NEOVITAMIN A AND VITAMIN A ALCOHOL IN COMMERCIAL FISH-LIVER OILS AND VITAMIN A CONCENTRATES

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THE spectroscopic method described in the Addendum, 1951, to the British Pharmacopœia, 1948, for the estimation of vitamin A in "Halibut-liver Oil, Concentrated Solution of Vitamin A, Concentrated Solution of Vitamins A and D and Products of Similar Properties" requires a determination of the absorption curve of the whole oil in *cyclo*hexane between 320 and 350 mμ. The value of $E^{1\text{ per cent.}}_{1\text{ cm.}}{}_{327\cdot5\,\text{m}\mu}$ can then be converted directly to I.U./g., provided two conditions are satisfied: first, the absorption maximum must be within the range 325 to 328 mμ, and secondly, intensities of absorption in the region 320 to 350 mμ expressed as decimal fractions of the maximum must not differ by more than 0·02 from similar ratios determined on the international standard with a compensating solution of the diluent oil as blank. If the second of these requirements is not met, the geometric correction of Morton and Stubbs[1,2,3,4] is applied and the corrected value of $E^{1\text{ per cent.}}_{1\text{ cm.}}{}_{327\cdot5\,\text{m}\mu}$ calculated from the formula—

$$E_{327\cdot5\,\text{m}\mu}\ (\text{corr.}) = 7(E_{327\cdot5\,\text{m}\mu} - 0\cdot405\ E_{312\cdot5\,\text{m}\mu} - 0\cdot595\ E_{337\cdot7\,\text{m}\mu}),$$

is taken instead of the gross.

Both these procedures are based on the assumption that the vitamin A is present in a form identical, spectroscopically over the material range of wavelength and biologically, with the all-*trans* vitamin A acetate of the international standard. No allowance is made for the presence of free vitamin A alcohol or of neovitamin A.

Vitamin A occurs in fish-liver oils in the form of esters of the higher fatty acids, only small amounts of the alcohol being present.[5,6,7,8,9,10,11] Concentrates prepared from fish-liver oils by molecular distillation or by the Solexol process[12] also contain little or no free alcohol. The esters of all-*trans* vitamin A have the same molar biological activity as the alcohol[13,14]; the Subcommittee on Fat Soluble Vitamins of the World Health Organisation[15] accepts the acetate and the alcohol as being identical in this respect. It is also generally believed that the spectroscopic properties of the higher esters are identical with those of the acetate, though published evidence on this point is lacking. The spectroscopic properties of the alcohol, however, are different; consequently, a different standard curve and a different correction formula must be used when a preparation containing all its vitamin A in the alcohol form is being dealt with. But the pharmacopœial monographs for concentrated solution of vitamin A and concentrated solution of vitamins A and D allow the use of vitamin A concentrates prepared by the now almost obsolete processes of saponification and partial saponification, and

vitamin A alcohol could legitimately form the whole or any proportion of the total vitamin in these preparations. Obviously, the presence of both alcohol and ester forms would complicate the method of estimation.

A further complication arises from the presence of *trans-cis* or neovitamin A which differs biologically and spectroscopically from the all-*trans* isomer. Neovitamin A was first isolated by Robeson and Baxter,[16,17] and they developed a method for estimating it in mixtures of the two isomers, making use of the difference in their rates of reaction with maleic anhydride. The results of estimations made on various fish-liver oils and concentrates by Robeson and Baxter and by other workers using their method are shown in Table I; the table includes figures for synthetic concentrates of uncrystallised material made by two different processes. Robeson and Baxter also showed that the anthraquinone carboxylate of either isomer could be converted to an equilibrium mixture containing 30 per cent. of the neo form. It is of interest that most of the samples in Table I contain the isomers in proportions not far removed from those found in the equilibrium mixture; in 27 of the 33 samples the neo isomer constitutes between 20 and 40 per cent. of the total.

The spectroscopic properties of neovitamin A have been described by Robeson and Baxter[17] and, in greater detail, by Chatain and Debodard.[18] A concentrate containing about 50 per cent. of neovitamin A, as natural esters with not more than 5 per cent. of all-*trans*, has been prepared by Dalvi and Morton.[19] The absorption curve of neovitamin A is displaced towards the long-wave side relative to all-*trans* and its molar extinction coefficient is lower. Moreover, Harris, Ames and Brinkman[20] found that neovitamin A has only 80 per cent. of the biological potency, as measured by rat-growth assays, of the all-*trans* form. Because of these differences, the presence of significant amounts of neovitamin A in oils and concentrates disturbs the spectroscopic determination of potency. If the ratio of the isomers in oils and concentrates were constant or nearly so, its presence could be allowed for by altering the requirement for the "purity" of the curve and adjusting the correction formula. The general problem is discussed by Cama, Collins and Morton.[1]

The present paper reports the results of neovitamin A and vitamin A alcohol estimations on 26 samples of fish-liver oils and natural vitamin A concentrates which passed through our hands in the autumn of 1951. These samples were drawn from large commercial batches and they represent a total of well over 3 million million I.U. of vitamin A. Our aim was to obtain information about the types of oils and concentrates at present used for pharmaceutical and veterinary purposes, and we were particularly interested in finding how the proportions of the isomers varied.

EXPERIMENTAL

Estimation of neovitamin A. We used the method of Robeson and Baxter[17]; a Hilger biochem absorptiometer with a vitamin A filter having maximum transmission at 605 mμ was used to measure the intensity of the blue colour produced by the reaction of vitamin A with antimony trichloride. The blue colour was measured after the maleic anhydride

had been allowed to react for 16 hours at 25° C. A blank solution without maleic anhydride was also tested. The percentage of neovitamin A was calculated from the formula:

$$\text{Per cent. neovitamin A} = \frac{100\,R - 5}{85} \times 100$$

where R = ratio of blue colour in the test solution to blue colour in the blank. Several experiments showed that the vitamin A solutions in benzene remained stable during the period of the reaction. It was also found that the presence of maleic anhydride had no effect on the blue colour when this was measured immediately after the anhydride had been added to the vitamin A solution in benzene.

Estimation of vitamin A alcohol. We used the method of Reed, Wise and Frundt.[8] A test of this method on three cottonseed-oil solutions containing known amounts of pure vitamin A alcohol and palmitate gave the following results for the percentage of total vitamin A present as alcohol: found 7·9, 18·1, 2·5; calculated 8·2, 19·1, 2·2. These solutions contained about 1 per cent. of total vitamin A.

The results of the estimations are given in Table II. Sample No. 13, one of 2 tunny-liver oils which had what appeared to be an abnormally low proportion of neovitamin A, was subjected to a more detailed examination; the rate of addition of maleic anhydride was measured at intervals over 16 hours on this sample and at the same time, for comparison, on a Solexol concentrate and on pure vitamin A acetate. To check the stability of the vitamin A, the corresponding blank solutions without maleic anhydride were also tested. The results are shown in Figure 1, which clearly indicates the difference between the 3 samples.

The average difference between duplicate neovitamin A estimations, done at different times, was 1·5 per cent. and the maximum 3·5 per cent.: both percentages are based on the total vitamin A.

Discussion

Neovitamin A. It will be noted that the proportion of total vitamin A as neovitamin A in 22 of the 26 samples is within the range 28 to 40 per cent., that is, fairly close to the equilibrium proportion. In 4 samples of fish-liver oil, however, the proportion is significantly lower. The low values for 2 tunny-liver oils are interesting, especially as the third tunny-liver oil we examined gave a figure of 37 per cent., and the only other figures for tunny-liver oils reported in the literature, those of Meunier and Jouanneteau,[21] are 55 and 46 per cent. It therefore seems that commercial batches of fish-liver oil may vary in the proportions of isomers they contain, though proportions of about 2 parts of all-*trans* to 1 of neo are the most common.

This variability makes the development of a standard spectroscopic method of vitamin A estimation applicable to all oils and concentrates a matter of difficulty, if the necessity of a neovitamin A determination on each sample is to be avoided. The B.P. method could not safely be altered in any case on the basis of our present knowledge of the properties

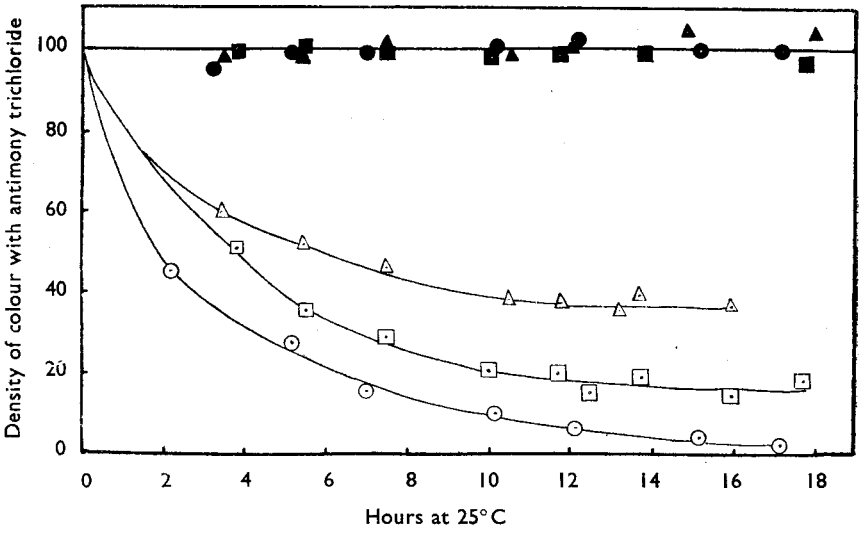

FIG. 1. Reaction of vitamin A with maleic anhydride
△ Solexol concentrate (25).
⊡ Tunny-liver oil (13).
⊙ All-*trans* vitamin A acetate.
● ■ ▲ Controls.

of neovitamin A, and it has perhaps the merit that it tends to under- rather than over-estimate the potency of many oils. There is something to be said, however, for the removal of the "tolerance" clause which allows the direct use of the 1900 factor for oils with curves close to that of the international standard; occasionally concentrates are encountered having curves which just come within the tolerance even though they contain nearly one-third of their vitamin A as the neo-isomer and consequently do not merit the 1900 factor.

We are only concerned here with the ratios of the isomers as found in commercial oils. It does not follow that the isomers occurred in the same proportions in the fish livers from which the oils were prepared. In the preparation of large batches of fish-liver oil, differences due to age, sex, size or other biologically important conditions of the fish tend to be obliterated and only an average product is obtained. Methods of extraction, too, may result in some isomerisation. Any investigation into the biological significance of the difference in the proportions of isomers would need to be done on fresh individual livers.

Because neovitamin A gives a more intense blue colour than the all-*trans* form,[22] results obtained by the method of Robeson and Baxter, in which no allowance is made for this difference, are somewhat higher than the true values; the true proportion is related to that found by the method as follows:—

$$x = \frac{100\,r}{100\,y - ry + r}$$

where x = true percentage of neovitamin A

r = percentage found by Robeson and Baxter's method

$$y = \frac{\text{Intensity of blue colour of neo}}{\text{Intensity of blue colour of all-}trans}$$

In the absence of a sample of pure neovitamin A the ratio, y, is difficult to determine, but comparison of a number of high-potency oils and concentrates with all-*trans* vitamin A acetate suggests that, for our absorptiometer and filter, it is in the region of 1·4; this means that an apparent value of 30 per cent. would correspond to a true value of about 23 per cent. We have not attempted to correct the results in Table II to allow for this difference; they are therefore too high, but they are directly comparable with the figures in Table I. All the figures may be regarded as upper limits.

TABLE I

NEOVITAMIN A IN FISH-LIVER OILS AND CONCENTRATES

(Data from the literature)

Reference	Type of oil or concentrate	Neovitamin A as percentage of total vitamin A
Robeson and Baxter[17]	U.S.P. Reference cod-liver oil	39
	Distilled vitamin A concentrates	39, 35
	Distillate from dogfish-liver oil	39
	Dogfish-liver oil	36
	Soupfin shark-liver oil	37
	Distillate from soupfin shark-liver oil	33
	Halibut-liver oil	32
	California Jewfish-liver oil	34
Karnovsky *et al.*[29]	Soupfin shark-liver oils:	
	Mixed oils	31
	Fat females	34
	Thin females	24
	Males	29
Meunier and Jouanneteau[21]	Shark-liver oil	18
	Argentine shark-liver oil	42
	Dakar shark-liver oil	42
	Spanish red-tunny-liver oil	55
	Red-tunny distillate	46
	Portuguese red-tunny-liver oil	46
Chatain and Debodard[18]	Molecular distillates	33, 31
Cama *et al.*[1]	High-potency oils	25, 24
Dalvi and Morton[19]	Solexol concentrates	23, 29, 24, 23
Cawley *et al.*[20]	Synthetic concentrates	40, 33
Schwarzkopf *et al.*[31]	Synthetic concentrates	37, 38, 41, 40

It may be noted here that we have little information about the other possible isomers of vitamin A, the *cis-cis* and the *cis-trans*. The possibility of their occurrence in fish-liver oils should not be overlooked, and if they were present, they might conceivably interfere with the Robeson-Baxter estimation. Little is known, too, of the effect on the estimation of vitamin A congeners and inhibitors of the blue colour.

Vitamin A alcohol. Table II shows that most of the samples contain little free vitamin A alcohol. 3 of the halibut-liver oils contain 7 or more per cent. of their total vitamin A as alcohol, but these are exceptional; it is quite likely that hydrolysis of some of the vitamin A esters occurred during the extraction of these oils. By careful extraction of the livers

TABLE II

NEOVITAMIN A AND VITAMIN A ALCOHOL IN SAMPLES

No.	Type of oil	$E^{1\%}_{1\,cm.}$ at 327·5 mμ	Percentage of total vitamin A	
			as vitamin A alcohol	as neovitamin A
1	Halibut-liver oil	55	2·1	37
2	" " "	17	2·3	35
3	" " "	23	10·8	31
4	" " "	34	7·0	18
5	" " "	15	7·0	32
6	" " "	13	0·2	32
7	" " "	15	1·3	33
8	" " "	25	1·5	39
9	" " "	13	3·0	28
10	" " "	50	4·0	31
11	" " "	24	2·2	18
12	" " "	52	2·4	35
13	Tunny-liver oil	301	1·1	12
14	" " "	340	1·3	11
15	" " "	348	0	37
16	Shark-liver oil	13	0	37
17	Fish-liver oil blend	6	1·3	33
18	" " " "	13	0	35
19	Molecular distillate	30	0	37
20	" "	130	0	29
21	" "	33	0	37
22	" "	129	1·5	33
23	" "	32	0	34
24	Solexol concentrate	31	1·2	36
25	" "	125	0	38
26	" "	130	0·9	37

it should be possible to avoid this hydrolysis and so produce oils containing not more than, say, 7 per cent. of total vitamin A as alcohol. We suggest that the B.P. monographs for halibut-liver oil, concentrated solution of vitamin A and concentrated solution of vitamins A and D should include such a limit test for vitamin A alcohol. It would, of course, be necessary to withdraw recognition from concentrates prepared by saponification and partial saponification as a source of vitamin A for the concentrated solutions.

This would ensure the removal of one likely source of error in the estimation of the vitamin A potency of these preparations. Moreover, it has been shown by various workers[14,23,24,25,26,27,28] that vitamin A alcohol is less stable than the esters. This is an additional reason for keeping the proportion of vitamin A alcohol as low as possible.

SUMMARY

1. Examination of 26 samples of commercial fish-liver oils and natural vitamin A concentrates showed that the proportion of total vitamin A present as neovitamin A varied between 11 and 39 per cent. when measured by the Robeson-Baxter method.

2. Because of the difference in the intensity of the blue colours produced with antimony trichloride by all-*trans* and neovitamin A, the Robeson-Baxter method gives results somewhat higher than the true values.

3. Most of the samples contained little or none of their vitamin A in the form of the free alcohol.

4. It is proposed that the B.P. monographs for halibut-liver oil, concentrated solution of vitamin A and concentrated solution of vitamins A and D should include a limit test for vitamin A alcohol.

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