LXVII. SOME FACTORS WHICH AFFECT THE ASSAY OF VITAMIN A BY THE SPECTROGRAPHIC METHOD.

By ROY JAMES MACWALTER.

*From the Department of Physiology and Biochemistry,
University College, London.*

(Received January 13th, 1934.)

THE physical methods of vitamin A assay have received much attention in the last few years but the trustworthiness of these determinations is still not clearly defined. Before discussing the present work, which deals with one aspect of the estimation of the vitamin, it may be of value to summarise briefly the present position.

The characteristic absorption spectrum of vitamin A is considered to be a broad continuous band with its head at 3280 Å. The intensity of the absorption at this wave-length in oils containing the vitamin has been found to agree very satisfactorily with the biological estimations [Coward *et al.*, 1931; 1932; Chevalier and Chabre, 1933]. This implies that constituents of the oil other than vitamin A absorb a proportional amount of light at 3280 Å.—or are transparent.

The blue colour obtained with the antimony trichloride reagent has been the subject of a large volume of work, and it now appears that the colour measured in the tintometer is the effect of two absorption bands, one at 5720 Å. and one at 6060 Å., whose positions are shifted by approximately 120 Å. towards the red in concentrated specimens. The intensities of these two bands have been compared with the intensity of absorption at 3280 Å. of the oil itself by Coward *et al.* [1931] and Gillam and Morton [1931], and it has been shown that while the intensities of the 5720 Å. band (in the antimony trichloride colour) and the ultra-violet band (in the oil itself) are always similar, the intensity of the 6060 Å. band does not appear to be so related. Furthermore, on oxidative treatment the latter band may be augmented in intensity leaving the other unaffected. As a result of their experiment, Heilbron *et al.* [1931] have suggested that only the band at 5720 Å. in the colour test is valid as a measurement of vitamin A activity.

The conditions which influence the intensities of these two bands of the colour are important in considering the validity of the blue test. The 6060 Å. band may be increased in intensity by keeping, by mild oxidative treatment with ozone, hydrogen peroxide and benzoyl peroxide and by saponification. It may be decreased in intensity by the addition of the antimony trichloride reagent in two stages, by the addition of pyrrole, indole, skatole or an unsaturated acid isolated from the saponifiable fraction of cod-liver oils [v. Eekelen *et al.*, 1933; Heilbron *et al.*, 1931]. The evidence points to the possibility of inhibition of the 6060 Å. band, but the improbability of inhibition of the 5720 Å. band. Assuming that the 5720 Å. band arises from vitamin A, it is necessary to adjust the conditions of conducting the "blue test" in order that the 6060 Å. band shall always bear the same relation to the 5720 Å. band.

For untreated cod-liver oils this appears to be the case, the ratio 6060 Å. band : 5720 Å. being 1.35 : 1, though considerable fluctuation about this mean value occurs (Gillam and Morton). If the absorption at 6060 Å. is caused to attain its maximum value by oxidative treatment a rather more constant ratio is attained (Heilbron *et al.*), but the mean value of the figures given the present writer finds to be 1.45 : 1 which differs from the previous ratio quoted by less than the estimated experimental error. This appears to the writer to indicate that in many cod-liver oils there can be very little inhibitor substance affecting the 6060 Å. band. In these oils saponification should not increase materially the absorption of the 6060 Å. band. In those cases where this band may be increased, we must infer that the ratio is less than about 1.40 : 1 and in order to attain the constant ratio, some means (*e.g.* saponification) must be used to enhance the 6060 Å. band. From these data it appears that in order that the colour test may measure variations in the 5720 Å. band, the determination must be made on the unsaponifiable matter in order that a constant ratio between the two bands of the colour test may be attained.

There is, however, another aspect of the problem. v. Eekelen *et al.* [1932; 1933], have stated that by an adsorption technique it has been possible to separate the two chromogens of cod-liver oils, which give rise to the two bands of the colour test. Only the fraction giving rise to the 6060 Å. band has been found to be active biologically. These experiments are at variance with the more circumstantial evidence which has been already summarised. The conclusions do not in any way affect the technique of making colour tests, however, since in each case the aim must be to cause the 6060 Å. band to attain its maximum value.

Measurements of vitamin A content on the basis of absorption at 3280 Å. involve, however, a purely empirical assumption that irrelevant absorption at this wave-length bears a constant relation to the relevant absorption for all oils or is negligible in comparison with it. With a view to attempting to estimate this irrelevant absorption, cod-liver oils have been aerated at 100° and the absorption determined at intervals. At the same time a sample of the oil was saponified and the absorption of the unsaponifiable material determined. In Fig. 1 a typical curve is shown for an oil of blue value 22 (B.P. test) referred to 20 % solution. Curve I which refers to the absorption at 3280 Å. of the oil on aeration may be regarded as compounded of two curves. By producing back the straight portion of the curve to the axis representing absorption and subtracting the two curves, curve III is obtained. From curve II which represents the absorption of the unsaponifiable matter after the oil has been aerated for varying times (referred to 1 % of oil) it is seen that the absorption attained a constant value after 5 hours, by which time no blue colour was obtained with antimony trichloride. This constant value may be taken as representing the absorption of unsaponifiable matter which is not vitamin A, and by subtracting it from curve II the true values for vitamin A should be obtained. This last curve is almost

Fig. 1.

identical with curve III which is logarithmic. It is, therefore, assumed that the extrapolated straight line in curve I represents the manner in which the absorption at 3280 Å. of the non-relevant part of the oil varies with time of aeration. If this is true, the intercept of this line on the axis representing $E_{1\text{ cm.}}^{1\%}$ provides a value for the absorption of the constituents other than vitamin A in the original oil, and in this case it amounts to approximately 40 % of the total absorption. The curves shown draw attention to the possibility of serious inaccuracies arising from the measurement of absorption at 3280 Å. as the sole criterion of vitamin A potency. Furthermore, the effect of previous ageing of the oil by oxidation probably has the effect of increasing the irrelevant, as well as decreasing the relevant, absorption at 3280 Å. The actual variation in absorption by oxidative change at room temperatures cannot of course be gauged by these curves since the temperature coefficients of the two reactions involved will probably be different. From the published figures of Heilbron *et al.* [1931] it appears that in some cases at least an actual increase of total absorption may occur after storing.

In Fig. 2 two further curves of aeration are given, curve I for an unusually potent cod-liver oil of blue value 112, and curve II for a weak oil of blue value 14. In each case a similar curve to that in Fig. 1 has been obtained, the linear

Fig. 2.

Fig. 3.

portions of which are approximately parallel and of similar initial values. Again, in the less potent oil the irrelevant absorption is of the order of 40 %, while in the potent oil it is approximately 12 %.

In a recent paper Dann and Moore [1933] have shown that the time of saponification is a factor which affects the absorption of the fatty acids from cod-liver oils. Edisbury *et al.* [1933] have suggested "cyclisation" as the cause. While a discussion of the chemical nature of the changes brought about by aeration treatment lies outside the scope of this paper, it is suggested that the increase in absorption at 3280 Å. by aeration may arise from a similar cause since the absorption at lower wave-lengths is increased very considerably, but by the

technique used it would not have been possible to detect the fine structure bands noted by these authors.

It would seem that the absorption at 3280 Å. can only be regarded as measuring the vitamin A activity for oils more potent than that shown in Fig. 2, curve I. For less potent oils the "non-vitamin A" absorption at 3280 Å. constitutes a considerable proportion of the total absorption. The very real parallelism, however, between the absorption in this region and the biological tests for normal cod-liver oils cannot be dismissed as fortuitous. It has been found that the oils which have been so far examined by the aeration technique contain approximately 40 % of irrelevant matter, as measured by the intercept, provided that the potency is of the order of 10–20 blue units. In these circumstances the measured absorption of the oil will be proportional to the amount of vitamin A present.

In Fig. 3, a curve for the blue values and absorption values of the unsaponifiable fraction of cod-liver oils has been drawn. Of all the oils which have been examined none has deviated from this curve by more than 12 %. Josephy [1933] has recorded some results of a similar nature.

While it is not practicable to adopt the aeration technique here described, it appears to the author desirable to determine the absorption upon the unsaponifiable matter from the oil rather than upon the oil itself. In this way errors arising from previous oxidation of the oil are eliminated and there only remains the error due to absorption at 3280 Å. of irrelevant unsaponifiable matter.

SUMMARY.

The effect of oxidative change on the absorption at 3280 Å. of liver oils is discussed in so far as it affects the assay of vitamin A by the spectrographic method. The need for saponification of the oil in order to avoid errors arising from previous oxidation is emphasised.

The author is indebted to Prof. Drummond for his interest in the work and his helpful suggestions.

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