A Histological Method for the Biological Estimation of Vitamin A

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There are considerable differences between physico-chemical and biological results of vitamin A estimations, owing to the fact that the 'analyst determines the proportion of a substance present, whilst the bio-assayist determines an effect' (Morton, 1942). The biological vitamin A value of food or pharmaceutical products is the sum of the values of all substances present having vitamin A activity, such as preformed vitamin A, either as the free alcohol or esters, and various carotenoid pigments. The response of the experimental animal to a given dose of test substance is dependent upon the absorption and utilization of vitamin A and its precursors, and is influenced by the digestibility of the food and the absence or presence of antioxidants. Physicochemical estimations of the vitamin A content of a product, on the other hand, depend in the first instance on determinations of intensity of absorption in the region 325–328 m μ , where vitamin A shows its maximal light absorption. Allowance may have to be made for irrelevant absorption.

To convert extinction coefficients, as determined by physico-chemical means, to biological units, a conversion factor has been adopted. This factor, however, is chiefly based on the results of the curative 'growth' method (Coward, 1947) which, although not specific for vitamin A, is more accurate than methods based on examination of the vagina or the eye (xerophthalmia). The results of various doses of vitamin A upon pathological changes in the central nervous system of the rat led Irving & Richards (1940) to suggest that such changes might form the basis of a prophylactic assay of vitamin A. At weaning, rats were placed on a vitamin A-deficient diet and graded doses of the vitamin were administered. When after 7 weeks the rats were killed, degeneration was found in those receiving 1 i.u. or less daily, but not in those receiving 1.5 i.u. or more, and the demarcation was extremely sharp.

The work presented here was undertaken primarily to determine the suitability of the method suggested by Irving & Richards as a prophylactic assay. Arising from this, the accuracy of the present-day values of the conversion factor was also investigated.

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Degeneration in the central nervous system due to vitamin A deficiency

Degeneration of the central nervous system in young swine, as a specific symptom of vitamin A deficiency, was described as early as 1914 by Hart & McCollum (1914; cf. Hart, Miller & McCollum, 1916). Their ration contained a high proportion of cereals, and it was believed that cereal toxicity was the cause of nervous degeneration. An unknown factor (vitamin A) 'associated with butter fat and certain other fats and found . . . in considerably higher concentration in the leaves . . . of alfalfa' had a protective action against nervous lesions.

Mellanby (1926) reported severe degeneration of the nervous system in puppies placed on high-cereal diets, and stressed the toxicity of cereals in the absence of fat-soluble vitamins. He suggested the name 'toxamin' for the harmful substance and ascribed its toxicity to interference with calcification of bones. His belief in the toxicity of cereals as the cause of degeneration was held for many years (Mellanby, 1930, 1931, 1934). Subsequent workers have not, however, supported it. Hughes, Lienhardt & Aubel (1929), who described nerve degeneration in pigs resulting from avitaminosis A, stated 'In no case, so far as we are aware, has anyone reported the finding of pathological changes in the nervous system accompanying avitaminosis A.' Zimmerman (1933) produced lesions in the nervous system with a diet containing no cereals. Finally, Irving & Richards (1938) replaced the cereal component of the diet by a mixture of tapioca starch and pure glucose (2:1) and proved that the belief in the toxicity of cereals was not justified.

Age is an essential factor in the production of nerve lesions in vitamin A deficiency. The main factors are the amount of vitamin A stored in the animal body at the beginning of the experiment and the effect of rapid growth in the early stages. This has been found by Suzman, Muller & Ungley (1932), Eveleth & Biester (1937), Wolbach & Howe (1925) and Wolbach & Bessey (1940, 1941).

That loss of weight alone does not account for paralysis or degeneration in the cord, was shown by several workers (Aberle, 1934; Wolbach & Bessey, 1940, 1942). Rats received a diet adequate in

vitamin A but restricted in amount, so that their weights paralleled those of litter mates on a vitamin A-deficient diet. All the rats died after approximately the same experimental period, but, in contrast with the controls, those receiving vitamin A or carotene showed no paralysis or degeneration of the cord. It was also demonstrated that neither tocopherol (Wolbach & Bessey, 1942) nor unsaturated fatty acids (Zimmerman, 1933; Zimmerman & Cowgill, 1936) affects the development of nervous lesions.

The general picture of vitamin A-deficiency degeneration in the central nervous system of the rat has been described by Irving & Richards (1937, 1938). Maximum degeneration was usually found in the funiculus praedorsalis at the level of, or just below, the pyramidal decussation. The degeneration gradually became less, and more diffusely spread, at lower levels in the cervical cord. Microscopical examination of a cross-section revealed the site of maximal degeneration in the anterior columns. As the severity of the lesions increased, the posterior columns and especially the column of Burdach, and the spino-cerebellar tracts, gradually became more affected, though the degeneration never reached the same intensity as in the funiculus praedorsalis. Irving & Richards (1937) stated that the 'uniformity with which the condition described occurs in every one of the rats examined is in striking contrast with the variability in pathological condition, and in the occurrence of epithelial metaplasia found in animals in advanced avitaminosis A. The fact that this degeneration in the medulla occurs with such uniformity before the weight curve is affected to any extent, suggests that it is one of the fundamental lesions of vitamin A deficiency.'

The causation of nerve degeneration in vitamin A deficiency. No worker has so far produced a theory which satisfactorily accounts for this. That vitamin A plays an important role in calcification of bones was pointed out by Copp & Greenberg (1945). They found that fracture callus was much smaller in the deficient rat than in the normal animal and the calcification was less active. Mellanby (1938, 1941, 1947) postulated that vitamin A deficiency upset the regulation of skeletal growth by means of interference with the activity of the osteoblasts and osteoclasts. On a surface where osteoclasts were normally active, their activity could be suppressed and that of the osteoblasts might predominate and vice versa. The resulting bony overgrowth caused pressure on the central nervous system and peripheral nerves by squeezing and stretching and resulted in degeneration due to mechanical injuries.

Wolbach & Bessey (1940, 1941, 1942) and Moore & Sykes (1941) agreed with Mellanby that degeneration was caused by mechanical injuries due to pressure. They, however, ascribed this to causes other

than bone growth. The bones ceased to grow with the establishment of vitamin A deficiency, which caused a disproportionate growth of the central nervous system in relation to its bony enclosure. Attention was also drawn to the possibility that vitamin A deficiency accelerated the growth of the central nervous system (Wolbach & Bessey, 1940).

Another factor that needs to be considered here is the increase in spinal fluid pressure that accompanies vitamin A deficiency, as was found by Moore & Sykes (1940, 1941) in bovines. It was suggested by these workers that inco-ordination and nervous lesions might be related to increases in the cerebrospinal fluid pressure. This was due to an increased volume of the cranial contents, caused by either a relative overgrowth as indicated by Wolbach & Bessey, or to bone overgrowth as postulated by Mellanby.

These data indicate that, whatever the mechanism of vitamin A-deficiency degeneration may be, it is still obscure. As far as the hypothesis of cessation of bone growth is concerned, the work of Orr & Richards (1934) and of Irving (1948) disproves it. Bone overgrowth and increased intracranial pressure have been definitely demonstrated. If, however, degeneration was due to any kind of pressure effect, an even distribution of demyelinated fibres would be expected in a cross-section of the cord and not the well-defined degeneration that has been found always to occur in the same tracts.

EXPERIMENTAL

Diet. The composition of the vitamin A-free diet was as follows: Casein, 17; potato starch, 47; arachis oil, 14.1; brewer's yeast, 17.2; salt mixture (McCollum 185), 4.7%.

Owing to post-war restrictions it was impossible to obtain cornstarch and so potato starch was used instead. The arachis oil was obtained in one-gallon tins and was of a good quality. The colour varied from a light straw colour to light brown and no vitamin A or carotenoids could be detected in it by the SbCl_5 test. Before using a new tin it was fortified with calciferol so as to obtain approximately 100 i.u. vitamin D/kg. of mixed diet. It contained enough tocopherol to give maximum vitamin A protection. The casein was extracted with 96% ethanol for about 7-8 hr. before using, care being taken not to overheat the casein during this treatment, in order to avoid diarrhoea in the experimental animals. It is said that rats require no ascorbic acid, but to be on the safe side one ground tablet, equal to 50 mg. vitamin C, was included in every 2 kg. of diet. The vitamin A-free food mixture (without the arachis oil) was mixed in quantities of 3 kg. at a time. Each day the necessary amount of this dry food mixture was weighed out and the corresponding amount of KI solution (0.64 g./l.) added, namely 0.5 ml. for every 70 g. of the former. The oil was rubbed in and afterwards enough water was added to make a stiff paste, which was crumbled up and put into the food pots.

Rats. Young rats of the Wistar Institute strain were employed. Litters of more than nine were reduced to that number at birth. The whole litter, including the mother, was

placed on the vitamin A-free diet 5 days before weaning, which was done at 22 days of age. This procedure had been adopted by Irving & Richards (1938), who found that it produced more consistent results, and that the test animals so treated showed signs of deficiency 7 or more days before those not put on the deficient diet before weaning.

As a general rule, the litters contained more females than males. To save time and material, two different preparations were tested simultaneously against the same standard and with three groups for every oil solution; each assay consisted of nine dosage groups. With a limited number of litters, it was not always possible to start such an experiment with equal numbers of males and females (5, 5) in each group. The distribution was, therefore, always carried out with the main object of starting an experiment with ten rats in each group which consisted as far as possible of five males and five females and to minimize the influence of litter variations.

Oils tested. Biological experiments were carried out on samples of the following, the spectroscopic characteristics (Table 8) of which were also determined first:

- (1) Vaalhaai (*Galeorhinus capensis*) liver oil.
- (2) Vitamin A acetate in arachis oil.
- (3) Vitamin A distillate in arachis oil.
- (4) Snoek (*Thyrstites atun*) liver oil.
- (5) Hake (*Merluccius capensis*) liver oil.
- (6) Tuna (*Germo albacora*) liver oil.
- (7) Dogfish (*Squalus* species) liver oil.
- (8) Stonebass (*Polyprion americanus*) liver oil.

Except for the vitamin A acetate and distillate the rest were natural fish-liver oils, where the vitamin A exists chiefly in the form of esters. Determinations of extinction coefficients were made with the Beckman spectrophotometer using cyclohexane as solvent and only in the case of the dogfish-liver oil was the estimation carried out on the unsaponifiable fraction. The vitamin A acetate and the distillate were dissolved in arachis oil to an *E* value of about 7 and the spectrophotometric estimations were carried out on the solutions.

Conduct of the experiment. To obtain the final dosing solutions, the oils were diluted in steps with arachis oil to a potency of about 60 i.u./g. as calculated from the $E_{1\text{ cm}}^{1\%}$ values using 1600 as conversion factor. Fresh dosing solutions were made up every 2 weeks from a solution of intermediate strength. All the oils were stored in the refrigerator in glass-stoppered bottles. International β -carotene standard and vitamin A acetate were used as biological standards and each experiment was a comparison between standard and test oil responses which were obtained simultaneously. The solution of crystalline vitamin A acetate was made up in pure arachis oil to an $E_{1\text{ cm}}^{1\%}$ value of 6.85. This was used as a standard of reference in a further diluted form after standardization against the International β -carotene standard. Thus the β -carotene preparation was accepted as the primary standard throughout. The U.S.P. vitamin A acetate standard was not used. The acetate referred to was a crystalline preparation obtained commercially.

Dosing started at the beginning of the experiment and vitamin A was administered orally every 3 days by means of a tuberculin syringe with a micrometer-screw attachment, which released a standard drop of 0.01 g. (= 0.6 i.u. vitamin A) per turn. Preliminary experiments showed that satisfactory results were obtained when vitamin A was administered every third day. The different dosage groups re-

ceived, therefore, 1, 2 and 4 turns per rat every third day, which was equal to approximately 0.2, 0.4 and 0.8 i.u. of this vitamin per day. These three levels were selected since it was expected that the dosage-response curve would be a straight line when the response was plotted against the log. of the dose. Owing to the possibility that the time of dosing might have an influence on the absorption and utilization of vitamin A and β -carotene, dosing was always carried out before feeding. The test period lasted for 35–42 days and the weights and eye conditions were taken as criteria as to when to stop the experiment, when the rats were killed with coal gas.

Histological examination of the central nervous system. In the demonstration of degeneration in the nervous system, most workers prefer to use the Marchi method, whilst Kultschitzky's stain and polarized light (Lee & Sure, 1937; Setterfield & Sutton, 1935) are less often applied. In the present experiments the Marchi technique was employed. Irving & Richards (1938) showed that, if proper precautions were taken, this method gave reliable and reproducible results; the method employed followed in the main that adopted by them. Though the Marchi technique is usually considered rather slow, a procedure was evolved whereby it was possible to embed eighty medullas in 1 day. A résumé of the method is therefore given for the benefit of other workers who may use this method of assay.

Rats were killed with coal gas in groups of about ten each. Part of the skin and the muscles on the back were removed to expose the skull and upper cervical vertebrae but without removing the ears, which had distinctive markings. The cranium and spinal canal were opened with great care to avoid injuring the medulla. Having finished that operation, the skull with the central nervous system inside was cut off from the rest of the body. Owing to the tension of the ligaments the cervical vertebrae were then pulled at an angle to the skull. To avoid fixing the medulla in that right-angled position, the axis of the skull was aligned with that of the spinal cord by inserting a sharpened match stick through the trachea into the mouth.

The skulls of the rats belonging to different dosage groups were placed in bottles containing 2.5% (w/v) aqueous $\text{K}_2\text{Cr}_2\text{O}_7$ solutions with corresponding labels. After 5 days the dissection was completed by removing the lower medulla and upper cervical cord (subsequently referred to as the medulla), which was transferred to the same strength of $\text{K}_2\text{Cr}_2\text{O}_7$ solution in a cork-stoppered, flat-bottomed specimen tube, where it remained for a further 5 days. The nervous tissue was very soft at that stage, and it was necessary to handle it with the utmost care to avoid squashing it between the fingers.

The 2.5% $\text{K}_2\text{Cr}_2\text{O}_7$ solution was next poured out and about 3 ml. of a mixture consisting of one part of a 1% (w/v) osmic acid solution in two parts of a 5.3% (w/v) solution of $\text{K}_2\text{Cr}_2\text{O}_7$, thoroughly mixed in a glass-stoppered measuring cylinder, was put into the tube containing the specimen. That solution was changed for a similar one after 5 days for the same period. The tissues were then washed, dehydrated in ethanol, cleared in CHCl_3 and embedded in paraffin.

Sections (10 μ) were cut serially starting from the cord end. Two out of every ten were taken and arranged in sequence on the microscope slide in two parallel rows of ten each. As a general rule, Marchi-stained nervous tissue is very brittle, but the use of the following precautions seemed to improve the method, and sections without any cracks

were obtained. Care was taken to perform the transference of the medullas from the CHCl_3 to the wax baths as quickly as possible, lest the tissues should dry and cracks appear by shrinkage. A vacuum oven was used for the embedding and it was found important to decrease the pressure very gradually as a rapid decrease also resulted in cracks.

Criterion of response. Irving & Richards (1938, 1940) indicated the degree of severity of the nervous lesions by numbers 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.

They added that the degree of degeneration represented by the numbers of 1 and 1 — was of no significance, since an exactly similar effect was observed in positive controls and stock rats, and was an artefact of the Marchi technique. This experience was not confirmed in the present work. No degenerated fibres could be detected under the low power of the microscope in stock rats and positive controls, and the slightest degeneration found in the experimental rats was, therefore, taken as an indication of the first vitamin A-deficiency symptom.

The classification used by Irving & Richards was found to be unsuitable for assay purposes since statistical methods could not be applied. It was found, however, that with a little experience the degeneration could be divided into six different stages, thereby increasing the accuracy of the estimation.

With the object of obtaining a positive curve of response it was decided to use the degree of protection against degeneration as the criterion of response. The value 0 was assigned to a section showing maximal degeneration to indicate that the degree of protection against degeneration was 0 in this case. A section with no degenerated fibres received the value 5 indicating full protection. (See Figs. 3–8, Pls. 6 and 7.)

For the classification of the slides, two microscopes with mechanical stages were used. Slides belonging to the same assay were first classified roughly with one microscope in different groups according to their 'stage of protection'. A standard slide, say of stage 4, was next put under one microscope and slides which were expected to show approximately the same protection were examined individually under the other microscope. By finding first the section in

Table 1. *Estimated degree of protection against myelin degeneration in male rats given by different doses of vitamin A*

(Each numbered experiment gives the result of assays carried out simultaneously. The International Standard was the β -carotene standard preparation, as issued by the National Institute for Medical Research, which was used after further dilution with pure arachis oil to a potency of 60 i.u./g. The vitamin A acetate solution ($E_{1\text{cm}}^{1\%}$ 6.85, see p. 630) was prepared from crystalline material. The letters (a), (b) and (c) below and in the following tables refer to duplicate or triplicate assays carried out on the same preparation. The potency of liver oils was roughly assessed by the gross $E_{1\text{cm}}^{1\%}$ value $\times 1600$. D.P. = degree of protection.)

Exp. no.	Preparation	Dose					
		0.2 i.u./day		0.4 i.u./day		0.8 i.u./day	
		No. males	D.P.	No. males	D.P.	No. males	D.P.
1	Internat. Std. (a)	3	1.5	4	5.0	4	10.0
	Vaalhaai liver oil (a)	3	2.0	3	4.5	4	9.5
2	Internat. Std. (b)	2	1.5	4	2.0	4	4.5
	Vaalhaai liver oil (b)	2	0.5	4	2.5	4	6.5
3	Vit. A acetate (a)	3	6.5	4	11.5	4	14.0
	Internat. Std. (a)	3	7.0	4	9.5	4	16.0
	Vit. A distillate (a)	3	8.5	4	9.0	4	16.5
4	Vit. A acetate (b)	5	10.0	5	10.5	5	16.5
	Internat. Std. (b)	5	9.5	5	9.5	5	15.0
	Vit. A distillate (b)	5	12.5	5	14.0	4	15.5
5	Snoek liver oil (a)	4	3.5	4	7.5	5	13.0
	Vit. A acetate Std. (a)	4	1.5	5	9.5	4	10.0
	Hake liver oil (a)	3	1.5	4	7.5	5	16.5
6	Snoek liver oil (b)	6	4.0	6	10.0	5	10.5
	Vit. A acetate Std. (b)	6	5.5	5	6.0	5	18.5
	Hake liver oil (b)	6	7.0	6	10.0	5	15.0
7	Tuna liver oil (a)	5	6.0	5	12.0	5	19.0
	Internat. Std. (a)	5	6.0	5	11.5	5	19.0
	Dogfish liver oil (a)	4	4.5	5	8.5	5	19.0
8	Tuna liver oil (b)	5	2.5	5	10.5	5	14.5
	Internat. Std. (b)	5	7.0	5	8.5	5	15.5
	Dogfish liver oil (b)	5	4.0	4	4.5	5	11.0
9	Stonebass liver oil (a)	5	1.5	4	2.0	5	13.0
	Vit. A acetate Std.	6	4.5	6	5.5	6	18.5
	Stonebass liver oil (b)	4	2.5	5	8.5	4	8.0
	Vaalhaai liver oil (c)	4	2.0	4	5.0	5	12.5
Totals		111	123.0	120	205.0	121	357.5
Weighted means			1.108		1.708		2.954

the two rows revealing the maximal degeneration and, secondly, by comparing the number and size of the black areas in this section with those of the standard under the first microscope, it was easy to recognize the actual stage of protection. If the black spots showed a similar intensity to that of the standard, that specific slide was taken as stage 4, while a little more degeneration denoted stage 3·5 and a little less stage 4·5. This procedure was repeated for every group. After a little experience had been gained, it was found that the classification of the same slides undertaken a few weeks later showed no significant difference.

RESULTS

Difference in response of males and females. Coward (1932) described the difference in response to vitamin A dosage of male and female rats. She found that males gave results with a greater standard deviation than that given by female rats. However, the steeper slope of the curve of response of the former caused them to give results of greater accuracy than those obtained with female rats. Brenner, Brookes & Roberts (1942) observed a significant sex difference in the liver storage of vitamin A in rats. 'The females stored and retained more vitamin A in the liver than the males, while the

males had higher blood values.' Using the fluorescence technique to demonstrate vitamin A in the

Fig. 1. Curve relating the degrees of protection of the central nervous system in male and female rats to the logarithm of the dose of vitamin A. —, females; ---, males.

liver, Popper & Brenner (1942) came to the conclusion that 'During depletion the livers of male rats

Table 2. *Estimated degree of protection against myelin degeneration in female rats given by different doses of vitamin A*

(See sub-heading to Table 1.)

Exp. no.	Preparation	Dose					
		0.2 i.u./day		0.4 i.u./day		0.8 i.u./day	
		No. females	D.P.	No. females	D.P.	No. females	D.P.
1	Internat. Std. (a)	6	6.0	5	17.0	5	21.0
	Vaalhaai liver oil (a)	6	12.0	5	10.5	5	20.5
2	Internat. Std. (b)	5	4.0	4	8.5	4	15.5
	Vaalhaai liver oil (b)	5	5.5	4	4.5	4	8.5
3	Vit. A acetate (a)	8	21.0	6	21.5	6	24.5
	Internat. Std. (a)	8	25.5	6	20.5	6	23.5
	Vit. A distillate (a)	8	23.5	6	18.0	6	25.5
4	Vit. A acetate (b)	5	11.0	5	17.5	5	22.0
	Internat. Std. (b)	5	14.5	5	19.5	5	22.0
	Vit. A distillate (b)	5	16.5	5	18.0	5	26.0
5	Snoek liver oil (a)	6	11.0	6	15.5	5	12.0
	Vit. A acetate Std. (a)	6	8.0	5	12.5	5	21.0
	Hake liver oil (a)	6	10.5	6	19.5	5	20.5
6	Snoek liver oil (b)	4	3.5	4	9.5	5	20.0
	Vit. A acetate Std. (b)	4	6.0	5	5.5	5	22.0
	Hake liver oil (b)	4	9.0	4	11.0	5	23.5
7	Tuna liver oil (a)	5	12.0	5	13.5	5	22.5
	Internat. Std. (a)	5	14.0	5	17.5	5	21.5
	Dogfish liver oil (a)	5	8.5	5	15.5	4	17.5
8	Tuna liver oil (b)	5	7.5	5	13.5	5	22.0
	Internat. Std. (b)	5	7.5	5	15.5	5	18.5
	Dogfish liver oil (b)	5	8.0	5	11.5	5	18.5
9	Stonebass liver oil (a)	5	2.0	6	17.5	5	20.5
	Vit. A acetate Std.	6	12.0	5	13.5	6	25.5
	Stonebass liver oil (b)	6	8.5	5	10.0	6	25.5
	Vaalhaai liver oil (c)	6	9.0	6	13.0	4	13.0
Totals		144	276.5	133	380.0	131	533.0
Weighted means			1.920		2.857		4.069

lose vitamin A faster than those of females. There is, however, no sex difference with respect to method of utilization, as judged from the histologic picture.' These observations are in accord with the results obtained with male and female rats as demonstrated in Tables 1 and 2.

The dosing solutions were made up so as to contain approximately 0.6 i.u. of vitamin A per 10 mg. When dosing every 3 days, the 0.2 i.u. per day group of rats received 10 mg. of this oily solution, while the 0.4 and 0.8 i.u. per day groups were given 20 and 40 mg., respectively. The degrees of protection of the individual rats were added together and appear in columns 4, 6 and 8.

By plotting the average results obtained in Tables 1 and 2 against the logarithms (to the base 10) of the doses the curves of response proved to be straight lines (see Fig. 1).

The average protection of females against degeneration was, however, $2.949/1.923 = 1.533$ times that of the males and it was, therefore, justifiable to assume that the minimum vitamin A requirement of male rats (about 9 weeks of age) was about 1.5 times that of females of the same age. Several workers have shown that the vitamin A requirement is a function of the body weight (Guilbert & Hart,

1935; Hart, 1940; Callison & Knowles, 1945; Paul & Paul, 1946). However, the latter alone cannot account for the sex difference in the daily requirement of this vitamin, or vice versa for the sex difference of protection against degeneration, since it is evident from the figures in Table 3 that the ratio of the body weights of male and female rats bears no relation to the protection ratio.

Curve of response. The sex difference in the response of rats to the same dose of vitamin A created a difficulty. It was impossible to use one sex only owing to the limitations in the breeding facilities. Two alternatives to solve the problem remained, namely, to keep the females longer on experiment until they reached a degeneration stage equal to that of the males, or to use equal numbers of males and females in all the groups. The former possibility was considered impracticable owing to the fact that it took more than 3 weeks to determine the stage of degeneration. With limited numbers of rats the latter alternative could not be followed.

To overcome this trouble of sex difference and to obtain results which might lend themselves readily to statistical analysis, Prof. S. J. Pretorius of Stellenbosch University suggested personally that an average factor should be calculated for each experi-

Table 3. Average weights of rats at the end of the test period

Preparation	Dose					
	0.2 i.u./day		0.4 i.u./day		0.8 i.u./day	
	Males (g.)	Females (g.)	Males (g.)	Females (g.)	Males (g.)	Females (g.)
Internat. Std. (a)	99	85	112	89	108	90
Vaalhaai liver oil (a)	101	84	111	87	102	93
Internat. Std. (b)	86	83	92	87	109	89
Vaalhaai liver oil (b)	84	85	97	84	113	87
Vit. A acetate (a)	135	105	140	99	151	116
Internat. Std. (a)	135	108	145	104	143	110
Vit. A distillate (a)	115	107	142	108	155	109
Vit. A acetate (b)	74	74	79	73	91	74
Internat. Std. (b)	70	73	80	70	94	82
Vit. A distillate (b)	72	76	78	77	98	74
Snoek liver oil (a)	81	86	100	83	113	85
Vit. A acetate Std. (a)	75	85	102	79	91	103
Hake liver oil (a)	85	82	95	86	103	88
Snoek liver oil (b)	88	69	94	83	109	90
Vit. A acetate Std. (b)	91	81	100	90	112	94
Hake liver oil (b)	93	79	91	84	105	87
Tuna liver oil (a)	100	80	102	85	111	88
Internat. Std. (a)	96	84	100	92	112	85
Dogfish liver oil (a)	99	81	109	89	112	88
Tuna liver oil (b)	91	81	99	84	106	94
Internat. Std. (b)	100	86	96	95	107	95
Dogfish liver oil (b)	93	81	105	89	111	99
Stonebass liver oil (a)	75	84	91	89	106	90
Vit. A acetate Std.	90	81	95	85	113	96
Stonebass liver oil (b)	88	82	84	89	114	81
Vaalhaai liver oil (c)	85	80	86	91	121	93
Totals	2441	2179	2626	2271	2910	2380
Averages	94	84	101	87	112	91
Ratio males/females	1.12		1.16		1.22	

ment by which to multiply the response of every individual female in that assay, so as to obtain a response corresponding to that of a male or vice

Table 4. *Estimated protection against myelin degeneration given by different doses of International Standard β -carotene*

(Protection is expressed in terms of the results on single rats, transformed as indicated in text to eliminate sex differences.)

	Daily doses of International Standard		
	0.2 i.u. D.P.	0.4 i.u. D.P.	0.8 i.u. D.P.
	1.0	1.5	3.5
	1.0	3.0	3.0
	0.5	1.5	3.5
	2.0	3.0	4.5
	1.5	2.5	4.5
	2.71	3.05	2.71
	0.68	3.05	2.37
	1.70	1.70	3.39
	3.50	1.70	3.05
	1.36	2.37	3.05
	1.0	0.0	3.0
	2.0	2.0	3.5
	0.5	2.5	3.5
	1.5	2.0	2.5
	2.0	2.0	3.0
	1.26	1.90	2.84
	0.32	1.90	1.26
	1.26	0.95	2.52
	0.95	2.84	2.84
	0.95	2.21	2.21
Totals	27.24	41.67	60.74
Weighted means	1.362	2.0835	3.037

versa. Such a factor represents the ratio of average response of males to average response of females in the present experiments. This suggestion was tested out and gave satisfactory results. Such transformed

results were used in calculating the vitamin A potencies of the different oils.

The log dose-response curve with this method was a straight line both for the β -carotene standard (Table 4) and vitamin A-containing oils (Table 5). The data of these two tables were used to plot the graphs in Fig. 2.

Fig. 2. Curve relating the degrees of protection of the central nervous system to the logarithm of the doses of β -carotene standard and fish-liver oils. —, β -carotene standard; ---, fish-liver oils.

Owing to the fact that the response of rats to a given dose of vitamin A is subject to fluctuations, 'it is absolutely essential that each test should be a comparison between the response given to a dose of the test substance and the response given to a dose of the standard at the same time' (Coward, 1947, p. 2). Experimental rats must be distributed in equal numbers between the biological standard and the substances to be tested and the slope of the response curve must be obtained from every individual test

Table 5. *Estimated degree of protection against myelin degeneration given by different doses of vitamin A*

Oil sample	Dose					
	0.2 i.u.		0.4 i.u.		0.8 i.u.	
	No. of rats	Value for group	No. of rats	Value for group	No. of rats	Value for group
Snoek (a)	10	9.76	10	16.34	10	24.96
Vit. A acetate Std. (a)	10	6.05	10	16.61	9	21.95
Hake (a)	9	7.47	10	18.63	10	28.18
Snoek (b)	10	6.17	10	15.89	10	22.93
Vit. A acetate Std. (b)	10	9.22	10	15.62	10	32.17
Hake (b)	10	12.58	10	16.82	10	29.62
Tuna (a)	10	14.15	9	21.15	10	35.25
Dogfish (a)	9	10.27	10	19.01	9	30.86
Tuna (b)	10	7.23	10	19.03	10	28.37
Dogfish (b)	10	9.05	9	11.75	10	22.67
Stonebass (a)	10	2.58	10	11.39	10	24.02
Vit. A acetate Std.	12	10.96	11	11.39	10	24.02
Stonebass (b)	10	7.09	10	13.89	10	21.71
Vaalhaai (c)	10	6.86	10	12.00	9	19.49
Totals	140	119.44	139	220.88	139	374.39
Weighted means		0.8531		1.5891		2.6934

itself. The slope from a previously constructed dosage-response curve cannot be used, because 'large changes of slope may occur with time, and... these changes do not occur in a random fashion but exhibit a secular or quasiperiodic tendency. The use of a fixed slope therefore introduces an uncontrolled error for which it is impossible to allow. Even if

results are summarized in Table 7. A comparison of the limits of error in this table and those given by Gridgeman (1944) and Irwin (1937) for the 3 weeks' curative 'growth' method indicates that this histological method for the bio-assay of vitamin A is as accurate as and possibly more accurate than the 3 weeks' curative 'growth' test.

Table 6. *Fluctuations in the slopes of the log dose-response curves*

Section no.	Test oil	— Standard	Test period	Slope *
1	Vaalhaai (a)	— Internat. Std.	2. iii. 47–18. iv. 47	0.786
2	Vaalhaai (b)	— Internat. Std.	24. iii. 47–6. v. 47	0.475
3	Vit. A acetate (a)	— Internat. Std.	27. iv. 47–29. vi. 47	0.563
	Vit. A distillate (a)	— Internat. Std.	27. iv. 47–29. vi. 47	0.547
4	Vit. A acetate (b)	— Internat. Std.	1. vi. 47–14. vii. 47	0.635
	Vit. A distillate (b)	— Internat. Std.	1. vi. 47–14. vii. 47	0.521
5	Snoek (a)	— Vit. A acetate Std.	21. vi. 47–11. viii. 47	0.838
	Hake (a)	— Vit. A acetate Std.	21. vi. 47–11. viii. 47	0.956
6	Snoek (b)	— Vit. A acetate Std.	21. vii. 47–7. ix. 47	0.993
	Hake (b)	— Vit. A acetate Std.	21. vii. 47–7. ix. 47	1.000
7	Tuna (a)	— Internat. Std.	12. viii. 47–25. ix. 47	0.954
	Dogfish (a)	— Internat. Std.	12. viii. 47–25. ix. 47	1.017
8	Tuna (b)	— Internat. Std.	14. ix. 47–23. x. 47	0.914
	Dogfish (b)	— Internat. Std.	14. ix. 47–23. x. 47	0.726
9	Stonebass (a)	— Vit. A acetate Std.	31. x. 47–12. xi. 47	0.970
	Stonebass (b)	— Vit. A acetate Std.	31. x. 47–12. xi. 47	0.815
	Vaalhaai (c)	— Vit. A acetate Std.	31. x. 47–12. xi. 47	0.819

* The values of these slopes are not of the same magnitude as those previously used.

regular records of slopes are kept, and their standard deviation over a considerable period of time calculated, this does not get over the difficulty. For the variations are not random, and so this standard deviation gives no clue to the error of the slope at any particular time' (Irwin, 1944). This author demonstrated further that it is faulty to take the position and slope of the response curve given by the doses of vitamin A standard as correct and to use that for calculating the potency of the test substance. The correct procedure is to find the slope of the best fitting straight line through all the observed points; 'that is to say, it assumes that apart from sampling error the slopes provided by the standard and the test substance are the same. The horizontal distance between the parallel straight lines then provides an estimate of the potency ratio.'

A similar method of calculation was used in this work and Table 6 illustrates the fluctuations which were found in the slopes of the response curves.

The slopes found for every section show very good agreement, but significant differences exist between slopes of different assays, e.g. section no. 3 and section no. 6. These data therefore confirm the statement of Coward quoted above.

As the log dose-response curve is a straight line, the statistical method described in the British Standards Institution Specification no. 911 (1940) was used to analyse the experimental data and the

Table 7. *Limits of error and conversion factors estimated for different vitamin A preparations*

(The average number of rats used in an assay of one oil was 30.)

Oil sample	Conversion factor	Average conversion factor	Limits of error (%)
Vaalhaai (a)	1805	—	84.2–118.9
(b)	1659	—	51.1–185.3
(c)	1391	1618	74.3–130.9
Vit. A acetate (a)	1818	—	52.6–151.2
(b)	1774	1796	54.6–179.5
Vit. A distillate (a)	1455	—	63.6–156.2
(b)	2459	1957	67.9–292.2
Tuna (a)	1776	—	75.6–132.1
(b)	1684	1730	78.4–126.8
Dogfish (a)	1557	—	75.1–130.4
(b)	1265	1411	69.4–135.8
Snoek (a)	1788	—	75.9–134.0
(b)	1234	1511	71.8–134.0
Hake (a)	1951	—	73.5–141.6
(b)	1683	1817	75.7–133.0
Stonebass (a)	1408	—	78.9–124.5
(b)	1538	1473	77.0–128.0

The given doses of both the vitamin A standard and the test oil were always in geometrical progression. This did not apply to the total vitamin A intake, owing to the fact that the rats received small but equal amounts of carotene derived from the

potato starch. Such an extra allowance, although small, would obviously tend to interfere with the log dose-response relationship. Therefore, a more satisfactory vitamin A-free diet would probably increase the accuracy of this method.

Correction for irrelevant absorption. Until recently it was believed that the full intensity of absorption at 328 m μ . was due to the presence of vitamin A. This assumption was proved to be unjustified, owing to the presence of irrelevant absorbing substances, even in high-potency oils and their unsaponifiable fractions.

The influence of the correction for irrelevant absorption, as suggested by Morton & Stubbs (1946, 1948) on the conversion factors obtained in this work, is illustrated in Table 8.

Table 8. *Influence of correction for irrelevant absorption on the conversion factor*

Preparation	Average* potency (i.u./g.)	<i>E</i>		Conversion factor	
		Observed	Corrected	Observed	Corrected
Vaalhaal liver oil	15,000	9.26	8.68	1618	1727
Vit. A acetate	12,300	6.85	6.80	1796	1810
Vit. A distillate	16,500	8.44	8.15	1957	2028
Tuna liver oil	129,900	75.10	67.50	1730	1925
Dogfish liver oil	5,710	4.05	3.60	1411	1586
Snoek liver oil	26,700	17.64	16.56	1511	1610
Hake liver oil	13,900	7.68	7.25	1817	1924
Stonebass liver oil	78,600	53.37	46.75	1473	1682
		Averages		1665†	1786

* International β -carotene preparation accepted as primary standard throughout.

† The average value for the liver oils only was 1593.

It is evident from the data in Table 8 that a conversion factor of 1600 is preferable for uncorrected *E* values and that the conversion factor of 2000 which is generally used in America, is definitely too high. A higher conversion factor should be used for corrected *E* values and the value of 1800 suggested by Morton & Stubbs (1948) is supported by these data.

DISCUSSION

The main objection to the use of a prophylactic method is that it takes longer to perform than the curative test. Time, labour and money, however, are saved at the feeding stage of this assay, seeing that the test period is not longer than 6 weeks, whereas the depletion period of the curative method takes about 5 weeks and the test period is at least 3 weeks in addition. Thus, the experimental periods for this prophylactic method and the curative test are 6, and at least 8 weeks, respectively.

The curative method has the further disadvantage that frequently rats have to be rejected at the end of the preliminary period, because their weight curves render them 'unsuitable' for the test. A further reduction in the number of available animals may result owing to losses by death during the depletion

period. The curative method is also carried out on animals which may differ widely in pathological condition, and this main source of error is avoided by using the prophylactic method. In this bio-assay no animals need to be discarded.

SUMMARY

1. Degeneration in the central nervous system is a characteristic symptom of vitamin A deficiency in rats. The Marchi technique is sufficiently accurate for following such degenerative changes in the myelin substance of the central nervous system, provided that it is properly used. With slight modifications in histological procedure it can be used for quantitative routine work.

2. A histological method for the biological estimation of vitamin A is described. If groups of young rats are kept under the same experimental conditions and receive graded doses of vitamin A over a suitable range, equal proportional increases in doses produce approximately equal average increases in protection against degeneration in the central nervous system.

3. There exists a significant difference between the response (protection against degeneration) of male and female rats to the same dose of vitamin A. This difference indicates that the minimum vitamin A requirement of male rats is about 1.5 times that of females of the same age. Although it is generally assumed that vitamin A requirement is a function of body weight, the latter alone cannot account for the sex difference.

4. The vitamin A potencies of vitamin A acetate, vitamin A distillate and six natural fish-liver oils were estimated by means of this histological method and corresponding conversion factors were calculated to test out the reliability of the present conversion factors.

5. The experimental data were analysed statistically with the result that the histological method seemed to be as accurate as, and possibly more accurate than, the 3 weeks' curative 'growth' test.

6. The calculated conversion factors indicate that 1600 is preferable to 2000 for uncorrected *E* values. A higher factor should be used for corrected *E* values.

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EXPLANATION OF PLATES 6 AND 7

Cross-sections of the medulla showing different stages of protection against degeneration.

Marchi-stained sections of the cord do not lend themselves readily to photography, especially at lower magnifications. The contrast of the normal photomicrograph is insufficient, especially for comparisons of the lower stages of degeneration. To overcome this trouble and to produce a greater degree of contrast, it was found necessary to transpose the negatives, which decreased the detail of the undegenerated structure and increased that of the degenerated tracts.

Figs. 3-8 show the six stages of protection against degeneration; (a) in each case, shows the entire spinal cord

($\times 14$) and (b) the corresponding anterolateral column ($\times 45$), where the degeneration on which the classification was based chiefly occurred.

It is evident from these photomicrographs that the first degeneration in vitamin A deficiency occurs in the funiculus praedorsalis and develops gradually in the columns of Goll and Burdach. Even in the second stage of protection the posterior columns are affected to only a very slight degree in comparison with the degeneration in the anterolateral columns. However, the severity of degeneration in the posterior columns increases rapidly in the later stages and reaches the same degree as in the anterior columns when there is no protection.