IV. THE RELATIVE VITAMIN A VALUE OF THE BODY AND LIVER OILS OF CERTAIN FISH.

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INTRODUCTION.

IN view of the fact that the biological method for the estimation of vitamin A is not only tedious and expensive, but is open to errors of a rather disturbing magnitude, the need for a more satisfactory method has always been keenly felt. In 1925 Rosenheim and Drummond described the AsCl_3 test as the basis of a quantitative method of assay, but the method was greatly improved by the use of SbCl_3 and other modifications introduced by Carr and Price [1926]. Drummond and his co-workers have presented evidence that these colour reactions are of practical value in the assay of vitamin A in liver oils, and support for this view has been brought forward by Rosenheim and Webster [1926, 1927], Willimott and Wokes [1927] and others. On the other hand some investigators do not regard the reaction as specific for vitamin A. Some of their objections have been dealt with by Drummond and Morton [1929] and have been shown to be based on unsatisfactory evidence, but those raised by Schmidt-Nielsen were, however, more serious, in that superficially it did not appear that the discrepancies could be attributed to obvious differences of technique.

The work recorded in this paper was undertaken for the purpose of examining the claims of Schmidt-Nielsen, and relates to a number of fish-body and -liver oils which have been studied both colorimetrically and biologically. While it was in progress, Norris and Danielson [1929] published data concerning colorimetric and biological tests of the vitamin A values of some Pacific coast salmon-body oils. They concluded that the results of the colorimetric assays agreed within reasonable limits with those obtained from the biological method. Both their colorimetric and biological values (3–10 units, United States Pharmacopoeia) are, however, so low that, in our opinion, their significance is not great.

Schmidt-Nielsen [1928, 1, 2, 3] stated that the body oils of sprat, mackerel and herring as well as the liver oil of the basking shark were, according to his biological tests, good sources of vitamin A, but that they did not react with

arsenic or antimony trichloride to give the characteristic blue colour. Generally speaking he found that a blue colour was given by the liver oils of bony fishes such as cod, tusk, ling and haddock, whereas the liver oils of most of the Elasmobranchs, sharks and rays, gave only a feeble reaction, the colour quickly changing to reddish or brown. On the other hand, the liver oil of salmon gave an intense blue colour with SbCl_3 (150–200 blue units on the Rosenheim-Schuster colorimeter), which apparently bore no relation to its vitamin A value as indicated by the animal tests. These results led him to conclude that there is no strict relation between the colour reaction and the biological assay for vitamin A, and that the blue colour reaction is probably due to substances which sometimes accompany the vitamins.

EXPERIMENTAL.

The body and the liver oils of salmon and herring were prepared in the laboratory by grinding the flesh or the livers with anhydrous Na_2SO_4 , and extracting the dry powder with ether in an atmosphere of nitrogen. The ether was removed in an atmosphere of nitrogen, and the oils were sealed in amber glass ampoules in which the air was replaced by the inert gas. Salmon body and liver gave deep orange-red oils, the yield being approximately 11.5 and 7.0 % respectively, whilst herrings gave a yellow body oil and a brownish-red liver oil in yields of approximately 7.6 and 10.0 %. The herring-liver oil had a distinct green fluorescence. Dr Schmidt-Nielsen also very kindly supplied us with samples of herring-body oil and a sample of the liver oil of the basking shark examined by him [Schmidt-Nielsen, 1928, 2; 1929]. These six samples were submitted to a comparative assay of vitamin A by the colorimetric and biological methods.

The colorimetric test was carried out according to Carr and Price's [1926] modification of Rosenheim and Drummond's method [1925], the colours being matched after 30 seconds. In the case of body oils which do not give a perceptible blue colour in 10 % dilution in chloroform, 0.2 cc. of the oil itself was used and is referred to as 100 % dilution in the table. A mean of at least three readings was taken. The original colour of the oil was also measured with the same colorimeter in suitable dilutions.

In the case of oils which show a very deep blue colour with SbCl_3 , it is often not possible to obtain a satisfactory match at 10 % dilutions. With the Rosenheim-Schuster colorimeter the most reliable readings are usually obtained by matching at dilutions which give 5–8 blue units. As the intensity of the blue colour is not strictly proportional to the degree of dilution, it is necessary to obtain five or six readings at different dilutions and plot an intensity-dilution curve, by reference to which 10 % dilution values can be interpolated for purposes of comparison. The values of the herring and salmon-liver oils were obtained in this manner.

The technique of the biological test employed was as follows.

Young rats at an average weight of 45–50 g. were put on a vitamin A-free basal diet consisting of

Purified caseinogen	...	20 parts
Purified rice starch	...	71 „
Salt mixture	...	4 „
Dried brewer's yeast	...	5 „

The antirachitic factor was supplied in the form of irradiated ergosterol prepared in the laboratory. A dose of 0.01 mg. of this preparation dissolved in ethyl oleate was given twice weekly to each rat throughout the preparatory and the testing periods.

The majority of the rats showed a steady weight after 4–5 weeks on the basal diet. They were then given measured supplements of the oils, which were administered directly into the mouth every morning. Ethyl oleate was used as the diluent, and the solutions were kept in brown glass ampoules of small capacity, and stored in a vacuum desiccator in a cold, dark cupboard. Fresh solutions were made every week or 10 days¹.

The tests of the supplements were carried on usually for 3 weeks in the case of rats which showed little or no growth, whilst they were continued for 4–6 weeks if the material under investigation induced appreciable growth. In the former case the supplement was changed to an adequate dose of another oil known to contain vitamin A, in order to demonstrate that the animal was still capable of growing. In a few cases, however, the rats rapidly declined in weight within a few days, with an onset of deficiency symptoms, and died before they could be saved by the administration of vitamin A.

The results of the colorimetric and biological tests are recorded in Tables I and II respectively, and the Figures 1–6 illustrate representative growth curves.

It will be evident from the data there given that the results are, on the whole, reasonably definite. The figures in the last column of Table I show that the SbCl_3 reaction is practically negative for the three body oils from salmon and herring, and for Dr Schmidt-Nielsen's preparation of the liver oil of the basking shark. The blue units given by herring-liver oil are of the same order as those of a good average sample of cod-liver oil, while those of the salmon-liver oil are about six times greater.

The results of biological tests are in satisfactory agreement with those of the colorimetric tests.

¹ The use of ethyl oleate as a diluent for this purpose was thought at first to be a great improvement in technique [Drummond and Morton, 1929]. It has been found unsatisfactory, however, in one respect. Probably owing to the presence of acids more highly unsaturated than oleic, autoxidative changes tend to occur and may involve the substance being tested. These can be prevented by replacing the natural antioxidant in olive oil by a substance such as quinol. In this investigation we guarded against deterioration of the solutions by making them up freshly every week. We have satisfied ourselves that the vitamin D content of the solution of irradiated ergosterol in ethyl oleate does not fall appreciably over a period of several weeks.

Table I. *Colorimetric tests.*

Nature of the oil	Dilution (%)	Colorimetric units of the oil	Colorimetric units with SbCl_3 reagent
Salmon-body oil	10	5.0 yellow 4.6 red	Yellowish red tint without any blue
	100	—	0.4 red 0.2 blue 1.5 yellow
Herring-body oil	10	—	Yellowish tint changing to red after 1-2 min.
	100	6.0 yellow 1.0 red	0.2 red 0.5 yellow 0.3 blue Red increasing after 1-2 min.
Herring-body oil (Schmidt-Nielsen)	100	0.6 red 2.8 yellow	0.2 red 0.6 yellow 0.3 blue After standing 2 min. 0.6 red 0.9 yellow 0.3 blue
			0.3 red 0.6 yellow No perceptible blue Forms a white precipitate
Basking shark-liver oil (Schmidt-Nielsen)	10	—	
	100	0.8 red 4.0 yellow	
Herring-liver oil	10	3.0 red 0.2 blue 0.2 yellow	11.2 blue — yellow
Salmon-liver oil	10	30.0 yellow 7.5 red	59.0 blue 13.6 yellow

Fig. 1. Salmon-body oil.

Fig. 2. Herring-body oil.

Fig. 3. Herring-body oil (Schmidt-Nielsen).

Fig. 4. Basking shark-liver oil (Schmidt-Nielsen).

Fig. 5. Herring-liver oil.

Salmon-body oil did not produce any appreciable amount of growth in any of the five rats employed, in doses of 10, 50 or 100 mg. Four of these rats showed a normal response when later the supplements were changed to 2 mg. of salmon-liver fat (Fig. 1). Rat No. 14, however, rapidly declined in weight, had acute ophthalmia of both eyes and died after 13 days. Ophthalmia was also acute in rat No. 10, of a moderate degree in rat No. 4, and only as a translucent film on the cornea of rats No. 15 and No. 16.

Table II. *Biological tests.*

Nature of the oil	Rat No.	Sex	Dose (mg.)	Total time of test (days)	Total change in weight (g.)	Average growth per week (g.)
Salmon-body oil	10	♂	10.0	27	- 4	- 1.0
	14	♂	10.0	13	- 11	- 5.9
	4	♂	50.0	17	+ 4	+ 1.6
	15	♂	100.0	20	- 6	- 2.1
	16	♂	100.0	20	+ 2	+ 0.7
Herring-body oil	37	♂	10.0	42	+ 10	+ 1.6
	42	♂	10.0	6	- 2	- 2.3
	57	♂	10.0	7	- 4	- 4.0
	34	♂	50.0	25	- 1	0.0
	36	♂	100.0	24	0	0.0
Herring-body oil (Schmidt-Nielsen)	33	♂	10.0	67	+ 9	+ 0.9
	56	♂	10.0	10	- 20	- 14.0
	71	♂	10.0	24	- 2	- 0.6
	30	♂	50.0	25	- 5	- 1.4
	25	♂	100.0	22	- 20	- 6.3
Basking shark-liver oil (Schmidt-Nielsen)	36	♂	1.0	10	- 10	- 7.0
	41	♂	2.0	16	- 14	- 6.1
	48	♂	2.0	65	+ 25	+ 2.7
	32	♂	10.0	23	- 10	- 3.0
	44	♂	10.0	10	- 5	- 3.5
Herring-liver oil	55	♂	10.0	15	- 14	- 6.5
	47	♂	20.0	16	- 8	- 3.5
	27	♂	2.0	19	+ 20	+ 7.3
	26	♂	4.0	19	+ 27	+ 9.9
	28	♂	4.0	19	+ 23	+ 8.4
Salmon-liver oil	5	♂	0.3	33	+ 14	+ 3.0
	6	♂	0.5	33	+ 22	+ 4.7
	21	♂	1.0	30	+ 30	+ 7.0
	23	♂	1.0	30	+ 31	+ 7.0
	13	♂	2.0	30	+ 30	+ 7.0
	24	♂	2.0	30	+ 41	+ 9.6
	4	♂	2.0	16	+ 20	+ 8.7
	15	♂	2.0	14	+ 17	+ 8.5
	16	♂	2.0	14	+ 19	+ 9.5
	32	♂	2.0	17	+ 20	+ 8.2
	41	♂	2.0	28	+ 28	+ 7.0

Fig. 6. Salmon-liver oil.

Herring-body oil was also tested in doses of 10, 50 and 100 mg. Some of the test rats showed a slight tendency to grow during the first two weeks, then became steady in weight and finally declined showing ophthalmia and other symptoms of vitamin A deficiency. Only rat No. 37 showed an appreciable

degree of growth by increasing 20 g. in the first 20 days of the test. Later its weight declined, and it finally died on the 42nd day, having acute ophthalmia of both eyes during the last week.

Herring-body oil obtained from Dr Schmidt-Nielsen was tested in the same doses as the other herring-body oil, but four of the rats showed no growth and generally rapidly declined in weight. Rat No. 33, however, like the one mentioned in the last paragraph, put on weight in the first 3 weeks, then steadied, declined and died with all the symptoms of vitamin A deficiency. In view of such abnormal cases it is wise to maintain the test for at least 5–6 weeks before conclusions regarding the vitamin A potency are reached. The importance of a long test period has been emphasised by Coward and Key [1928] and Drummond and Morton [1929].

The liver oil of the basking shark was tested in doses of 1, 2, 10 and 20 mg. and gave negative results. Some of the rats after 3 weeks of declining weight were given 2 mg. of salmon-liver oil and promptly showed a very rapid response in growth (Fig. 4).

The herring-liver oil gave normal growth in doses of 2–4 mg. (Fig. 5). The limited quantity of the oil did not permit further tests with smaller doses or for longer periods.

The salmon-liver oil was as potent biologically as was to be expected from its colorimetric reaction. As the tests presented in Table II will show, it gave appreciable growth in doses of 0.3 and 0.5 mg. per day and a very good response in 1.0 and 2.0 mg. doses (Fig. 6).

DISCUSSION.

The biological results are, we think, in reasonably good agreement with the colorimetric tests. The three body oils of herring and salmon, and the liver oil of the basking shark neither gave a positive colorimetric test nor showed activity in the biological tests. An average sample of cod-liver oil which induces normal growth by a dose of 2–4 mg. per day shows colorimetrically about 10 blue units in 10 % dilution. The herring-liver oil with 11.2 blue units is found to be biologically active in a dose of 2 mg. The salmon-liver oil which shows 59.0 blue units is active in 0.3 mg. dose. The results agree with each other as well as can be expected, having regard to the variations to which the biological test is liable [Drummond and Morton, 1929].

The results of the colorimetric tests are similar to those recorded by Schmidt-Nielsen, except that he obtained 150–200 blue units for his sample of salmon-liver oil. In view of the probability that Schmidt-Nielsen refers his units to 20 % dilutions, as was originally proposed by Carr and Price [1926], it must be borne in mind that the intensity of the blue colour is not in strict mathematical relation to the dilution employed. In higher dilutions the curve slopes away from the intensity axis, *i.e.* the observed intensity is lower than the calculated one. This point has also been emphasised by Wokes and Willmott [1927, 1, 2]. If an oil gives 10.0 blue units in 1 % dilution, it will usually

give values of the order of 60–65 units in 10 % dilution and not 100 units as would be expected, though the steepness of the curve varies with the individual oils. The apparent disagreement between the two colorimetric tests for salmon-liver oil may arise because Schmidt-Nielsen refers his units to 20 % dilutions and obviously calculates them from a lower dilution.

The biological results of Schmidt-Nielsen deviate very widely from ours. His animal tests led him to assign 50–100 units (U.S.P.) to a number of samples of herring-body oils, and 100 units (U.S.P.) to the sample he so kindly sent us for a comparative test. Our examination of this sample failed to detect the presence of appreciable amounts of vitamin A in doses as high as 100 mg. The sample of the basking shark oil prepared by him in June 1925, and tested biologically in his laboratory in the beginning of 1926, gave him a value of 10,000 units (U.S.P.). Tested again in the middle of 1927, its biological value was only 500–1000 units (U.S.P.), whilst the colour test applied about the same time was negative. This same oil was tested by us in June 1929, and, as reported above, yielded both colorimetrically and biologically negative results. The sample of salmon-body oil tested by Schmidt-Nielsen was active to a degree represented by 20–30 units (U.S.P.) whilst our sample was inactive in doses up to 100 mg. His sample of the salmon-liver oil contained 600 units (U.S.P.) while the test carried out by us indicated an activity of the order of over 3000 units (U.S.P.).

Recently Schmidt-Nielsen [1929] has published additional evidence in support of his view that there is no agreement between the colorimetric and the biological tests. These experiments cover several additional examples, namely, the body oil of halibut and the blubber oil of whale, both showing negative colorimetric tests and 30–40 biological units (U.S.P.): the liver oils of rabbit-fish and the little spinous dog-fish giving 0.2–1.5 blue units and 300 biological units (U.S.P.): and the liver oils of salmon and halibut giving 200 and 700 blue units respectively. In his first experiments Schmidt-Nielsen found the biological activity of these last mentioned oils to be of the same order as the common cod-liver oil, but in this communication he states that on extending his animal experiments to smaller doses they were found to be considerably richer in vitamin A, and one of them (halibut-liver oil) gave as much as 25,000 units (U.S.P.).

Schmidt-Nielsen's results can be grouped into two categories. In the first place there are the oils which, according to him, give a negative colour reaction, but a varying, though appreciable, degree of biological activity. Such are the body oils of herring, sprat, mackerel, halibut and salmon, the blubber oil of whale, and the liver oils of the rabbit-fish, dog-fish, and basking shark. Some of these have only a very small degree of activity, 20–40 units (salmon and halibut-body oils and the blubber oil of whale). Others are rather more active, 50–100 units (herring-body oils), whilst a third group possesses a moderate degree of activity, 300–1000 units (liver oils of the rabbit-fish, dog-fish and basking shark).

The other class consists of the liver oils of certain bony fishes, *e.g.* salmon and halibut, which give a very intense blue colour reaction but show a comparatively low biological activity.

We have confirmed Schmidt-Nielsen's observation that the oils in the first category yield very weak or negative colorimetric tests. It is between the results of his biological tests and ours that the serious discrepancy arises, and it is not a simple matter to arrive at a satisfactory explanation, unless details of the tests employed in the two laboratories are closely compared, especially in regard to the breeding and diet of the stock animals, the length of the test, and range of doses tested, the number of animals employed to test each dose and the variations in response of individual animals. It is important, however, to remember that unless rather large numbers of animals are employed for each test, the results of biological experiments cannot be fairly expressed in such units as those proposed by the United States Pharmacopœia. According to this unfortunately premature method of assay, a 10 mg. dose is equivalent to 100 units and one of 12 mg. to 80 units, if they both induce a weekly growth of the order of 2-4 g. In our experience it is a difficult enough matter in many cases to differentiate between the responses given by 10 and 20 mg. doses, and it is impossible to attach importance to values based on differences which would not bear critical examination.

In view of the observations made in this laboratory on the sensitivity of the biological test [Drummond and Morton, 1929] the values of 20, 30 and 40 units given to some oils by Schmidt-Nielsen have, in our opinion, no real significance.

They can be interpreted as indicating that the oils in question are of low vitamin A value, but it is impossible to regard them in any sense as quantitative.

Actually, we are inclined to attribute many of the apparent discrepancies between results of the biological tests carried out by Schmidt-Nielsen and those obtained by us to his applying the U.S.P. method of evaluation to unsuitable cases, namely those of oils of low vitamin A content.

In view of the errors associated with the colorimetric assay, to which attention has been drawn, and those of very much more serious dimensions that are almost inseparable from the biological test, unless very large numbers of animals are used, it seems unreasonable to seek an exact quantitative relation between the two methods. The nature of the relationship has been considered in a previous study [Drummond and Morton, 1929], and, bearing in mind the limitations of both methods, we consider that the results of the biological and colorimetric tests described in the present paper are in satisfactory agreement.

In the biological tests, cases are sometimes encountered in which an appreciable amount of growth is observed during the first week or two in which the supplement is given. The growth is, in these cases, not maintained and is followed by a rather sharp decline and the sudden onset of deficiency symptoms. Such animals probably carry with them into the testing period a reserve of the vitamin which is not entirely depleted during the preparatory period.

The disagreement between Schmidt-Nielsen's colorimetric and biological tests of the oils in the second category, namely, halibut and salmon-liver oils, is easily explained. It has been shown above that the results of his colorimetric tests, having been calculated from low dilutions, are much higher than their real values. The complete results of his biological experiments are not yet available, but in his later communication referred to above [Schmidt-Nielsen, 1929] he has stated that these oils which he first believed to be of the same order of biological activity as a common cod-liver oil, having now been tested in smaller doses, prove to be much richer in vitamin A than he had expected. In the case of halibut-liver oil he obtained growth in daily doses of 0.04 mg. (25,000 units U.S.P.), which appears to be in good agreement with its colorimetric value.

SUMMARY.

Schmidt-Nielsen, on the basis of a comparative colorimetric and biological assay for vitamin A of a number of fish oils, has claimed that there is no relation between the results obtained by the two methods, and consequently concluded that the blue colour reaction with AsCl_3 or SbCl_3 is not specific for vitamin A.

Six samples of fish-body and -liver oils, including two samples kindly supplied by Dr Schmidt-Nielsen, have been examined both colorimetrically and biologically for vitamin A. The results of animal tests agree within reasonable limits with those of the colorimetric method, although we make no claim that the colour reaction is specific.

The disagreement between Schmidt-Nielsen's results and ours is due possibly to differences of technique, and to his attempting to express the results of the biological test, which is liable to wide variations, in terms of the units proposed by the United States Pharmacopœia.

This is especially liable to lead to erroneous results when oils of low vitamin A content are examined.

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