CXI. THE VARIATION OF THE EXTINCTION COEFFICIENT OF VITAMIN A WITH SOLVENT

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THE ultra-violet absorption spectrum of vitamin A, with its characteristic maximum near $328\text{m}\mu$, is now used extensively as a means both for identification and determination of the vitamin in the various natural sources in which it occurs. When, however, intensity of absorption of light is used for the quantitative determination of any absorbing entity in solution it has to be borne in mind that the intensity of absorption is not an exclusive property of the solute but also depends somewhat upon the nature of the solvent. In spectrographic determinations of vitamin A this source of discrepancies has usually been avoided in the past, and comparative results have been obtained, by using the same solvent whenever possible (most commonly alcohol). It is, however, sometimes convenient to use media other than alcohol for these determinations as, for instance, when Carr-Price tests are being carried out on the same sample in chloroform solution and only limited quantities of material are available.

It was therefore decided to determine the ratios of the intensities of absorption of vitamin A in several typical solvents, i.e. alcohol, chloroform, benzene and light petroleum. In view of the fact, however, that the vitamin is usually present in natural products in only minute amounts, e.g. order 0.03 % in cod liver oil and 0.001 % in butter fat, it was necessary to determine whether the chemical substances constituting the natural environment of the vitamin cause any variation in the light-absorbing properties of the solute-solvent complex.

For these reasons we have separately considered vitamin A in four different environments:

(a) Fat and sterol-free fish liver oil concentrates prepared by distillation or chromatography and containing some 50–70 % of the vitamin.

(b) The gross fat-free unsaponifiable matter of fish liver oils containing sterols as the major impurities (vitamin A content order 10–25 %).

(c) Fish liver oils consisting mainly of glycerides with only relatively small amounts of vitamin A (0.5–3 %, or more rarely 10 %, in halibut liver oils; 0.1–0.5 % in most other fish liver oils).

(d) Butter fat and butter unsaponifiable matter. The vitamin content of the former is so low (order 0.001 %) that it is only accurately measurable after concentration in the unsaponifiable matter (order now 0.1–1.0 %).

In order to convert the intensities of absorption obtained experimentally into absolute amounts of vitamin A the intrinsic absorption of this substance provisionally used in a number of previous investigations has been the value $E_{1\text{cm.}}^{1\%}$ $328\text{m}\mu$ (alcohol) = 1600, due to Carr & Jewell [1933]. The vitamin has since been concentrated further however; thus Karrer & Morf [1933] have reported a concentrate having $E_{1\text{cm.}}^{1\%}$ $328\text{m}\mu$ = 1700, whilst Holmes & Corbett [1937] have recently isolated the vitamin as a solid having $E_{1\text{cm.}}^{1\%}$ $328\text{m}\mu$ = 2100 (alcohol).

Since our experiments were completed we have learned that Smith *et al.* [private communication, 1938] have also obtained data on the variation of the extinction coefficient of vitamin A with solvent although the variations found do not appear to be so large as those reported here.

EXPERIMENTAL

The absorption spectra determinations were carried out with a Hilger E 3 quartz spectrograph in conjunction with a Spekker photometer, standard methods being used throughout.

Solvents.

Ethyl alcohol. Absolute benzene-free solvent refluxed over metallic sodium and redistilled.

Light petroleum. High grade benzene-free solvent, B.P. 60–80°, redistilled.

Benzene. Pure thiophene-free solvent as obtained commercially.

Chloroform. Pure anaesthetic material washed with water, dried and distilled, yielding a product still containing traces of alcohol.

RESULTS

(a) *Concentrates.* These were all prepared from halibut liver oil (several different specimens) by hot saponification followed by removal of sterols by crystallization from alcohol, first at 0° and then at –60°, the product being finally purified by high vacuum distillation [cf. Heilbron *et al.* 1932], or chromatographically on alumina or lime [Karrer & Morf, 1933; Castle *et al.* 1934].

Four portions of the same concentrate were weighed off at the same time and each portion was diluted to the concentration required for the absorption spectra measurements (order 0.001 %) with one of the four solvents chosen. The absorption spectrum was then determined, the measurements being made immediately after dissolution in order to lessen deterioration of the vitamin [cf. Holmes & Corbett, 1937]. The results of five such groups of evaluations on several different concentrates are enumerated in Table I.

Table I. *Variation of the extinction coefficient of vitamin A with solvent*

(Saponified concentrates only. Numerical values = $E_{1\text{ cm.}}^{1\%}$ *)

Solvent	Wave-lengths of maxima m μ	Concentrates					Mean values relative to alcohol = 1600	Mean values of fifteen separate determinations	Gross mean values
		1	2	3	4	5			
Ethyl alcohol	325	1025	1100	1210	1290	1290	1600	1600	1600
Light petroleum (B.P. 60–80°)	325	950	900	1170	1180	1190	1450	—	1450
Benzene	331	860	800	1100	1140	1090	1330	—	1330
Chloroform	332	816	800	1100	1060	1060	1300	1340	1330

* $E_{1\text{ cm.}}^{1\%} = \log I_0/I$ for a 1 cm. cell of 1 % solution. I_0 = the intensity of the incident light and I = the intensity of the transmitted light.

In order to obtain a better mean value the extinction coefficients were re-determined for alcohol and chloroform only (i.e. the solvents showing the extreme variations). Fifteen experiments in all were made, some by weighing off the same concentrate into the two solvents separately, and some by making a strong solution of the concentrate in light petroleum and diluting it largely with either alcohol or chloroform to the required strength. The mean ratio of

the extinction coefficients¹ for alcohol and chloroform, respectively, as given by these fifteen experiments was 1600 : 1340 and the mean of the whole twenty experiments gives the final alcohol : chloroform ratio as 1600 : 1330 (cf. Table I).

It is thus obvious that if the solvent is other than ethyl alcohol, when using absorption spectra methods for the determination of vitamin A in any given saponified material, the extinction coefficient specific to vitamin A in the particular solvent must be used for the calculation of absolute concentration.

In view of the fact that since these experiments were carried out vitamin A has been concentrated further, the question arises whether alcohol : chloroform intensity ratios obtained on concentrates containing only 60–70 % of the vitamin would be valid for the pure material. Whilst there is no evidence available on this point it is to be noted that owing to the extreme difficulty of obtaining and keeping vitamin A of the purity attained by Holmes & Corbett [1937] concentrates of the order of purity discussed here are those most commonly met in practice.

(b) *The effect of sterols on the ratio of the extinction coefficients of vitamin A in alcohol and chloroform.* In order to determine the effect of the gross non-vitamin A portion of the unsaponifiable matter of halibut liver oil upon the extinction coefficients of vitamin A in alcohol and chloroform respectively, three samples of the fat-free unsaponifiable matter were prepared from different halibut oils. They contained some 25 % of vitamin A, the remainder being mainly sterols with smaller amounts of other impurities. The extinction coefficient of each sample was determined twice at different times in both alcohol and chloroform, at concentrations between 0.005 and 0.02 %. The values obtained were as follows:

Unsaponifiable fraction	$E_{1\text{ cm.}}^{1\%}$ alcohol	$E_{1\text{ cm.}}^{1\%}$ chloroform	$E_{1\text{ cm.}}^{1\%}$ chloroform taking alcohol value as 1600
1 (a)	489	391	1280
(b)	424	358	1350
2 (a)	358	290	1300
(b)	341	285	1340
3 (a)	456	378	1330
(b)	442	380	1370
		Mean value 1600 : 1328	

This mean value is so close to that already found for the purified concentrates (1600 : 1330) as to provide additional support to this latter value whilst at the same time showing that the gross non-vitamin A portion of the unsaponifiable matter of halibut liver oil has no effect on the alcohol : chloroform extinction coefficient ratio of vitamin A.

(c) *Liver oils.* On account of the fact that the non-vitamin portion of fish liver oils (mostly glycerides with some sterols and other alcohols) is always in very large excess compared with the vitamin A present (order from 30 : 1 in rich halibut oils to 3000 or 4000 : 1 in average or poor cod liver oils) as well as the probability that the vitamin occurs at least partially as an ester, it might be expected that the fatty medium would exert some "solvent-effect" upon the extinction coefficient of the vitamin A even when the nominal solvent is alcohol or chloroform.

We have therefore considered the case of liver oils apart from the fat-free concentrates prepared from them. The results may be summarized briefly as follows: The extinction coefficient of vitamin A as it occurs in liver oils varies

¹ The term "extinction coefficient" as used throughout this paper equals $E_{1\text{ cm.}}^{1\%}$. This is however not quite the usual meaning of the term (cf. Twyman & Allsopp, 1934).

with the solvent used for the determination but the difference between the values for any two solvents appears to vary for different oils. Further, the difference between the values for alcohol and chloroform solutions appears to be distinctly less than the corresponding figure found for concentrates. It would thus seem that the presence of oil in the solution lessens the difference between the extinction coefficients in the two solvents. That this smoothing-out of differences is probably due to a raising of the extinction coefficient for chloroform rather than a lowering of the alcohol value seems probable from the fact that spectrophotometric evaluations of the vitamin A content of halibut liver oils, either directly in alcohol or via the antimony trichloride blue test, give closely comparable values for the absolute concentration even when these are calculated from standards obtained on fat-free concentrates only [Lovern *et al.* 1933].

Some typical results on oils will indicate the kind of variation obtained in our experiments but, in view of the apparent difference in individual oils, average values for the extinction coefficients in different solvents would scarcely seem to be significant when the medium contains oil.

Table II. *Relative intensities of absorption (328 m μ) of liver oils in different solvents*

	Halibut liver oil 1	Halibut liver oil 2	Mixed halibut and cod liver oils (1 : 2)
$E_{1\text{ cm.}}^{1\%}$ 328 m μ (alcohol)	66.5	68	23.5
Alcohol taken as:	100	100	100
Light petroleum	98.5	101	89
Benzene	83.4	—	84
Chloroform	92	—	85

(d) *Butter*. The spectrophotometric method provides a useful means of assaying the vitamin A in butter via its unsaponifiable fraction but not on the original fat [Heilbron & Morton, 1930; Gillam *et al.* 1933], and both alcohol and chloroform have been used as solvent for this purpose [Baumann & Steenbock, 1933; Gillam, 1934]. It was therefore decided to examine a number of typical butters to ascertain whether the differences in extinction coefficient already found for vitamin A concentrates in alcohol and chloroform, respectively, persisted in the presence of large excess of the unsaponifiable matter of butter.

For this purpose eighteen butters of widely differing origin and vitamin A content were obtained and the intensity of absorption due to the vitamin was determined in both alcohol and chloroform by the method of Gillam [1934]. Taking the mean value of $E_{1\text{ cm.}}^{1\%}$ for alcohol as 1600 the mean value for chloroform was found to be 1540 (cf. Table III). The errors in the determination of vitamin A in butter are much larger than in the case of a liver oil or concentrate, on account of the fact that the active substance is present in much smaller amount and further because carotene is also present and the values have therefore to be corrected for this absorbing constituent. Thus, although it appears that the non-vitamin A portion of the unsaponifiable matter of butter tends to smooth out the differences in extinction coefficient characteristic of vitamin A in alcohol and chloroform solutions, respectively, part at least of this apparent smoothing-out effect may possibly be due to the relatively large experimental error inherent in the method as applied to butter.

In the course of these experiments we also examined a number of colostrum fats and richer butters [cf. Semb *et al.* 1934; Gillam *et al.* 1936] and the results are shown in Table III.

Table III. *Comparison of the values obtained in the spectrophotometric determination of vitamin A in butter using alcohol and chloroform, respectively, as solvents*

Normal butter fats				Colostrum and richer butter fats			
Butter no.	Vitamin A mg. per 100 g. fat (basis $E_{1\text{ cm.}}^{1\%} = 1600$)		Ratio alcohol/chloroform values	Butter no.	Vitamin A mg. per 100 g. fat (basis $E_{1\text{ cm.}}^{1\%} = 1600$)		Ratio alcohol/chloroform values
	Alcohol	Chloroform			Alcohol	Chloroform	
1	1.31	1.31	1.00	1	7.3	5.95	1.22
2	1.33	1.47	0.90	2	4.86	3.86	1.26
3	1.45	1.53	0.95	3	3.23	3.1	1.04
4	1.53	1.55	0.99	4	4.8	3.9	1.23
5	0.70	0.64	1.09	5	3.45	2.8	1.23
6	1.04	0.93	1.11	6	3.75	3.23	1.16
7	1.35	1.22	1.10	7	1.91	1.76	1.08
8	0.57	0.51	1.11	8	1.95	1.73	1.13
9	0.97	0.85	1.14	9	9.6	8.7	1.10
10	0.54	0.57	0.94	10	10.0	8.9	1.11
11	1.29	1.28	1.00	11	8.74	6.2	1.41
12	0.48	0.48	1.00				
13	0.69	0.69	1.00				
14	0.61	0.61	1.00				
15	0.70	0.64	1.09				
16	0.57	0.54	1.05				
17	1.35	1.22	1.10				
18	1.04	0.93	1.11				
			1.038				1.18

Alcohol : chloroform = 1600 : 1540

Alcohol : chloroform value 1600 : 1360

When considered separately from the ordinary butters the relative values for the extinction coefficients of the vitamin A present (mean of eleven samples) work out at 1600 : 1360. Thus for especially rich summer butter and colostrum fat the extinction coefficient differences are approximately the normal ones found for concentrates either with or without sterols (*v. supra*).

Comparison of the results obtained on ordinary butters with the values for colostrum fats makes it appear probable that in the former the approximate equality of the extinction coefficients of the vitamin A present, when the unsaponifiable matter is dissolved in alcohol or chloroform, is due to the depression of the higher alcohol value towards that characteristic of chloroform solutions. This seems probable from the fact that when vitamin A is present in greater amount (as in colostrum fat and richer butter fats) the values for alcohol and chloroform begin to separate and approach the normal ones for uncontaminated vitamin A in these solvents.

The mean values for the extinction coefficient of vitamin A in various natural media—as determined above—can therefore be summarized as follows:

	$E_{1\text{ cm.}}^{1\%}$ value on the basis of $E_{1\text{ cm.}}^{1\%} = 1600$ for pure vitamin A in alcohol		$E_{1\text{ cm.}}^{1\%}$ value on the basis of $E_{1\text{ cm.}}^{1\%} = 2100$ for pure vitamin A in alcohol	
	Alcohol	Chloroform	Alcohol	Chloroform
Concentrates (sterol-free)	1600	1330	2100	1740
Concentrates (+ gross sterols)	1600	1330	2100	1740
Halibut liver oils	1600	Variable	2100	Variable
Butters (normal and poor)	1400	1350	1840	1770
Colostrum fats and especially rich butters	1600	1350	2100	1820

SUMMARY

The relative intensities of light absorption of a number of purified vitamin A concentrates have been determined in the solvents ethyl alcohol, light petroleum, benzene and chloroform. Taking the value for alcohol as 1600 the other values average 1450, 1330 and 1330 respectively.

When the values are determined in the presence of the gross unsaponifiable matter of halibut liver oil the ratio 1600 : 1330 for alcohol : chloroform remains unaltered. In the presence of the fatty components of the liver oils, however, the extinction coefficient in chloroform approaches that obtained for alcohol solutions much more closely than in the case of the fat-free concentrates. The ratio appears to vary somewhat with different oils.

The question of the spectrophotometric determination of vitamin A in butter and colostrum fats in different solvents has also been examined and discussed.

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