LXV. A CRITICAL EXAMINATION OF METHODS OF EVALUATING VITAMIN A BY MEANS OF THE GROWTH OF RATS.

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WHEN Steenbock and Nelson [1923] made the important deduction that the absence of vitamin D acts as a limiting factor in growth, it was clear that much of the past work, in which substances had been tested for their value in vitamin A by means of the growth of rats fed on a diet deficient in both the fat-soluble vitamins A and D, could no longer stand. Steenbock showed that the rat's reserve of vitamin A usually long outlasts that of vitamin D and that, for the first 5 or 6 weeks of its life on a diet deficient only in these two vitamins, growth is limited by the lack of vitamin D; if vitamin D is supplied, growth is restored, only to be limited again later when the rat has exhausted its supplies of vitamin A. Tests for vitamin A, which were in use before these facts were realised [cf. Zilva and Miura, 1921], must often have been made when the rat was deficient in vitamin D but still had a reserve of vitamin A, and a growth response which was really due to an addition of vitamin D would be ascribed to the action of vitamin A. Other tests may have been made when the rat had exhausted its reserves of both vitamins A and D; when this was the case the addition of a substance not containing them both would fail to restore growth and the substance would be returned as deficient in vitamin A, although it really might have contained it. It is impossible to interpret the old results, since knowledge does not exist as to the state of depletion of the rats which were then used, and one is forced to regard them all as dubious.

Sherman and Hessler [1927] maintain that their results are not affected in this way, because their experimental animals have always had a reserve of vitamin D which outlasted their reserve of vitamin A; this difference from Steenbock's animals they explain as being due to the fact that their stock animals receive milk while Steenbock's receive alfalfa. But Steenbock's theory was originally based on experiments made by one of the present writers [Hume, 1922] and by Goldblatt and Soames [1922] upon rats taken from a breeding stock which received milk in its diet. Indeed, it is safe to say that, at the Lister Institute, rats bred in the ordinary routine way have not been encountered whose behaviour suggested that their reserve of vitamin D was greater

than that of vitamin A. It is therefore very unsafe to rely upon any rat having such a constitution. Sherman and Hessler admit that, in spite of the peculiar constitution of their rats, it is probably wiser to supply vitamin D, when testing for vitamin A, and it is now generally recognised that this should be done.

Since Steenbock's discovery, he and a number of others have devised and used various ways of supplying vitamin D to the rats used in assaying vitamin A. In 1924 [Steenbock, Nelson and Black, 1924] he published his first paper, dealing with the actual technique of the determination of vitamin A. He administered vitamin D in the form of direct irradiation of the rat for 10 minutes daily. The irradiation was given from the start of the experiment, as soon as the rats were placed on the basal diet, and so were the various doses of alfalfa to be tested. The paper does not, however, go much beyond establishing the theory of the method. For one sample of alfalfa, it is seen that normal growth was established when the alfalfa was added as 4 %, 2 % and 0.98 % of the diet, but no dose is shown which yielded subnormal growth. On the other hand, a sample of weathered alfalfa gave no growth superior to that of controls when added to the diet in the same three percentages.

The writers, wishing to make a careful quantitative estimation of the vitamin A content of two samples of spinach, modified Steenbock's technique somewhat and devised the one used by Chick and Roscoe [1926, 2] for estimating the value of some different samples of milk. The modification consisted in feeding the rats for some weeks on a diet deficient in both vitamins D and A, and then giving vitamin D alone for a short period, until weight began to decline, and at that point adding the substance to be tested for vitamin A. For various reasons it was thought that this modified method would have advantages over Steenbock's original one. In the method as used by Chick and Roscoe, vitamin D was supplied by direct irradiation in most of the experiments, but in some of them it was provided by irradiating the hardened cottonseed oil of the basal diet. The latter modification was, however, not in use at the time when the tests on spinach, about to be described, were made; it was only introduced later as the result of those tests.

In the tests on spinach, three doses, 0.2, 0.4 and 1.0 g., of spinach were given to groups of rats, which had ceased to grow and had begun to show a decline on a diet deficient in fat-soluble vitamins, and which were receiving daily irradiation with a mercury vapour quartz lamp. It was believed that when the spinach was added the rats would show a resumption of growth, proportional to the dose of spinach given, but this did not prove to be the case. Throughout the range of doses of two samples of spinach, no consistent difference in the behaviour of the rats was found. There were considerable individual and sex differences (cf. Fig. 1), but in almost all groups one or more rats died from respiratory trouble, and of the rest most showed normal growth for a time, but then the curve flattened off before the rats had reached the weight at which growth normally slackens. A series of experiments was therefore set on foot

to find an explanation of this behaviour and to seek to ascertain how vitamin A might better be estimated. The resulting experiments extended over a considerable period, and while they have been in progress a number of other workers have published accounts of methods of testing for vitamin A and records of tests made. These are, however, more conveniently discussed after the present experiments have been described.

EXPERIMENTAL.

Young rats were used, bred at the Lister Institute, which were of 40 to 50 g. weight at the beginning of the experiment, and the usual precautions were taken with regard to sex and litter in their distribution amongst the experimental groups. Each rat was kept separately in a glass jar and bedded on sawdust.

The diet used throughout, except when modifications are especially described, was as follows:

Inactivated caseinogen	...	...	300 g.
Starch	...	...	750 g.
Hardened cottonseed oil	...	...	225 g.
Salt mixture	...	...	75 g.
Marmite	...	...	75 g.
Lemon juice	...	...	75 g.
Distilled water	...	...	900 cc.

The salt mixture was as follows:

Sodium chloride	...	...	51.9
Magnesium sulphate	...	...	164.0
Sodium dihydrogen phosphate	...	...	104.1
Dipotassium hydrogen phosphate	...	...	286.2
Calcium phosphate	...	...	162.0
Calcium lactate	...	...	390.0
Ferric citrate	...	...	35.4

Irradiation was carried out by means of a mercury vapour quartz lamp (Ulviarc), which had been in use for about a year. Rats were exposed to it for 10 minutes six times a week at a distance of about 50 cm. Hardened cottonseed oil was melted and exposed for 30 minutes at a distance of 33 cm. in a thin layer.

Series I. Experiments with spinach as source of vitamin A.

The object of the experiment was to test the value for vitamin A of the same samples of specially grown spinach as were tested for vitamin D by Chick and Roscoe [1926, 1]. The spinach (*Spinacea oleracea*) was that described by them (p. 146) as "summer-grown spinach, May—October, 1925," of which there were two kinds "open" and "frame" spinach. The "open" spinach was raised in the open ground; the "frame" spinach was grown in a well-ventilated

frame, glazed with Crookes's "A" glass. This glass has the property of absorbing ultra-violet radiations of wave-length shorter than 3780 Å., while transmitting 88 % of the visible light.

Three doses, 0.2, 0.4 and 1.0 g., of each kind of spinach were given daily, except on Sundays.

The rats received the diet deficient in both vitamins A and D for a period of from 35 to 40 days. Irradiation with the mercury vapour quartz lamp for 10 minutes daily was then applied and the growth rate at once increased, but after a short time again slackened or actually began to decline. The dose of spinach was then given daily with the result shown in Fig. 1. In all the groups,

Fig. 1. Weight curves of rats on a diet deficient in fat-soluble vitamins, vitamin D given as direct irradiation after 5 weeks. Doses of 0.2, 0.4 and 1.0 g. spinach, grown out-of-doors and under a frame, given daily 3 weeks later.

except those receiving 0.4 g. of "open" or 1.0 g. of "frame" spinach, one or two animals died, due to respiratory disease contracted before the spinach was administered, and these deaths must be discounted in evaluating the whole result. The surviving animals in the various groups grew normally for about 40 days after the beginning of the administration of the dose of spinach, but after that the growth curve began to flatten, regardless of the weight to which they had attained or of the dose of spinach which was being administered. No group of rats showed a consistently subnormal rate of growth immediately after the first administration of the spinach, and it might therefore be argued that none of the doses was minimal, but if that were so the question still remains, why the rats did not continue to grow normally until they were adult. As regards the vitamin A value of the two samples of spinach, it is fairly safe

to conclude that there was no great difference between them; had culture in the absence of ultra-violet light greatly reduced the content of vitamin A, this would have been revealed by the non-recovery of rats on the 0.2 g. dose of "frame" spinach. It is not, however, possible to draw a more exact quantitative conclusion. The result, as far as it goes, therefore, is in accordance with that of Coward [1927], who found that the short ultra-violet rays from a mercury vapour quartz lamp, used in conjunction with the visible rays, had no influence on the ultimate amounts of vitamin A contained in the plant tissues investigated.

After the rats had been receiving the spinach doses for some 10 weeks, various additions to the diet were made to see if restoration of growth could be brought about. In some instances these were successful, more particularly when 2.0 g. of wheat germ or 5 drops of cod-liver oil were given daily; additions of iodine, thyroid extract, irradiated cottonseed oil or oxidised cod-liver oil did not appear to confer any benefit.

On post-mortem examination most of the rats showed some degree of respiratory disease, the inception of which probably dated from the preliminary period on deficient diet, but which had also progressed during the period of spinach feeding. Many rats also showed abnormal eyes, suggestive of the early stages of xerophthalmia; even rats receiving a daily dose of 1.0 g. of spinach were so affected, but the effect was undoubtedly in part due to the daily exposure to the mercury vapour lamp.

As the results in testing the spinach were so unsatisfactory further experiments were planned to seek an explanation of them.

Series II. Experiments with butter as source of vitamin A.

In the next series of experiments butter was used as a more convenient source of vitamin A, since it could be stored and the same sample used throughout the experiment. New Zealand butter was chosen; since the cows are at pasture all the year round, it was thought that New Zealand butter was likely to furnish a material of high and tolerably constant vitamin A content. Doses of 0.2 and 0.5 g. daily were given.

It was thought possible that in the last series of experiments the rats had suffered from the constant daily exposure to the mercury vapour lamp. Some rats, therefore, in the series received direct irradiation, while others were given the hardened cottonseed oil of the basal diet, irradiated as already described. At first 5 drops of this were given to each animal individually, but it was soon clear that such an amount was insufficient, and after 10 days the whole of the hardened cottonseed oil (about 10 % of the diet) used in making up the basal diet was irradiated, and vitamin D was thus supplied in the basal diet. The amount supplied in this way was always found to be sufficient.

A series of rats was also included which received no additional vitamin D beyond that contained in a daily dose of 0.2 or 0.5 g. of butter.

It was thought possible that the long preliminary period on deficient diet without vitamins A or D might have a deleterious effect upon the health of the rat and that this injury might ultimately show itself in the failure of the rats to grow normally until they were adult; a comparison was therefore made between the growth of a series of rats kept for a time on the deficient diet and then given vitamin D and finally vitamin A, as in the previous experiment, and that of a series which received the basal diet together with vitamin D and the experimental dose of vitamin A from the very start of the experiment.

There were therefore 12 groups of rats, the weight curves of which are shown in Figs. 2 and 3, and which received treatment as follows:

Group 1.	Irradiated cottonseed oil and 0.5 g. butter, from start of experiment
Group 2.	0.2 g. " " " "
Group 3.	Direct irradiation 0.5 g. " " " "
Group 4.	0.2 g. " " " "
Group 5.	No addition of vitamin D 0.5 g. " " " "
Group 6.	0.2 g. " " " "
Group 7.	Preliminary "depletion", then irradiated cottonseed oil, 0.5 g. butter
Group 8.	0.2 g. " "
Group 9.	Preliminary "depletion", then direct irradiation, 0.5 g. butter
Group 10.	0.2 g. " "
Group 11.	Preliminary depletion, no added vitamin D, 0.5 g. butter
Group 12.	" " " " 0.2 g. "

On reference to Figs. 2 and 3 and on comparing the growth in the groups which received vitamin D as irradiated cottonseed oil (1 and 2, Fig. 2, and 7 and 8, Fig. 3) with that in the groups which received it by means of direct irradiation (3 and 4, Fig. 2, and 9 and 10, Fig. 3), it is seen that there is no difference between the comparable sets which can be attributed to the different mode of administration of vitamin D. As in the case of the rats which received the various doses of spinach in Exp. 1, there was no quantitative difference in the growth of rats receiving daily doses of 0.2 or 0.5 g. butter, after a period of depletion (groups 7, 8, 9, 10, Fig. 3); the growth curve showed the same peculiar character with resumption of growth followed by a premature flattening, whether the rats were irradiated directly or received irradiated cottonseed oil. The flattening of the weight curve was therefore not due to over-irradiation. Leigh-Clare [1927] has also shown that rats suffer no ill-effect from prolonged irradiation if their eyes are shaded, and Goldblatt and Moritz [1926] showed that direct irradiation and irradiated fat are about equally good for supplying vitamin D to rats. The use of the irradiated cottonseed oil is, however, on the whole to be recommended as more convenient, since it does not bring about the abnormal condition of the eyes which direct irradiation does, and an accurate appraisalment of the eye condition, as influenced by vitamin A deficiency, is therefore not interfered with. This method of supplying vitamin D was also preferred by Chick and Roscoe [1926, 2] in a part of their experiment to evaluate the vitamin A in milk for the same reason.

Of the rats receiving vitamin D from the start (groups 1, 2, 3 and 4, Fig. 2), though there was no difference between the weight curves of those receiving vitamin D as direct irradiation and those receiving it in the diet, yet there was

a definite difference between those receiving 0.2 g. of butter and those receiving 0.5 g. in either category. In each instance those receiving the smaller dose showed an earlier slackening of the rate of growth than did those to which the larger dose was given; in fact, when vitamins A and D were given from the beginning of the experiment, there were signs that the growth response of the rats varied quantitatively with the dose of vitamin A given, which it had failed to do when the experiment began with a period of depletion. Even so, the

Fig. 2. Weight curves of rats receiving additions of 0.5 or 0.2 g. butter daily. Groups 1 and 2 received irradiated cottonseed oil as source of vitamin D. Groups 3 and 4 were irradiated directly and groups 5 and 6 received no extra addition of vitamin D.

growth of rats receiving 0.5 g. of butter daily was not fully normal, for the growth rate slackened prematurely, but not so early as did that of rats which received 0.2 g. of butter. It was, however, clear that in any further experiment there was more hope of obtaining a quantitative result when vitamins A and D were administered from the start than when they were administered after a period of depletion.

The explanation why quantitative results could not be obtained after a period of depletion was still not clear. The doses of butter given were evidently not sufficient to maintain normal growth after the rats' own reserves were exhausted, either because of an insufficiency of vitamin A or of some other factor. Yet those same doses of butter were able to restore normal growth for a time in rats already depleted of their reserves. The simplest explanation

would be that some other food factor was lacking and was acting, by its absence or inadequacy, as a limiting factor in growth. In the last series of experiments (Series I) some recovery had been obtained when wheat germ or cod-liver oil was added to the diet, and in the present series (Series II) increased butter and yeast extract seemed to have some beneficial effect, results pointing to an insufficiency of vitamin A, B or E in the diet. If the insufficiency were still one of vitamin A after the additions of butter had been made, the non-

Fig. 3. Weight curves of rats on a diet deficient in fat-soluble vitamins, receiving doses of 0.5 and 0.2 g. butter after a preliminary depletion period of about 9 weeks. Vitamin D given after the first 8 weeks as irradiated hardened cottonseed oil to groups 7 and 8, and as direct irradiation to groups 9 and 10. Groups 11 and 12 received no extra addition of vitamin D.

quantitative growth of the rats after a period of depletion still remained unexplained; this might, however, be due to a pathological condition initiated during the earlier period on deficient diet. Most of the rats at autopsy were found to have some degree of lung disease, and Macy and co-workers [1927, 1] have pointed out that when once a pathological condition of the upper part of the respiratory tract has been established by the use of a diet deficient in vitamin A it seems to be ineradicable, although the diet may be changed and the rat appear to thrive for a time. In the next series of experiments, therefore, the use of a preliminary period of depletion was abandoned.

The four remaining groups (5, 6, Fig. 2, and 11 and 12, Fig. 3) of the present experiment, in which 0.2 g. and 0.5 g. of butter were given from the start of

the experiment and after a depletion period, but without any addition of vitamin D, show how necessary it is that vitamin D should be specially given in all attempts to test for vitamin A. Groups 11 and 12, Fig. 3, reproduce the method of Zilva and Miura [1921]; in all four groups the growth is depressed, compared with that of groups which received corresponding doses of butter together with vitamin D. The insufficiency of vitamin D is therefore limiting the growth and obscuring any reaction which would be given by addition of vitamin A.

Series III. Experiments with butter and cod-liver oil as source of vitamin A and with doubled ration of vitamin B.

In the third series of experiments the various possibilities just sketched were examined and a special attempt was made to secure normal growth up to maturity in both sexes, as it was felt that no basal diet could be regarded as really satisfactory for the estimation of vitamin A unless it gave such growth when vitamin A in sufficient quantity was added as the sole addition.

As has been stated, the use of a depletion period was abandoned and vitamins A and D were supplied throughout from the start of the experiment; irradiated cottonseed oil was again used as the source of vitamin D.

Groups of experiments were devised to test whether the failure of rats in the previous experiments to grow to maturity could have been due to insufficiency of vitamin A, B or E. To test the first possibility, a group receiving a daily dose of 1.0 g. butter was included, as well as groups receiving 0.5 and 0.1 g. To test the possibility of a deficiency of vitamin B, one series received a basal diet in which the amount of marmite had been doubled. To test the third possibility, groups were included which received 5 drops of cod-liver oil, and 0.5 g. butter together with 5 drops of cod-liver oil. While neither butter nor cod-liver oil is rich in vitamin E [Evans and Burr, 1927] yet butter is richer than cod-liver oil, which is almost devoid of it; if, therefore, growth were being limited by insufficiency of vitamin E and not of vitamin A the effect of administering cod-liver oil should be much inferior to that of administering butter.

The following groups were included:

Group 13.	Irradiated cottonseed oil from start and 1.0 g. butter
Group 14.	" " " " " 0.5 g. "
Group 15.	" " " " " 0.1 g. "
Group 16.	" " " " " 0.5 g. butter and 5 drops cod-liver oil
Group 17.	" " " " " and 5 drops cod-liver oil
Groups 18-22	as groups 13-17, but with double the amount of yeast extract in the basal diet

Figs. 4 and 5 show the results. In this series a large preponderance of females was used, only one animal in each group being a male. All through the present investigation it has been found much harder to produce continuously normal growth in females than in males; the weight curves of normally fed females, in any case, flatten off earlier than do those of males, so that there is a less range of growth over which differentiation of the doses can be displayed. For both these reasons females are therefore less suitable for an evaluation by

Fig. 4. Weight curves of rats on a diet containing irradiated hardened cottonseed oil as source of vitamin D, and receiving doses of 1.0, 0.5 and 0.1 g. butter from the start of the experiment. Groups 18, 19 and 20 received a double ration of vitamin B in the form of marmite.

Fig. 5. Weight curves of rats on a diet containing irradiated hardened cottonseed oil as source of vitamin D, and receiving doses of 5 drops cod-liver oil, and 0.5 g. butter and 5 drops cod-liver oil. Groups 21 and 22 received a double ration of vitamin B.

growth. In the present experiments, however, it was felt that the end would not be satisfactorily attained unless normal growth could be evoked in females as well as in males by the addition of vitamin A to the basal diet.

On reference to Figs. 4 and 5 it is seen that on the whole the growth of the groups receiving the double doses of marmite is better than that of the groups receiving the single ration. The difference is not very great, but the only male in the whole series growing to 300 g. in the 5 months of the experiment (No. 304, group 18, Fig. 4), and the only females growing to 200 g. (No. 339, group 18, Fig. 4, and No. 340, group 21, Fig. 5) were in the double marmite series. This growth represents very good normal growth [cf. Macy *et al.* 1927, 1, Chart III]. The eyes of rats receiving the double marmite ration, together with high rations of vitamin A, also approached most closely to the normal. It was found very difficult to obtain rats with consistently perfectly normal eyes, by which is meant eyes that are fully exophthalmic and showing the blue line of the sclerotic all round the outside of the eyeball.

The three different doses of butter, whether combined with the single or the double marmite diet, gave growth results graded proportionally to the dose of butter given. The larger the dose, the longer was normal growth maintained. The individual variation in performance is great, but the general result is clear. Two of the excellent examples of growth referred to as occurring on the double marmite diet represented the growth of rats which were also receiving 1.0 g. of butter daily and were the best performances of any rats on the butter rations.

The behaviour of the rats which received cod-liver oil or butter and cod-liver oil (Fig. 5) was very uneven. Group 16, which received 5 drops of cod-liver oil and 0.5 g. of butter, added to the single marmite diet, was particularly poor, while the corresponding group, 17, which received 5 drops of cod-liver oil only, showed a good result. The latter group only contained two animals, but both were litter mates of two animals in group 16, so that the result must be taken as one of individual variation. Group 21, which received the double marmite ration, together with cod-liver oil and butter, showed an excellent result, approaching very closely that shown by the group which received double marmite and 1.0 g. butter daily and superior to that shown by the group which received double marmite and 0.5 g. butter. The cod-liver oil therefore seemed to be quite as effective in promoting growth as was the butter, and the beneficial effect of both must consequently be ascribed to their content of vitamin A and not, on the part of the butter, to its content of vitamin E. In any case it would appear, from the work of Evans and Burr [1927] and of Kennedy and Palmer [1926], that vitamin E should have been adequately supplied by the hardened cottonseed oil of the basal diet.

The general conclusion, therefore, to be drawn from the last series of experiments is that, when vitamin A is added in sufficient quantity (1.0 g. of butter daily) to the basal diet containing irradiated cottonseed oil, normal growth can be obtained in the rat when the addition is made from the start of the experiment. When doses less than sufficient are given, the weight curve flattens off after a period directly proportional to the size of the dose of vitamin A given. The individual variation in growth even among rats of the

same litter is, however, great and a clear-cut result cannot be expected when only a small number of animals is used. When an increased ration of vitamin B is given, the growth of a series of groups receiving different doses of vitamin A is transferred to a higher level, but the relation between the amounts of growth in the different groups is not altered. Growth is not so limited by the insufficiency of vitamin B in the diet used that the result, in attempting to evaluate vitamin A, is in any way obscured, but when the increase in vitamin B is made normal growth is more easily secured.

DISCUSSION.

From their own work the writers were inclined to conclude that pathological processes, set on foot during the preliminary depletion period, were responsible for the premature slackening of growth which ensued when vitamin A had been administered for some weeks. But from a scrutiny of the work of others they concluded that this was not so.

In the present series of experiments 0.5 g. daily was the largest dose of butter administered after a depletion period, whereas it was found necessary to give 1.0 g. daily in order to produce normal growth without a depletion period. Macy *et al.* [1927, 2] mention that a larger dose of vitamin A is needed to produce normal growth after a depletion period than when the vitamin is given from the start of the experiment, and Steenbock and Coward [1927] were successful in obtaining normal growth when four yellow corn seeds daily were given after a depletion period. It would therefore appear that it is not impossible to obtain normal growth to maturity, after a depletion period, if a large enough dose of vitamin A is given, and had the writers given doses of 1.0 g. or more of butter daily, they would probably have obtained normal growth.

The question next arises, how can vitamin A best be evaluated? From the present three series of experiments the writers are inclined to recommend that tests for vitamin A should be made by giving a basal diet containing irradiated hardened cottonseed oil as the source of vitamin D, together with a range of doses of the substance to be tested for vitamin A, from the start of the experiment. The use of the double ration of vitamin B does not appear to be essential, as it simply serves to shift the whole series of weight curves to a slightly higher level, and to secure normal growth with a somewhat smaller dose of vitamin A than would otherwise be the case, as has just been explained.

All laboratories differ in the diet on which their rats are bred and, consequently, in the growth and behaviour of those rats when they are placed upon a deficient diet, so that it is not possible to lay down any universal rule that a certain growth performance should be correlated with a certain standard dose of vitamin A; but, for the rats which the writers are in the habit of using, they would regard the attainment of 300 g. by males and of 200 g. by females, at the age of 6 months, as an optimum performance which a sufficient dose of the material to be tested for vitamin A should be able to produce. This, however, demands an experimental period of 5 months, which is unpractically

long, but if lower doses are used a result would probably be apparent in 3 months. An early result is in any case impossible to attain, as the rats' own reserves may take from 6 to 8 weeks to exhaust, and before that has happened no quantitative result, on any system, can be looked for.

Where an early result is desired and the substance to be tested is believed to be deficient or wholly lacking in vitamin A, a purely qualitative result may be obtained by using a preliminary depletion period, and seeing whether the substance to be tested is capable of bringing about recovery after vitamin A deficiency has set in. If the dose given is wholly ineffective the rat dies, but if it contains vitamin A the rat recovers. This method was used by the writers [Hume, Smith and Smedley-MacLean, 1928] in testing yeast-fat, and the result was held to be a satisfactory proof that yeast-fat does not contain vitamin A. Drummond, Coward and Handy [1925], Moore [1927] and Rosenheim and Webster [1927] have used the method in this way.

Certain of the results of other workers show that by giving sufficiently small doses it is possible to obtain subnormal growth, graduated to the dose of vitamin A given, in the first few weeks after the depletion period, and before the premature flattening begins. Chick and Roscoe [1926, 2], in testing various samples of cow's milk by the same method as was used by the present writers in testing spinach, drew their conclusions only from the first 3 or 4 weeks of growth, after the first administration of the test doses of vitamin A. The growth during that period they found to be quantitatively related to the dose of vitamin A given, but they have stated (verbal communication) that after the first 3 or 4 weeks they found that growth always tended to slacken off, regardless of the dose of vitamin A given. No doubt they were confronted with the same phenomenon as were the present writers. 3 cc. was their highest dose of pasture milk and the rats receiving it showed normal growth for the first 4 weeks after depletion. Such a dose equalled about 0.15 g. of milk-fat, and the writers used 0.2 g. of pasture butter as their minimum dose after a depletion period and obtained normal growth initially; their results are therefore in agreement with those of Chick and Roscoe and none of their doses was small enough to show subnormal growth in the initial period after depletion. The writers did not follow up the possibilities of basing a method of testing vitamin A upon the varying growth in the first 3 or 4 weeks after the depletion period, as they became too much interested in investigating the cause of the premature flattening in the weight curve after that first 3 or 4 weeks.

Golding, Soames and Zilva [1926] obtained similar results to those obtained by Chick and Roscoe, using the same method. They tested butter from cows fed in various ways and obtained subnormal growth in the first 4 weeks after the depletion period, when as much as 0.6 g. of butter from cows on a poor diet was given daily to the rats. They only give summary curves of growth, but there is indication of premature flattening in some cases.

Amongst American workers, Steenbock, Nelson and Black's [1924] first outline of a technique has already been referred to, but it did not take definite

shape until 1927, when Steenbock and Coward [1927] published a full account of a method in which vitamin D was given from the start of the experiment, and the doses of material to be tested for vitamin A after a preliminary depletion period. They show curves of rats (p. 771) which grew to maturity, when four yellow corn seeds were given daily, after a period of depletion. In this and a subsequent paper by Coward [1927], a number of weight curves are set out of rats which received different doses of various cereals or germinated cereals. In a great many of them there was no recovery when the test material was given, showing that it was lacking in vitamin A; some of the curves show subnormal growth on small doses and some show early normal growth followed by premature flattening [Coward, 1927, Chart X, p. 795], such as the writers have described. It would almost appear from a study of these weight curves, and of the results obtained by the present writers and by Chick and Roscoe [1926, 2], that when a depletion period is used, and various doses of vitamin A covering a wide range are given, the growth response of the rat is as follows.

(1) With a very small dose of vitamin A, such as one yellow corn seed daily [Coward, 1927], there is no recovery and death ensues.

(2) With somewhat larger doses, such as two yellow corn seeds daily [Coward, 1927], or less than 0.1 g. pasture butter daily (present paper), or less than 3 cc. milk (0.15 g. milk-fat) from pasture cows [Chick and Roscoe, 1926, 2], there is recovery with subnormal growth, which is quantitatively related to the dose of vitamin A for the first few weeks, but later ceases to be so, the growth curve flattening off.

(3) With still larger doses, such as 0.5 g. pasture butter daily (present paper), 1.0 g. spinach daily (present paper), or two wheat shoots irradiated for some hours on 4 or 5 successive days [Coward, 1927], normal growth is produced in the first few weeks after the depletion period, but is followed by a premature flattening of the weight curve.

(4) With still larger doses, such as four yellow corn seeds daily [Coward, 1927] and perhaps a daily dose of 1.0 g. or more of pasture butter, though this dose was not tested by the present writers, after a depletion period, normal growth is obtained up to maturity.

When, however, vitamin A is given from the start of the experiment without any preliminary depletion of the animal's reserves, there is normal growth to maturity if the dose of vitamin A is large enough, and, if it is not large enough, the period for which normal growth is maintained is proportional to the amount of vitamin A given. This latter behaviour is easily intelligible, but it is not easy to understand why, after the rat's reserves have been exhausted, a certain dose of vitamin A should be able to evoke growth at a normal or a subnormal rate for a time, but not up to maturity. It might be that the daily supply needed is much larger when the rat is larger, or, much more probably, that the sudden remedying of a deficiency is met by the organism with a tremendous initial response which it is unable long to sustain.

Macy and co-workers [1927, 2], working on human milk, and Outhouse,

Macy and co-workers [1927], on cow's milk, show curves which fall into line with the above scheme. They include one series in which no depletion period was used and in which the growth on 1.5 cc. and 2 cc. of human milk was compared. On 2 cc. normal growth was produced up to maturity, but on 1.5 cc. normal growth ceased after about 8 weeks; the result given is clear and is comparable with that obtained by the writers when different doses of butter were given from the start of the experiment. When a depletion period was used, Macy found that larger doses of milk were needed to produce normal growth; there is then much greater variation between the individual curves and there is not a sharp differentiation between the doses. Most of her curves seem to be of the type in which normal growth occurs initially, followed by premature flattening, though with some of the larger doses normal growth takes place. These workers suggest a variety of other symptoms of vitamin A deficiency, the date of incidence of which might be used in the quantitative evaluation of vitamin A; such, for instance, are the appearance of xerophthalmia or of cornified cells in the vaginal smear, but to get a sharp result from the study of these symptoms would appear to the writers to be even harder than to get such evidence from a method of growth comparison.

The method of Sherman and Munsell, as employed by Sherman and Hessler [1927], has already been referred to. They regard the addition of vitamin D to the diet as perhaps unnecessary, but a wise precaution, and make their standard the restoration of growth to a rate of 3 g. weekly, after a depletion period. They show an estimation of vitamin A in carrot on these lines. They use a large number of animals, about two dozen in each group, but they only give figures for the average growth increment for one experimental period of 8 weeks. The data are thus insufficient for any analysis of the curves; presumably they are working with doses which are only just sufficient to bring about recovery and restore a very slow subnormal rate of growth. The same method was used by Quinn and co-workers [1927] to examine the vitamin A value of green plant tissue other than leaves, but they gave no vitamin D in any form, so that their results cannot be regarded as satisfactory.

Dye and co-workers [1927] made use of a method with a preliminary depletion period and irradiated basal diet. They give only average curves in which the sexes are not differentiated nor the range of variation of behaviour shown. With 0.3 g. of lettuce leaf daily their curve is of the type showing normal growth initially, after depletion, followed by premature slackening after 5 weeks. Supposing lettuce leaf to have somewhat the same content of vitamin A as spinach leaf, this result agrees with that obtained by the writers with spinach. Smaller doses or doses of less potent material gave subnormal growth with premature flattening of the curve.

Dutcher and co-workers [1927] used an irradiated basal ration, with a depletion period of 5 weeks, followed by 5 weeks' feeding of the test ration. They gave doses of from 1.0 to 3.0 cc. of variously treated milks, and got subnormal growth in varying degrees. They show no curves and only give average

figures in which sexes are not differentiated, so that the results cannot be analysed; but premature flattening might not be apparent in 5 weeks.

Supplee and Dow [1927] used a depletion period in estimating the vitamin A in milk and had groups of rats irradiated directly, receiving irradiated basal diet and receiving no addition of vitamin D beyond that contained in any milk supplement given. They obtained much better growth when milk supplements were given alone than when they were given with extra vitamin D in any form; they do not explain this curious result, which makes their work difficult of interpretation.

Bethke and co-workers [1927], testing vitamin A in various samples of egg-yolk, used a depletion period, with direct irradiation. They show a series of curves in which there is a great deal of individual variation. The growth responses for the different doses range from non-recovery, through recovery with subnormal growth, to recovery with normal growth. Some curves show signs of the beginning of premature flattening. A survey of the results of other workers thus seems to support the conclusions which the writers have drawn as to the possible types of growth response.

One of the really great difficulties in the present type of work lies in the individual variability in performance of the experimental animals, even when every known precaution is taken in breeding uniformly and in selecting litter mates. This difficulty is particularly stressed in the recent Report to the League of Nations on the biological assay of cod-liver oil [1928] and in an extension of the same piece of work by Leigh-Clare and Soames [1928]. The report deals with work from a number of laboratories and points out that even a moderate degree of accuracy can only be hoped for when a large number of animals is used, so as to eliminate the effect of the wide deviation of some individuals. The practical difficulties are thus still further enhanced by the need for large numbers of animals to be used. In that Report it is laid down by the Accessory Food Factors Committee (of the Medical Research Council and Lister Institute) that the method should include a preliminary depletion period, during the whole or part of which vitamin D should be given in the form of direct irradiation or of irradiated foodstuff. This method was adopted by the four laboratories who undertook to make the test independently. The laboratories were in tolerable, but by no means complete, agreement in their results, and both in the Report itself and in the extension of it by Leigh-Clare and Soames [1928] the want of accuracy in the results is stressed. No individual growth curves of rats are given, so that the individual variation in behaviour is not shown, but it is to this factor, as has already been mentioned, that the inaccuracy is ascribed. Difficulties such as troubled the writers when testing for vitamin A in spinach do not seem to have been encountered by these workers, nor indeed has any reference to them been found in the literature.

From the writers' own results and from an analysis of the work of others it would appear that the best hope in attempting an estimation for vitamin A is either to give the test dose from the start of the experiment and seek a dose

which produces a given amount of growth in a given time, taking growth to 300 g. for males and 200 g. for females at 6 months of age as that which an optimum dose ought to give, or to seek such doses as will, after a depletion period, give subnormal growth, graded to the dose, for the first few weeks. This latter method gives a very wide individual variation, the rats are in indifferent health as the result of the depletion period, and the danger exists that all the doses selected may lie within the range which produces normal growth followed by premature flattening. This range may be wide, including doses differing by 500 %, as the writers found in the case of spinach.

SUMMARY.

(1) Experiments are described in which doses of 0.2 to 1.0 g. of two kinds of spinach were tested for vitamin A, by the growth of rats. A depletion period was used and vitamin D was given as direct irradiation. All doses of spinach gave normal growth curves for a time, followed by premature flattening and no quantitative result was obtained.

(2) Similar experiments were made with doses of 0.2 and 0.5 g. of butter, given with and without a depletion period, and with irradiated hardened cottonseed oil and direct irradiation as sources of vitamin D. There was no difference in the results yielded by the two sources of vitamin D and a normal growth curve, with premature flattening, was obtained with both doses of butter after a depletion period. Without a depletion period, the result was quantitative, normal growth being continued for a period directly proportional to the dose of vitamin A.

(3) Further experiments were made with doses of 0.1, 0.5 and 1.0 g. butter, and some with cod-liver oil, without a depletion period. One series received a double ration of vitamin B. Normal growth to maturity was obtained with 1.0 g. butter and the double ration of vitamin B, and the growth on 0.5 g. and 0.1 g. was proportionately less, according to the dose. The series on the single ration of vitamin B gave the same result but on a slightly lower level throughout. The results with cod-liver oil indicated that the only limiting factor in growth had been the amount of vitamin A and not any other dietary factor.

(4) It was concluded from the present experiments and from the results of other workers that the growth response of the rat to a series of doses of vitamin A after a depletion period is as follows. (a) Very small or no dose. No recovery. (b) Small doses. Subnormal growth, graded quantitatively to the dose; later, premature slackening. (c) Larger doses. Normal growth for a time, followed by premature slackening. (d) Optimal dose. Normal growth to maturity. The peculiar character of the growth under (b) and (c) is regarded as a disproportionate response to a supply of vitamin after depletion, which cannot be maintained subsequently.

(5) It is suggested that evaluation of vitamin A is best made by dispensing with the depletion period, or if it is used, as being quicker, by employing only doses which are likely to give the responses (a) and (b).

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