A Re-examination of Halibut-liver Oil. Relation between Biological Potency and Ultraviolet Absorption Due to Vitamin A

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(Received 3 March 1947)

Rather more than ten years ago, the Vitamin A Sub-Committee of the Accessory Food Factors Committee (Medical Research Council) organized large-scale co-operative biological assays on halibut-liver oil, with the object of ascertaining as accurately as possible the relationship between vitamin A potency and ultra-violet absorption at 328 $m\mu$.

Two fairly large samples of oil were obtained and mixed in equal proportions. One sample was presented by Parke, Davis and Co., and the other, made by I. Spencer and Co. of Aberdeen, was obtained through the Torry Research Station, Aberdeen. The mixed oil was used by various groups of workers for feeding tests on properly prepared rats, using the responses to known dosages of the 1934 Standard β -carotene solution issued by the Director of Biological Standards (National Institute for Medical Research), as the fundamental criterion of vitamin A activity. The two oils (denoted hereunder as the P.D., and S. oils, respectively) were tested spectroscopically at Liverpool in 1936, and sealed samples in brown glass ampoules are still available and bear the following information on the labels:

Parke, Davis halibut-liver oil	Spencer halibut-liver oil
24. iii. 36. M.R.C.	23. iii. 36. M.R.C.
$E_{1\text{ cm.}}^{1\%}$ { 617 $m\mu$. 230 583 $m\mu$. 121 328 $m\mu$. 74.4	$E_{1\text{ cm.}}^{1\%}$ { 617 $m\mu$. 143 583 $m\mu$. 74.5 328 $m\mu$. 47

617 and 583 $m\mu$. refer to the SbCl_3 colour test and 328 $m\mu$. to the ultra-violet absorption.

The biological data were gathered together and a statistical examination was made (Hume, 1937, 1939; Irwin, 1944). For the mixed oil, the estimated biological activity expressed in i.u./g. (1 i.u. equivalent to 0.6 μg . β -carotene) divided by the intensity of absorption at 328 $m\mu$., expressed in terms of $E_{1\text{ cm.}}^{1\%}$, was 1570. In other words, when $E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$. equals 1, the potency is 1570 i.u./g.

At that time it was believed that if the absorption curve obtained for a solution of a rich oil was the same as that obtained for an equivalent solution of its unsaponifiable fraction there was no irrelevant absorption, i.e. the full intensity of absorption was due to the vitamin. It is now found that the belief is not necessarily, nor even usually, justified.

A later co-operative experiment under the same auspices as the previous one made use of a carefully prepared solution of vitamin A β -naphthoate. This substance was available in crystalline form and, apart from the presence of a small amount of vitamin A_2 , the specimen used must have been nearly pure. Statistical study of the relationship between biological potency (assessed by comparison of responses with responses to Standard β -carotene) and ultra-violet absorption at 328 $m\mu$. (corrected for any contribution traceable to the β -naphthyl moiety) led to a conversion factor of 1770 (Hume, 1943; Irwin, 1944). A similar assay with cod-liver oil, led to a conversion factor of 1820 (Hume, 1939; Irwin, 1944).

The difference between the conversion factors of 1570 and 1770 has since remained difficult to explain, and the assessment of absolute potency as distinct from relative potency has not been free from uncertainty. A re-examination of the two samples of halibut-liver oil in 1946 has gone far to improve the position.

METHODS

The oils were dissolved in purified *cyclohexane* and their absorption curves determined using a Beckman photo-electric spectrophotometer. The SbCl_3 colour test (Carr-Price reagent) was carried out by measuring intensities of absorption at 617 and 583 $m\mu$. using a Hilger-Nutting visual spectrophotometer. The following data were obtained:

	P.D. oil	S. oil
$E_{1\text{ cm.}}^{1\%}$ { 617 $m\mu$.	172	145
583 $m\mu$.	95	77
328 $m\mu$.	70.65	46.2 (ampoule <i>a</i>)
		47.2 (ampoule <i>b</i>)

By comparison with the 1936 tests the S. oil had kept very well, but the P.D. oil had undergone appreciable change.

It has been shown (Morton & Stubbs, 1946) that for vitamin A preparations free from detectable irrelevant absorption:

$$E_{313\text{ }m\mu} = E_{338.5\text{ }m\mu},$$

and $E_{328\text{ }m\mu} / E_{338.5\text{ }m\mu} = 1.166.$

The above criteria were satisfied by the purest preparations of crystalline vitamin A acetate dissolved in *cyclohexane*. In the paper cited a simple method for estimating irrelevant absorption at 328 $m\mu$. was proposed and tested. By

applying the method to the values of E at 313, 328 and 338.5 $m\mu$. obtained for the two oils in *cyclohexane*, the corrected intensities of absorption were

	P.D. oil	S. oil
$E_{1\%}^{1\text{ cm.}} 328\text{ }m\mu$. (corr.)	46.9	40.2 (a) 40.0 (b)

Now the complete absorption curve for vitamin A over the wave-length range 250–370 $m\mu$. is known with considerable accuracy (Morton & Stubbs, 1946) and the intensities of

curve may at once be constructed. When this is done for the P.D. and S. oils (Figs. 1, 2) it becomes possible, by subtracting the corrected curve from the observed curve, to obtain the complete curve for irrelevant absorption. In so doing, the only assumption made is that between 313 and 338.5 $m\mu$. this is sensibly linear.

RESULTS

Inspection of the subtraction curve in Fig. 1 (P.D. oil) reveals the presence of several maxima at positions coinciding with those already known to be characteristic of the so-called cyclized or anhydro-vitamin A. The corresponding subtraction curve in Fig. 2 (S. oil) shows very much less intense irrelevant absorption, and the narrow maxima are only barely detectable. In the P.D. oil nearly one-third of the gross absorption at 328 $m\mu$. is irrelevant in the sense that it is not due to vitamin A, whereas in the S. oil the correction is only about one eighth of the gross value. Since the latter oil shows no detectable change over the ten-year period of storage, it may be assumed that the irrelevant absorption has remained unchanged.

It is therefore justifiable to argue that a more satisfactory conversion factor for vitamin A can be obtained from the results of the original co-operative assay on halibut-liver oil by dividing the observed potency by the corrected value of $E_{1\%}^{1\text{ cm.}} 328\text{ }m\mu$. Unfortunately, the known potency applies to a mixture of the two oils in equal proportions, and the amount of correction which should be applied to the original value of $E_{1\%}^{1\text{ cm.}} 328\text{ }m\mu$. for the P.D. oil is not known. However, it seems that the two oils were originally very similar spectroscopically since there was very good agreement between the two samples for the ratios $E 617\text{ }m\mu./E 583\text{ }m\mu$. and $E 617\text{ }m\mu./E 328\text{ }m\mu$. as shown in Table 1. These ratios also provide further confirmation for the assumption that the S. oil has changed very little over the ten-year period of storage.

Table 1. *Relative intensities of absorption in the SbCl_3 colour test and at 328 $m\mu$. for two samples of halibut-liver oil, in 1936 and 1946*

Oil	$E 617\text{ }m\mu./$ $E 583\text{ }m\mu$.	$E 617\text{ }m\mu./$ $E 328\text{ }m\mu$.
P.D. (1936)	1.90	3.09
S. (1936)	1.92	3.05
P.D. (1946)	1.81	2.43
S. (1946)	1.88	3.14

Since, then, the two oils were originally similar, the correction for irrelevant absorption which is now found necessary for the stable S. oil, should be applied to the original spectroscopic data on both oils. If this had been possible the conversion factor

Fig. 1. Absorption spectrum of 'P.D.' halibut-liver oil 0.00932 % in *cyclohexane*, 1 cm. cell. Sample stored in sealed ampoules 1936–46. ———. Observed. ———. Corrected curve. Difference curve.

Fig. 2. Absorption spectrum of 'S.' halibut-liver oil 0.0127% in *cyclohexane*, 1 cm. cell. Stored in sealed ampoules 1936–46. ———. Observed. ———. Corrected curve. Difference curve.

absorption at various wave-lengths (5 $m\mu$. intervals) have been recorded on a scale such that $E 328\text{ }m\mu$. is 1.0. Given any value for $E 328\text{ }m\mu$. (corr.) the complete corrected

as determined by the co-operative assay would have been $1570 \times 46.7/40.1 = 1828$. A value of 1830 for the conversion factor, obtained from assays on halibut-liver oil now falls into line with the value of 1770 obtained from the assays on crystalline vitamin A β -naphthoate, and 1820 obtained using cod-liver oil. In the latter case, the spectroscopic measurements were carried out on the non-saponifiable fraction of the oil, the irrelevant absorption in which is in general almost negligible (Morton & Stubbs, 1946).

The subtraction curves in Figs. 1 and 2, indicate the presence of anhydro-vitamin A (λ_{max} 351, 371 and 392 $m\mu$.), with a suggestion of further selective absorption below 300 $m\mu$. The validity of the subtraction procedure depends, however, upon a rather high degree of accuracy not only in the curves for the P.D. and S. halibut-liver oils, but also in the standard vitamin A curve, and furthermore the assumption of linearity in the irrelevant absorption between 313 and 338.5 $m\mu$. is crucial. It would be more satisfactory, both as regards the accuracy of $E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$. (corr.), and the characterization of the absorbing contaminants, if the latter could be separated from the vitamin A and the entire argument checked. The results of two different fractionation procedures are valuable in this respect.

The low temperature fractions (1-8) show little evidence of the presence of vitamin A, but their absorption spectra possess well-defined fine structure with the most prominent band at λ_{max} 367-370 $m\mu$., indicating the presence of anhydro-vitamin A. These fractions also show selective absorption below 300 $m\mu$. (λ_{max} 272 and 262 $m\mu$.) in qualitative agreement with the subtraction curves. (The spectroscopic data obtained in 1939 on the original mixed oils suggest that the P.D. oil had probably started to deteriorate, since the absorption bands of anhydro-vitamin A appear as well-marked inflexions.)

(ii) The oils (1 g. of each) were saponified and the unsaponifiable matter extracted quantitatively with redistilled ether. The solvent was removed and the residue taken up in purified light petroleum. This solution was then extracted 12 times with aqueous ethanol (83 ml. absolute ethanol, 17 ml. water). The petroleum fraction was made up to 50 ml. and dried with anhydrous sodium sulphate. A portion was diluted with cyclohexane for spectroscopic examination. The aqueous ethanol fraction was diluted with a large volume of water and exhaustively extracted with ether. After removal of the ether from the extract, the residue was transferred to cyclohexane for spectroscopic examination. The results are shown in Table 3.

Table 2. *Molecular distillation of a mixture of two halibut-liver oils (P.D. and S.)*

Fraction	Temp. (° C.)	Wt. (g.)	Ultra-violet absorption maxima ($m\mu$.)			Intensities of absorption ($E_{1\text{ cm.}}^{1\%}$)		
			(a)	(b)	(c)	(a)	(b)	(c)
1	80	3.7	367	273	263	24	4.4	3.7
2	90	5.08	370	272	262	36.6	5.6	4.8
3	100	4.18	369	272	262	46.6	7	6
4	110	5.02	369	272	262	50	7.5	6.25
5	120	4.60	368	272	262	38	6.5	5
6	130	4.37	369.5	272	262	21.16	4.8	3.8
7	140	4.83	370	~272	—	9	3.0	—
8	150	4.32	~370	328	—	3	8.66	—
9	160	4.12	~389	328	—	1.4	11.25	—
10	170	4.66	—	328	—	—	23.5	—
11	180	4.28	—	328	—	—	57	—
12	190	3.54	—	328	—	—	109.3	—
13	200	3.22	—	328	—	—	166.7	—
14	210	3.42	—	328	—	—	224	—
15	220	3.80	—	328	—	—	205	—
16	230	3.73	—	328	—	—	158.7	—
17	240	5.88	—	328	—	—	86.6	—
18	250	6.28	—	328	—	—	37.5	—
Original oil			~391 ~368	328	—	14 29	64.5	—

(i) The mixed oils (91.9 g.) were mixed with 60 ml. of constant yield oil and the mixture subjected to molecular distillation by Dr T. H. Mead of British Drug Houses, Ltd. Table 2 shows the spectroscopic results obtained in 1939 on the resulting fractions.

It will be seen that the vitamin A is almost quantitatively extracted by the 83 % ethanol, giving an absorption curve requiring very little correction and agreeing very well with the corrected values of $E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$. obtained for the original oils. The light

Table 3. *Partition of halibut-liver oil unsaponifiable fractions between light petroleum and 83 % aqueous ethanol*

	P.D. oil		S. oil	
	Petrol fraction	83 % ethanol fraction	Petrol fraction	83 % ethanol fraction
$E_{1\text{ cm.}}^{1\%} 328\text{ m}\mu.$ Gross (calc. on original oil)	16.8	48.25	2.9	39.4
$E_{1\text{ cm.}}^{1\%} 328\text{ m}\mu.$ Corrected (calc. on original oil)	3.1*	45.5	0.6*	37.8

* Quantitative validity doubtful owing to questionable linearity of irrelevant absorption.

Fig. 3. Absorption spectrum of 'P.D.' halibut-liver oil; unsaponifiable fraction partitioned between 83 % ethanol and light petroleum; petroleum fraction 0.04 % in *cyclohexane*, 1 cm. cell.

Fig. 4. Absorption spectrum of 'S.' halibut-liver oil; unsaponifiable fraction partitioned between 83 % ethanol and light petroleum; petroleum fraction 0.2 % in *cyclohexane*, 1 cm. cell.

petroleum fraction showed the characteristic absorption of anhydro-vitamin A (Figs. 3, 4). The application of the correction procedure to these curves indicated the presence of only very small amounts of vitamin A.

DISCUSSION

The application of photoelectric spectrophotometry to the examination of the halibut-liver oils used in the large-scale co-operative assays of 1936 has shown that the original values of $E_{1\text{ cm.}}^{1\%} 328\text{ m}\mu.$, ascribed solely to the absorption of vitamin A, should have been subjected to some correction for absorption due to other constituents of the oils. When this irrelevant absorption is allowed for by measurements at three wave-lengths, the original estimate of 1570 for the conversion factor is raised to 1830, whilst the irrelevant absorption is shown, by subtraction of the vitamin A contribution from the observed curves, to be due mainly to anhydro-vitamin A. The validity of the correction procedure can be demonstrated by fractionation of the oils.

The principal significance of these results is that a single conversion factor can be applied to spectroscopic measurements on fish-liver oils, on unsaponifiable fractions of fish-liver oils and on crystalline vitamin A esters, provided that proper correction is made for irrelevant absorption. The potencies in i.u./g. so obtained will then be in accord with the biological estimates, provided that the biological assays are carried out under strictly comparable conditions. Thus, the conversion factor of 1800 suggested by the present work only applies to the particular conditions of diet, dosage level, etc. under which the three co-operative assays were carried out; changes in these conditions might well lead to a conversion factor appreciably different from 1800.

If, then, the expression of vitamin A potency in terms of i.u./g. is to persist, and general usage suggests that it should, a conversion factor of 1800 would seem to be desirable, on condition that the values of $E_{1\text{ cm.}}^{1\%} 328\text{ m}\mu.$ are fully corrected for irrelevant absorption. This is not likely to give rise to any serious discrepancies at the present time, when the more precise evaluation of $E_{1\text{ cm.}}^{1\%} 328\text{ m}\mu.$ due unequivocally to vitamin A is not generally applied, since $E_{1\text{ cm.}}^{1\%} 328\text{ m}\mu.$ for the majority of commercial fish-liver oils requires a correction of approximately 12 %. That is, the potency obtained by multiplying $E_{1\text{ cm.}}^{1\%} 328\text{ m}\mu.$ (gross) by the present conversion factor of 1600 is close to that obtained by multiplying $E_{1\text{ cm.}}^{1\%} 328\text{ m}\mu.$ (corrected) by 1800. Whilst this is true for the majority of fish-liver oils, the exceptions obviously cannot be ignored, so that a spectroscopic method of assay must eventually be universally adopted which makes proper allowance for irrelevant absorption. When such a method is generally applied, the actual value of the conversion

factor is not of fundamental importance, since the corrected value of $E_{1\text{ cm}}^{1\%}$ 328 m μ . is just as informative. The use of the 1800 factor would preserve continuity since it forms a link between the co-operative assays and the 1934 International Standard β -carotene.

SUMMARY

1. A re-examination of the two halibut-liver oils used in the co-operative assays of 1936 has been carried out by photo-electric spectrophotometry. The results show that the original spectroscopic data required some correction for

absorption at 328 m μ . by constituents other than vitamin A.

2. When such a correction is applied, the conversion factor relating $E_{1\text{ cm}}^{1\%}$ 328 m μ . to potency in i.u./g. is raised from 1570 to 1830, which agrees well with the values of 1770 obtained for crystalline vitamin A β -naphthoate and 1820 for cod-liver oil, and justifies a round figure of 1800.

3. The results of fractionation experiments on the oils support the method of correction for irrelevant absorption.

4. The significance of the results with respect to the spectrophotometric assay of vitamin A is discussed.

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The Choice of a Chelating Agent for Inactivating Trace Metals

1. A SURVEY OF COMMERCIALY AVAILABLE CHELATING AGENTS

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(Received 6 January 1947)

The important part played by various heavy metals in metabolic processes is well recognized. In studies of such processes, the removal of part or all of a particular metal from active participation in a biological system may be desired. Microchemical reagents, particularly the chelating agents evolved in the last 30 years (Morgan & Drew, 1920; Morgan & Burstall, 1936), seem likely to prove valuable tools for such purposes and some such applications have already been made.

The iron-deficient medium devised by Waring & Werkman (1942) is an interesting example. These authors saturated a synthetic bacteriological medium with oxine (8-hydroxyquinoline) and then extracted it with chloroform, thus removing heavy metals in the form of oxine complexes; traces of manganese, zinc and copper were then added. This produced a medium that was deficient in iron and which supported the growth of certain bacteria in proportion as iron was added.

It is not easy to make a good choice of micro-analytical reagents in biological work because there are practically no data available as to which metals these reagents will inactivate under physiological conditions. Little help can be obtained from the analytical literature which usually stipulates that the solutions to be tested must first be boiled, or treated with strong acid or alkali, or even that all organic matter should be removed.

The present studies are intended as a contribution towards exploring this gap in knowledge. Their aim is to discover which reagents give visible signs of reacting with a series of biologically important metals at 37° and pH 7.3. The investigation is confined to those substances believed to act through chelation and hence it excludes a few reagents of comparable delicacy such as hydrogen sulphide and some oxidizing agents. The results of the present work show that several reagents which are widely used in analytical practice to remove traces of metal from solution are ineffective at pH 7.3 and 37°. Other reagents are found to retain their efficacy, even at great dilution and to show more or less specificity for the various metals.

METHOD AND MATERIALS

The metals were used in the form of sulphates (acetates in the case of calcium and lead) of analytical reagent quality. One ml. of a 0.0004M solution of the metal was mixed with 2 ml. of 0.1M-borate buffer of pH 7.3. One ml. of 0.004M-reagent was then added and the mixtures incubated in stoppered flasks at 37° for 24 hr. This made a final strength of 0.0001M-metal and 0.001M-chelating agent. Usually two molecules of chelating agent combine with one ion of metal so that there is a fivefold excess of chelating agent present in most cases. Although such a large excess may not have been necessary in every case, it was borne in mind that the