CCXIX. THE DETERMINATION OF VITAMIN A.

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It is recognised by all workers in the vitamin field that rats which have ceased to grow on a diet deficient in vitamin A respond to a given dose of that factor to a varied degree. The variations have, in the main, been attributed to variations in the breed of the rats, in the pathological conditions of the animals when considered ready for a test, and in the diet of the colony from which the experimental animals were taken, variations which occurred even when this diet had been kept as nearly uniform as possible. The present paper is an attempt to demonstrate how they may be overcome for the purpose of making a quantitative comparison of the potency of two vitamin-containing substances.

The first experiment.

It has been the practice of many, when testing substances like cod-liver oil, to add one daily dose of the substance to the diet of two or three rats, a second, larger dose to the diet of two or three more rats and a third and even a fourth larger dose to the diet of further small groups of rats. It is generally admitted that the behaviour of each animal does not change strictly according to the size of the dose administered, and that some rats receiving the higher doses resume growth less promptly and grow at a slower rate than those receiving the lower; but it is rarely realised how widely different the responses of different animals may be, so different indeed as to throw suspicion on results with a small group of rats in which there is an appearance of a response graded to the dose. Workers are much inclined, we think, to accept results as satisfactory in an experiment on a few animals in which there is some gradation according to the dose, and to discard others as inexplicable where the gradation is not apparent. There is, of course, no justification for this. We give an example in Fig. 1 which illustrates how different the response of different animals may be. Of two rats prepared in the same way on a vitamin A-free diet containing Glaxo "casein," one rat gained 38 g. in 10 weeks on a daily dose of 2·5 mg. cod-liver oil, whereas the other lost 1 g. in the same time on a daily dose of 15 mg. These irregularities may dominate the picture at the close of the test so much that it is impossible to detect any gradation of the growth response at all.

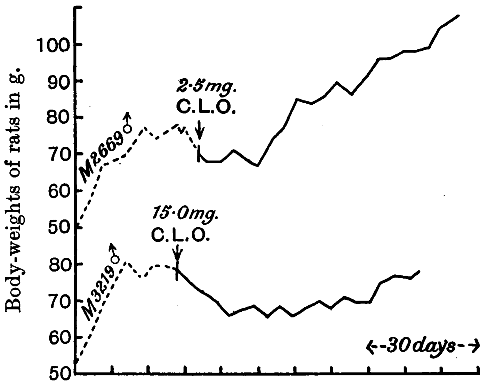

Fig. 1. Exp. 1. Growth curves of two rats showing the wide variation in response to cod-liver oil.

Our first object, therefore, was to determine under what conditions it was possible to obtain a growth response in different groups of animals which should be graded according to the size of the dose of cod-liver oil. It became necessary to find a means of making a sum of the responses of the animals of a group in order to compare it with that of a different group of animals receiving a different dose. The first attempt to do this followed the very simple plan of counting the growth response of each animal as positive or negative according as it had gained 10 g. in 5 weeks or had not. This response is the basis of the definition of the unit of vitamin A activity prescribed by the United States Pharmacopoeia. This plan, though simple, involved a considerable extension of the work ordinarily undertaken in vitamin A examinations. Instead of treating each curve of growth resumption as a result in itself, we proposed to treat it merely as one positive or negative out of a large group of such curves. We, therefore, used approximately 30 animals in each group, each animal in a group being given the same dose of a particular sample of cod-liver oil, which will henceforward be described as oil A. There were in all, in this first experiment, in which Glaxo "casein" was used in the basal diet, 11 groups of about 30 rats each. For every one of the 330 rats, an ordinary weight chart was plotted. The test period from the time when each rat had ceased to grow and when the daily dose of oil A was first administered was 10 weeks.

The diet used in preparing the rats was as follows:

Vitamin-free caseinogen (Glaxo)	15 %
Dextrinised rice starch	73
Dried yeast	8
Salt mixture (Steenbock's 40)	4

This was exposed in thin layers (200 g. over 4 sq. ft.) to the rays of a quartz mercury vapour lamp at a distance of 2 ft. for 30 minutes, the lamp running at 2·5 amps., 130 volts. This generated ample supplies of vitamin D. We made no attempt to divide litters among the different groups. We completed each group as the rats became steady in weight, assuming that they would be as nearly as possible in the same physiological condition when considered by the same workers to be ready for the test. Greater accuracy may be obtainable by putting one animal of each litter into each group, but we have not investigated this point.

In Fig. 2 is shown the extraordinary result which was obtained when

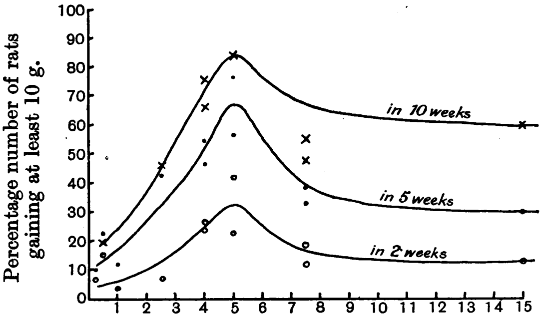

Fig. 2. Exp. 1. The relation of growth response to dose of cod-liver oil. The percentage number of rats in each group that gained at least 10 g. weight in 2, 5 and 10 weeks respectively. "Vitamin-free casein" (Glaxo) in diet.

11 groups, each of about 30 rats, were examined in this way. In Table I is given the order in which the doses were tested. The significance of the points

Table I. *Order and times of testing the different doses.*

Dose in mg.	Date test began	Dose in mg.	Date test began
0·5	Aug. 1928	7·5	July 1929
0·25	Nov. 1928	7·5 (second test)	Aug. 1929
1·0	Feb. 1929	15·0	Sept. 1929
5·0	Apr. 1929	4·0 (second test)	Oct. 1929
2·5	May 1929	5·0 (second test)	Jan. 1930
4·0	July 1929		

on the curves is best explained by an illustration. To each of one group of 28 rats, a daily dose of 2·5 mg. of oil A was administered. Of these rats it was found that two, or 7 %, had gained at least 10 g. at the end of 2 weeks. Twelve rats, or 43 %, had gained at least 10 g. at the end of 5 weeks, and 13 rats, or 46 %, had gained at least 10 g. at the end of 10 weeks. It may be noted at once how little difference there was between the number of rats gaining 10 g. at the end of 5 weeks and at the end of 10 weeks.

We expected, of course, that as the dose of cod-liver oil increased, so the percentage of rats gaining at least 10 g. would increase until the figure of 100 % was reached. Actually the percentage rose, as shown, up to a dose of

5 mg. only. Above this, however, the percentage fell and the dose of 7·5 mg. was apparently less effective than those of 4 and 5 mg. This was an extraordinary result to have reached as a result of an exhaustive research on over 300 animals. At first sight it meant that vitamin A, as present in cod-liver oil, was not an agent which promoted growth in proportion to the amount of it present in the dose. Every estimation of vitamin A which has ever been made, however, assumes that the growth response is, up to a certain limit, dependent on the amount of vitamin. It seemed that some might attempt to explain the observations on the ground that this sample of cod-liver oil possessed some obscure toxic property which had developed as time had passed, and which had interfered with the true effect of the higher doses. Accordingly we tested the doses of 7·5 mg., 4·0 mg. and 5·0 mg. again, and also examined the effect of a daily dose of 15 mg. With slight differences, the previous results were confirmed, and the dose of 15·0 mg. produced an effect not appreciably

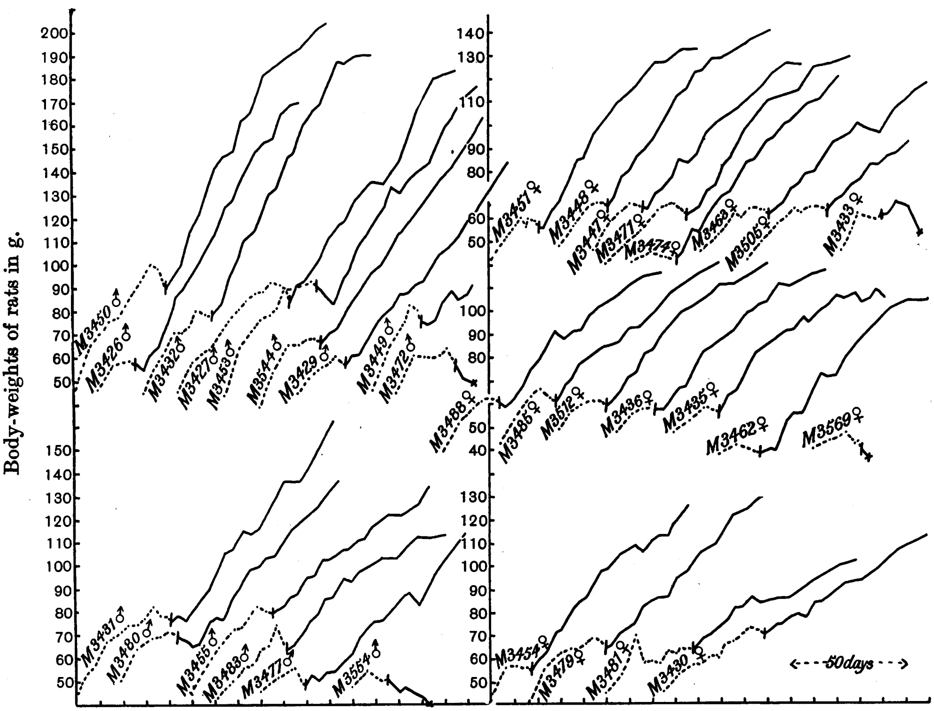

Fig. 3 A. Exp. 1. To show the variation in response to a dose of 5·0 mg. cod-liver oil in 34 rats (15 ♂, 19 ♀) during a 10 weeks' test with extracted "light white casein" (B.D.H.) in the diet. The standard deviation in this group was 12·1.

worse than that of 7·5 mg. This excluded, in our opinion, the possibility of any toxic effect. On the other hand it abundantly confirmed the finding that even limited growth was not resumed on this diet by a majority of rats when given an excess of vitamin A.

At this stage of the investigation we had realised from separate experiments

[Coward, Key and Morgan, 1929; Coward, Key, Morgan and Cambden, 1929] that the basal diet described was not always sufficient, together with vitamin A, to produce normal growth, in contrast to a diet in which an extracted caseinogen was used instead of the Glaxo "casein." This extracted caseinogen, which was "light white casein" bought from B.D.H. and extracted by ourselves with alcohol and ether, provided a basal diet on which rats grew rapidly when the diet was supplemented with a small dose of cod-liver oil. Accordingly we prepared a set of rats on a diet similar to the other one in all respects except that it contained "light white casein" (B.D.H.) which we extracted as required once with cold alcohol, and four times with cold ether (200 g. in 1–1·5 litres solvent) in jars with frequent shaking, 24 hours each extraction. As the rats became steady in weight they were used for a test of 5·0 mg. of the oil. The result of this test together with the result on 5·0 mg. on the first diet containing Glaxo "casein" is shown in detail in Figs. 3 A and B. It is

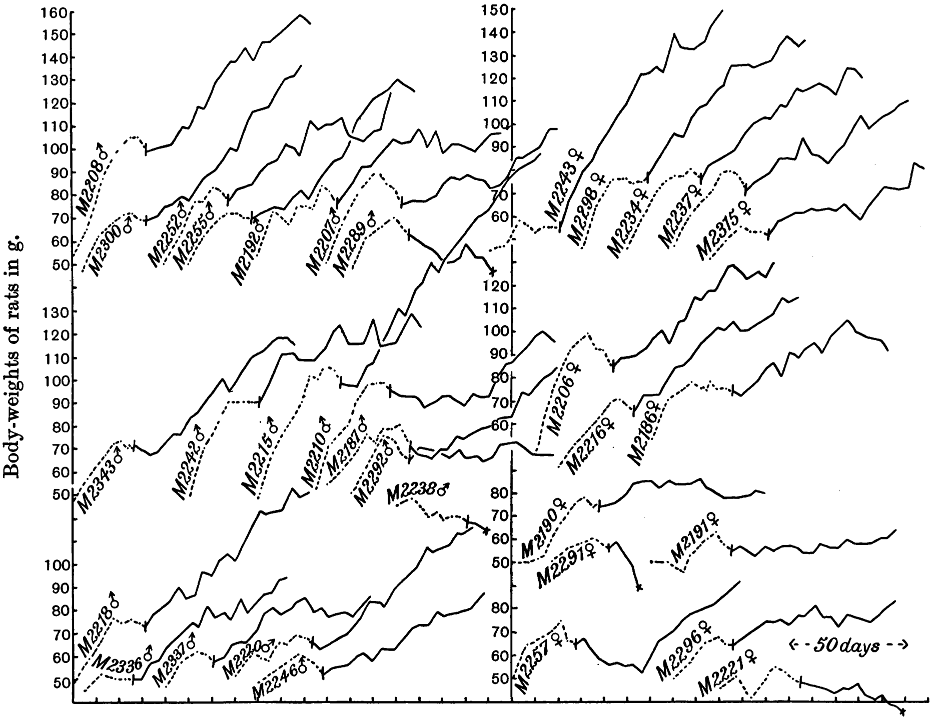

Fig. 3B. Exp. 1. To show the variation in response to a dose of 5·0 mg. of the same oil in 33 rats (19 ♂, 14 ♀) but with "vitamin-free casein" (Glaxo) in the diet. The standard deviation in this group was 16·8.

obvious from a survey of the individual curves that this response is far better than that on the diet containing vitamin-free caseinogen (Glaxo). 93 % of the rats of this group of 34 gained at least 10 g. in 5 weeks, whereas the highest percentage to make such a gain on the other diet was 77 in the first group that was given 5·0 mg. The results of the first experiment are summarised in Table II. We considered this result additional evidence that the "light white

Table II. *Exp. 1. To show the growth response of groups of rats to different doses of cod-liver oil, "vitamin-free casein" (Glaxo) in the diet.*

Dose of C.L.O. mg.	No. of rats	Date test began	No. dead in 1 week	No. dead in 2–5 weeks	% increasing 10 g. in 5 weeks	Mean increase in 5 weeks in g.	$\sigma=\sqrt{\frac{\Sigma d^2}{n}}$	$\frac{\text{Log}_e M_2 - \text{Log}_e M_1}{K_2 - K_1}.100$
0·25	29	Nov. 1928	0	4	10	−3	10·3	−0·14100
0·5	29	Aug. 1928	3	3	23	3·5	10·7	0·13866
1·0	36	Feb. 1929	4	3	12	−0·7	11·4	−0·02832
2·5	39	May 1929	11	1	43	4·0	12·4	0·16514
4·0	38	July 1929	8	4	47	10·5	18·3	0·40086
4·0	32	Oct. 1929	3	1	55	12·7	11·8	0·44046
5·0	33	Apr. 1929	2	0	77	21·5	16·8	0·74332
5·0	30	Jan. 1930	1	2	57	12·5	11·3	0·39787
7·5	33	July 1929	0	7	33	10·3	14·1	0·33388
7·5	32	Aug. 1929	1	1	39	7·3	12·0	0·29478
15·0	33	Sept. 1929	3	2	30	6·7	11·0	0·28918
5·0 Cas. B.D.H. in diet	34	Oct. 1929	2	3	93	40·7	12·1	1·3976

casein" has some growth-promoting property which the vitamin-free caseinogen (Glaxo) does not have. As it was clearly impossible to get reliable indications of differences of vitamin A potency by the use of the vitamin-free caseinogen (Glaxo) and the stock of rats that we had available, we decided to repeat the whole experiment, using a diet containing extracted "light white casein" (B.D.H.) instead of the vitamin-free caseinogen (Glaxo), hoping that we should obtain, with some dose, 100 % of the animals of a group gaining at least 10 g. in 5 weeks, and that the curve of response to different doses would be steep enough to give fairly accurate comparisons (say to within 25 or 50 %) between different samples of oil.

The second experiment.

A second sample of the same batch of cod-liver oil was obtained from Messrs Allen and Hanbury and used throughout this experiment with a diet containing extracted "light white casein" (B.D.H.). Doses were tested in the order given in Table III.

Table III. *Order and times of testing the different doses in Exp. 2.*

Dose (mg.)	Date test began	Dose (mg.)	Date test began
2·5	Nov. 1929	0·25	Jan. 1930
7·5	Dec. 1929	1·0	Apr. 1930
20·0	Dec. 1929	1·5	May 1930

The result is plotted in Fig. 4. Curves of the percentage number of rats gaining 20 and 30 g. respectively in 5 weeks are also plotted. The steeper parts of the curves are again plotted in Fig. 5 on a more extended scale. The result is very different from the one obtained in the first experiment and shows at once that it is possible to get a growth response graded to the dose of vitamin A. Thus, although actual measurements of vitamin A

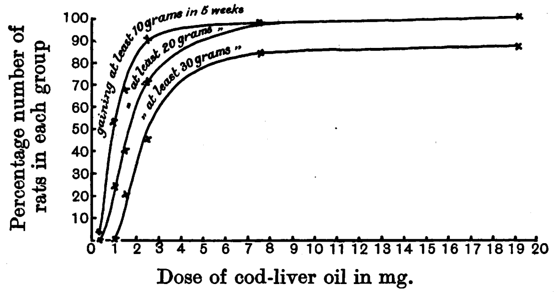

Fig. 4. Exp. 2. The relation of growth response to dose of cod-liver oil. The percentage number of rats in each group that gained at least (*a*) 10, (*b*) 20 and (*c*) 30 g. in 5 weeks. Extracted "light white casein" (B:D.H.) in the diet.

cannot be made without a standard of reference, yet comparisons between different substances with respect to their vitamin A potency may be drawn

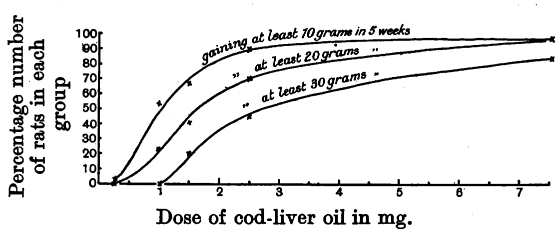

Fig. 5. Exp. 2. Fig. 4 expanded to a more useful form.

with a degree of accuracy equal to that obtained in many assays made by biological methods.

Other methods of measuring the response.

It was obvious that these curves failed to make anything like full use of the data obtained. In Tables II and IV are collected the results of the two experiments, giving the following information.

1. *Number of rats dying during the test.*

The number of rats dying during the first week of the test bears no relation to the dose of cod-liver oil given. Considering the varying and unknown degrees of severity of the pathological conditions of the animals, this is not surprising. These rats have been ignored in all calculations. Some workers discard those that die in the second week also. We have counted those that died in the second to the fifth week, and found in the second experiment a well-marked relation between the number dying during this period and the dose of cod-liver oil given. It is shown in Fig. 6 that it may be possible to devise a method based on a curve of this kind for comparing the vitamin A potency of different substances. The fact that the corresponding figures in Exp. 1 do

Table IV. *Exp. 2. To show the growth response of groups of rats to different doses of cod-liver oil, extracted "light white casein" (B.D.H.) in the diet.*

Dose of C.L.O. mg.	Total no. of rats	Date test began	No. dead in 1 week	No. dead in 2–5 weeks	Rats alive at end of 5 weeks	% increasing 10 g. in 5 weeks	Mean increase in 5 weeks g.	$\sigma = \sqrt{\frac{\Sigma d^2}{n}}$	$\frac{\text{Log}_e M_2 - \text{Log}_e M_1}{K_2 - K_1} . 100$
0·25	31	Jan. 1930	2	15	14 (7 ♂, 7 ♀)	3	−11·5	13·75	−0·3583
1·0	37	Apr. 1930	7	6	24 (17 ♂, 7 ♀)	53	13	8·60	0·37837
1·5	35	May 1930	5	3	27 (10 ♂, 17 ♀)	67	17·1	14·8	0·52620
2·5	32	Nov. 1929	1	1	30 (19 ♂, 11 ♀)	90·3	27·7	10·6	0·81349
7·5	31	Dec. 1929	0	1	30 (18 ♂, 12 ♀)	97	48·2 {♂ 55·7, ♀ 37·0}	14·3 {♂ 10·3, ♀ 10·4}	1·3139 {♂ 1·4846 Init. wt. 81·8, ♀ 1·0391 „ 84·2}
20·0	32	Dec. 1929	1	0	31 (15 ♂, 16 ♀)	100	45·4 {♂ 53·0, ♀ 37·2}	14·3 {♂ 13·9, ♀ 9·11}	1·2349 {♂ 1·3835 Init. wt. 85·1, ♀ 1·0601 „ 83·75}

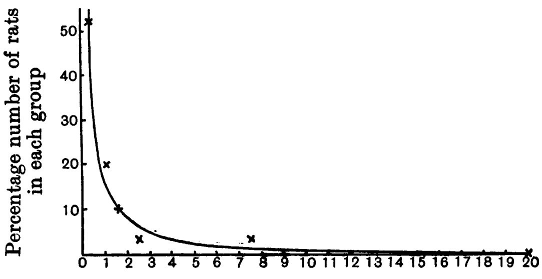

Fig. 6. Exp. 2. The relation between dose of cod-liver oil and the percentage number of rats in each group that died during the second to the fifth week.

not show a similar correlation with dose, would appear to lend additional support to the view that many of the rats in Exp. 1 were not suffering from a deficiency of vitamin A only.

2. *The total growth of all animals of each group* has been expressed in various ways.

(*a*) A composite curve of all the animals of a group may be found by averaging their weights when first given the dose and again at weekly intervals during the test. If a rat dies during the test, the curve may be broken at the week of its death. It ends at the point where it dies, this point including its last weight, and a fresh curve is begun at the same point of time, calculated from the average weight of the remaining rats. The general slope of the curve represents the full growth response of all animals of the group.

The composite curves of all groups of animals in both the experiment with Glaxo "casein" and the experiment with extracted "light white casein" are shown in Fig. 7. They depict quite clearly the differences in response to similar doses obtained by using the different preparations of caseinogen in the basal diets. The curves obtained by the use of Glaxo "casein" and different doses of oil are very little different on the lower doses, and are practically alike on the higher doses. It would be impossible to make comparisons over the very small range of difference shown by these curves.

It is quite obvious, however, that there are greater differences between the

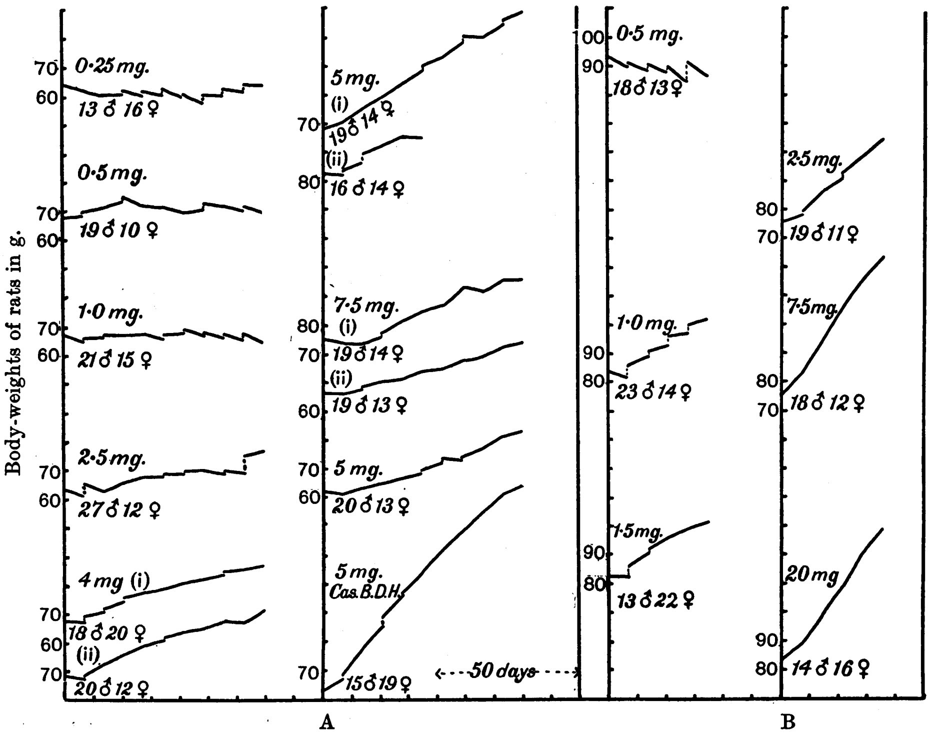

Fig. 7. Composite growth curves of groups of rats on different doses of cod-liver oil. A. Exp. 1. "Vitamin-free casein" (Glaxo) in diet. B. Exp. 2. Extracted "light white casein" (B.D.H.) in diet.

curves obtained by the use of a similar series of doses of oil when extracted "light white casein" is used. The series of curves is useful to indicate the differences in dosage which are responsible for differences in slopes of the composite curves obtained. It is perhaps surprising to note that the dose of oil A (7·5 mg.) which produced the greatest growth (20 mg. produced no more than 7·5 mg.) is, in fact, seven and a half times as great as that (1·0 mg.) which produced slight growth (13 g. in 5 weeks).

(*b*) A curve has been plotted in Fig. 8, relating the mean increase in weight in a group in 5 weeks to the dose administered. It demonstrates very clearly that, up to a point, the growth response increases with the dose of vitamin A given. It appears to be the most direct and complete way of relating response to dose given and, on the lower doses, the slope is steep enough to give results comparable in accuracy with those of most biological measurements. It ignores differences that may be due to differences in initial weight[1] of animals or groups of animals. Actually, in the experiment, the average initial weights of the groups of animals were 75–85 g.; that is they were sufficiently alike to make it unnecessary to allow for the differences.

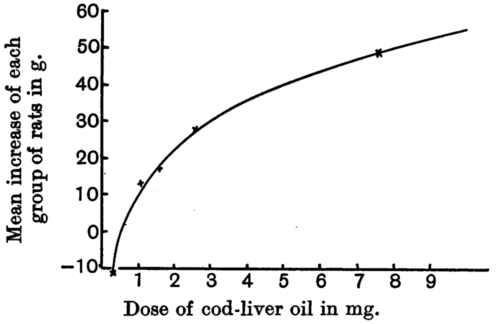

Fig. 8. Exp. 2. The relation between dose of cod-liver oil and the mean increase in weight of each group of rats. Extracted "light white casein" in diet.

The curve drawn in Fig. 8 fits the equation $y = 12{\cdot}1 + 40{\cdot}2 \log x$, which may be used instead of the actual curve for evaluating doses of vitamin A responsible for mean increases in further experiments. The best accuracy would be obtained by giving such a dose as would bring one on to the part of Fig. 8 where the fraction $\frac{\text{standard deviation}}{\text{mean increase} \times \text{slope of curve}}$ is a minimum, which is perhaps at about 20–30 g. mean increase.

(*c*) The curve relating the growth as calculated from the formula

$$\frac{\text{Log}_e M_2 - \text{Log}_e M_1}{K_2 - K_1} . 100$$

to the dose of cod-liver oil is slightly smoother than the curve of mean increase. This formula (in which M_1 is the weight of the group of animals at the beginning of the experiment, M_2 the weight at the end and $(K_2 - K_1)$ the duration of the experiment in days) takes into account the fact that animals grow more slowly as they approach maturity. The curve is shown in Fig. 9.

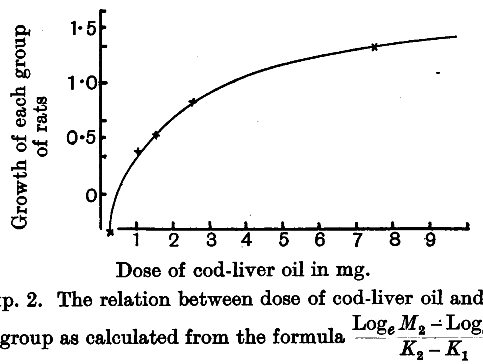

Fig. 9. Exp. 2. The relation between dose of cod-liver oil and the growth of each group as calculated from the formula $\frac{\text{Log}_e M_2 - \text{Log}_e M_1}{K_2 - K_1} . 100$.

[1] The weight at which the rats have ceased to grow on the vitamin A-free diet and have been given the dose of cod-liver oil.

The sequence of testing the different doses is of interest. The fact that the response was graded to the dose, in spite of the doses not having been tested in order of magnitude, indicates that the response was independent of the season at which the test was made. In other words, there is no seasonal variation in the response of animals to the giving of vitamin A.

THE DEGREE OF ACCURACY OBTAINABLE BY THE USE OF SMALLER GROUPS OF ANIMALS.

The smoothness of the curve of reference given in Fig. 8 is a general indication of the degree of accuracy attainable by the use of 30 animals on a dose. We may, therefore, accept it as the expression of the true relation between response and dose of vitamin A.

In order to determine the degree of accuracy attainable by the use of a smaller number of animals, we have divided our results on the rats used in the making of the curve into groups of (*a*) 5 animals and (*b*) 10 animals. They were taken strictly in order as they occurred on the record sheets without selection or rearrangement of any kind. The mean increase for each group was calculated and, by reference to the curve, the corresponding dose of cod-liver oil was determined. For example, the mean increases of the different groups of 5 animals each, given 1·0 mg. oil A, were 9, 14, 11·4 and 15·2 g. respectively. By reference to the curve, it was found that the doses of cod-liver oil corresponding to the same mean increases when 30 rats were used in each group were 0·9, 1·2, 1·05 and 1·3 mg. respectively. But the actual dose was always 1·0 mg.; therefore, the percentage errors were 10, 20, 5 and 30 respectively. The figures for all the groups on the various doses are given in Table V.

In the 21 groups of 5 animals each, the errors of one result, and possibly a second, were more than 50 but less than 100 %. The errors of 10 others were 20–50 %, and the errors of the remaining 9 were 0–10 %.

In the 10 groups of 10 animals each, one error was 32 %, three errors were 21–30 %, three were 11–20 % and three were 0–10 %.

Two further results of interest in this connection are quoted in Table VI. Two oils, B and C, were tested by this method, each in two different doses. The figures for oil C are particularly striking. 19 rats given a dose of 1 mg. oil C increased in weight in 5 weeks by an average amount of 22·5 g. But this mean increase would have been given by 1·95 mg. of the oil A (see curve, Fig. 8). Hence oil C is 1·95 times as potent as oil A. A second test of oil C, on a dose five times as great, gave in 17 rats a mean increase of 56 g. in 5 weeks. This increase would have been given by 10·2 mg. of oil A. Hence oil C is $\frac{10{\cdot}2}{5{\cdot}0} = 2{\cdot}04$ times as potent as oil A. Here are two estimations of oil C in terms of oil A, which differ by only $\pm$ 2·5 %, a degree of accuracy as great as that of any other biological estimations.

Thus our actual results showed a degree of accuracy (*a*) generally within 50 % (19 times out of 21) of the true value by the use of 5 rats on a test,

Table V. *To show the variation in result obtained by using smaller groups of animals.*

Each group of 30 animals used in the making of the curve has been divided into groups of (*a*) 5 and (*b*) 10 animals. By referring the mean increase of each small group to the curve, the dose apparently given to produce this increase is found. It is called the "found" dose. Its difference from the actual dose is stated as % error.

	5 animals in each group				10 animals in each group			
Actual dose mg.	Group	Mean increase g.	Found dose mg.	% error	Group	Mean increase g.	Found dose mg.	% error
1·0	1	9·0	0·9	-10	1	11·5	1·05	+5
	2	14·0	1·2	+20	2	13·3	1·2	+20
	3	11·4	1·05	+5				
	4	15·2	1·3	+30				
	5 (4 only)	16·25	1·4	+40				
1·5	1	19·8	1·65	+10	1	19·1	1·65	+10
	2	18·4	1·55	+3·3	2	20·3	1·75	+16·7
	3	29·2	2·8	+87				
	4	11·4	1·05	-30	3 (7 only)	9·6	0·95	-37
	5	12·2	1·1	-27				
	6 (2 only)	3·0	0·6	-40				
2·5	1	27·6	2·5	0	1	29·4	2·9	+16
	2	31·2	3·1	+24	2	32·6	3·3	+32
	3	31·2	3·1	+24	3	21·2	1·85	-26
	4	34·0	3·55	+42				
	5	26·2	2·4	-4				
	6	16·2	1·4	-44				
7·5	1	42·6	5·6	-25·3	1	42·1	5·5	-26·7
	2	41·6	5·35	-28·7	2	47·9	7·2	-4·0
	3	49·4	7·75	+3·3	3	54·6	9·7	+29·3
	4	46·4	6·8	-9·3				
	5	49·2	7·7	+2·7				
	6	60·0	12·0?	+60·0?				

Table VI. *To show the close agreement of results obtained by testing two different doses of an oil and referring the mean increases of the groups to the curve of the original oil.*

Oil	No. of rats	Actual dose mg.	Mean increase of wt of group in 5 weeks g.	Dose of oil A which produced the same mean increase mg.	Potency of oil in terms of oil A %
B	19	1	7·7	0·78	78
	9	4	29·5	2·83	71
C	19	1	22·5	1·95	195
	17	5	56·9	10·2	204

(*b*) always (10 times out of 10) within 33 % by the use of 10 rats, and (*c*) within 2·5 % (2 out of 2 times) by the use of about 20 rats. The data are too few for us to make a suggestion of the probable error of any determination with any given number of animals. We have calculated the standard deviation of the individuals in each group of 30 animals according to the formula $\sigma = \sqrt{\frac{\Sigma d^2}{n}}$, where σ is the standard deviation of the individuals, d is the difference from the mean of the group, and n is the number of animals in the group.

These calculations are included in Tables II and IV. In order to determine the standard deviation of the mean of the whole group we applied the formula

$$\epsilon_m = \sqrt{\frac{\Sigma d^2}{n(n-1)}}.$$

This is a formula which has been used in many biological estimations, the probability being (*a*) 2/3 that a similar estimation on a similar group of animals would give a result within $\pm \epsilon_m$ of the truth, and (*b*) 21/22 that the result would be given within $\pm 2\epsilon_m$ of the truth. When we applied this formula to our small groups of 10 animals, we found that we should have to expect a result 1 out of 22 times either 50 % below or 100 % above the real dose. Actually we have found a much greater degree of accuracy than this. All the 10 results on the 10 groups of rats fell within 33 % of the true dose, but, after all, with only 10 groups no great stress can be placed on this result.

Irwin, Brandt and Nelson [1930, 1] have lately reported an analysis of results obtained in their laboratory which it is of interest to note here. They have accepted the contention that two mean increases differ significantly only if the difference between them is three times the standard deviation of the difference, and from this they have determined that, in their colony, the difference between two means must be at least 20·45 g. to be significant, and that in order to get this difference each group must consist of 20 animals. They have not, however, examined their figures with a view to discovering whether a smaller difference than three times the standard deviation of the difference between the means is significant, and it seems probable, from their statement concerning the source of their figures, that they have not had a sufficient number of animals on one dose of one particular substance to make such an analysis possible.

Discussion.

Three points which have emerged from the work here described require some discussion. In the first place we have found that it is impossible to make any estimation of vitamin A when Glaxo "vitamin-free casein" is used. It is unsafe, of course, to say that this finding must hold good for other laboratories, for differences in the basal diet or in the diet of the breeding colony may make good the deficiency that we have detected in ours. We suspected the existence of this deficiency after 6 months' use of Glaxo "casein" in these laboratories and found within a few months that it could be made good by the substitution of "light white casein," but it has taken nearly 3 years to show that the inclusion of "light white casein" in our basal diet makes possible a reasonably accurate estimation of vitamin A. It is quite possible to get large numbers of results on single rats like those shown in Fig. 3 B which might lead anyone to conclude that the basal diet used was the best one available, but we believe that the only way to make certain of this is to demonstrate it by some means such as we have used and depicted in Figs. 5, 6, 8 and 9.

The second point which requires comment is the demonstration that a relation exists between the size of the dose administered to a group and the percentage of animals dying in the period from the second to the fifth week of the administration of cod-liver oil. We believe the results shown in Figs. 6 and 8 indicate that there is the closest relation between the growth-promoting power of vitamin A and the anti-infective power as described by Green and Mellanby [1929], and that a measure of the one is a measure of the other. The essence of the view put forward by Green and Mellanby was that since rats, dying on a shortage of vitamin A, die suffering from infections in different parts of the body, it is to be concluded that the absence of vitamin A means the absence of an anti-infective vitamin on which the resistance of the body depends. We have found that when rats have ceased to grow on a vitamin A-free diet, the addition of small daily doses of cod-liver oil to the diet does not prevent some rats from dying during the next 5 weeks, while it enables others to resume some growth. The rats which die, when examined *post mortem*, show the same abscesses as those described by Green and Mellanby. As the doses of cod-liver oil given to different groups of rats increase, the proportion of rats which die diminishes, the proportion which resume growth increases, and also the mean increases of the groups are greater. Thus the first effect of the addition of the smallest doses of cod-liver oil to groups of rats may be described as the anti-infective effect, by which the proportion which die in 5 weeks is steadily reduced. We see that as the dose increases, this effect passes, by insensible gradations, into the growth-promoting action, and we conclude that, for purposes of measurement at any rate, the anti-infective property and the growth-promoting property are merely different aspects of the same thing.

The third point of discussion arises from the finding by Irwin, Brandt and Nelson [1930, 2] that, in their experiments, of the three factors, total food eaten, initial weight and experimental period, the total food eaten has by far the greatest influence on the gain in weight of the animals. They suggest, therefore, that some technique for the regulation of the food intake of the animals would lead to less variability in response. We have not made records of the daily intake of basal diet in these experiments, but it would appear to be unnecessary for our purpose to do so, as we have obtained comparable results on small numbers of animals without considering this factor.

SUMMARY.

A method is described by which the vitamin A content of two substances may be compared, using two groups of rats, there being five or preferably ten rats in each group.

Each group of rats is fed on vitamin A-free diet until growth ceases and each rat within the group is then given the same daily dose of the substance to be tested. The mean increases in weight of the two groups are calculated.

These mean increases are then referred to a curve which relates mean increase to dose of a particular sample of cod-liver oil. The relative potency of the two substances is calculated from the doses of cod-liver oil corresponding to the mean increases of the groups used in testing these substances.

When 10 rats are used in a group, the potency of a substance can usually be estimated with an error of less than 30 %.

The curve relating dose to mean increase of weight in a group cannot be assumed to be applicable to all vitamin A-free diets. It was found, indeed, that the use of one type of caseinogen made it impossible to construct such a curve at all.

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