XXXVII. A DISCREPANCY BETWEEN BIOLOGICAL ASSAYS AND OTHER METHODS OF DETERMINING VITAMIN A. II

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THE idea that vitamin A is a single sharply defined chemical entity suffered a set-back when it was discovered that the fundamental biological criteria could be satisfied by several carotenoids obviously different from the active principle of liver oils. This complication was mitigated, on the theoretical side, by the demonstration that β -carotene and certain structurally related lipochrome pigments are capable of transformation, within the body, into the vitamin A of liver and are therefore to be regarded as provitamins. The routine biological test does not differentiate provitamins from vitamin but on the practical side this particular non-specificity is relatively unimportant because, according to available evidence:

- (1) The activity of vegetable products is due entirely to provitamins.
- (2) The activity of liver oils is uninfluenced by provitamins, which are present only in small traces.
- (3) Methods applicable to the determination of carotenoids (e.g. absorption spectra in the visible region) and ultra-violet spectroscopy as applied to vitamin A permit separate, fairly accurate evaluation of the contributions to the total growth-promoting effect of provitamins and vitamin where they co-exist in comparable amounts, as in milk, butter, eggs, blood, etc.

It has hitherto been assumed that among all the possible degradation products of carotenoids only one, vitamin A, possesses biological activity and that any subsequent change (e.g. oxidation) results in a complete loss of activity. On the score of simplicity these assumptions have much to commend them, but they possess no necessary validity. In the study of vitamin D it was for a time reasonable to believe that ergosterol was unique as provitamin D and that calciferol was the unique antirachitic agent. Convincing evidence has since made such a view quite untenable and a plurality of provitamins D and antirachitic agents is now established. This experience renders it all the more necessary to examine carefully the assumption of the uniqueness and strict homogeneity of the alcohol $C_{20}H_{29}OH$ isolated from liver oils.

Vitamin A, as thus known, has definite characteristics. The richest concentrates are apparently homogeneous and reproducible and exhibit:

- (i) An absorption maximum at 325–328 $m\mu$ of intensity $E_{1\text{ cm.}}^{1\%}$ 1600.
- (ii) Chromogenic properties towards antimony trichloride, with the specifications:
 - (a) Blue value 80,000–100,000 Carr-Price units.
 - (b) Absorption maxima at 617–620 $m\mu$ ($E_{1\text{ cm.}}^{1\%}$ 5000) and 583 $m\mu$ ($E_{1\text{ cm.}}^{1\%}$ 2600).

- (iii) A biological value of the order 1.7–2 million International Units per g.
- (iv) An elementary analysis corresponding with $C_{20}H_{29}OH$.

The assumption that the foregoing characteristics are exhibited either exclusively by a single isomeric form of the alcohol $C_{20}H_{29}OH$ or equally by all isomerides, and that related compounds are inert in every sense, involves a set of constant ratios between the various characteristics; in particular $\frac{\text{Bio-assay}}{E_{1\text{ cm.}}^{1\%} 328\text{ m}\mu}$ should provide a constant conversion factor. The value of 1600 recommended by the International Conference of 1934 represents a useful compromise, but careful estimates of the conversion factor on different preparations and in different laboratories may vary over the range 700–2500 and it remains uncertain how far the discrepancies are due to inaccuracy in the biological assays and how far to other contributory causes of a more fundamental nature. The discrepancies appear, however, to be outside any known experimental error. Heterogeneity in the growth-promoting material might be indicated by:

(1) A normal absorption spectrum accompanied by abnormally high¹ biological activity.

(2) Displacement of the maximum in the ultraviolet absorption accompanied by biological activity in excess of the amount corresponding with the proportion of "328 m μ " vitamin A present.

(3) Similar anomalies involving the antimony trichloride colour test.

The first two indications would probably be superior to the third, in view of the known sensitivity of the colour test to inhibitors and other foreign material [e.g. Morton, 1932; and several others]. Morgan *et al.* [1935], in the first paper of this series, have already raised the question of heterogeneity, and the present work supplements the evidence previously adduced.

Some 3 years ago, in attempting to remove inert or feebly active material from sterol-free mammalian liver oil concentrates, it was found that aqueous alcohol (83%) readily dissolved the main chromogenic constituent, leaving behind about half the original material as an insoluble fraction possessing a lower ultraviolet absorption and blue value. Further quantitative experiments on these lines have shown that the alcohol-soluble fraction corresponds very closely with the physical and chemical criteria for vitamin A; whereas the insoluble portion accumulates quite appreciable quantities of a substance characterized by an ultraviolet maximum at 285–290 m μ , and an abnormal colour reaction. Both fractions showed biological activity of a high order (cf. Table I, Figs. 1–4).

The biological value of an alcohol-insoluble fraction could be predicted within the limits of error which apply to more normal materials by assuming that the gross absorption at 328 m μ afforded a measure of vitamin A content. In order to investigate more fully the material absorbing at 285–290 m μ it was decided to attempt a more complete separation. Two procedures suggested themselves:

(A) Selective adsorption of the insoluble fraction, following the chromatographic technique of Tswett.

(B) More exhaustive extraction with 83% alcohol.

(A) Aluminium oxide prepared according to the recommendations of Brockmann was used as adsorbent and several fractions were obtained, particulars of

¹ An unduly low bio-assay is capable of similar interpretation but may alternatively be attributed to the presence of inactive chromogenic material.

Fig. 1. Mammalian liver oil concentrate A. The dotted curves are an attempt to resolve the main curve into two components one of which is the absorption spectrum of vitamin A; if this curve, drawn to scale, is subtracted from the main curve, a third curve with a maximum at 285–290 $m\mu$ is obtained. A better separation was achieved in some of the subsequent experiments, but most of the curves can be so resolved.

Fig. 2. Mammalian liver oil concentrate B.

Fig. 3. Commercial cod-liver oil concentrate.

Fig. 4. *Johnius hololepidotus* liver oil.

Table I. (Cf. Figs. 1-4)

	Bio-assay i.v.	Blue value Carr- Price units	Spectroscopic data			Bio-assay Blue value	Bio-assay $E_{1\text{ cm.}}^{1\%}$ 328 m μ
			$E_{1\text{ cm.}}^{1\%}$ values at				
			620	583	328 m μ		
Fractions soluble in 83% alcohol							
Mammalian liver oil. Concentrate A	410,000	22,700	1330	760	430	18	950
			No band at 328 m μ . Max. at $E_{1\text{ cm.}}^{1\%}$ 316 m μ 480				
Mammalian liver oil. Concentrate B	906,000	49,000	2430	1345	720	18.5	1260
			Nearly normal, but band also seen at 315 m μ				
Commercial cod-liver oil. Concentrate	630,000	35,700	1450	840	480	17.7	1310
			Normal, apart from inflexion at 315 m μ				
Concentrate from liver oil of <i>Johnius</i> <i>hololepidotus</i>	1,025,000	50,600	2520	1320	800	20.2	1280
			Doubtful inflexions 315 and 340 m μ . Otherwise normal				
Fractions insoluble in 83% alcohol							
Mammalian liver oil. Concentrate A	226,000	7,000	460	285	230	32.3	1000
			No band at 328 m μ . Max. at $E_{1\text{ cm.}}^{1\%}$ 290 m μ 385				
Mammalian liver oil. Concentrate B	112,000	2,700	320	310	180	41.5	620
			No band at 328 m μ . Max. at $E_{1\text{ cm.}}^{1\%}$ 290 m μ 270				
Commercial cod-liver oil. Concentrate	<37,000	1,075	55	51	52	<34	<700
			Curve shows fine structure. Main max. $E_{1\text{ cm.}}^{1\%}$ 279 m μ 58				
Concentrate from liver oil of <i>Johnius</i> <i>hololepidotus</i>	20,000	565	38.7	24.1	13.4	37	1500
			Actual maxima $E_{1\text{ cm.}}^{1\%}$ 284 m μ 11.2 324 m μ 13.5				

which are given in Table II. The second layer was the most interesting, in that the material showed a strong absorption band at 286 m μ and a new maximum at 496 m μ in the colour test. Iodine values were determined on each fraction. From the results it seems improbable that squalene-like hydrocarbons were present, as they would be among the least strongly adsorbed components and the lower layers would accordingly show rising iodine values. They may, of course, have been in the very small fraction which passed right through the column, but their absence from the fractions examined may be safely inferred.

During adsorption there is always the possibility of artefacts as several workers [Gillam & El Ridi, 1936; Heilbron *et al.* 1934] have found to be the case for carotene and vitamin A. In order to make sure that the material responsible for the 496 m μ band had not been produced in this manner, but is actually present in the alcohol-insoluble fraction, an attempt was made to effect separation by more rigorous exhaustive extraction with aqueous alcohol.

(B) This procedure involved placing the sterol-free concentrate (14,500 blue value) in a cylinder (50 ml.) filled with 83 % ethyl alcohol, and blowing nitrogen

Table II

Sample	Weight (g.)	Colour	Iodine value	Blue value
Untreated fraction	2.5	Reddish brown	191	—
Top layer	0.726	Red	211	2480
Second layer	0.489	Reddish yellow	154	610
Third layer	0.500	Light yellow	136	2600
Lowest layer	0.402	Very pale yellow	129	3000
Not adsorbed	0.012	Colourless	—	250

Spectroscopic data $(E_{1\text{ cm.}}^{1\%})$ values at the wave-lengths specified)

Top layer	Second layer	Third layer	Lowest layer
Colour test:			
606 m μ 216	600 m μ 236	600 m μ 420	
This band divided on standing into two submaxima	575 m μ 161	575 m μ 295	Fraction lost
620 m μ 183			
583 m μ 162			
495 m μ 106 (ill-defined)	495 m μ 225	495 m μ 158	
Ultraviolet:			
328 m μ 120 (No max.)	328 m μ 100 (No max.)	328 m μ 140 (No max.)	
289 m μ 260 (Max.)	286 m μ 260 (Max.)	286 m μ 200 (Max.)	

through the mixture in order to effect thorough stirring. After 5 min., the concentrate was allowed to settle and the supernatant liquid decanted. The process was repeated with 10–12 fresh portions of the solvent, until no trace of yellow colour was discernible in the upper layers. The alcoholic solutions were centrifuged when necessary and any insoluble residue replaced in the cylinder. Both fractions were dried at 100° in a stream of nitrogen.

	Blue value	$E_{1\text{ cm.}}^{1\%}$ 620 m μ	594 m μ	583 m μ	496 m μ	290 m μ	328 m μ
83% alcohol-soluble fraction: 5.638 g.	24,500	1340	—	740	—	—	440 (Good band)
83% alcohol-insoluble fraction: 4.592 g.	1,160	—	180	—	172	240	80 (No band)

The blue value and the spectroscopic data on the alcohol-soluble portion revealed nothing of interest and the fraction was not examined further. The insoluble fraction, however, was strikingly abnormal in that it (a) showed no 620, 583 nor 328 m μ bands, (b) was unaffected by 7-methylindole in the colour test, (c) exhibited maxima at 594 and 496 m μ (rapidly fading) in the colour test, and 290 m μ in the ultra-violet absorption spectrum, (d) exhibited well-defined narrow bands of the type associated with cyclized vitamin A, (e) possessed marked biological activity, 17,900 I.U. per g.

DISCUSSION

It seems highly improbable that sufficient unchanged vitamin A, $C_{20}H_{29}OH$, was present to account entirely for the last-mentioned bio-assay. Perhaps the most sensitive qualitative test for vitamin A in the presence of other chromogenic and chromophoric substances is the development of narrow absorption maxima at 333, 350, 369 and 392 m μ after standing for 20 min., at room temperature,

in *N/30* alcoholic hydrogen chloride solution [Edisbury *et al.* 1932]. This anomalous fraction already showed two of these maxima and an inflexion indicated the presence of a partially obscured third (Fig. 5), but there was

Fig. 5. Mammalian liver oil concentrate.

complete failure to respond further to the test, in spite of a favourably low intensity of absorption in this region. On the basis of a bio-assay of 17,900 i.u. per g. and assuming unchanged vitamin A to be the sole biologically active constituent of the fraction, the contribution of vitamin A to the absorption at 328 $m\mu$ would be between 9 and 18.¹ Previous experience has shown that on cyclization the whole vitamin absorption curve is displaced some 40 $m\mu$ towards the visible, so that the intensity at 369 $m\mu$ approximates to the original intensity at 328 $m\mu$. In this instance an increase of the order of 50% in the intensity at 369 $m\mu$ was to be expected; actually no change was observed. Taking into consideration all the evidence, including the absence of a 620 $m\mu$ band from the colour test, it seems highly improbable that this fraction contained more than a trace of vitamin A, if any. There are three other possible sources of the observed activity: (i) the "285–290 $m\mu$ " product, which may or may not be directly associated with the 496 $m\mu$ band in the colour test; (ii) fully cyclized vitamin A, (iii) some hitherto unidentified transparent (or completely masked) material. These possibilities must be much more fully explored before even a tentative identification of the active constituent can be made. It may be pointed out that unless the cyclized product itself possesses biological activity at least as high as the vitamin, it is impossible to reconcile the bio-assay of 17,900 i.u./g. with the observed intensities of the narrow bands in Fig. 5. (Edisbury *et al.* [1932], showed that the cyclization does not influence the order of *E* values.) For the first possibility (the 285–290 $m\mu$ product) the ratio Bio-assay/ $E_{1\text{ cm.}}^{1\%}$ 290 $m\mu$ is only of the order 75, i.e. about 1/20th the factor associated with vitamin A. A

¹ $\frac{\text{Bio-assay}}{\text{Conversion factor}} \frac{17,900}{2,000}$ or $\frac{17,900}{1,000}$ leads to $E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$ 9 or 18.

substance related to $C_{20}H_{29}OH$, but containing one ethenoid linkage less, would probably exhibit max. 285–290 $m\mu$ $E_{1\text{ cm}}^{1\%}$ somewhat <1600, but of the same order. It would also probably be chromogenic toward antimony trichloride.

Karrer *et al.* [1933] reported chromatographic separation of a rich concentrate into two fractions, one of which absorbed at 270 $m\mu$, whilst Castle *et al.* [1934] and other investigators obtained, by partial oxidation of vitamin A, fractions absorbing at 275–280 $m\mu$. It is conceivable that in some natural products the gross absorption at 328 $m\mu$ may be appreciably enhanced by the presence of such materials as the Karrer, Castle or “285–290 $m\mu$ ” substances, resulting in the prediction, from the observed absorption at 328 $m\mu$, of a fictitiously high activity which would not be supported by subsequent bio-assay. While this possible source of error can have little practical effect on the vast majority of spectroscopic assays, especially in view of the fact that anomalous absorption is comparatively rare and easily recognized, it may be significant in these circumstances. We propose to pursue this matter further, and also to study the possible biological activity of the fully cyclized material.

SUMMARY

In continuation of earlier work [Morgan *et al.* 1935] on the discrepancies between biological and physico-chemical methods of vitamin A assay, rich concentrates have been separated into two fractions by exhaustive extraction with aqueous 83 % alcohol. The soluble fractions generally correspond fairly closely with the physical and chemical criteria usually accepted for vitamin A. The insoluble fraction is highly abnormal, possessing much greater biological activity than would be anticipated from the blue value, and exhibiting a maximum at 285–290 $m\mu$ in the ultraviolet absorption spectrum, often without even an inflexion at 328 $m\mu$. The most interesting fraction, one derived from a mammalian liver oil concentrate, contained no detectable vitamin A (no bands at 620, 583 or 328 $m\mu$; no change in spectrum on treatment with alcoholic hydrogen chloride) but was biologically active (17,900 I.U. per g.) and had the following spectral characteristics:

Colour test $E_{1\text{ cm}}^{1\%}$ 594 $m\mu$ 180, 496 $m\mu$ 172; ultraviolet absorption $E_{1\text{ cm}}^{1\%}$ 290 $m\mu$ 240; also some minor bands indicating cyclized products. It is not known whether the biological activity is associated with the constituent showing these characteristics, but if so, it has only 1/20th of the activity of vitamin A, and is not considered to be the cause of the major discrepancies encountered in fish-liver oils. Similar but less striking fractions have been obtained by chromatographic adsorption.

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REFERENCES

- Castle, Gillam, Heilbron & Thompson (1934). *Biochem. J.* **28**, 1702.
- Edisbury, Gillam, Heilbron & Morton (1932). *Biochem. J.* **26**, 1164.
- Gillam & El Ridi (1936). *Biochem. J.* **30**, 1735.
- Heilbron, Gillam & Thompson (1934). *J. Soc. chem. Ind., Lond.*, **53**, 980.
- Karrer, Walker, Schöpp & Morgan (1933). *Nature, Lond.*, **132**, 26.
- Morgan, Edisbury & Morton (1935). *Biochem. J.* **29**, 1645.
- Morton (1932). *Biochem. J.* **26**, 1197.