A COMPARISON OF PHYSICAL AND CHEMICAL METHODS WITH BIOLOGICAL ASSAY OF VITAMIN A*

By T. K. Murray and J. A. Campbell

From the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Canada

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In 1946, Morton and Stubbs proposed their correction procedure for the spectrophotometric determination of vitamin A[1]. Since then it has been adopted as an official method by the U.S. Pharmacopeia[2] and the Association of Official Agricultural Chemists[3]. Both bodies specify that the correction be applied only after saponification. The British Pharmacopœia[4] has also adopted this method but requires saponification only for cod-liver oils, the correction being applied directly to whole oil solutions of halibut-liver oil and concentrates. Since 1951, the Food and Drug Laboratories have used a method essentially the same as that of the U.S.P. XIV[5].

Although used extensively, the method also has been subjected to criticism. Gridgeman[6] discussed the theory of the three-point correction very fully and concluded that direct evidence of its validity is needed. Cama, Collins and Morton[7], in a very comprehensive paper, have stated that the correction procedure gave erroneously low values. They supported this statement by the results of detailed analysis of a cod-liver oil and two high potency oils. For good quality, low potency oils, they recommended the use of uncorrected *E* values, measured on unsaponifiable extracts, with a correction made for vitamin A_2 only. These authors believed that the application of the correction to solutions of whole oils of this type gave low results, unless a suitable adjustment for the presence of *neo*vitamin A was also made. In the analysis of high potency oils they recommended the use of solutions of the whole oil, with correction of the results by the application of two or more formulæ. They stated that results obtained in this way also required adjustment for the presence of *neo*vitamin A. The correction procedure when applied to unsaponifiable extracts gave erroneously low results, apparently because the irrelevant absorption was not linear. They suggested that the non-linear irrelevant absorption was caused by isomerism during saponification. The criterion used by these authors was chemical purity as determined by a comprehensive analysis of 3 oils. In reaching these conclusions they assumed that *neo*vitamin A was equal in biological potency to all-*trans* vitamin A and that vitamin A_2 had no biological activity. Both Cama *et al.*[7] and Bagnall and Stock[8] have pointed to the need for further data regarding these points. Swann[9] discussed the determination of vitamin A in cod-liver oil and stated that corrected *E* values, as determined on the unsaponifiable matter, underestimated this type of oil. This conclusion

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was reached as a result of comparisons with other methods, including a chromatographic procedure.

Melnick *et al.*[10] also reached the conclusion that the geometric correction, applied to unsaponifiable fractions of high potency oils, gave low results. They based their conclusions on a comparison with rat growth assays and attributed the low results obtained by the U.S.P. method to the presence of *neo*vitamin A and vitamin A_2. Unpublished results of a similar comparison conducted by an informal committee of the U.S.P.[11] indication of over-correction to the extent reported by Melnick *et al.* conservative estimate of biological potency. There was, however, no indication of overcorrection to the extent reported by Melnick *et al.*

Analysts concerned with market samples frequently have to take into account the presence of oxidised vitamin A as well as *neo*vitamin A and vitamin A_2. In some samples slight oxidation will be indicated by a decrease in the absorption at 325 mμ accompanied by a relative increase in the absorption at 310 mμ. More extensive oxidation will result in a shift of the maximum absorption from 325 mμ so that the application of the correction is no longer valid. This problem has been discussed by Bagnall and Stock[8], who pointed out that while the practice of multiplying gross E values by a factor of 1600 may be useful when dealing with fresh oils, it could lead to very erroneous results with oxidised samples. Since these samples are generally below label claim, the problem is important in practice if an exact potency must be assigned.

Another aspect of the correction procedure that has been criticised, particularly by manufacturers, is that it is unsuitable for routine analysis because of the length of time required to complete an assay. The elimination of either saponification or correction would do much to simplify the procedure. Swann[9] has reported that for cod-liver oils there is fair agreement between E (gross) $\times$ 1600 and E (corr.) $\times$ 1900 as determined on the whole oil. He intimates, however, that results obtained in this manner are high. Bagnall and Stock[12] have reported that for halibut-liver oils the agreement is good between E (gross) $\times$ 1600 and the geometric correction. As noted above, this is not suitable for samples which have become oxidised.

Finally, there appears to be one other point deserving mention at this time. The U.S. Pharmacopeia has described a single method for all pharmacopœial substances. The British Pharmacopœia, on the other hand, gives considerable freedom, particularly with regard to the saponification of concentrates. Cama *et al.*[7] have proposed a scheme by which, if assumptions made are correct, a very precise and accurate estimate of the true vitamin A content may be obtained. Such methods as the latter, however, are not readily adapted to routine analysis where hundreds of samples are being assayed. For most effective control, uniformity of procedure, while possibly sacrificing the ultimate in accuracy, has much to recommend it as far as governmental control or industrial transactions are concerned.

Since the correction procedure has been widely adopted for routine control, and since there have been few published comparisons involving

this method, it seemed of prime importance to determine the relative merits of the Morton–Stubbs method as an indicator of biologically active vitamin A under conditions of routine analysis. Any discussion of chemical methods for the determination of vitamin A is based on certain assumptions of biological activity. One of these assumptions involves the biological potency of *neo*vitamin A which has not, as yet, been definitely established. It seemed important, therefore, to obtain further data on this point. It was also of interest to determine the effect of oxidised vitamin A on the various assay procedures. This paper presents the results of studies on these problems.

Methods

The biological potencies were estimated by the vaginal smear method developed in this laboratory by Pugsley, Wills and Crandall[13], and by the U.S.P. XIV growth assay. 3 dosage levels for both the standard and sample were used in all assays and the results of each assay were

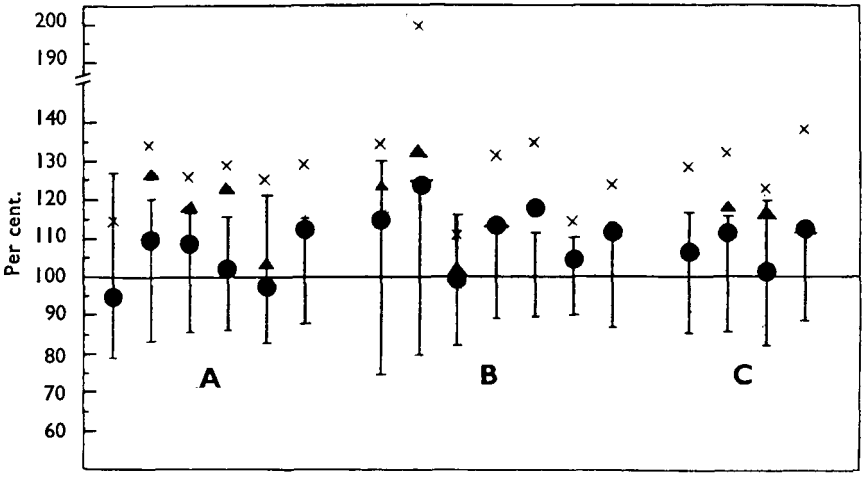

Fig. 1. A comparison of the corrected, uncorrected and antimony trichloride methods with vaginal smear assays of Vitamin A on cod-liver oils, concentrates and halibut-liver oils. Bioassay potency is plotted as 100 per cent.

A. Cod-liver oils.
B. Concentrates.
C. Halibut-liver oils.

I Biological.
● Corrected.
× Uncorrected.
▲ Antimony trichloride.

calculated from the data on 48 to 60 rats. The vaginal smear method was chosen for the majority of the comparisons because of its relatively greater precision, the speed with which an assay could be completed and because several assays could be completed on one group of rats. Calculations of potency in the growth assays were based on figures obtained by considering the weights of rats at each week of test as suggested by Bliss and György[14]. The U.S.P. XIV methods were followed for the corrected spectrophotometric and colorimetric determinations which were

made using a Beckman model D.U. spectrophotometer and Evelyn colorimeter respectively. Uncorrected results were calculated by multiplying the uncorrected E_{325} by 1900, 1600 or 1450 as indicated in the text. The samples used in this study were pharmaceutical grade cod- and halibut-liver oils and concentrates, most of which were retail products obtained from manufacturers.

Experimental Results

Comparison of methods. Samples of cod- and halibut-liver oils and concentrates were assayed in duplicate, spectrophotometrically and colorimetrically. The results were compared with biological potencies as determined by the vaginal smear method. In cases where there was a wide difference between results, the biological assay was also done in duplicate. The results of these assays are shown in Figure 1 and in Table I. Except for one halibut-liver oil and two concentrates, all the corrected spectrophotometric results fall within the fiducial limits of the bioassay. It should be noted that there does not appear to be any tendency for the Morton and Stubbs procedure to overcorrect. In fact, the corrected spectrophotometric results are, on the average, 3·9 per cent. higher than the bioassay for cod-liver oils, 7·9 per cent. higher for halibut-liver oils and 12·3 per cent. higher for concentrates. If the factor of 1820, which has been reported by Cama *et al.*[7] to be more correct for vitamin A alcohol, had been used, the difference would have been about 0, 4 and 8 per cent. respectively.

TABLE I

VITAMIN A CONTENT OF FISH OILS AS DETERMINED BY SEVERAL METHODS

	Potency expressed as I.U./g. or capsule					
	Spectrophotometric		Antimony trichloride	Biological assay (vaginal smear)		
Sample	*E* gross × 1900	*E* corr. × 1900		Potency	Confidence limits (P = 0·05)	
Cod-liver oils ..	2220	1840	—	1940	1530	2470
	2250	1840	2120	1680	1400	2020
	3530	3040	3300	2790	2390	3260
	2370	1860	2260	1830	1590	2120
	1090	850	960	870	720	1050
	2250	1960	—	1750	1530	2010
Concentrates ..	3900	3340	3610	2910	2220	3810
	53,600	32,900	35,400	26,800	21,300	33,600
	55,900	50,500	50,800	50,500	41,700	61,100
	70,400	60,800	—	53,600	47,700	60,500
	215,000	188,000	—	15,900	143,000	177,000
	211,000	187,000	—	177,000	16,100	195,000
	120,000	103,000	—	90,900	79,800	104,000
Halibut-liver oils	5290	4370	—	4100	3520	4800
	66,700	56,000	59,500	50,300	43,400	58,400
	4850	4010	4610	3940	3270	4740
	92,400	75,200	—	66,800	59,400	75,000

The uncorrected results, on the other hand, are outside the limits of the bioassay except in the case of one cod-liver oil. The percentage by which the uncorrected results exceeded the bioassay for cod-liver oils was 26·2 per cent., for halibut-liver oils 30·6 per cent. and for concentrates 36·3 per cent. The antimony trichloride values fell in an intermediate

position and are within the fiducial limits of the bioassay in the case of one cod-liver oil, one halibut-liver oil and two concentrates. The extent to which the colorimetric results over-estimated the biopotencies were, for cod-liver oils 17·6 per cent., for halibut-liver oils 17·7 per cent. and for concentrates 18·9 per cent. The colorimetric results have been shown by an analysis of variance to differ significantly ($P = 0{\cdot}01$) from the corrected spectrophotometric results.

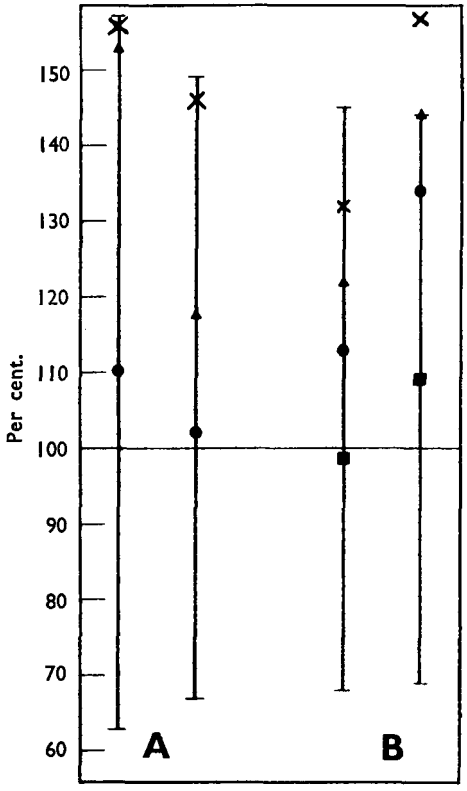

FIG. 2. A comparison of corrected, uncorrected and antimony trichloride methods with bioassays of Vitamin A in cod-liver oils and concentrates. Growth assay potency is plotted as 100 per cent.

A. Cod-liver oils.
B. Concentrates.
I. Biological, growth.
■ Biological, vaginal smear
● Corrected.
× Uncorrected.
▲ Antimony trichloride.

Figure 2 shows the results of similar comparisons in which the rat growth assay was used in place of the vaginal smear method. The first two samples in this comparison were low potency cod-liver oils of the type used for animal feeding. The other two oils were concentrates which had been assayed previously by the vaginal smear method. The relation between potencies as determined by chemical and physical assays and the growth assay agree well with those shown in Figure 1. The corrected values are again the best estimate of biological potency and there is no tendency for the Morton and Stubbs procedure to over-correct. Agreement between potencies estimated by the growth and vaginal smear assays was good.

Potency of neo*vitamin A.* The suggested over-correction by the Morton and Stubbs method has been attributed, in part, to the presence of *neo*vitamin A. Since the results discussed above show no tendency towards over-correction, it was of interest to determine the potency of *neo*vitamin A using the vaginal smear assay. A sample of crystalline *neo*vitamin A acetate in Wesson oil and a sample of crystalline *neo*vitamin A alcohol*

* Samples of *neo*vitamin A kindly supplied by Dr. Norris D. Embree, Director of Research, Distillation Products, Ind., Rochester, N.Y.

were diluted with corn oil and assayed against all-*trans* vitamin A acetate. The results of these assays, shown in Table II, are in good agreement with the values reported by Harris, Ames and Brinkman[15], who found a potency for *neo*vitamin A of 75·6 per cent. of all-*trans* vitamin A when measured by the liver storage method and 83·2 per cent. by the growth assay. The potencies reported here appeared to agree better than would be expected from the confidence limits of the individual assays.

TABLE II

POTENCY OF *neo*VITAMIN A ACETATE ON A PER UNIT *E* VALUE BASIS RELATIVE TO ALL-*trans* VITAMIN A ACETATE

Assay	Sample	Percentage of all-*trans* A	I.U./g.	Limits in per cent. (P = 0·05)
1	Acetate in oil RL 330	71·3	2,377,000	83·5–119·5
2	,, ,,	74·3	2,477,000	84·7–118·0
3	Alcohol crystals 817 B	73·0	2,433,000	89·5–111·7
Mean (logarithmic)		72·0	2,400,000	91·6–109·1

Presence of oxidised vitamin A. Oxidised vitamin A is one of the constituents most commonly encountered in routine analysis of vitamin A in retail products. It was therefore of interest to determine whether the correction procedure gave reliable results when applied to a partially oxidised oil. For this purpose a sample of cod-liver oil was subjected to progressive oxidation by bubbling oxygen through it at 90° C. As the potency dropped, samples were removed, flushed with nitrogen and refrigerated until biological, spectrophotometric and colorimetric assays were carried out. The results of these assays are shown in Figure 3. It will be noted that there is good agreement between the results of the corrected spectrophotometric assay and those of the biological assay in the two samples to which the correction could be validly applied. The correction could not be applied to the third sample because the absorption maximum had shifted from 325 mμ.

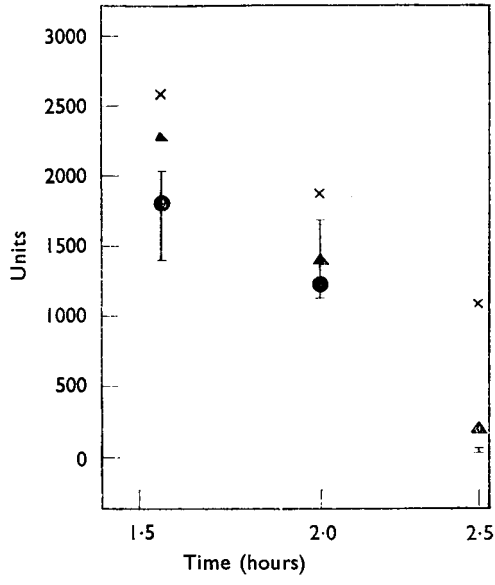

FIG. 3. A comparison of methods for the determination of vitamin A in an oxidised cod-liver oil.
I Biological.
● Corrected.
× Uncorrected.
▲ Antimony trichloride.

The uncorrected results are outside the limits of the bioassay in all

cases, while the antimony trichloride method gave values that fell between the corrected and uncorrected potencies. When the oxidation had reached the point at which the correction could not be applied, neither the antimony trichloride nor the uncorrected spectrophotometric figures was a good estimate of biological potency. This latter observation is supported by unpublished data on other oils in which extensive oxidation had taken place.

Determinations on whole oil solutions. Another of the criticisms of the spectrophotometric method, as it is now used, is lack of adaptability to routine analysis. The elimination of saponification, if practicable, would do much to simplify this method. It was of interest, therefore, to compare results obtained by the U.S.P. XIV method with those obtained by applying the correction directly to solutions of the whole oil. The solvent chosen for this work was *cyclo*hexane, which is used in the method

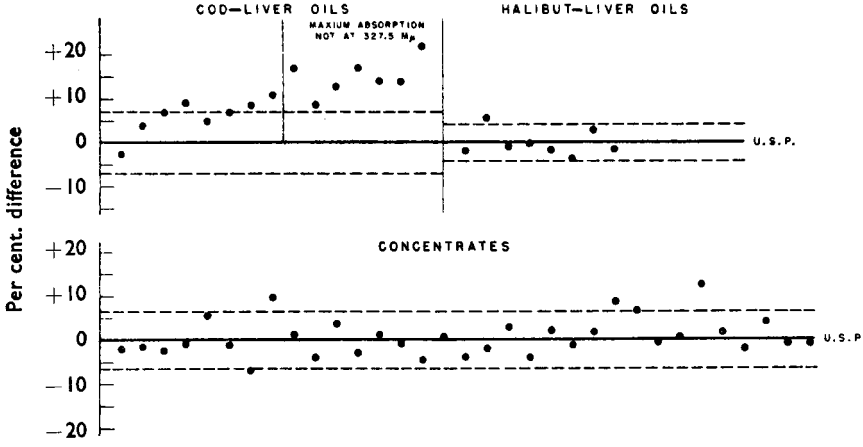

FIG. 4. A comparison of the corrected potency of fish oils determined on the whole oil and non-saponifiable basis. Base line is U.S.P. method non-saponifiable basis.

of the British Pharmacopœia for this determination. The appropriate formula for vitamin A ester was derived from the absorption curve of the Canadian Reference Standard vitamin A acetate. The wavelengths at which absorption was 6/7 maximum were found to be 312·5 and 337·7 mμ with the maximum at 327·5 mμ. These figures agree with those published by Cama *et al.*[7] and it follows that the correction formula is $7(E_{327\cdot5} - 0\cdot405E_{312\cdot5} - 0\cdot595E_{337\cdot7})$. The E value at 327·5 was found to be 5·11, so that the appropriate conversion factor was 1958 rather than 1900, which was used in determinations of the unsaponifiable fraction in *iso*propanol.

Samples of pharmaceutical grade cod-liver oil, halibut-liver oil and concentrates were assayed in duplicate, on different days, by the application of the appropriate correction formula to absorption readings of unsaponifiable fractions in *iso*propanol, and of whole oil solutions in *cyclo*hexane. The comparison of these results is shown in Figure 4.

The cod-liver oils fell into two categories, those in which the absorption maxima of the whole oil solutions occurred at or near 327·5 mμ and those to which the correction could not be validly applied because maximum absorption occurred between 320 and 325 mμ. It may be noted that using the regular 3-point correction, no distinction would be made between these oils since the absorbancy at 310 mμ in all cases was less than at 327·5 mμ. The absorption maxima of all unsaponifiable fractions occurred at 325 mμ. In neither group was there good agreement between the results of the two methods, the difference between them being significant at $P = 0{\cdot}01$. This indicated that it was not possible to apply the correction directly to cod-liver oils. In the case of concentrates and halibut-liver oils there was no significant difference between the results of the two methods. Only 3 samples fell outside the limits of error (2 × S.E. of the difference between duplicate determinations), the maximum deviation being 12·6 per cent. and the average deviation a fraction of 1 per cent. If, as Cama *et al.*[7] have reported, the conversion factor of pure vitamin A acetate in *cyclo*hexane is 1920, the values reported here should be reduced by 2 per cent.

Elimination of correction. The potencies of the same group of oils were also estimated by multiplying gross *E* values by a factor, without first correcting for irrelevant absorption. Since all samples examined contained irrelevant absorption it was obvious that the factor of 1900 was too large. The factor of 1600 which, according to Morton[16] is as good as could be chosen, was therefore used. For cod-liver oils it was soon found that, as judged by the U.S.P. XIV method, the conversion factor of 1600 was too high. Consequently a factor of 1450, obtained by calculating the average factor required to arrive at the corrected potency, was used for cod-liver oils while 1600 was retained for halibut oils and concentrates. The results obtained in this manner were compared with those found by the U.S.P. method and the analysis of variance of the logarithms of the data from this comparison is shown in Table III.

TABLE III

ANALYSIS OF VARIANCE OF VITAMIN A ACTIVITY (IN LOGARITHMS) AS MEASURED BY THE U.S.P. AND GROSS *E* × 1600 BASIS

Source of variation	Halibut-liver oils			Concentrates			Cod-liver oils		
	D.F.	Mean square	F.	D.F.	Mean square	F.	D.F.	Mean square	F.
Methods ..	1	0·000351	3·98	1	0·000249	2·18	1	0·000434	1·38
Samples ..	5	1·850703	20,971*	9	0·755005	6617*	11	0·179199	572*
Method X samples ..	5	0·000237	2·68	9	0·001151	10·08*	11	0·0016100	5·14*
Error ..	12	0·00008825		20	0·0001141		24	0·00031291	

* Significant at P = 0·01.

It will be noted that none of the mean squares for methods was significant which shows that the factors chosen were of the right magnitude. An examination of the mean squares for the method X sample interaction shows that this was significant at $P = 0{\cdot}01$ for cod-liver oils and concentrates. This indicated that some cod-liver oils and concentrates were

over-estimated, while others were under-estimated by the elimination of the correction. The halibut-liver oils which were tested were sufficiently uniform in irrelevant absorption that a similar estimate of potency was obtained on the uncorrected and on the corrected basis. This is in agreement with results published by Bagnall and Stock[12].

Discussion

One of the most serious criticisms which has been made of the Morton–Stubbs correction, as applied in the U.S.P. XIV method, is that it under-estimates the true potency of samples. The results of comparisons of methods which have been carried out in this laboratory give no indication of under-estimation of biological activity by the U.S.P. spectrophotometric method.

In the case of cod-liver oils, the geometric correction applied to unsaponifiable fractions gave results which were in excellent agreement with the biological potencies as measured by the vaginal smear method. Uncorrected results on the same oils were, on the average, 26·2 per cent. higher than the potencies determined by biological assays. Similar results were obtained when two low potency cod-liver oils were assayed spectrophotometrically, colorimetrically and by the rat growth assay. These results appear at variance with those of Cama *et al.*[7], who report that, while the correction applied to unsaponifiable extracts leads to erroneously low results, uncorrected results on the same fraction gives results which need only be corrected for the presence of vitamin A_2. Neither do the results reported here agree with those of Swann[9], who reported that the correction applied to unsaponifiable fractions led to low results.

The biological potencies of halibut-liver oils and of concentrates were also in good agreement with the potencies obtained by the U.S.P. XIV method, in fact, almost all samples of this type were slightly over-estimated. These results, too, do not agree with the conclusions of Cama *et al.*[7] More surprisingly they do not appear to agree with the results of Melnick *et al.*[10], who based their conclusion that the U.S.P. XIV method under-estimated the biological potency of concentrates on rat growth assays. However, it should be noted that, of the 3 laboratories which carried out biological assays reported by Melnick *et al.*[10], one laboratory reported results which were, in every case, higher than those found by multiplying gross *E* values by a factor of 1894. The average biological value of 5 oils reported by this laboratory was 11 per cent. greater than the average uncorrected value of the same 5 oils. These results are obviously biased towards high values and should, for purposes of comparison, be disregarded. The biological results from the other 2 laboratories varied above and below the corrected spectrophotometric results, the average biological results were, in one case, exactly equal to the average corrected value, and in the other, 8·8 per cent. higher. No indication of the error of these assays was given, and it must be concluded that, with the exception of the results from one laboratory, the data of Melnick *et al.*[10] are actually in good agreement with those reported here.

They are also in agreement with those found by the U.S.P. Informal Committee[11].

It is interesting to note that the relation reported here between uncorrected spectrophotometric results and bioassay data was in excellent agreement with that found by Chilcote, Guerrant and Ellenberger[17]. They found that the uncorrected spectrophotometric results exceeded the biological potencies of 28 fish oils by an average of 30·6 per cent. The antimony trichloride method gave results 18·8 per cent. higher than biological potencies. These authors measured biological activity by means of the growth assay and did not report corrected spectrophotometric results.

Some of those who have found that the procedure of Morton and Stubbs introduced too great a correction have sought a partial explanation in the fact that *neo*vitamin A is under-estimated whenever the geometric correction is applied. If, as has been assumed, *neo*vitamin A has the same potency as all-*trans* vitamin A, the spectrophotometric over-correction of *neo*vitamin A would amount to 14 per cent. if measured in *cyclo*hexane and 20 per cent. in *iso*propanol. Using the vaginal smear assay, the potency of *neo*vitamin A has been found to be 72 per cent. of that of the all-*trans* form. This is in good agreement with the potency found by Harris *et al.*[15] by the liver storage method, but somewhat lower than the figure of 83·2 per cent. reported by the same authors for the growth assay. If, as now seems likely, the potency of *neo*vitamin A is accepted as being 17 to 28 per cent. less than that of all-*trans* vitamin A, then the need for adjusting results upward by a factor of 1·04 to allow for its presence, as suggested by Cama *et al.*[7], will have disappeared. If any adjustment need be made it would appear to be in the downward direction, especially if the solvent is *cyclo*hexane.

The fact that *neo*vitamin A is evidently not as potent biologically as had been supposed does not fully explain the difference between the results reported here and those of Cama *et al.*[7] and Swann[9]. The reasons for this difference are not at once evident. Obviously the approach to the problem has been made from different angles. While we also find that the correction applied to unsaponifiable fractions gives lower results than any other physical or chemical method, there is not the slightest indication of over-correction in our comparisons as judged by biological assays. The possibility of some unknown factor influencing either or both the chemical and biological assays cannot be excluded. For example, the relative utilisation of various esters is not known precisely. The fact that such a difference exists indicates that very careful consideration should be given to the whole problem of the estimation of the vitamin A assay before the biological assay is discarded completely. Since the unit of measurement is still the International Unit, indicating specific activity, the question seems of considerable importance.

Cama *et al.*[7] have recommended that concentrates and halibut-liver oils be assayed on the whole oil basis, since saponification followed by correction apparently led to low results. In the samples we have tested we have been unable to demonstrate a significant difference between the

results obtained by these methods. Occasionally oils were encountered in which the whole oil corrected values were as much as 12 per cent. higher than the corrected value measured on the unsaponifiable fraction. In our experience, however, these oils appeared to be exceptions to the general picture. When the correction was applied to whole oil solutions of cod-liver oils, the results were significantly higher than the corrected results of unsaponifiable fractions, which have already been shown to be in excellent agreement with biological potencies.

The practice of multiplying gross E values by a factor to obtain biological units appears to be applicable only to halibut-liver oils. Concentrates and cod-liver oils seemed too variable in their content of irrelevant absorption to be handled in this manner. Moreover, the factor of 1600 which has been proposed for this method was too high for the cod-liver oils we have examined, the average factor required by these oils being 1450.

It would appear from these data that similar results can be obtained by applying a correction to either whole oil solutions or unsaponifiable fractions of concentrates or halibut-liver oils. Similar results may also be obtained on halibut-liver oils by multiplying gross E values by 1600. However, for the sake of uniformity, and particularly for control purposes, it is considered preferable to adopt, as far as possible, one method for the routine estimation of vitamin A. The correction procedure applied to the non-saponifiable fraction seems to be most widely applicable.

Summary

1. The Morton and Stubbs correction procedure for vitamin A has been widely adopted as an official method, but few data are available to indicate its validity as determined by direct comparison with the biological method. Accordingly, the results of vitamin A assays by existing physical and chemical methods have been compared with each other and with the results of biological assays.

2. The procedure of Morton and Stubbs, as applied in the U.S.P. XIV method, afforded a more accurate estimation of the biological potency of market samples of fish-liver oils than did either the antimony trichloride method or uncorrected spectrophotometric measurements. The correction also yielded the best estimate of the biological potency of a partially oxidised cod-liver oil.

3. Application of the Morton and Stubbs correction to the optical density of whole oil solutions of halibut-liver oils and concentrates yielded results which were similar to those found on the non-saponifiable basis. When applied to solutions of cod-liver oils, the correction yielded results significantly higher than when applied to non-saponifiable fractions of the same oils. The practice of multiplying uncorrected E values by 1600 did not indicate accurately the vitamin A content of cod-liver oils or concentrates, but appeared to be satisfactory for halibut-liver oils.

4. The potency of *neo*vitamin A has been found to be 72 per cent. of that of all-*trans* vitamin A when assayed by the vaginal smear method.

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