29. THE INFLUENCE OF GRADED DOSES OF VITAMIN A UPON PATHOLOGICAL CHANGES IN THE CENTRAL NERVOUS SYSTEM OF THE RAT, WITH SUGGESTIONS FOR A PROPHYLACTIC ASSAY OF THE VITAMIN

BY J. T. IRVING AND M. B. RICHARDS

From the Rowett Research Institute, Aberdeen

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THE quantitative determination of vitamin A is a problem which has formed the subject of much research. The unsatisfactory nature of the biological assay, when carried out by the usual curative method, has led to many attempts to find a reliable chemical or physical method for the determination. Good results seem to have been recently obtained with the spectrophotometric method, and Barthen & Leonard [1937] consider that this method can be adopted as an alternative for the biological test in determining the vitamin A content of fish liver oils. According to other authors, colour and vitameter tests are only supplementary to the biological test and cannot replace it [e.g. Black *et al.* 1938; Haines & Drummond, 1938; Møllgaard, 1938]. Hence the necessity for a reliable method of biological assay still exists.

In discussing results obtained by the curative method, reference is continually being made to the large margin of error inherent in the method. Richards & Simpson [1934] adduced much evidence regarding the unreliability of the method and showed that the differences in the pathological condition of the animals at the end of the depletion period are such that variations in response to vitamin A dosing are inevitable. A survey of the literature since that date indicates that little attention has yet been paid to this question of the pathological condition of the animals and to the impossibility of securing uniformity of condition at the time when the curative test begins. Freudenthal [1938] supports the view that the curative method is unreliable and advocates the preventive method, but most workers seem content to assume with Munsell [1938] that it is possible to obtain results with the growth method (i.e. curative method) "that have as high a degree of accuracy as can be expected of procedures in which animals are used as reagents". We are prepared to admit that the degree of accuracy may be as high as can be expected when the test animals are depleted of vitamin A before use; but it seems highly probable that much greater accuracy could be attained if a method were adopted which would ensure greater uniformity in the experimental material. A prophylactic test, starting from the time of weaning, on young rats in which the reserves of vitamin A have been kept low, seems to offer more prospect of success. It is objected that a prophylactic test would require much longer time to carry out, and this may be true where "increase in weight" is taken as the criterion in the assay of vitamin A. It would not be difficult to bring forward objections to the use of this criterion, and apparently other methods suggested, such as the "xerophthalmia" method and the "vaginal contents" method, do not possess advantages which outweigh the convenience of the "increase in weight" method [Coward, 1938]. The necessity for a new criterion seems therefore to be indicated.

Richards & Simpson [1934] suggested that a protective method should be adopted with the object of finding the dose of vitamin A necessary to keep the animal in healthy condition as evidenced by post-mortem examination. Subsequent work at this Institute makes it possible now to amplify this suggestion in a more practicable manner. Irving & Richards [1938] have shown that, in young rats on a vitamin A-free diet, degeneration occurs in the *funiculus praedorsalis* in the *medulla oblongata* with a constancy which is in marked contrast with the variability in pathological condition met with in other parts of the body. Since the degeneration is progressive and increases in severity with time from about 21 days onwards, it seemed possible that, by giving graded doses of vitamin A to the young animals and killing all members of a series at the same time, one might get a gradation in the severity of the lesion corresponding with the gradation of the dose.

EXPERIMENTAL

A preliminary experiment was carried out on the above lines, giving daily doses of 0, 1, 2, 4, 8 and 16 I.U. to six series of male litter-mates, which were fed on our usual vitamin A-free diet. In addition, two rats received doses of 3 I.U. and two received 6 I.U. A vitamin A concentrate in arachis oil, supplied by Glaxo Laboratories Ltd., was used for these tests. The rats belonging to five of the series were killed after 47 days on experiment, and those belonging to one series after 42 days, when the negative control animal was nearly moribund. The histological work was carried out by the method already described by Irving & Richards [1938]. The results for this preliminary experiment are given in Section 1 of Table 1, in which the degree of severity of the lesions is indicated by a number.

Table 1. *Degree of severity of lesions in the medulla of male rats after varying doses of vitamin A, all animals of a litter being killed on the same date*

The severity of the lesions is indicated by a number according to the following scheme:

Stage 1-: a very few fibres degenerated.

Stage 1 : a few scattered fibres degenerated.

Stage 2 : well-marked degeneration.

Stage 3 : very marked degeneration.

Stage 4 : heavy degeneration.

Section	Litter	Days on diet	Dose of vitamin A in International Units										
			0	$\frac{1}{3}$	$\frac{2}{3}$	1	1.5	2	3	4	6	8	16
1	A	47	4			2-3		1	1	1		1-	1-
	B	47	4			3		1		1-		?1-	1-
	C	47	4			1		1-		1		1-	1-
	D	47	4			2		1-	1-	1-	1-	1-	1-
	E	47	4			2		1-		1-	1-	1-	1-, 1-
	F	42	4			2		1		1-		1-	
2	C ₁	50	3-4	4	3	2	1-						
	D ₁	42	4	—	2-3	1-	1-	1-					
	F ₁	52	—	3	3	3	1	1-					
	H	49	4	4	—	3	1	—	1				
	I	52	3-4	3	3	2	1	1					
3	E ₁	52	4	4	4	2-3	1	1-	1				
	G	49	3-4	4	2	2-3	1-	1-	1				

Food *ad lib.*

Food intake equalized for litter

It was found that the range of doses of vitamin A given in this experiment was unsuitable for showing the desired gradation in the lesions. Well-marked degeneration was present in all except one of the animals receiving 1 unit, but the

lesion was absent from all those which had received 2 or more units. (It is to be noted that the degree of degeneration represented by the numbers 1 and 1- is of no significance since the few scattered fibres thus denoted are found equally in positive controls and stock rats.)

The experiment was therefore repeated on similar lines but using smaller doses of vitamin A, viz. 0, 1/3, 2/3, 1, 1.5, 2 and, in a few cases, 3 units (Table 1, Section 2). In a further experiment two series of male rats had the food intake controlled so that all rats of one litter received the same amount of food; the results of these two series are shown in Section 3 of the table. The rats included in Sections 1 and 2 received food *ad lib*. The animals of each series were killed when the negative control was practically moribund.

It will be seen that in six of the seven series in Sections 2 and 3 there was a sharp demarcation between rats receiving 1 unit of vitamin A and those receiving 1.5 units. The lesion, which was still well marked in rats receiving the smaller dose, had disappeared from rats receiving 1.5 units. These animals were killed after periods of 49-52 days on experiment. In one series which was killed after 42 days, the demarcation occurred between the doses of 2/3 and 1 unit. It is possible that this difference may be due to the shorter period on experiment, but it is to be noted that of the litters in Section 1 killed at 47 days, the animal of litter C receiving 1 unit did not show a definite lesion in the medulla, while the corresponding animal of litter F, killed at 42 days, showed quite marked degeneration. The difference is therefore more probably due to individual variation. The control of the food intake, which was equalized for litters E and G (Section 3), apparently made no difference to the results, the demarcation for these two litters also occurring sharply between 1 and 1.5 units.

DISCUSSION

A study of the data in Table 1 makes it clear that the severity of the lesion in the medulla in a prophylactic biological test is well adapted for evaluating the vitamin A content of a food substance. The constancy of the change at various dosage levels of vitamin A is in great contrast with the variability in weight response reported in animals examined by the curative method [Richards & Simpson, 1934; Coward, 1938 (p. 17)]. The working out of the exact method of procedure for comparing an unknown with a standard substance may be left to those to whom routine assay is a matter of importance. The details of our technique in regard to the breeding of the rats and the histological method adopted are to be found in our earlier paper [Irving & Richards, 1938], in which also are given our reasons for considering the Marchi technique reliable, if due precautions are taken. The procedure is simple and, although it may take 4-5 weeks before the final result is obtained, the stages before the cutting of the sections occupy little time and the whole process can be carried out as a simple working routine. After a little practice the maximum degree of degeneration present in any series of sections can be rapidly assessed.

It is possible that the time required might be reduced by using the short Marchi technique described by Stewart [1936]. We have not actually tried out this technique as we have found our own method very satisfactory and have felt no urgent need for hastening the results. We prefer to avoid formalin fixation in carrying out the Marchi method, but those to whom quick results are of importance might find the method of value.

Objection has been raised that the prophylactic test takes longer to perform than the curative method. It has to be remembered that in the method here suggested time and money are saved at the feeding stage of the experiment.

Most of the animals in our tests were killed after 47–52 days of feeding, but it is probable that for routine assay 42 or even 35 days would be preferable. Our previous work showed that after 35 days on the vitamin A-free diet all rats, both male and female, whose vitamin A intake had been restricted before weaning, showed stages 3 to 4 of the degeneration. If the rats receiving graded doses were killed at this stage, it seems likely that a better differentiation might be secured between the 0, 1/3 and 2/3 unit doses. When the animals are killed at a later stage, as in our tests, the degeneration tends to be equally severe for all these doses. In a curative assay the rats may well take 35 or more days for the preliminary period alone and they have then to be continued for a test period of 3–5 weeks. The total time taken for a prophylactic assay by this method need thus be little more than is required for an ordinary curative test.

It has to be remembered also that rats in a curative test frequently have to be rejected after the preliminary period because their weight renders them “unsuitable” for the test, and in the test period there may be losses by death which also reduce the number of available animals. In the results reported above no animals were rejected for any reason, and there would indeed be no need for any rejections in a test of this type.

It must not be assumed that we consider 1.5 units a sufficient dose, when given prophylactically from weaning, to meet the young rat's requirement of vitamin A. We have found from recent work on the teeth [Irving & Richards, 1939] that the rat's requirement for vitamin A seems to increase with age, since a dose which is sufficient to keep the teeth normal at 7 weeks proves inadequate if the animal is kept for over 17 weeks on the diet. It is thus impossible to state at present what is the amount of vitamin A required to bring an animal to maturity. The results quoted above indicate, however, the daily amount needed to avert the lesion of the medulla during the early period of rapid growth.

SUMMARY

1. Rats were placed at weaning on a vitamin A-free diet and given graded doses of vitamin A. They were killed after about 7 weeks and their medulla was examined by Marchi's method. Degeneration was found in the *funiculus praedorsalis* in those receiving 1 i.u. or less per day but not in those getting 1.5 i.u. or more, the demarcation being extremely sharp.

2. It is suggested that this procedure might be adapted to form the basis of a prophylactic assay of vitamin A. The main source of error in the curative method, in which the test is carried out on animals which may differ widely in pathological condition, is thereby avoided. Further, the procedure is simple and quick enough to be suitable for routine assay.

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