. It may be a sounder method than the former, and it confirms the conclusion of Sherman and Burtis [1928] who made their calculation by a different method from either of those given in this paper.

Table II.

Test	♂			♀		
	Average weight at beginning of test period g.	Increase in weight in 5 weeks g.	Abscissa corresponding to increase in weight	Average weight at beginning of test period g.	Increase in weight in 5 weeks g.	Abscissa corresponding to increase in weight
1	95.1	11.4	1.00	75.0	9.25	0.77
2	91.3	9.3	0.90	76.3	14.5	1.20
3	90.0	16.1	1.24	91.0	13.7	1.12
4	76.7	7.8	0.85	79.7	6.7	0.62
5	98.6	20.6	1.53	81.0	17.4	1.52
6	—	—	—	78.4	15.4	1.29
7	69.7	14.7	1.17	67.7	13.3	1.08
8	87.6	29.8	2.33	83.5	25.5	3.01
9	73.0	42.0	4.08	91.2	26.0	3.14
10	84.0	19.6	1.46	88.4	26.0	3.14
11	94.7	8.0	0.86	88.4	18.8	1.71
12	73.7	26.3	1.99	57.9	20.9	2.04
13	77.9	18.1	1.36	70.1	19.1	1.76

We then compared the average initial weights of the groups of rats in the several tests with the average increases in weight in 5 weeks and also with the abscissae of the curve of response corresponding with these average increases in weight (Table II). It is, however, evident that differences in initial weight do not account wholly for the big differences in response of the different groups of animals to the same dose of vitamin A.

SUMMARY.

Thirteen tests of the vitamin A content of a particular sample of cod-liver oil have been made on groups of rats, each group containing 10–12 animals. The tests were made successively and extended over about 17 months. A five-fold variation in result was found.

The variation in result is not a seasonal one. It is apparently not due to changes in the basal diet. It is not due to variations in the weights of the rats when they were first given the doses of cod-liver oil, or to differences in rate of growth in the preparatory period during which the rats' reserves of vitamin A were being exhausted. The variation must, therefore, be due to some influence not yet determined.

It is obviously essential that the vitamin A content of a substance should be determined not simply by the response of a particular group of animals to a given dose of the substance, but by a comparison of the response of this group with the response of another group to a dose of some standard of reference, the two tests being carried out simultaneously.

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