CLXI. A DIET WITHOUT CASEINOGEN FOR USE IN THE DETERMINATION OF VITAMIN A.

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IN the direct determination of vitamin A by a feeding test it is necessary to use a diet which is very deficient in vitamin A but which needs no other supplement to allow animals receiving it to grow at a rate of the same order as that of animals on a satisfactory diet of mixed foods such as is used for maintaining a breeding colony. The source of protein in the diets recommended for use in vitamin A determinations has always been caseinogen, a protein derived from milk, and therefore likely to be contaminated with vitamin A. Many workers consider it necessary to submit the caseinogen to a fairly prolonged heat treatment to destroy the vitamin A, but Coward *et al.* [1929; 1930; 1931] found that a diet containing heat-treated caseinogen as the source of protein was deficient in some factor other than vitamin A, so that after growth on the diet had ceased, supplements of cod-liver oil at any level failed to produce growth approaching the normal rate. They found it impossible to make any estimations of vitamin A from experiments in which a diet containing such caseinogen was used, because of the absence of a consistent gradation between the dosage of vitamin A and the growth response. A diet containing an untreated caseinogen ("light white casein"), however, was not deficient in the unknown factor; much better growth was obtained during the test period than could be obtained in experiments with the diet containing heated caseinogen, and the growth response was graded to the dosage of vitamin A so that satisfactory determinations of vitamin A could be made.

"Light white casein" contains an appreciable amount of butter fat (Culhane [1933] quotes determinations of 0.6 to 4.5 % with an average of 2.5 %), and it might be expected that this would supply sufficient vitamin A to interfere seriously with the development of symptoms of vitamin A deficiency; but in Coward's experience no such difficulty was met with, and it was found that "light white casein" defatted with alcohol and ether showed no advantage over the untreated caseinogen. Culhane [1933], however, concluded from a comparison of the effects of diets containing "light white casein," or heated caseinogen, on the development of ophthalmia that "light white casein" contains a considerable amount of vitamin A.

Bacharach [1933] found that the time required for the arrest of growth on a diet deficient in vitamin A, the weight reached before the growth arrest and the degree of recovery produced by doses of vitamin A were the same whether heated caseinogen or "light white casein" were used in the diet, but neither diet supplemented with vitamin A gave normal growth.

In our experience with a vitamin A-deficient diet similar in composition to that described by Coward, we were unable to obtain the early growth arrest

such as is obtained in her laboratory. Examinations of extracts from the livers of newly weaned rats failed to reveal the presence of sufficient quantities of vitamin A to account for the prolonged survival of rats on the deficient diet, and it seemed likely that our rats were obtaining significant quantities of vitamin A from the caseinogen. Unwilling to resort to the use of heated caseinogen because of the experience reported by Coward, and because A. F. Morgan [1931] has shown that strong heating damages proteins and makes them nutritionally unsatisfactory, we devised a diet containing no caseinogen, by using defatted meat meal and coconut meal to supply protein. The preliminary experiments were so encouraging that after some time the use of the caseinogen-containing diet was discontinued, and the new diet is now used in all our routine assays of vitamin A. Rats receiving the new diet show permanent growth arrest in about four weeks; they respond well to curative treatment with small doses of vitamin A, and the growth response is steeply graded to the dosage.

The present paper describes the technique for the determination of vitamin A as used in this laboratory, with a statistical treatment of the results so far available. The behaviour of rats on the new diet and the accuracy obtainable are compared with the results obtained with rats of the same colony fed on the diet containing caseinogen.

DIETS.

The rats used are albino rats of Wistar Institute strain. The breeding stock are fed on a cooked diet as previously described [Morgan, R. S., 1932]. The diet containing caseinogen was similar to that described by Coward *et al.* [1930]:

"Light white casein" (B.D.H.)	...	...	15 %
Starch	...	...	73 %
Dried yeast	...	...	8 %
Salt mixture (Steenbock's No. 40)	...	...	4 %
Vitamin D	...	...	8 units per week

Originally the starch was supplied as raw rice starch and the diet was cooked with water to a stiff jelly before feeding. Later the raw starch was replaced by ground flaked rice (cooked over rollers) and the diet was fed dry. This change did not affect the performance of the rats.

The diet without caseinogen is as follows:

Ground flaked rice	...	...	48 %
Coconut meal	...	...	30 %
Defatted meat meal	...	...	10 %
Dried yeast	...	...	8 %
Salt mixture (Steenbock's 40)	...	...	4 %
Vitamin D	...	...	8 units per week

A large stock of dried meat meal was specially prepared. It was dried with a minimum exposure to heat in order to avoid damage to the protein. Quantities were defatted from time to time as required in a small solvent-extraction plant.

CONDUCT OF EXPERIMENTS.

Litters are not reduced in size and they are weaned on to the deficient diet at 23 days of age unless, as occasionally happens, the average weight is then less than 25 g., when they are left with the mother for a further week. All the rats of any one litter intended for test are started on experiment on the same day. Pairs of rats are selected of the same sex and as nearly as possible of the same

weight; one of each pair receives a daily dose of the test substance and the other receives a comparable dose of the international carotene standard. Arachis oil is the solvent used for dissolving the carotene standard and for diluting oils to be assayed. It is obtained direct from our own refinery; on receipt, quinol is added, and the oil is allowed to stand at room temperature for a day with

Fig. 1. Typical growth curves of litters from vitamin-A tests showing the difference between the behaviour of rats on the diet with casein and that of rats on the diet with meat meal and coconut meal as sources of protein.

=====	carotene standard	=====	carotene standard
-----	red palm oil 1	-----	butter 1
.....	red palm oil 2		butter 2

occasional shaking. Under these conditions the oil absorbs about 0.05 % quinol. It is then stored in a refrigerator. A strong solution of the carotene standard is prepared by dissolving a weighed quantity in a little benzene, adding arachis oil and evaporating the benzene under reduced pressure. Sub-dilutions of this solution for the purpose of dosing are prepared from time to time. The stability of carotene dissolved in this way is satisfactory. Judging by Lovibond

matches on dilutions with chloroform, we found no significant loss of carotene from a solution after storage in a refrigerator for six months.

In the experiments from which the data presented in this paper have been taken, a test period of five weeks has been used, but the animals have been continued on test for a further week and the growth has been expressed as the average of the growths made in 4, 5 and 6 weeks.

In the experiments with the diet containing caseinogen, animals would sometimes still be growing slowly after they had been receiving the deficient diet for 13 weeks, though others might have died much earlier. It was impossible to wait for a permanent arrest of growth before beginning a test, because this was sometimes not obtained, and we adopted the practice of starting the rats of a litter on test after about 5–7 weeks on the deficient diet and keeping some animals as negative controls. Usually these negative controls would survive the test period and make some growth, but it was nearly always less than the growth given by the lowest dose of standard or test substance.

In experiments with the diet without caseinogen, the rats cease to grow in about four weeks from weaning. They are started on test as soon as the growth rate has begun to slacken; this happens for all the rats of a litter at about the same time, and incipient xerophthalmia is usually apparent throughout the litter. It is not advisable to wait for a decline in weight, for many of the rats may then be lost; they may fail to respond to quite large doses of vitamin A and die within a few days of the beginning of the test; or even if they survive, their growth rate may be determined rather by the seriousness of their condition at the beginning of the test than by the dosage of vitamin A, so that even a positive control receiving about 20 units per day may grow at a very subnormal rate.

The difference between the behaviours of rats on the diets with and without caseinogen is shown in Fig. 1 which gives the growth curves of two typical litters.

RELATION BETWEEN GROWTH-RESPONSE AND DOSAGE; INTERPRETATION OF ASSAYS.

On both the vitamin A-deficient diets that have been used in this laboratory there is a definite increase in the growth-response with increase in the dosage of vitamin A, but this increase is much more striking in the experiments with the diet without caseinogen than in the experiments with the diet with caseinogen.

Diet with caseinogen. For the interpretation of experiments when the diet with caseinogen was used sufficient data were not available to construct a curve showing the average relation between growth-response and dosage of the standard preparation. Coward *et al.* [1930; 1931] showed that for their colony, using practically the same vitamin A-deficient diet, the relation was given by the equation:

$$\text{growth-response} = c + k \log \text{dose},$$

and a similar relation has been assumed in the interpretation of our experiments.

Since it is our custom to feed the test substance and the standard at at least three levels of dosage, it was possible to calculate our own values of the constant k in the above equation from the average difference between the growth-responses of two animals of the same litter receiving doses of the same substances in the ratio of 2 to 1. These average values were as follows:

Difference in growth-response (average at 4, 5 and 6 weeks) corresponding to a dose ratio of 2 to 1.

Bucks (37 values)	12.05 g.	$k = 12.05 / \log 2 = 40.0$
Does (54 values)	8.4 g.	$k = 8.4 / \log 2 = 27.9$

If the growth-response is measured as the actual growth in 5 weeks instead of as the average in 4, 5 and 6 weeks, the values of k become: bucks 37.6, does 26.1. These values would be expected to be somewhat lower than those obtained by Coward *et al.*, because in nearly all our experiments the negative controls made some growth, and if this is attributed to vitamin A in the diet, it follows that when the doses given to two rats are in the ratio of 2 to 1 the total vitamin A received will be in a lower ratio, *viz.*: $(2+x)/(1+x)$, where x is the amount of vitamin A supplied by the diet.

The values of k given by Coward *et al.* [1931] were originally: bucks 50.3, does 27.4, but with the accumulation of further data, they were modified [Coward, 1933] to: bucks 45.81, does 27.0.

Diet without caseinogen. The smallest dose of the standard carotene that we use is 0.5 unit. This is often insufficient to allow the animals to survive the full test period, but some growth-response is usually given at the beginning of the test. In such a case the growth-response is recorded up to the maximum weight obtained. This practice is justified by the observation that when a substance has been fed at two levels both failing to allow full survival, the maximum growth-response is still graded to the dosage.

The inter-litter variation in growth-response is very great; the response to a dose of 2 units in one litter may exceed that of the positive controls in another litter. The international carotene standard has not yet been fed to a sufficient number of rats at a sufficient variety of levels to allow a satisfactory average curve relating growth-response and dosage to be drawn. The average values so far obtained are given in Table I.

Table I. *Average values of the growth-response produced by doses of the international carotene standard.*

Dose	Bucks		Does	
	Growth-response in g. averaged at 4, 5 and 6 weeks	No. of animals	Growth-response in g. averaged at 4, 5 and 6 weeks	No. of animals
0.5 unit	23.4	12	24.8	26
0.75 unit	45.7	13	31.4	22
1 unit	55.3	64	42.3	43
2 units	76.7	53	59.6	7
Positive controls (about 20 units)	94.5	22	60.3	12

Soon after the new diet was first introduced, however, it became evident that the difference between the growth-responses produced by 0.5 unit and 1 unit was greater than the difference between the growth-responses produced by 1 unit and 2 units. The average values of the difference in growth-responses between animals of the same litter receiving doses of the standard, or of substances undergoing assay, in the ratio of 2 to 1 have been calculated separately for the higher levels and the lower levels of dosage. They are as follows:

	Bucks	No. of values	Does	No. of values
Higher levels	22.8	45	11.0	9
Lower levels	35.9	19	16.7	27

It is evident therefore that the growth-response does not increase so rapidly with the dosage as is required by the equation

$$\text{growth} = c + k \log \text{dose}$$

and that the relation will be better expressed by some such equation as

$$(\text{growth})^n = c + k \log \text{dose},$$

where n is greater than unity.

For the purpose of interpreting assays, n has been taken as 2, *i.e.* the square of the growth is taken as proportional to the logarithm of the dose. The average values of the difference in the squares of the growth-response between animals of the same litter receiving doses in the ratio of 2 to 1 are:

	Bucks	Does
Higher levels	3100	1050
Lower levels	3590	1248

and thus the values for the two levels agree with each other much more closely than when the differences in the growth-responses themselves are considered.

Considering all values together, including the values that can be derived from the difference in the squares of the growth-response between animals receiving doses in the ratio $\frac{1}{2}$ to $\frac{3}{4}$, or $\frac{3}{4}$ to 1, the average values of the increment in the square of the growth produced by doubling the dose are:

Bucks (70 values)	3235
Does (46 values)	1310

By means of these values, the average growth-responses produced by various doses of the standard (Table I) can be averaged at 1 unit as 54.7 for the bucks and 42.4 for the does. The full equations relating growth to dosage in units of standard are then calculated as:

$$\text{Bucks } (\text{growth})^2 = 2992 + 10,730 \log \text{dose}$$

$$\text{Does } (\text{growth})^2 = 1798 + 4,350 \log \text{dose}.$$

The curves relating growth to dosage given by these equations are plotted in Fig. 2, which shows also the actual points taken from Table I. These curves are

Fig. 2. Relation between growth response and dosage of vitamin A in vitamin A tests. The points are the average values given in Table I.

not designed to fit the points; their slopes have been determined from the average values of the increment in the square of the growth produced by doubling the dose, and the average values of the growths produced by various doses of the standard preparation have been introduced merely to give the positions of the curves. Two features of the curves require some comment. (i) The curve for bucks gives an imaginary value for the growth of rats receiving 0.5 unit of

standard, while the average growth given in Table I is 23·4 g. Even if adequate data were available, however, it would be difficult to calculate a satisfactory average value of the growth response produced by 0·5 unit, since when a rat receiving 0·5 unit loses weight and dies, it is impossible to tell whether the dose was insufficient for the rat, or whether it was in such a weakened condition that it would have died even if the dose had been 2 or 4 times as large. (ii) Both curves show the growth as increasing indefinitely with increasing dosage, while it is evident that an upper limit is set to the growth by the maximum growth capacity of the rat; but if the curves were actually limited by the average growth of positive controls, which is approximately equal to normal growth, many individual values obtained would lie above the curve at all levels of dosage. In practice the relation between growth and dosage given by the curve holds satisfactorily from slight growth up to the growth produced by doses of about 4 units; it is not claimed to be valid for doses above about 4 units.

The method of interpreting an assay is made clear by an example (Table II).

Table II. *Assay of a sample of cod-liver oil, blue value = 10·5, blue value via unsaponifiable matter = 24·2.*

Litter	Dose units	Growth in g.		Dose mg.	Growth in g.	
		Bucks	Does		Bucks	Does
822	0·5	14·7	—	1·11	46	—
824	1	—	43	2·22	—	37
	2	88·3	—	4·44	97	—
826	1	57·7	—	2·22	73·3	—
828	0·75	—	21	1·67	—	40
830	1	—	47·3	2·22	—	59
	2	81·7	—	4·44	89·3	—
832	0·5	—	19·7	1·11	—	35·3
	1	67·3	—	2·22	88	—
	2	88·7	—	4·44	93·3	—
834	1	—	30	2·22	—	57
839	1	34·3	—	2·22	79·7	—
	2	56·3	—	4·44	84	—
841	0·75	—	43·3	1·67	—	59·7
	1	—	57·7	2·22	—	67
843	0·75	—	42·7	1·67	—	57·7
	1	62·3	—	2·22	75	—
	2	82	—	4·44	100·3	—

The pair differences of the squares of growth, *i.e.* the differences between the squares of figures on the same horizontal line in the above table are:

Cod-liver oil <i>minus</i> standard.	
Bucks	Does
1900	— 480
1612	1159
2044	1244
1299	670
3215	2349
1881	1689
5176	1160
3886	1506
1656	—
3336	—
Averages	2601 1162

These mean pair differences correspond to dose ratios calculated from the equations relating growth response to dosage thus:

$$\begin{aligned}\text{Bucks: Dose ratio (2.22 mg. oil)/(1 unit standard)} \\ &= \text{antilogarithm of } 2601/10,730 \\ &= \text{antilogarithm of } 0.242 \\ &= 1.746;\end{aligned}$$

i.e. 2.22 mg. of oil = 1.746 units of standard, and the assay according to the bucks is 785 units per g.:

$$\begin{aligned}\text{Does: Dose ratio (2.22 mg. oil)/(1 unit standard)} \\ &= \text{antilogarithm of } 1162/4350 \\ &= \text{antilogarithm of } 0.267 \\ &= 1.849;\end{aligned}$$

i.e. the assay according to the does is 830 units per g. The average assay is calculated from the weighted average of the logarithms of the dose ratios (0.242 for the 10 pairs of bucks and 0.267 for 8 pairs of does), but, for reasons given below, 1 pair of bucks is considered equivalent to 2 pairs of does. The weighted average of the logarithms of the dose ratios is thus:

$$\begin{aligned}(0.242 \times 20 + 0.267 \times 8)/28 &= 0.249 \\ \text{Dose ratio} &= 1.774 \\ \text{Assay} &= 800 \text{ units per g.}\end{aligned}$$

CALCULATION OF ERRORS.

Diet with caseinogen. Coward [1932; 1933] calculated the average accuracy of a determination of vitamin A from the variation within a group of rats each of which received the same dose. The standard deviation from the mean growth in a five-week test was given in the later paper as 15.37 g. for bucks and 11.93 g. for does. In a test in which the growth-response produced by a substance of unknown potency is compared with that produced by the standard, the standard deviation of the difference between two observations is required. This value would be expected to be $15.37\sqrt{2} = 21.7$ g. for bucks and $11.93\sqrt{2} = 16.9$ g. for does. Three times the probable error of the difference between two means in an experiment in which 10 animals received the standard and 10 received the test substance can then be calculated as:

$$\begin{aligned}2 \times 21.7/\sqrt{10} &= 13.7 \text{ g. for bucks,} \\ 2 \times 16.9/\sqrt{10} &= 10.7 \text{ g. for does.}\end{aligned}$$

From Coward's equations relating growth to dosage, these errors correspond to dose ratios of:

$$\begin{aligned}\text{antilog } 13.7/45.81 &= 1.99 \text{ for bucks,} \\ \text{antilog } 10.7/27.0 &= 2.49 \text{ for does,}\end{aligned}$$

i.e. the percentage errors corresponding to three times the probable error in a determination of vitamin A with two groups of 10 animals each are

$$\begin{aligned}&+ 99 \text{ or } - 50 \% \text{ if all the animals are bucks, or} \\ &+ 149 \text{ or } - 60 \% \text{ if all the animals are does.}\end{aligned}$$

In our own experiments using practically the same diet as that used by Coward *et al.*, in which we obtained the same order of increase in growth with

increase of dosage, the average values of the difference between the growth of two animals of the same litter receiving doses in the ratio of 2 to 1 were

for the bucks 12.05 g. (37 values), standard deviation = 13.02 g.,
for the does 8.4 g. (54 values), standard deviation = 10.87 g.

These standard deviations should be about the same as the standard deviation of the difference between two observations. Three times the probable error of the mean pair difference of ten pairs is calculated as

$$\begin{aligned} 2 \times 13.02 / \sqrt{10} &= 8.25 \text{ g. for bucks,} \\ 2 \times 10.87 / \sqrt{10} &= 6.89 \text{ g. for does.} \end{aligned}$$

Taking values of k in the equations relating growth to dosage as 40.0 for bucks and 27.9 for does, these errors correspond to dose ratios of

$$\begin{aligned} \text{antilog } 8.25/40.0 &= 1.61 \text{ for bucks,} \\ \text{antilog } 6.89/27.9 &= 1.77 \text{ for does,} \end{aligned}$$

i.e. the percentage errors corresponding to three times the probable error in a determination of vitamin A with 10 pairs of animals are

$$\begin{aligned} &+61 \text{ or } -38 \% \text{ if all the animals are bucks, or} \\ &+77 \text{ or } -44 \% \text{ if all the animals are does.} \end{aligned}$$

These values are very much smaller than those calculated from Coward's values of the standard deviation of the mean growth of a group of animals receiving the same dose of vitamin A. The difference between the two calculations represents mainly the gain in accuracy that is to be expected by confining consideration to litter-mate pairs started on test on the same day. Admittedly the second calculation is based on fewer data than the first, but in view of the elimination of inter-litter variation it would be expected to give much lower values.

Diet without caseinogen. Since, in the experiments with the new diet containing meat meal and coconut meal as sources of protein, it has been found that the growth is graded much more steeply to the dosage than in the experiments with the diet containing caseinogen, it would be expected that a further reduction of error would be obtained. The average differences between the squares of the growth of animals of the same litter receiving doses in the ratio of 2/1 were calculated as

$$\begin{aligned} &\text{for bucks 3235 (70 values), standard deviation} = 2100, \\ &\text{for does 1310 (46 values), standard deviation} = 1005. \end{aligned}$$

From the equations relating the square of the growth to the logarithm of the dosage, given above, the values of the dose ratio corresponding to three times the probable error of the mean pair difference in an experiment with 10 pairs of animals are calculated as

$$\begin{aligned} &\text{for bucks, antilog } 2 \times 2100/10,730 \sqrt{10} = 1.33, \\ &\text{for does, antilog } 2 \times 1005/4350 \sqrt{10} = 1.40, \end{aligned}$$

i.e. the percentage errors corresponding to three times the probable error are

$$\begin{aligned} &+33 \text{ or } -25 \% \text{ for 10 pairs of bucks, or} \\ &+40 \text{ or } -29 \% \text{ for 10 pairs of does.} \end{aligned}$$

A second calculation of the errors to be expected in experiments with the new diet has also been made from the mean variance of the pair difference determined in each test.

For the bucks, the sum of the squares of the deviations from the mean pair difference in each test was 374,320,000; the number of pairs was 193 and the number of tests 38. The standard deviation is given as

$$\sqrt{\{374320000/(193-38)\}}=1554,$$

from which the percentage error corresponding to 3 times the probable error is calculated as

$$+24 \text{ or } -19 \% \text{ for 10 pairs of bucks.}$$

For the does, the sum of the squares of the deviations from the mean pair difference in each test was 103,120,000; the number of pairs was 149 and the number of tests 35. The standard deviation is therefore

$$\sqrt{\{103120000/(149-35)\}}=950,$$

and the percentage error corresponding to three times the probable error is

$$+37 \text{ or } -27 \% \text{ for 10 pairs of does.}$$

These errors are smaller than the errors calculated from the variation in the difference between the squares of the growth of litter-mates receiving doses in the ratio of 2/1. Two factors operate in this reduction of the calculated error: (i) in the last calculation the variation is in the difference between litter-mates selected to be as nearly as possible of the same weight at the beginning of the experimental period, while in the first the variation is between litter-mates not so selected, and (ii) in the second calculation the curve relating growth to dosage is assumed to be absolutely correct, *i.e.* no account is taken of error introduced in the interpretation of an assay by reference to a curve which may not be strictly applicable to the group of rats under consideration. Even if the curve were constructed from data obtained from several thousands of rats so that it represented very accurately the mean behaviour, some error from this source might still enter an assay; for just as some litters grow more rapidly than others, so the growth of some may be more steeply graded to dosage than that of others. The magnitude of the error derived from this source will depend entirely on the magnitude of the mean difference between the growths produced by the test substance and the standard, from which the assay will be calculated. If this difference is zero, *i.e.* if the comparative doses of test substance and standard produce equal average growth, the error is eliminated, because the standard curve of relation between growth and dosage is not required. The relation between growth and dosage given in this paper is, for bucks:

$$(\text{growth})^2 = c + 10,730 \log \text{dose};$$

if for a certain assay group of rats the true relation should be

$$(\text{growth})^2 = c + 9000 \log \text{dose},$$

the error introduced by interpreting the assay by reference to the standard relation would be 0 for a dose ratio test-substance/standard=1, but if the true dose ratio were 2/1, an error of -11 or +12 % would be introduced in the interpretation of the assay, and if the true dose ratio were 4/1 the error would be -20 or +25 %.

The second calculation of errors therefore gives the expected error in a test in which the comparative doses of the test substance and the standard produce nearly equal growth. The first calculation gives the expected error in an assay in which the doses of test substance supply about twice as much vitamin A as the comparative doses of standard. The above calculations of the errors expected in

a determination of vitamin A are summarised in Table III. The number of pairs of bucks required to give an assay of the same accuracy as that obtained with 10 pairs of does has been calculated from the limits of error given by each method, and the figures are given in the last column.

Table III. *Summary of the calculations of the errors expected in vitamin A determinations.*

Diet	Basis of calculation	Upper limit of error (%) corresponding to 3 times the probable error in an assay in which 10 rats re- ceive the test substance and 10 receive the standard		No. of pairs of bucks required to give an assay of the same accuracy as that given by 10 pairs of does
		Bucks	Does	
Diet with caseinogen	The variation within groups of rats receiving the same dose of vitamin A [Coward, 1933]	99	149	5.7
„	The variation in the growth difference between litter- mates receiving doses in the ratio 2/1	61	77	6.9
New diet without caseinogen	The variation in the differ- ence between the squares of growth of litter-mates receiving doses in the ratio 2/1	33	40	7.1
„	The variation in the differ- ence between the squares of growth of litter-mate pairs, one of which receives the test substance and the other the standard	24	37	4.4

Coward [1932] has already pointed out that to get equal degrees of accuracy, about twice as many does as bucks must be used in a test. Our own figures confirm this finding. For this reason, in the interpretation of an assay in which both bucks and does have been used, one pair of bucks is considered equivalent to two pairs of does. For the same expenditure of labour, however, greater accuracy may be obtained by using bucks only in vitamin A tests; litters at weaning may be separated into bucks and does, the bucks being taken for vitamin A tests and the does for some other purpose, such as vitamin D tests, for which they are as suitable as bucks.

Coward [1933] has reported that the error of an assay with a 3-week experimental period is only slightly greater than that of an assay with a 5-week experimental period. The relation between the errors of 3-week and 5-week assays has also been calculated from the records of experiments in which the new diet without caseinogen has been used. The mean difference between the squares of the growth-responses in three weeks of two rats receiving doses in the ratio 2/1, is

for bucks: 1785, standard deviation 1332,
for does: 606, standard deviation 563.

The equations relating growth-response to dosage are

$$\begin{aligned}\text{Bucks: } (\text{growth})^2 &= 1521 + 5930 \log_{10} \text{dose,} \\ \text{Does: } (\text{growth})^2 &= 900 + 2013 \log_{10} \text{dose.}\end{aligned}$$

The percentage error, corresponding to 3 times the probable error, of a test with 10 pairs of animals, is calculated from these figures as +39 for bucks, or +50 for does. By comparison with the values calculated in the same way from the growth-responses averaged at 4, 5 and 6 weeks, it is found that 14 pairs of rats in a 3-week experiment will give an assay of about the same accuracy as that given by 10 pairs in (4, 5, 6)-week experiment. By a similar calculation it is found that if the growth-response in 6 weeks is considered, the number of pairs of rats required to give the same accuracy is 10.6.

DISCUSSION.

The diet described in this paper, with meat meal and coconut meal as sources of protein, is in our experience very satisfactory for use in the determination of vitamin A. It is so deficient in vitamin A that animals receiving it show a permanent growth arrest in about 4 weeks, while animals of the same colony receiving a diet with 15 % of "light white casein" may continue to grow for 10 weeks or more; and when supplemented with vitamin A, it allows growth of the same order as that of rats of the breeding colony receiving the stock diet. This is seen by the performance of positive controls given about 20 units of vitamin A daily (1 drop of oil of 56 blue value).

Bucks.

22 positive controls, average growth-response=94.5 g.

Average weight at beginning of experimental period=76 g.

Average age at beginning of experimental period=49 days.

From the average growth curves of rats of the breeding colony, the growth of bucks averaged at 4, 5 and 6 weeks after they attain a weight of 76 g. (at 41 days) is 91 g.

Does.

12 positive controls, average growth-response=60.3 g.

Average weight at beginning of experimental period=71 g.

Average age at beginning of experimental period=49 days.

From the average growth curves of rats of the breeding colony, the growth of does averaged at 4, 5 and 6 weeks after they attain a weight of 71 g. (at 43 days of age) is 62 g.

Since the diet only needs to be supplemented with vitamin A to give normal growth (at least for the periods considered here), it does not appear to be deficient in any factor essential to growth, other than vitamin A. The only ingredient of the diet originally in use that is omitted from the new diet is "light white casein," and since the new diet produces a much earlier development of symptoms of vitamin A deficiency than did the original diet, it appears likely that the difference is indeed due to the presence of vitamin A in "light white casein" in sufficient amount to hinder the development of vitamin A deficiency in animals of our stock.

The observation that the difference in growth between animals receiving doses in the ratio of 2/1 is greater in the experiments using the diet without caseinogen than it is in the experiments using the diet with caseinogen, also supports the view that the diet without caseinogen is more deficient in vitamin A than the other; for if two rats receive doses in the ratio of 2/1 and they also obtain some vitamin A from the diet, the ratio of the total amounts of vitamin A received will be less than 2/1, and therefore the growth difference will be less than if the diet had supplied no vitamin A. In our experiments using the diet with caseinogen the negative controls usually showed some growth, but the

does survived the full experimental period more often than the bucks; a growth of 25 g. during the experimental period was quite usual, and generally the negative controls resembled rats receiving 0.5 unit daily in the experiments using the diet without caseinogen. If it is assumed that the caseinogen was actually supplying about 0.5 unit of vitamin A daily, the growth-responses that would be produced by doses of the standard may be calculated from our equations relating growth-response to total vitamin A (Fig. 2) as follows:

Table IV. *Relation between growth-response and dosage expected if diet supplies about 0.5 unit of vitamin A daily.*

Dose	Total vitamin A received including 0.5 unit daily from the caseinogen	Growth-response calculated from the curves given in Fig. 2	
		Bucks (g.)	Does (g.)
No dose	$\frac{1}{2}$ unit	0	22.1
$\frac{1}{4}$ unit	$\frac{3}{4}$ "	28.2	30.1
$\frac{1}{2}$ "	$\frac{5}{4}$ "	40.7	35.4
$\frac{3}{4}$ "	1 "	54.7	42.4
1 "	$1\frac{1}{2}$ units	69.9	50.7
2 units	$2\frac{1}{2}$ "	85.3	59.4

From the figures in Table IV it is seen that using a diet supplying about 0.5 unit of vitamin A daily, it would be expected that within the range 0.25 to 2 units the growth-response would be related to the dosage (excluding the vitamin A obtained from the diet) by

$$\text{growth} = c + k \log \text{dose.}$$

The increments in growth-response produced by successively doubling the dose from 0.25 unit to 2 units are approximately constant and are calculated from the above figures as 14.0 g., 15.2 g. and 15.4 g. for bucks, and as 7.0 g., 8.3 g. and 8.7 g. for does. These values are of the same order as those actually obtained in our experiments with the caseinogen diet (12 g. for bucks and 8.4 g. for does).

If 15 % of caseinogen in the diet supplies about 0.5 unit of vitamin A daily to rats eating about 10 g. of food per day, the caseinogen should contain $\frac{1}{3}$ unit per g. Summer butter-fat, according to our assays, contains about 18 to 25 units per g., and the amount of butter-fat required to be present in caseinogen to account for this potency would be 1.4 %. Culhane, as quoted above, gives the average fat content of "light white casein" as 2.5 %, so that a fair margin for loss of vitamin A from the fat during the manufacture and storage of the caseinogen can be allowed for.

Apart from the question of the composition of the diet, it is suggested that the following conditions should be observed for the most accurate assay of vitamin A.

(i) The carotene standard and oils to be tested should be dissolved in an oil in which they are stable over periods of several months. Arachis oil obtained direct from the refinery and left to stand over quinol fulfils this condition.

(ii) The growth of rats receiving the test substance should be compared with that of rats receiving the carotene standard, each rat being compared with another of the same litter and as nearly as possible of the same weight, started on test on the same day. If one rat of such a pair dies at the beginning of the test period, its control is not considered in interpreting the assay.

(iii) The comparative doses of the test substance and the carotene should be selected, if necessary after a preliminary trial, to give nearly equal average growth-responses.

SUMMARY.

A vitamin A-free diet not containing caseinogen has been used in the routine assay of vitamin A. This diet unsupplemented with vitamin A produces a permanent arrest of growth in about four weeks, and growth at the normal rate can then be restored by the addition of vitamin A. Animals of the same colony given a diet with 15 % of "light white casein" as the source of protein may grow for 10 weeks or more, before showing a permanent growth arrest. The difference is attributed to the presence of vitamin A in the "light white casein."

When the above diet with defatted meat meal and coconut meal as sources of protein is used, the growth is graded steeply to the dosage.

From a review of the results so far obtained it is concluded that the growth-response is related to the dosage of vitamin A (in international units) approximately by the equations:

The square of the growth-response = $2992 + 10,730 \log_{10} \text{dose}$ (for bucks),

or

$1798 + 4,350 \log_{10} \text{dose}$ (for does).

The growth-response is taken as the growth in g. averaged at 4, 5 and 6 weeks after the beginning of the test period. In carrying out an assay, the test substance and the carotene standard are fed at three or four levels, and the growth of each rat receiving a dose of test substance is compared with that of a rat receiving the standard, of the same litter, the same age and as nearly as possible the same initial weight, started on test at the same time.

From the results so far available it is calculated that the limits of error corresponding to three times the probable error (*i.e.* the error expected once in 22 tests) in an assay with 10 pairs of rats are:

(i) If the comparative doses of standard and test substance supply nearly equal amounts of vitamin A

–19 to +24 % for bucks,
–27 to +37 % for does.

(These values are calculated from the results of tests with 342 pairs of rats.)

(ii) If the comparative doses of standard and test substance supply amounts of vitamin A in the ratio of about 2/1

–25 to +33 % for bucks,
–29 to +40 % for does.

(These values are calculated from the results of tests with 116 pairs.) These errors are much lower than those obtained in assays with a diet containing caseinogen.

Coward's findings [1932; 1933] that about twice as many does as bucks are required to obtain assays of the same degree of accuracy, and that the error of an assay with an experimental period of 3 weeks is only slightly greater than that of an assay with an experimental period of 5 weeks, are confirmed.

Arachis oil is the solvent used for dissolving the carotene standard and for diluting oils to be assayed; it is obtained direct from the refinery and allowed to stand over quinol. No loss of carotene dissolved in this solvent and stored in a refrigerator has been observed during six months.

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