XX. VITAMIN A AND CAROTENE

XV. THE INFLUENCE OF THE VITAMIN A RESERVE ON THE LENGTH OF THE DEPLETION PERIOD IN THE YOUNG RAT

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THE ability of the animal to store vitamin A has been inferred by many workers, from the early days of vitamin research, in order to account for the different survival periods of animals bred or reared on diets containing different amounts of the vitamin when subsequently restricted to a diet deficient in the vitamin. More recently the discovery of colorimetric [Rosenheim & Drummond, 1925; Carr & Price, 1926] and spectroscopic [Takahashi *et al.* 1925; Morton & Heilbron, 1928] tests for the vitamin has permitted direct confirmation of the presence of the vitamin, often in very high concentration, in the livers of animals which have received either vitamin A or carotene [Moore, 1930].

Opinion is more divided, however, as to the quantitative extent to which the reserve of vitamin A may influence the behaviour of the animal, and particularly the experimental rat, when given a diet deficient in the vitamin. Thus Richards & Simpson [1934] have suggested that "the theory of the animal's capacity to store reserves of vitamin A rests on a somewhat insecure experimental foundation", and that the "theory of the reserve stores and the practice of breeding so as to reduce these reserves in experimental animals seems to require further examination". Orr & Richards [1934] are of the opinion that "the animal's capacity for storing reserves of vitamin A is much less than is generally supposed, and that the preliminary or depletion period during which it is assumed to be using up these reserves, is in reality the time taken for the outward manifestation of symptoms of disease which may be already far advanced".

The work of Guilbert & Hinshaw [1934] on chickens and turkeys has proved that there is a close correlation between the liver reserves, as measured by the antimony trichloride reaction, and the time of survival of pen-mates when restricted to a diet deficient in the vitamin. Moreover, both in these animals and in cattle [Guilbert & Hart, 1934] the vitamin A reserves in individuals killed after varying periods on a deficient diet become gradually smaller. The evidence in the case of the rat is less straightforward. Baumann *et al.* [1934] have certainly shown that rats given single doses of halibut liver oil may survive for prolonged periods, increasing with the amount of the oil given, on a diet deficient in vitamin A, and that progressive falls in the liver reserve may be observed in rats kept on the deficient diets for increasing periods. Much other work, however, seems to have been directed towards the establishment of exceptions rather than the general rule. Thus in experiments by Dann [1932] on young rats which had acquired low vitamin A reserves (0-45 I.U.) during suckling, no simple relationship could be traced between the extent of the reserve and survival period upon a deficient diet. Baumann *et al.* also noted that in young rats the survival period

was longer than could be predicted from the extent of the vitamin A store alone. They even observed prolonged growth in the apparent absence of stored or dietary vitamin A. It is therefore clear that the extent of the vitamin A reserve is not the sole factor in determining the time of survival.

Work by ourselves [Davies & Moore, 1935] has pointed to another apparent inconsistency in the economy of the vitamin. Very high reserves, sufficient to support the supposed requirements of the animal for a theoretical period of about a century, may be accumulated by adult rats when given diets rich in the vitamin. The rate of dissipation of these reserves, however, may sometimes be much greater than the apparent physiological requirements seem to demand, actually amounting in the experiment reported to a loss of 98% of the total reserves within 3 months.

The results obtained in the present work fill a gap in our knowledge by showing that a moderately large reserve of vitamin A, such as may be accumulated by a young rat if allowed access to a diet rich in the vitamin for a short period at the end of lactation, may be sufficient to increase the depletion period from a few weeks to several months. Further proof is thus afforded of the necessity of keeping down the vitamin A reserves of the young rat if satisfactory vitamin A determinations are to be carried out.

EXPERIMENTAL

At the time of the experiment two breeds of rats were available in this laboratory. (1) A piebald strain, used for general work, and bred and reared on a diet of bread, milk, wheat, corn, marmite, carrots, greenstuffs and liver. This diet, designed to ensure maximum strength and fertility, is extremely rich in sources of carotene and vitamin A and leads, in the adult rat, to very high liver reserves of this vitamin. (2) A Wistar albino strain receiving a diet of maize meal, whole wheat meal, skimmed milk powder, dried yeast, wheat germ, calcium phosphate and salt mixture, with whole milk during lactation. These rats were kept for experiments on vitamins A and D, and both the mothers and young had very low reserves of the former vitamin.

Groups of young rats of ages 5-7 weeks, and of about the same initial weights, were taken from each breeding group at a time which afforded a period of 2-4 weeks during which they were able to eat the maternal diet for themselves. Some were killed at once and the vitamin A reserves determined by the antimony trichloride method. The total liver reserves of four members of group I were 1650, 2250, 2250 and 3000 I.U., mean 2290 I.U. Three members of group II gave values of 25, 25 and 30 I.U., mean 27 I.U.¹ The remaining rats of each group were given a diet deficient in vitamin A of the following composition: extracted Glaxo caseinogen 20%; rice starch 30%; cane sugar 30%; arachis oil 15%; salt mixture 5% + dried yeast 10%; radiostol (vitamin D) 1 drop per rat per week. The rate of exhaustion of the vitamin A reserves in each group was examined by means of weight increase curves and by killing groups of three animals at intervals for estimation of the liver reserves by the antimony trichloride method. The results obtained are given in Fig. 1.

In group I, except in the case of certain individuals which will be mentioned below, growth was continued for about 150 days. The livers of animals killed before this point gave positive results in the antimony trichloride test (42 days, mean total reserve 540 I.U., 84 days, mean 275), but at 175 days, when serious

¹ Blue units as determined in the antimony trichloride reaction have been converted into international units (I.U.) by multiplying by 0.6 [Moore, 1937].

weight declines had occurred in two out of the three rats killed, uniformly negative results were obtained. The surviving three rats were each given one drop of halibut liver oil daily. In two instances slight increases in weight were observed, and were maintained over a period of 59 days. The third rat declined in weight, and was killed in moribund condition after receiving the halibut liver oil for 50 days. Strongly positive colour reactions were observed in the livers of all three rats which had received halibut liver oil (mean total reserve 8000 I.U.).

Fig. 1. The effect of a substantial vitamin A reserve in increasing the depletion period in rats restricted to a diet deficient in vitamin A.

In group II the rats reacted as would be expected in a well-planned experiment for the estimation of the vitamin, growth ceasing in most instances after a period of about 5 weeks. The livers of rats killed after 35 or 41 days all gave negative colour tests. Two rats were each given a single dose of 4 drops of halibut liver oil, which caused a sharp resumption of weight increase. On killing strongly positive liver reactions were observed.

Instances of abnormal behaviour in group I. The weight increases shown by most animals in this group, together with the results of the antimony trichloride tests at autopsy, indicate clearly that the vitamin A reserves were not exhausted

until about 150 days on the deficient diet had elapsed. Nevertheless five out of the original sixteen members of this group (excluding those killed to determine the initial vitamin A reserves) died after various intervals within this period. Colorimetric estimations of the liver reserves were carried out in 4 cases, with positive results. In those cases in which post mortem examinations were carried out death appeared to be due to intestinal stoppage. A similar condition, with a septic focus, was found to be the cause of the failure of rat 14 (Fig. 1) to respond when dosed with halibut liver oil. A list of the animals showing abnormal behaviour is given in Table I (p. 176).

The breeding of rats for use in vitamin A determinations. The work of Dann [1932, etc.] in this laboratory has shown that only small amounts of vitamin A are transferred from the mother rat to her young during pregnancy and lactation, even when the diet is extremely rich in this factor. We have recently applied this principle to the breeding of rats suitable for use in vitamin A tests with satisfactory results. The procedure adopted has been to give the mother a diet rich in vitamin A, in practice the same diet as given to our piebald rats, until the birth of the litter. A diet low in vitamin A (e.g. light white caseinogen 25 %, starch 50 %, arachis oil 20 %, salts 5 % + dried yeast 15 %, radiostol (vitamin D) 6 drops per week) is then given to the mother during lactation and to the young at weaning.

When subsequently restricted to a diet completely deficient in the vitamin, young rats bred under these conditions invariably cease to gain weight after 3-4 weeks, and death usually follows rapidly if a source of the vitamin is not promptly administered. The scheme possesses two advantages over that adopted for the breeding of the rats of Group II in the present work. (1) The health of the mother is not impaired by constant restriction to an inferior diet. (2) It is not necessary to keep a separate colony of rats for the breeding of animals to be used in vitamin A tests.

DISCUSSION

The reality of the vitamin A reserve. When the weight increase curves obtained in the above experiments are studied in conjunction with the results of the colour tests applied at autopsy little room is left for doubting either the reality of the vitamin A reserve or its influence in determining the time of survival during restriction to a diet deficient in the vitamin. Two criticisms might however be directed against the conclusiveness of the present experiments. In the first place it might be argued that the difference in the depletion periods might be due to the use of piebald rats in group I and of albino rats in group II. Our own experience, however, suggests that this explanation is incorrect, since piebald rats show depletion periods of the ordinary length when precautions against the accumulation of high reserves have been taken. Secondly it might be argued that the shorter depletion period in group II was related not to the smaller vitamin A reserves in this group, but to a diminution of the general health and vitality as the result of a breeding diet generally inferior to that in the other group. In our experience there is some justification for the criticism of Richards and Simpson [1934] that the use of breeding diets calculated to restrict the vitamin A reserves may sometimes lead to general weakness in the young animal. Since however the administration of vitamin A to depleted animals in group II caused rapid increases in weight, this explanation cannot hold good in regard to the present work.

Whilst it is plain that the vitamin A reserve, as determined colorimetrically in the liver, is of fundamental importance in determining the length of the

depletion period, it must be emphasized that no direct proportionality between the vitamin A reserve and time of survival can be deduced. Dann [1932] and Baumann *et al.* [1934] have shown that rats having no measurable reserves may survive for considerable periods. It seems probable that, in addition to the time necessary for the dispersion of the vitamin A reserve, the rat can exist for some time on vitamin A present in the tissues either at a concentration too low to be detected by the antimony trichloride reaction as usually applied, or in some form which does not give this reaction. This period is probably succeeded by the stage during which, as Orr and Richards suggest, the characteristic lesions due to the deficiency are in process of development. As a result we may expect the total time of survival to be made up of a variable period during which the measurable reserve is exhausted, and of another period during which life is sustained in the absence of any measurable reserve. Even if no other complicating factors had to be considered the existence of the latter period would rule out the possibility of a linear relation between the liver reserve and the time of survival.

Abnormal behaviour in certain rats during depletion of the vitamin A reserves. Whilst the behaviour of most of the rats in group I was consistent with their having high reserves of vitamin A, the cases of abnormal behaviour given in Table I are of interest as instances of death occurring before the exhaustion of

Table I. *Rats in group I showing abnormal behaviour*

No. of rat	Body weight			Period of survival on deficient diet days	Total vit. A reserve I.U.	Autopsy findings
	Init.	Max. g.	Final			
17	63	—	—	4	900	Swollen intestine
18	56	58	58	16	120	—
19	72	98	74	26	—	—
7	55	79	79	42	190	Swollen intestine
20	55	84	74	114	190	Volvulus of intestines. Swelling noticed at 56 days
14	78	247	160	224	7500	Intestinal stoppage with sepsis

N.B. The weight increase curves of rats 7 and 14 will be found in Fig. 1. For simplicity the curves of rats 17, 18, 19 and 20 have been omitted.

the reserves. In two cases (rats 17 and 18) death occurred shortly after starting on the basal diet. In neither instance was there any initial weight increase, as observed in animals reacting normally, and even without an examination of the liver reserves one would not have hesitated in ascribing the cause of death to individual failure in adaptation to subsistence on the basal diet. With rats 19, 7 and 20, however, which died after receiving the basal diet for 26, 42 and 114 days, respectively, the case is different. All these rats showed some initial growth, and if colorimetric determinations of the liver reserves at autopsy had not been carried out death might reasonably have been attributed to vitamin A deficiency, following in the usual course of events on the exhaustion of the reserves. Since adequate reserves of vitamin A were found to be present we must conclude either that death occurred through some cause other than vitamin A deficiency, or that the reserves present in the liver were for some reason not available for metabolism. This second alternative has been suggested by Mellanby [1934] as a possible explanation of the presence of substantial reserves in human subjects dying from diseases in which low reserves might have been expected. From the results at autopsy it appears that the immediate cause of death, in at least two cases, was intestinal lesions. In rat 16 an abdominal swelling was noticeable for

several weeks before death. An intestinal stoppage with sepsis also accounted for the continued decline of rat 14 which failed to respond to a very high dosage of halibut liver oil. Since a high vitamin A reserve was found at autopsy there can be no doubt as to the efficient absorption of the vitamin.

These results support the assertion of Richards & Simpson [1934], that the occurrence of incurable lesions may lead to inaccurate results in routine determinations of vitamin A. It appears indeed that such lesions may occur not only in authentic vitamin A deficiency, but also occasionally when adequate reserves of the vitamin are still present. This inherent defect appears to present a barrier against the refinement of the curative procedure to any high degree of accuracy. In the absence of positive evidence, however, it is doubtful whether the prophylactic procedure advocated as an ideal by Richards & Simpson could be made to give better results. In adopting a prophylactic method the worker would have to contend not only with careful standardization of the initial reserve of vitamin, but with the exact definition of the "first class condition" which Richards & Simpson suggest as a criterion of vitamin A sufficiency. Under these circumstances the curative procedure appears for the present to be the only reliable method for the biological estimation of the vitamin. It seems advisable, however, to do everything possible to minimize inaccuracies caused by animals sustaining lesions so serious as to interfere with their growth responses. This could be effected (*a*) by avoiding any undue delay in dosing, and (*b*) by performing autopsies on animals showing weight increases abnormally low for their groups, with a view to their exclusion from the calculation of the mean response in the event of severe lesions being found.

SUMMARY

1. Two groups of young rats were taken at a point when they had been eating for themselves for a few weeks. Members of each group were killed and the vitamin A reserves of their livers determined by the antimony trichloride method. In agreement with the composition of the two diets used during breeding low reserves were found in one group, high reserves in the other.

2. The remaining rats from both groups were given a diet deficient in vitamin A. The exhaustion of the vitamin A reserves was studied both by colorimetric determinations on the livers of members killed at intervals and by following the weight increase curves. In the group having low reserves weight increase ceased after 5 weeks, and negative results in the antimony trichloride reaction were obtained at this point. In the group having high reserves weight increase continued for several months. Successive falls were however observed in the reserves of animals killed at intervals, and in most cases definite regression in weight had occurred after 25 weeks. The antimony trichloride reactions at this point were negative.

3. After the exhaustion of the reserves members of each group were given halibut liver oil. Weight restoration resulted in most cases, and the liver reactions were invariably strongly positive.

4. These results reaffirm the reality of the vitamin A reserve and emphasize the importance in routine vitamin A determinations of using rats which have been reared under conditions ensuring low reserves.

5. Although the general behaviour of the rats in each group was consistent with the extent of their reserves, some instances of anomalous behaviour were observed in rats with high reserves. Thus several deaths occurred while substantial liver reserves, as measured colorimetrically, were still present. Moreover,

after depletion one rat in this group failed to respond to treatment with halibut liver oil, although an intense antimony trichloride reaction was observed at autopsy. The failure to respond, and most of the cases of premature death, were due to intestinal lesions. In vitamin A determinations animals reacting abnormally should be rejected from calculations if lesions sufficient to account for the abnormality are found at autopsy.

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