CLXXIII. THE CURATIVE METHOD OF VITAMIN A ASSAY.

BY MARION BROCK RICHARDS AND BEATRICE WEIR SIMPSON.

From the Rowett Research Institute, Aberdeen.

(Received July 2nd, 1934.)

THE curative method of vitamin A assay, which is still the method most widely used for the biological estimation of vitamin A, is thus summarised in the Medical Research Council's Report on "Vitamins" [1932]: "In a curative test, the animals are allowed to exhaust their reserves of vitamin A, as indicated by cessation of growth, after which doses of the test substance are administered for about 5 weeks. . . . Comparisons are drawn between doses which give equal average increments in weight." The method is apparently simple, but reference to the literature makes it evident that in practice the results obtained are far from satisfactory. Serious discrepancies occur not only among the results obtained by workers in different laboratories, but even among those obtained in one laboratory by the same workers using carefully standardised procedure. Thus Drummond and Morton [1929], in analysing the results obtained for the vitamin A content of a series of oils, give the case of two litter-mates of which one lost 24 g. in 4 weeks while the other, on the same dose of cod-liver oil, gained 34 g. in 5 weeks. Such extreme variations are by no means infrequent in their occurrence and could probably be paralleled by most workers from their own data.

The history of vitamin A assay is largely a record of attempts made to eliminate such discrepancies. In order to secure uniformity in the experimental animals stress has been laid on the necessity of using litter-mates for comparison, and of reducing the reserve stores of vitamin A in the young by careful regulation of the mother's diet. Certain workers find it preferable to take the occurrence of xerophthalmia rather than "cessation of growth" as an indication of depletion of the animal's reserve stores of vitamin A. Coward *et al.* [1930] have emphasised the importance of the type of caseinogen used in the basal diet. They found that by substitution of extracted "light white casein" for the "vitamin-free casein" previously used, it was possible to obtain greater uniformity in the results and to get growth-response graded to the dose of vitamin A.

The failure of all such attempts to effect the necessary improvement in results, the growing dissatisfaction with the method among experienced workers and their striking lack of agreement in regard to details of technique are indicated clearly by papers published in 1933 from three different English laboratories. Thus Culhane [1933], in reporting work with various basal diets, states that rats on her vitamin A-free diet invariably develop xerophthalmia, whereas Bacharach [1933] finds the onset of xerophthalmia so uncertain that it is useless as a criterion of avitaminosis-A. Further, Bacharach's results, "in their bearing on the source of protein in vitamin A-free diets, do not confirm those reported by Coward," and Culhane from her experiments comes to the conclusion that Coward's "light white casein" contains some vitamin A. Of even greater significance is a communication by Coward *et al.* [1933], in which they report the

great variations they have themselves found in the apparent vitamin A value of a sample of cod-liver oil, when tested at different times. Their numerical values for one sample, expressed as abscissae of the curves of response relating weight increase to dose of vitamin A, range from 0.73 to 3.48. After investigating possible causes of the variation in response, such as seasonal influence, changes in basal diet and differences in the weight of the rats when depleted of vitamin A, the authors conclude that the variation must be due to "some influence not yet determined."

From a review of the literature it would appear that some fundamental source of error in the technique of the assay has been overlooked. The outline of the method given shows that the technique is based entirely on the "growth-promoting property" of vitamin A, "cessation of growth" being regarded as the criterion of depletion of the reserve stores, and "resumption of growth" after addition of a supplement as an indication of the presence of vitamin A in that supplement [Medical Research Council, 1932]. The significance of the pathological conditions that arise from vitamin A deficiency does not seem to have been taken sufficiently into account. Obvious signs of disease such as xerophthalmia and lung trouble have received attention, while other more dangerous but less apparent symptoms have been almost ignored. The occurrence of diverse pathological conditions has been reported by numerous workers, notably by Green and Mellanby [1928], but apparently those who have been interested in the relationship of vitamin A to disease have not as a rule been concerned with the quantitative estimation of the vitamin, while the assay workers have not recognised fully the extent to which their results can be affected by the varied nature of the symptoms arising in the absence of vitamin A.

In the course of a number of assays, by the curative method, of the vitamin A content of various samples of fish oils and dried milk, we have accumulated much evidence regarding the unsatisfactory nature of the curative test. In addition to many discrepancies of the types frequently reported in such tests, difficulties of another nature have been encountered. Much of the evidence could not be reconciled with current ideas regarding the growth-promoting activity of vitamin A and the cessation of growth that is said to occur after the animal's reserve stores of the vitamin have been depleted. It was clear that in many cases, particularly in the dried milk experiments, the weight curve did not give a reliable indication of the state of depletion of the animal, and that increase in weight after administration of a supplement could by no means invariably be ascribed to the vitamin A content of that supplement. Consequent on these observations experiments were carried out in this Institute which proved that there is in reality no cessation of growth in vitamin A deficiency [Orr and Richards, 1934]. The animal continues to increase in size, as determined by skeletal development, even when it ceases to increase in weight, and the fall in weight which eventually occurs is simply the result of the pathological conditions due to the vitamin deficiency.

The present paper is a summary of experimental data bearing on various points of technique in the curative method. The results illustrate the errors which may arise from taking the weight curve either as an index of vitamin A depletion in the preliminary period, or as a measure of vitamin A potency in the test period. There is little room for doubt that the cause of many of the reported discrepancies lies in the different pathological conditions of the experimental animals at the end of the preliminary period.

EXPERIMENTAL.

Weight curve during the preliminary period.

The weight curve during the preliminary period has been taken by most workers to indicate the state of depletion of the animal's reserve stores of vitamin A. As these stores become exhausted the weight curve tends to flatten, and dosing with vitamin A is begun when it is clear that weight increase has ceased. Considerable importance has been attached in curative tests to the actual weights attained by the rats during the preliminary period, and it is customary to reject from the test animals which grow beyond a certain limit. The Medical Research Council's Report states that "suitable" animals cease to grow at about 100 g. weight. Coward *et al.* [1932, 1] consider that the rats should cease to grow at 80–100 g. and they regard 110 and 120 g. as the permissible maxima for females and males respectively.

We have found that the course of the weight curve during the pre-period is influenced very significantly both by the composition of the basal diet, quite apart from its lack of vitamin A, and by the type of experimental animal used. On a given basal diet, the weight level at which the curve tends to flatten or fall suddenly will be higher for a vigorous animal than for one of a more weakly type, while the use of a more complete diet will tend to raise the weight level of all the rats.

Composition of the basal diet. In selecting the basal diet the tendency of assayers seems to have been to direct attention chiefly to the necessity of using a diet completely devoid of vitamin A, and to ignore the equally important requirement that "all growth-promoting factors other than the one under test should be abundantly supplied" [Medical Research Council, 1932]. Unless this requirement is satisfied, retardation in the rate of growth will not necessarily be due to lack of vitamin A, and increase in weight may be caused by the administration of other missing factors. While the theoretical ideal may as yet be impossible of attainment, in view of our present imperfect knowledge of nutrition, the diet should at least be so chosen as to include all known essential factors other than vitamin A. Throughout this series of experiments, except in a preliminary oil assay, we have used with slight modifications the vitamin A-free diet recommended by Sampson *et al.* [1932]. This diet (Diet L), which includes fat and a high proportion of dried yeast, seems to approximate more closely to the theoretical ideal than some of the basal diets in use.

As an illustration of the extent to which the weights attained by the rats in the pre-period can be influenced by the composition of the basal diet, the following data may be given. In a preliminary oil assay, in which the basal diet used (Diet I) consisted of caseinogen 20, rice starch 70, salts 5 and yeast 5, with radiostol as source of vitamin D, 14.4 % of a lot of 104 rats exceeded the limits of weight set by Coward and should therefore have been rejected as "unsuitable" for experiment. In a later assay, in which basal Diet L was used, 57 % of a batch of 51 rats of similar breeding should have been rejected by the same standards. Diet L consisted of caseinogen 18, wheat starch 50, cotton-seed oil 15, salts (McCollum 185) 5, dried yeast 18.3 and lemon juice 5, with a small amount of potassium iodide solution and sufficient water to make into a stiff paste. Vitamin D was supplied as radiostol (0.02 ml. per rat twice weekly). That the superior "growth-promoting" properties of Diet L are not due to any accidental inclusion of a source of vitamin A may be seen from the weight curves of 12 rats which served as negative controls in this series of experiments (Fig. 1). These animals received throughout, after weaning at 21–25 days, the basal diet together

with a supplement of 0.075 g. of dried separated milk (Diet L + M). The nature of the curves and the lengths of the survival periods (28–53 days for the males and 47–70 days for the females) furnish strong evidence that the amount of vitamin A, if any, in the basal diet and in this amount of dried milk is entirely

Fig. 1. Negative controls. Basal diet supplemented with 0.075 g. "unfortified milk," i.e. dried separated milk, which gave a negative result when tested colorimetrically for vitamin A. KD = killed in moribund condition; D = died.

negligible. In addition to the effect on the weight curves, the substitution of Diet L for Diet I caused a marked improvement in the general condition of the animals. In rats fed on Diet L there were none of the sores on feet, tail, shoulders and neck which were of frequent occurrence in the earlier experiment. There is little doubt that Diet I was too low in vitamin B, and that the improved results on Diet L must be attributed largely to its exceptionally high proportion of yeast, amounting to about 20 % of the dry constituents. The inclusion of fat is probably also a factor of importance, since Culhane [1933] has shown that body sores occur more readily on a fat-free diet.

Type of experimental animal. No adequate reason seems to have been given for rejecting from the test animals which grow beyond the prescribed limits of weight during the preliminary period. Even among litter-mates it frequently happens that from the beginning certain individuals show greater vitality than others. These rats have a tendency to maintain appetite and weight-increase beyond the level at which the less vigorous litter-mate with its more capricious appetite tends to fall off. The practical result of the weight limitation imposed would thus appear to be to eliminate from experiment the more vigorous animals. Such animals may possibly be less likely to give the desired response to vitamin A dosage, but this seems scarcely sufficient cause for rejecting them, and for confining the tests to animals of a less vigorous type. The minimum effective dose of vitamin A determined for a weakly rat of poor growth rate will not necessarily be effective for a more vigorous animal, and a biological method cannot be regarded as satisfactory if its results are applicable only to a limited class of animals of a subnormal standard.

Weight curve during the test period.

In the curative method increase in weight during the test period is regarded as an indication of the presence of vitamin A in the substance tested. Even small temporary increases in weight occurring when a vitamin A-free diet has been replaced by another basal diet have been taken as proof that the second diet

contains some vitamin A. Little attention has been paid to the fact that with animals kept for some time on one ration, practically any change in the diet will stimulate appetite, temporarily at least, and if the changed diet be particularly palatable, the effect on the weight curve may be of considerable duration, even though the added substance is devoid of vitamin A.

Results of this type occurred in an assay of "fortified milk," *i.e.* dried separated milk to which had been added before evaporation a vitamin A concentrate. A blue value test carried out on the ether extract of the milk sample had indicated that apparently only a small amount of the vitamin A potency of the added concentrate had been retained during evaporation. When the biological test was carried out by the usual curative procedure (Curative method I), *i.e.* giving the milk as a supplement after a preliminary period on the basal diet, the weight curves in a number of cases seemed to show the presence of a considerable amount of vitamin A in the "fortified milk," and no fewer than 20 of the 30 rats on the three doses fed (0.025, 0.050 and 0.075 g. milk) survived the test period of 5 weeks. The condition of the rats, however, made it evident that the amount of vitamin A present must have been small. Xerophthalmia when present was not cured, and in spite of considerable increases in weight, respiratory trouble developed later in several cases. In view of the obvious ill-health of the animals, it was felt that the prolonged survival periods and the increases in weight were not truly indicative of the amount of vitamin A in the "fortified milk," but were to be ascribed partly to the food value of the milk and partly (possibly chiefly) to the stimulating effect of the milk supplement on appetite. For administration the milk powder was mixed with water, and the requisite doses, which were given from a graduated pipette, were invariably taken with great avidity by the rats, even when they were practically moribund.

In order to eliminate the effect of the stimulus to appetite caused by the sudden addition of this extremely palatable supplement, the assay was repeated, using a slightly modified technique (Curative method II). Each rat received during the preliminary period a dose of "unfortified milk" (*i.e.* dried separated milk) equal in amount to the dose of "fortified milk" it was to receive during the test period. Thus any stimulus to appetite due to the milk supplement was present throughout the experiment, and any weight increase found after substituting "fortified milk" for "unfortified" could therefore be more legitimately ascribed to the vitamin A potency of the former. The striking difference in results brought about by this slight modification in procedure is shown in Fig. 2. In each row the first 3 curves are weight curves obtained by the ordinary curative method, while the remainder were obtained by the Curative method II. In place of the prolonged survival periods and the weight increases obtained by Curative method I, there is a marked preponderance of curves of the sharply peaked type. Only two out of 36 rats survived the test period of 5 weeks, and of these survivors one (graph not included in Fig. 2) was then unable to respond to a large daily dose of vitamin A concentrate. It survived only 10 days longer, and *post mortem* examination revealed tongue abscess and advanced pyelitis. In the other survivor (Rat 2441), which was killed after 95 days' dosing with vitamin A, the caecum was found to be highly distorted and perforated by an abscess.

The marked contrast between the results obtained in these two assays of "fortified milk" served to bring into prominence the mistaken assumptions on which the curative technique is based. In the first assay the ill-health of the animals made it clear that the weight-increases in the test period could not be regarded as indicative of vitamin A potency, and in the second assay, in a large

number of cases there was no sign of the occurrence of the growth plateau which is supposed to indicate the depletion of the animal's reserve stores of vitamin A. *Post mortem* examination of the rats showed the presence of disease of widely varying types, and it was obvious in many cases, especially where the weight

Fig. 2. Assay of "fortified milk" (F.M.) by the curative method.

Method I: Basal ration during preliminary period; supplement of "fortified milk" in test period.

Method II: Basal ration + "unfortified milk" during preliminary period; basal ration + "fortified milk" in test period.

curve was very sharply peaked, that disease must have been already far advanced while the weight curve was still rising steadily. It seemed evident that deficiency of vitamin A does not affect the process of growth *per se*, but that the inevitable sequel to such deficiency is the onset of disease, the effect on the weight curve being merely a secondary and incidental manifestation. The occurrence of a growth plateau seems to be more or less fortuitous, and in many cases no effect on the weight curve may be apparent until after ineradicable disease has been established.

Examples of the sharply peaked type of curve occur not infrequently even in ordinary curative tests, and it will usually be found that in such cases death ensues without any apparent response to the dose of vitamin. It is significant that any addition to the basal diet which renders it more complete seems to increase the number of such curves. Thus, for example, when Drummond *et al.* [1925] modified their technique of vitamin A assay by introducing a source of vitamin D into the diet, they found a tendency for curves to be sharply peaked

instead of falling off gradually. In our experiments a similar effect was produced by the change from Basal diet I to Diet L. The exceptionally high proportion of such curves recorded in the second assay of "fortified milk" might possibly therefore be an indication that the milk supplement has acted as a stimulus to appetite by supplying some unknown deficiency in the basal ration. On the other hand we have had many instances of sharply peaked curves, especially in males of the more vigorous type, when no milk supplement was given with the basal ration, while less vigorous rats, even when receiving a milk supplement, have shown a tendency to give flatter curves. Apparently the more vigorous the rat and the more complete the diet, the greater is the probability that a sudden decline in weight will set in without the usual premonitory flattening.

Estimation of the end of the depletion period.

One of the main difficulties in carrying out a curative assay is to determine when to begin administration of the vitamin. "Cessation of growth" is the criterion given in the M.R.C. Report, while in Sherman's technique, which seems to be that adopted by most American workers, the instructions are to "begin the test period a few days after growth has ceased on the basal diet, and about one day before the appearance of distinctly sore eyes. . . . Have the animals thoroughly depleted of surplus vitamin A but not in a seriously pathological condition when the experimental period is begun" [Sherman and Burtis, 1928].

The occurrence of sharply peaked curves, such as those shown in Fig. 2, makes it clear how unsatisfactory are any instructions based on the course of the weight curves. In those cases the dose of vitamin A given was small, but even when a considerable or large dose is given, many instances of similar curves are found (Fig. 3 *a*). In such cases it is evident that no dose of vitamin A, however large, could have effected a cure. The impossibility of controlling the severity of the pathological conditions seems self-evident.

Certain workers have found it useful to take the ophthalmic reaction as an indication of depletion [*e.g.* Steenbock and Coward, 1927], but as has already been noted, there is a lack of unanimity regarding the invariability of its occurrence. If it does happen to occur, xerophthalmia is undoubtedly more reliable than the weight curve as an indication of avitaminosis-A. Certain data from one of our experiments are of interest in this connection. Pairs of litter-mates were placed on deficient diets, one rat receiving Diet L and the other Diet L + M. In spite of considerable differences in the weight curves during the preliminary period, the first signs of xerophthalmia when it did occur, appeared in litter-mates on approximately the same dates (Fig. 3 *b*). In the case of rats 3144 and 3145, the former, in which the eyes remained unaffected for a further period of 12 days, showed symptoms of lung trouble on the same day as xerophthalmia was first noted in its litter-mate. Reference to the graphs in Fig. 3 *b* shows that if these rats had been used in a curative test, with xerophthalmia as the index of depletion, dosing would have been begun in most cases several days sooner than would have been the case if the weight curve were taken as the index.

In estimating the end of the depletion period individual workers apparently use their own discretion in determining when to begin dosing, and in this one point there is room for a variability which may exert a powerful influence on the results obtained. It is obvious that the earlier dosing begins, the greater is the chance that the pathological conditions present will still be amenable to treatment, and the better will be the weight response. The weight curves of the two

Fig. 3 a. Graphs illustrating the difficulty of estimating the end of the "depletion" period.
Chief *post mortem* findings:

- 2668. Ballooning of intestines and caecum; pneumonia.
- 2692. Gastric haemorrhages; inanition.
- 2670. Stomach—keratosis and ulceration of non-glandular part; punctuate petechial areas all over glandular part.
- 2609. Caecum ballooned, 2 ulcers; peritonitis; stomach—many small ulcers.
- 2657. Ballooning of stomach and intestines; pneumonia.
- 2354. Pneumonia.
- 2420. Ballooning of stomach and intestines. (Noted before death.)
- 2437. Large tongue abscess; inanition.

Fig. 3 b. Estimating the end of the "depletion" period. X=appearance of xerophthalmia; L=symptoms of lung trouble.

series of male rats given in Fig. 4 illustrate the variations in response which accompany variations in the length of the preliminary period. In the first series each rat received the same dose of a vitamin A concentrate, after preliminary periods varying from 24 to 40 days. In the second series each rat received 2.2 mg.

of a good cod-liver oil after preliminary periods of 29–42 days. In both series it will be seen that where dosing was begun fairly early, *i.e.* after about $3\frac{1}{2}$ – $4\frac{1}{2}$ weeks on the basal diet, the weight response was in general satisfactory, and on *post mortem* examination at the end of the test period in most cases no macro-

Fig. 4. Variations in response accompanying variations in the length of the preliminary period. The 13 rats of the first series each received 0.46 mg. of a vitamin A concentrate; the 10 rats of the second series received 2.2 mg. cod-liver oil. The dotted lines indicate the early stage at which the weight graph begins to diverge from the normal course.

scopic lesions were found. On the other hand, where dosing had been delayed until 34–42 days, the weight response was much poorer, and *post mortem* examination revealed the usual macroscopic evidence of disease, even after 5 weeks' dosing with vitamin A. Comparison of the *post mortem* findings given in Table I with the course of the weight curves in Fig. 4 shows how close is the connection between the weight response of the animal and its pathological condition. In the second series rat 2664, which died without any indication of response to the dose of vitamin, forms a good example of the risks inherent in the curative method. Even when dosing is begun as early as 31 days, the damage done may happen to be irreparable.

Table I. *Effect on pathological condition of varying the length of the depletion period in curative assays. (Cf. Fig. 4.)*

No. of rat	Length of depletion period Days	Chief <i>post mortem</i> findings (macroscopic) (after 5 weeks' test period)
2438	24	No macroscopic lesions
2727	30	"
2701	30	"
2715	30	"
2702	31	"
2477	31	Inflammation of caecum
2653	32	No macroscopic lesions
2608	34	Stomach—2 healed ulcers. Caecum—ulcer
2520	35	Caecum—ulcer, and patch of chronic inflammation
2587	37	Caecum very badly ulcerated. End of ileum ulcerated all over. Inflammation of small intestine. Stomach—much keratosis, 4 ulcers
2421	40	Abscess on head. Large lung abscess
2569	40	Tongue abscess. Caecum—chronic ulceration. Inflammation of ileum
2565	40	Lungs badly infected. Intestines inflamed. Caecum ballooned
2716	29	Stomach—healed ulcer
2731	29	No macroscopic lesions
2664	31	Died after 9 days' dosing. Lungs infected. Inflammation of small intestine, including duodenum. Caecum ballooned
2707	32	No macroscopic lesions
2454	35	Large tongue abscess. Bladder abscess. Died after 8 days' dosing
2480	36	Duodenum inflamed. Entire caecum inflamed and distorted
2393	36	Large tongue abscess. Lung abscess. Caecum—large perforated ulcer
2458	39	Caecum—two ulcers
2416	40	Peritonitis. Caecum—healed perforated ulcer
2345	42	Stomach—keratosis. Duodenum—ulcer

From these data it is evident that variations of even a few days in the length of the depletion period may cause wide discrepancies in the response of the animal to vitamin A dosage, and it is probable that such variations are the cause of some at least of the differences in the results obtained by different workers. Thus Dutcher *et al.* [1927] reported that they could get weekly gains of only 8–9 g. on 3 ml. of cow's milk, whereas Macy *et al.* [1927, 1] had got gains of 12–13 g. They suggested that the environment and diet of the cows might account for the difference, but a study of their technique indicates that the length of their preliminary period exceeded that given by Macy and co-workers by about 7–9 days, a prolongation which would have a very significant effect on the animal's response. The latter authors, who were evidently well aware of the dangers involved by the use of an extended preliminary period, advocated that curative feeding should commence before definite xerophthalmia and maintenance or loss in weight were observed, to avoid dealing with grossly pathological animals.

Diversity of pathological conditions after preliminary period.

Apart from the discrepancies that may arise from variations in the length of the preliminary period, a further source of error obviously lies in the wide diversity of the pathological conditions that may result from the vitamin A deprivation. As an illustration of this diversity we give in Table II the chief *post mortem* findings for the rats in the second milk assay, whose weight curves were included in Fig. 2.

Table II. Post mortem findings in rats from second milk assay, showing diversity in pathological condition after depletion. (Cf. Fig. 2.)

2378	Tongue abscess; ballooning of stomach and intestines; peritonitis
2562	Ballooning and inflammation of entire intestine
2604	Inflammation of duodenum; pyelitis; cystitis
2423	Submaxillary abscesses; stomach—ulceration and keratosis; cystitis
2440	Tongue and submaxillary abscesses; stomach—keratosis, haemorrhage; caecum—perforated ulcer
2600	Tongue abscess; lungs infected; pus in seminal vesicles
2435	Pyelitis; cystitis
2561	Pyelitis; duodenal haemorrhages
2520	Stomach—keratosis; caecum ballooned; small intestine inflamed
2572	Neck and tongue abscesses; stomach—ulceration; caecum—inflammation; pyelitis
2589	Stomach—keratosis; caecum—inflammation; cystitis
2616	Stomach—keratosis; duodenum and small intestine highly inflamed; pus in seminal vesicles
2380	Caecum ballooned; pyelitis
2577	Pyelitis; bladder—ballooned, abscess
2606	Pyelitis; stomach—keratosis and ulceration; caecum—ulcer and inflammation
2388	Stomach and intestines ballooned
2573	Stomach—keratosis and inflammation
2441	Stomach—keratosis and ulceration; caecum—perforated ulcer

A study of these findings brings into prominence one of the most radical defects of the curative method. In a biological test it is essential that the experimental animals at the commencement of the experiment should be as nearly alike in all respects as possible, and in vitamin A work much stress has been laid on the importance of securing uniformity in the animals at weaning. In a curative test, however, the real beginning of the experiment is not the time when the rats are first placed on the deficient diet, but the end of the "depletion" period, when vitamin A dosage is begun. This is the point, therefore, at which the animals should be in similar condition, if similarity in response is to be expected. It is significant that all assay workers reject from their tests animals which die during the early stages of dosing. Drummond and Morton [1929] reject all rats which die in the first 3 weeks. Coward *et al.* [1930] reject all those which die in the first week, since the number of these bears no relation to the dose of vitamin given. "Considering the varying and unknown degree of severity of the pathological condition of the animals," it is stated, "this is not surprising." It is to be noted, however, that these varying pathological conditions affect not only the rats which die during the first week of the test but also the survivors, and on the growth, or more correctly the weight-increase, of these survivors the calculations of vitamin A potency are based. It is obvious that the response of such animals to a dose of vitamin A must depend largely on the nature and severity of the pathological conditions present, and that these dissimilarities in the experimental material when dosing begins cannot fail to produce variations in the response of individual rats to the same dose of vitamin A. The various discrepancies reported by numerous assayers admit of easy interpretation if the possible pathological condition of the animal is taken into account.

Persistence of pathological conditions.

We have selected from our data numerous examples of "anomalous responses" which seem similar in type to results reported in the literature. These are presented along with the main findings of the *post mortem* examinations which were carried out on the rats, either when they became moribund as the result of the deficiency, or at the end of the 5 weeks' test period.

(1) *Appreciable growth at first, followed by sudden decline.* Ahmad and Drummond [1930] reported cases of this kind. Again, Culhane [1933] has stated that occasionally rats on a vitamin A-free diet "died rather suddenly without any obvious cause." In Fig. 5 the first 3 curves represent rats in which

Fig. 5. Anomalous responses in vitamin A curative tests. Initial response followed by a decline. This type of result has been ascribed to incomplete depletion of the vitamin reserve during the preparatory period. The pathological conditions arising from the avitaminosis seem to be a more probable explanation.

Chief *post mortem* findings:

- 2393. Large tongue abscess; abscess in lung; caecum—large abscess, perforated.
- 2377. Pyelitis; lungs badly infected.
- 2593. Lungs full of chronic abscesses.
- 2351. Large tongue abscess; keratosis of stomach; ballooning of stomach and intestines.
- 2446. Chronic abscess in lung; chronic ulceration of caecum.
- 2728. Pyelitis of right kidney; extravasation of urine retroperitoneally.

"appreciable growth" was maintained for 11, 13 and 16 days after dosing began. In the remaining 3 curves the weight response was excellent for even longer periods, *viz.* 24–26 days, after which death occurred suddenly without any "obvious cause," *i.e.* with no outward signs of vitamin A deficiency. A study of the main *post mortem* findings given below the diagram seems to furnish an adequate explanation for the occurrence of weight curves of these types. In our opinion the sudden decline following an initial response is not the result of the complete exhaustion of hitherto undepleted reserves of vitamin A, as suggested by Ahmad and Drummond [1930]. It is due rather to the presence in the animal at the end of the preliminary period of some ineradicable pathological condition, which has increased in severity while the improvement of other conditions has caused a temporary increase in weight. It is to be noted that cases may occur in which the outward appearance of the animal gives no indication of its real condition. Thus rat 2728, which died of advanced pyelitis, was apparently in perfect health 26 days after dosing began and became practically moribund in one night.

(2) *Subnormal growth response.* Among the different types of response to vitamin A dosage tabulated by Hume and Smith [1928] there is included

“recovery with subnormal, or normal, growth for a time, followed by premature flattening.” Coward and Key [1928] report similar results and include “continuous subnormal response” as a further type. Bacharach [1933] also speaks of “the frequent failure of the animals, after they have ceased to grow, to respond to even relatively large doses of the vitamin with anything approaching normal growth. It is generally held that this is due to some inadequacy in the basal diet.” In Fig. 6 are included a number of curves which may be regarded as illustrative

Fig. 6. Persistence of pathological conditions after avitaminosis-A may account for the varying types of response to vitamin A dosage. These graphs show varying degrees of “subnormal response,” such as have been described by Hume and Smith, Coward and Bacharach.

Chief post mortem findings:

- 2594. Chronic inflammation of caecum.
- 2592. Ulceration of caecum.
- 2507. Chronic inflammation of caecum; perforated ulcer, now closed.
- 2576. Several healed ulcers in stomach.
- 2650. Inflammation of lower end of intestine.
- 2484. Ulceration of caecum.
- 2598. Pyelitis; small haemorrhagic pits in stomach.
- 2520. Ulceration of caecum, and patch of chronic inflammation.
- 2736. Pneumonia.
- 2439. Large ulcer in caecum.
- 2352. Keratosis of stomach.
- 2418. Keratosis of stomach; lymphatics much enlarged; lower end of ileum congested.
- 2524. Chronic inflammation of caecum; lymphatics enlarged.
- 2477. Chronic inflammation of caecum.
- 2497. Chronic inflammation of caecum.
- 2494. Pyelitis in left kidney.
- 2541. Inflammation of caecum, and 3 ulcers.
- 2456. Chronic inflammation of stomach.

of these types. We have found that “continuous subnormal response” is very frequently associated with inflammation or ulceration of the caecum. The premature slackening which may follow a period of subnormal or normal growth is undoubtedly due to the persistence of pathological conditions dating from the

preliminary depletion period. Hume and Smith were inclined to draw this conclusion from their own work but apparently rejected it after a scrutiny of the work of others. "Some inadequacy in the basal diet" seems a less adequate explanation of the failure to attain normal growth than the fact of the persistent pathological conditions arising from the vitamin A deficiency. Even when the animals have responded with practically normal growth performance during the test period, *e.g.* rats 2456, 2541 and 2494, it will be seen that such conditions as inflammation of the stomach, ulceration and inflammation of the caecum and pyelitis may still persist.

We have evidence which shows that the pathological conditions due to vitamin A deficiency may persist even after several months on normal diet. In Table III are given data for 5 rats which, after being used in vitamin A tests,

Table III. *Persistence of pathological conditions due to avitaminosis-A after several months on normal diet.*

No. of rat	Period on normal diet months	<i>Post mortem</i> findings
580	8½	Pleurisy; ulceration of stomach; small ulcer in duodenum; peritonitis round caecum
1631	3	Chronic abscess involving most of left lung; areas of consolidation in right lung
1538	4	Chronic abscess in apex of right lung, and consolidated patches in rest of same lung; caecum—perforated ulcer, partially healed
1588	4	Caecum—two chronic ulcers
1706	3	Caecum—perforated ulcer, healed

had been placed on normal diet for periods varying from 3 to 8½ months. These animals had appeared to be in good condition at the end of the vitamin test, but as they had afterwards failed to attain "normal growth performance" on normal diet, they were killed and examined for macroscopic lesions.

In other two rats examined after 3 and 4½ months on normal diet no definite macroscopic lesions were found, but it is significant that even in these cases, and in that of rat 1706, in which the main lesion was a healed ulcer in the caecum, the growth performance and condition of the animals remained subnormal. It was pointed out by Macy *et al.* [1927, 1] that if the preliminary period were too prolonged, advanced pathological conditions might be established which could not be ameliorated by vitamin adjuvants. They found that generalised infections resulting in the localisation of pus in the upper part of the digestive and respiratory tracts could not be cured by feeding methods, regardless of the richness of the diet in vitamin A. Few assayers, however, seem to have realised the significance of such results.

This persistence of pathological conditions that have been induced by vitamin A deficiency, taken in conjunction with their diverse nature, forms the strongest argument against the curative method of assay. In a curative test vitamin A can influence growth only indirectly, by effecting improvement in the pathological conditions, and it is obvious that no consistency in results can be expected when these conditions may vary so widely not only in their nature but in their intensity and amenability to treatment. It has been shown that with a comparatively short depletion period there is more likelihood of a good response to vitamin A dosage, but even a considerable reduction in the length of this period cannot ensure similarity in the condition of the experimental animals.

Early occurrence of pathological conditions in vitamin A deficiency.

The results of the second milk assay, recorded in Table II and Fig. 2, seemed to indicate that varied pathological conditions must have been present in the animals before the weight curve had shown any marked flattening. This point was therefore investigated in a later experiment, in which a number of rats were killed, not in moribund condition, after periods of 3-7 weeks on vitamin A-free diet. The results were as follows:

No. of weeks on diet	3	4	5	6	7
No. of rats examined	19	18	20	8	5
No. classed as "normal," <i>i.e.</i> with no macroscopic lesions	3	6	0	0	0

It will be seen that of 37 rats killed after 3 and 4 weeks, *i.e.* before they would be considered to be "depleted" of vitamin A according to the usual criteria, there were only 9 in which no macroscopic lesions were detected. In rats killed from 5 weeks onwards none were classed as normal. The conditions found included ulceration and haemorrhagic points in the glandular part of the stomach, inflammation of the small intestine, especially the duodenum, and inflammation or ulceration of the caecum. These findings are of particular interest in view of the instructions given in the M.R.C. Report for carrying out a prophylactic assay of vitamin A: "Doses of the material to be tested are given either from the beginning of the feeding with the experimental diet or from about 3 weeks after, when the rats' reserves of vitamin A have been partially exhausted." The evidence given above shows that a test in which dosing is begun after 3 weeks on the basal diet cannot be regarded as prophylactic but is essentially of the curative type.

A close study of the weight curves during the preliminary period of a curative test shows that in many cases divergence from normal begins to show very early. For a rat growing normally this part of the weight curve should be practically a straight line. In Fig. 4, where the earlier course of the weight curve has been continued by means of a dotted line, it will be seen that divergence begins after periods of 8-18 days on the basal diet, except in the case of rat 2565, where the normal course was followed for 26 days. For the 23 examples shown in this figure, the average time of divergence is 13.5 days. It seems very probable therefore that these effects on the weight curve are an indication that diseased conditions had already begun to develop even at this early stage.

Reserve stores of vitamin A in young rats.

The early onset of disease in young rats deprived of vitamin A gives rise to serious doubt regarding their supposed reserve store of the vitamin and the time necessary for its depletion. According to Green and Mellanby [1928] there is no doubt that these stores rapidly disappear. They found that even in the case of rats dying at an early stage, before any great pathological change had occurred, liver extracts gave negative results when tested colorimetrically for vitamin A. Dann [1932] showed that the amount of vitamin A in the livers of rats at birth was small and was unaffected by the amount of carotene eaten by the mother before or during pregnancy, although the amount at weaning could be increased by feeding the mother with a diet rich in carotene. It was found, however, that the weight of the young at weaning and their weight and age when growth ceased on a vitamin A-free diet appeared to be independent of the amount of vitamin A in the mother's diet during pregnancy or lactation, except when she was almost depleted of vitamin A. This would appear to indicate that any extra

stores of vitamin A acquired before weaning are rapidly depleted and have no practical effect on the length of the preliminary period. There seems therefore to be little justification for the practice commonly adopted of restricting the mother's intake of vitamin A during breeding, in order that the reserve stores of vitamin in the young rats may be kept low. There is no doubt that when rats so bred are placed on a vitamin A-free diet, their weight curves will tend to flatten at a lower level than those of animals bred on a more liberal system, but no clear proof appears to have been given that this difference is due to differences in the vitamin A reserve stores of the young rats, as is generally assumed [Smith and Chick, 1926]. The possibility has apparently been overlooked that differences in the levels at which the weight curves flatten may be due simply to differences in the vigour and health of the animals. That the system of breeding adopted tends to produce an inferior type of rat is indicated by the falling off in breeding capacity reported by various workers. Thus Smith and Chick [1926] state that litters after the third are small and irregular, and Scheunert and Schieblich [1933] report that they have been unable to maintain a supply of standard young rats on any of the standard diets in the literature. Cramer [1923; 1924] showed how restriction of the vitamin supply in early life may lead to imperfect development in rats without necessarily producing obvious ill-health. Similarly, Macy *et al.* [1927, 2] found that advanced tissue changes characteristic of vitamin A deficiency appeared very early (6-12 days) in rats whose mothers had received inadequate vitamin A, and the evidence indicated the probability that these changes had begun during lactation. There seems therefore to be a serious danger that the approved system of breeding may result in producing young rats which, if not already suffering from incipient vitamin A deficiency, are at least sub-normal in regard to their standard of health, and that animals considered "suitable" for vitamin A tests are not merely low in vitamin A reserves but low in vitality.

The facts that macroscopic evidence of disease was found in 75 % of the rats examined after 3-4 weeks on the deficient diet and that the weight curves begin to diverge from normal at as early a time as 13.5 days indicate that any reserve stores of vitamin A in the young rat are depleted much more rapidly than is generally assumed. It is probable that histological examination might reveal evidence of the effects of the deficiency at a still earlier stage. The whole question of the "reserve store" of vitamin A seems to require further investigation. In the case of the young animal the chief argument in favour of the existence of these reserve stores appears to be the work of Korenchevsky and Carr [1924]. These authors found that excess of the fat-soluble factor in the mother's diet during pregnancy caused a marked decrease in the disorders of general nutrition produced in the young by a diet deficient in fat-soluble factor. It does not follow from this, however, that "variation in the state of nutrition of the young rats" is synonymous with "variations in the reserves" of the fat-soluble vitamins possessed by the young animals at the beginning of the experimental period [Smith and Chick, 1926]. If the results of Korenchevsky and Carr are taken in conjunction with the finding of Dann that the vitamin A content of the liver at birth is unaffected by the amount of carotene in the mother's diet during pregnancy, they show rather that an ample supply of the fat-soluble factors is essential in the mother's diet during pregnancy to ensure the production of healthy offspring.

Sherman and Cammack [1926] made a quantitative study of the storage of vitamin A in older animals, of ages 2-6 months, which, after weaning, had been given varying amounts of vitamin A before being placed on the deficient diet.

The extent of storage was judged by determining the lengths of the survival periods when the animals were subsequently deprived of vitamin A. This assumption that the length of the survival period is an index of the amount of vitamin A previously stored by the animal cannot be accepted without question. Even in animals of similar breeding, and with exactly similar opportunities for storing vitamin A before being placed on the vitamin A-free diet, wide variations may be experienced in the length of the survival period. In one experiment in which 64 rats were given the deficient diet immediately after weaning, we found that the first death occurred after 34 days, while the last of the 64 rats was killed, moribund, after 79 days on the diet. A similar difference in survival period, in spite of previous equality in opportunity for vitamin A storage, was found in the case of older rats. For 16 rats which had previously been on normal diet and which were placed on the vitamin A-free diet at the age of $5\frac{1}{2}$ -6 months, we found that the survival periods ranged from 158 to 320 days. In one experiment of Sherman and Cammack rats were transferred at the age of 6 months to the deficient diet after being kept from weaning either on the stock diet B or on this diet with additions of 1, 2 or 4 % of cod-liver oil. For rats on these diets the average survival periods, as estimated from the diagram, were apparently 164, 268, 328 and 340 days. No information is given regarding the number of rats included in the respective groups, but it will be noted that the range of variation in survival period we have found within our one group is practically identical with that found between the extreme groups in Sherman and Cammack's experiment. Moreover, it is obvious that in giving such large percentages of cod-liver oil, food constituents other than vitamin A are being added in considerable amount to the diet. Yet the increased length of the survival period is ascribed solely to increased storage of vitamin A. It would appear that the theory of the animal's capacity to store reserves of vitamin A rests on a somewhat insecure experimental foundation.

The system of breeding rats considered "suitable" for vitamin tests thus introduces another potential source of variability into the curative method. The system produces subnormal animals, but there is no way of ensuring that even in one laboratory the degree of subnormality will be the same for all the rats. Still less probable is it that animals reared in different laboratories will be in exactly similar condition.

From the evidence given it seems clear that efforts to develop a satisfactory technique for vitamin A assay along curative lines are futile. The records of the last 5 years show that even with the utmost care experienced workers have been unable to obtain consistent results, and the difficulties inherent in the method seem to be insurmountable. It is impossible to secure uniformity in the experimental animals after a period of deprivation of vitamin A and therefore impossible to secure uniformity of response in the test period. The only hope of evolving a satisfactory biological test would appear to be to adopt a protective method, starting off with young healthy animals of first class breeding, and endeavouring to find the dose of vitamin A which will keep the animal in healthy condition, as evidenced by *post mortem* examination. It may well be that the dose required will prove to be considerably higher than has been considered necessary for satisfactory growth, since good weight curves are not incompatible with the presence of disease. It is recognised that prophylactic tests will be even more lengthy and laborious than curative tests, but at least there seems to be a chance that the results obtained will be more consistent and reliable. Hume and Smith [1928] advocated the adoption of a test of the prophylactic type, but they offered it only as an alternative to a curative test, and although the suggestion has met

with some approval [*e.g.* Drummond and Morton, 1929], it has not been generally adopted. These authors pointed out that Hume and Smith still found rather disturbing variations when the test was made by supplying the substance under investigation from the beginning. Where the doses employed are insufficient, variations in response may naturally still be expected, since the pathological conditions developing will still tend to vary in nature and degree of severity.

Various workers have made comparisons of the colorimetric, spectrographic and biological methods for the determination of vitamin A, but the results reported show the same lack of agreement as has been found in all vitamin A assays. In some cases [*e.g.* Schmidt-Nielsen, 1929; Norris, 1931] it is stated that the biological values do not agree at all with those obtained by the chemical or physical tests. In other cases it has been found that agreement is tolerably good, considering the wide variations to which the biological test is liable [*e.g.* Ahmad and Drummond, 1930]. Biological and chemical values seem to agree more closely if the unsaponifiable fraction is used instead of the crude oil [*e.g.* Wise and Heyl, 1932; Coward *et al.* 1931; 1932, 2]. The latter authors find that the spectrographic test for the 328 $m\mu$ absorption band gives a better indication of biological activity than the antimony trichloride test.

Since the errors of the curative biological method are even greater than has apparently been realised, it seems possible that the tolerably good agreement already found between the biological results and those obtained colorimetrically or spectrographically might become much closer if the biological test were carried out on prophylactic lines. If such agreement could be established, the necessity for the biological test would disappear.

SUMMARY.

Difficulties encountered in the curative method of vitamin A assay are discussed. It has been found that discrepancies of varied types may be explained by the differing pathological conditions of the animals at the end of the preliminary "depletion" period. These conditions occur much earlier than has been generally supposed and may be far advanced before any indication of their existence is seen in the weight curve. The response of the animal to vitamin A dosage is determined by the nature and severity of the pathological condition present. Since uniformity of condition cannot be ensured in spite of all precautions, variations in response are inevitable. It has been shown that pathological conditions induced by avitaminosis-A may persist after weeks of dosing with a large amount of vitamin A, or after several months on normal diet. The diversity of these conditions and their frequent lack of amenability to treatment constitute sources of error in the curative method which it seems impossible to eliminate.

The theory of the "reserve stores" of vitamin A and the practice of breeding so as to "reduce these reserves" in the experimental animals seem to require further examination. The effect of restricting the mother's intake of vitamin A appears to be not merely a lowering of the reserve stores of the vitamin in the young animal but a diminution in its health and vitality.

It is suggested that any biological assay of vitamin A to be satisfactory must be of the prophylactic type. Its object should be to determine, not the amount of vitamin A which will produce fairly satisfactory weight curves in animals of inferior type, but the amount which will keep first-class animals in first-class condition, as evidenced not only by the weight curves, but by *post mortem* examination of the tissues.

The authors wish to express their indebtedness to Dr J. B. Orr for his interest and helpful advice throughout this investigation.

REFERENCES.

- Ahmad and Drummond (1930). *Biochem. J.* **24**, 27.
Bacharach (1933). *Biochem. J.* **27**, 5.
Coward and Key (1928). *Biochem. J.* **22**, 1019.
— Key, Dyer and Morgan (1930). *Biochem. J.* **24**, 1952.
— Dyer, Morton and Gaddum (1931). *Biochem. J.* **25**, 1102.
— Cambden and Lee (1932, 1). *Biochem. J.* **26**, 679.
— Dyer and Morton (1932, 2). *Biochem. J.* **26**, 1593.
— Key and Morgan (1933). *Biochem. J.* **27**, 873.
Cramer (1923). *Lancet*, i, 1046.
— (1924). *Lancet*, i, 633.
Culhane (1933). *Biochem. J.* **27**, 69.
Dann (1932). *Biochem. J.* **26**, 1072.
Drummond, Coward and Handy (1925). *Biochem. J.* **19**, 1068.
— and Morton (1929). *Biochem. J.* **23**, 785.
Dutcher, Honeywell and Dahle (1927). *J. Biol. Chem.* **75**, 85.
Green and Mellanby (1928). *Brit. Med. J.* ii, 691.
Hume and Smith (1928). *Biochem. J.* **22**, 504.
Korenchevsky and Carr (1924). *Biochem. J.* **18**, 1313.
Macy, Outhouse, Long and Graham (1927, 1). *J. Biol. Chem.* **73**, 153.
— — Graham and Long (1927, 2). *J. Biol. Chem.* **73**, 175.
Medical Research Council (1932). *Spec. Rep. Series*, No. 167.
Norris (1931). *Bull. Basic Sci. Res.* **3**, 249.
Orr and Richards (1934). *Biochem. J.* **28**, 1259.
Sampson, Dennison and Korenchevsky (1932). *Biochem. J.* **26**, 1315.
Scheunert and Schieblich (1933). *Biochem. Z.* **263**, 444.
Schmidt-Nielsen (1929). Quoted by Ahmad and Drummond (1930)
Sherman and Burtis (1928). *J. Biol. Chem.* **78**, 671.
— — Cammack (1926). *J. Biol. Chem.* **68**, 69.
Smith and Chick (1926). *Biochem. J.* **20**, 131.
Steenbock and Coward (1927). *J. Biol. Chem.* **72**, 765.
Wise and Heyl (1932). *J. Amer. Pharm. Assoc.* **21**, 1142.